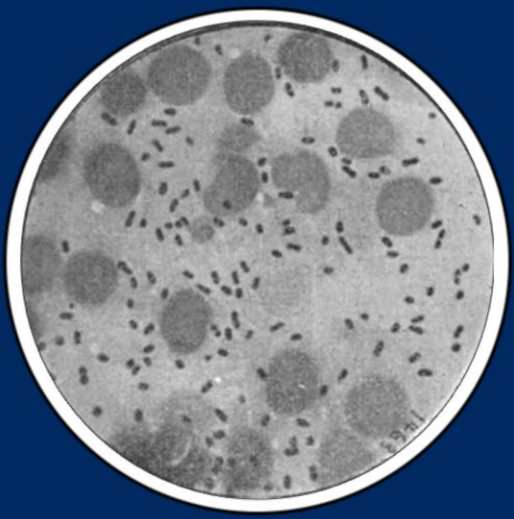
Vladimir A. Gorshkov-Cantacuzene

The Opera on the Plague



Vladimir A. Gorshkov-Cantacuzene

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Recommended by the Academic Council of the State Research Center for Applied Microbiology and Biotechnology as a textbook (manual) for in-depth study by students of medical, biological and veterinary higher educational institutions, as a manual for students of professional training courses (post-graduate course) on work with pathogens of especially dangerous infections

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Ο γιατρός είναι φιλόσοφος *
Ιπποκράτης

Dedicated to those closest to me:
my mother and my brother

* Greek "the physician is a philosopher". Hippocrates.

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On the 130th anniversary of
the discovery of the Yersinia pestis

Human life and knowledge are said to originate in the realm of Ranginui, the heavenly father. At least that's what the Māori, the indigenous people of Aotearoa (New Zealand), believe. They also believe that long ago his son, the forest god Tāne, ascended to the abode of Te Tihi-o-Manono, the highest of the twelve heavens, called Te Toi-o-ngā-rangi. There he received three baskets of knowledge and gave them to the people: Te Kete Tuatea (the basket of light) holds the knowledge already discovered; Te Kete Tuauri (the basket of darkness) holds the knowledge not yet discovered; Te Kete Aronui (the basket of pursuit) holds the knowledge to which man now aspires.

It is generally believed that infectious agents are something detached from ordinary life. They are studied as part of an independent world, separate from the human world, at best concerning a particular individual and usually only during illness. However, these tiny, not visible to the naked eye "pathogenic biological agents", which we contemptuously call them from the height of their grandeur, are not lonely rocks of the Faroe Islands. They literally created the reality around us. They are the ones who radically changed the course of all human history, instilling animal fear in the "crown of nature's creation". They are the true prima donnas of opera of the La Scala of life.

This opera (It. "work, labour") is probably one of the first experiments in considering the causative agents of particularly dangerous bacterial infections within the "human world", within what we call our everyday life: what is and once was. The author invites each reader to look at the familiar world from a different angle and, in this regard, humbly asks, firstly, to read this book (and not to teach or take notes), and secondly – and this is important! – to make every effort to imagine what the narrative is about. It doesn't matter so much what level of preparation you have. It doesn't matter whether you are quick to piece together a complex puzzle of information into a coherent picture or slow. What is important is that you let it sink in and live the story, be-

cause medicine is not only a science, but also an art that is characterised by elegance and romance.

Embark on this amazing journey for Te Kete Aronui with enthusiasm of Odysseus, fearlessness of a Maori warrior, belief in your own strength and success, and tenacity of a cat that wakes me up every morning demanding to be given food.

ACT I

Monstra nigrantis





Basilica of Santa Maria della Salute in Venice

1 UNDE ORIGO…

A Man enters Dorsoduro, separating the opposite bank with its carnivals, its Piazza San Marco and Palazzo Ducale, from the marble vaults of the Basilica of Santa Maria della Salute, which stands majestically at the entrance to the Venetian Grand Canal. He is dressed in a black overcoat, and his dark-blond hair is already showing some grey. His dull brown eyes are scorched by the mad whirlwind of time. He walks slowly across the mosaic floor. When he reaches the composition depicting flowers surrounding a copper disc in the middle of the octagon, under the dome, above which the Mother of God in the armour of a sea general[1] rises majestically, he slowly kneels down and bows before it. On the disc is visible the inscription, "*Unde origo, inde salus*"[2]. Like a silent chime, the events of the terrible epidemic that took place in the Serene Republic and claimed the lives of many of its inhabitants awaken in memory. The reason for the installation of the gondola-supported bridge on the tame waters of the Adriatic Sea, on which every year on 21 November, the Doge of the Republic, the true Lord of the Seas, betrothed to the sea according to ancient tradition, walks from his magnificent palace to bow his head in the centre of the octagon in the same humble manner in gratitude for salvation. It seems that these words, as a symbol of heroism and courage, of a challenge to death itself, are swirling in the air, filling the space and rushing upwards to the very dome...

This disease has accompanied mankind on a long historical path. Genetic material of its causative agent is found even in fossilised remains 5000 years old, and references to it are found in the earliest written records. Several terrible pandemics and many epidemics were caused by it, leaving such an indelible trace in the memory of mankind that even some epidemics caused by other agents have long been attributed to it. She spared neither the rich nor the poor. She did not fade into oblivion like many others, and the shadow of her greatness still hovers in the air, reflecting off buildings in narrow canals to the songs

[1] Ven. "Capitano generale da Mar"; the elective office of commander-in-chief of the navy in time of war.

[2] Lat. "where the source is, thence [came] salvation".

of gondoliers, with the rarest of flashes instilling terror at the possibility of once again throwing the world we are accustomed to into chaos.

Its name is the Plague.



A brass disc with the inscription "Unde origo, inde salus"

* * *

The year is 1629, and it is eleven years since the Republic has been involved in the Thirty Years' War. Venetian troops are retreating into northern and central Italy under the onslaught of the German and French armies. They do not yet know that, in saving their lives, they are carrying inland an enemy far more terrible than the gunpowder and steel they had captured in the city of Mantua. By October, the plague had already arrived in Milan. Although effective anti-epidemic measures including quarantine, restriction of trade and movement of citizens had a positive effect, their subsequent hasty relaxation for the sake of traditional carnivals in March 1630 led to a sharp deterioration of the situation in the form of a new outbreak. The result was the death of 60,000 people, i.e. 61 per cent of the city's population. This story clearly demonstrates an important law of epidemiology: you can't rush to relax anti-epidemic measures during an epidemic, especially when the cause is a traditional carnival. Yes, it was beautiful, but for many it was then the last in their lives.

In the same year, the plague reached Venice, 60 kilometres to the east. But it was brought not by the army, but by the ambassador of the Duke Charles I Gonzaga of Mantuan, whose name history has not preserved. At first he was hastily isolated on the island of Lazaretto (about which more details will be given below), but, having decided that such a high-ranking person should not stay in isolation with mere mortals, he was transferred to the monastery of San Clemente, where a residence was specially built. The builders were from the Dorsoduro area, which is where infection began to spread, and which is why it is where the Basilica of Santa Maria della Salute now stands, built by Doge Nicolo Contarini as a fulfilment of the vow solemnly made to the Virgin Mary the Saviour (Salute) in the Piazza San Marco in front of her icon Theotokos of Nikopea (Victorious Lady). It was the first time the Doge publicly removed his headdress[1]. *Unde origo, inde salus*[2].

[1] A traditional headdress, analogue of a crown; in Ven. zoia – "precious stone". It was a tall cap with a horn on the back, decorated with precious stones. According to contemporaries, "*its beauty and size allowed to consider [it] as the most important part of the treasury*". According to tradition, the Doge had to wear the headdress at all times.

[2] The symbolism of what is happening lies also in the fact that Mary is the reason for the existence of Venice itself as spiritually "conceived" on the same day as the Son of God

The epidemic raged for a year, killing 46,000 people (33% of the city's population), which, given its very compact location and high population density, was, without exaggeration, the lowest figure in Europe, while this plague epidemic was probably the largest on the Italian peninsula, claiming more than a million lives. This success in countering the infection was the result of a unique system of sanitary and epidemiological measures that emerged and developed in Venice, so grandiose that some of its elements are still in use today.

As a centre of international trade, compactly located on the islands, the Republic of Venice was quite vulnerable to infectious diseases, but the Republic's Health Service quickly realised that the only way to protect the city (and, consequently, business) was to divide people into healthy and sick people. The latter were isolated, including the suspicious, the contact and the infected. Already at the dawn of the XV century, in 1423, a small island Lazzaretto was used for isolation of plague patients, on which since 1249 stands the church of St Mary of Nazareth, which apparently gave the island its name (Nazaretum-Nazzaretto became Lazzaretto). On the other hand, the island may have received its name from St Lazarus, who was considered to be the patron saint of the brothers of the Order of the same name, who were engaged in the care of the sick. The first hospital for appestati (plague victims) was located on the island, where the patients were housed and the so-called "plague" physicians worked. However, they were rarely physicians by training, but were more often apothecaries (pharmacists) authorised to treat plague patients. This system was widespread in Europe and was primarily due to the economic and physical difficulty of training and certifying physicians. Recall at least the Anatomical Act passed by the English Parliament in 1832, which allowed the dissection and examination of corpses for anatomical purposes. It was thanks to him that in 1858 the world saw the famous anatomy textbook by Henry Gray, illustrated by Henry Vandyke Carter, which by now has gone through more than 40 editions and raised many generations of excellent physicians. To be fair, apothecaries were not used as a "cheaper resource"; on the contrary, the "plague" occupation was voluntary and quite lucrative. The

(it is believed that the city was founded on 25 March, the day on which the conception of Jesus Christ is celebrated). This is why the disc with the inscription is placed clearly below the statue of Mary, who is clad in the armour of the commander-in-chief who leads Venice in battles against enemies, epidemics and evil.

building where the sick were treated was called "lazaret" after the name of the island, which gave its name to other similar establishments. Another island, called Lazaretto Nuovo, was set up for those who were ill, as well as those who had contact with the sick. With the emergence of the "new" island, the "old" island was called Lazzaretto Vecchio, and under these names they still exist today, although they are no longer used for their intended purpose.

To assess the effectiveness of separating the sick from the healthy, let us remember the famous physician Michel de Notredame (Nostradamus)[1], who fought the plague epidemic of 1546 in Aix-en-Provence (south-eastern France). He considered the most effective measure to be the rapid removal of dead bodies (so that the disease did not "pass" from one sick person to another) along with providing the sick with fresh air and plenty of water. In addition, he practised oral administration of rose petal infusion. And there is a certain logic in this. The fact is that rose petals have antibiotic activity. Experience[2] has shown that the antibiotic activity of extracts from rose petals (*Rosa damascena*) depends on the colour of petals and the type of microorganism. *Micrococcus luteus* is the most sensitive, but sensitivity to extracts from red and yellow petals is higher than to extracts from white petals. *Bacillus subtilis* is the least sensitive to all extracts. Measures of Nostradamus were so effective that he received a pension for life in gratitude for them.

It should be noted here that the very idea of the possibility of "transfer" of a disease from a sick person to a healthy person, apparently, belongs to the physician Grigoris[3], who lived in Cilician Armenia in the 12-13'th centuries. Grigoris came to the conclusion that some diseases can be transmitted in three ways: by direct contact with a sick person; by using objects that had already been used by the sick person (i.e. the household contact route); and by being in the same room with a sick person (i.e. the airborne route). These progressive ideas became the

[1] 1503–1566; the French physician, pharmacist, poet, astrologer, diviner and alchemist.

[2] Zhang W., Abdel-Rahman F. H., et al. Natural resistance of rose petals to microbial attack. J Environ Sci Health B. 2011; 46(5):381–93; DOI: 10.1080/03601234.2011.572502. Here and hereafter, whenever an important discovery or experience is mentioned, a reference to the relevant publication will be given so that everyone can consult it if they wish. It is given in a different font for ease of perception.

[3] Գրիգորիս, ?-?; the Armenian physician. Author of the treatise Analysing the Nature of Man and His Ailments.

property of European science only in the XVI century thanks to the works of the famous physician Girolamo Fracastoro (Fracastorus)[1], who, developing the ideas of Grigoris, in 1546 suggested that epidemics are caused and spread by tiny "contagions"[2] (however, he perceived them more as chemical elements than living beings). It is to him belongs the introduction of the term "infection" into medicine. Unfortunately (or maybe fortunately), Fracastoro did not live to see the truth of his ideas with his own eyes until the next plague epidemic just 22 years later. History has not preserved for us evidence of whether Nostradamus was familiar with ideas of Grigoris (most likely not) and whether he had any communication with Fracastoro (it is noteworthy that his work on "contagions" was published in the year of the sad events in Aix-en-Provence), but the conjectures of these three physicians prove that the idea of contact infection was already "flying in the air", becoming the catalyst for an important anti-epidemic measure – *isolateon of the sick*.

From the beginning of the XV century, bearing in mind all the epidemics that had occurred (including those brought by Genoese sailors), the Venetians, fearing the introduction and spread of new ones, required ships to stay in port for 40 days before entering the port. This period was purely religious in nature and was a reminder of the temptations of Jesus Christ in the desert. And although the designated period did not have any medical and epidemiological justification, it should be understood that all these effective (and seeming to modern man logical and simple) anti-epidemic measures were introduced before mankind came to understand the mechanisms of spread of infections and the concept of incubation period, that is, they were foreseen intuitively be-

[1] 1478–1553; the Venetian physician and writer.

[2] Along with this theory there was the miasna theory (Greek μίασμα – "pollution", "filth"), i.e. "night air", which are vapours of decay processes and other impurities (e.g. vapours of marshes). Penetrating into the air, these vapours are inhaled by humans and cause diseases. From the time of Hippocrates, Galen and Ibn Sina (Avicenna) until the discovery of microorganisms this theory was the most widespread, and the construction of sewerage systems justified by it only contributed to its approval. It is worth noting that the existence of this theory contributed to the appearance of masks for "plague doctors", whose beaks were filled with herbs and cotton wool, i.e. protection of the respiratory organs was carried out.

fore the emergence of scientific basis. Ibn Sina[1], better known as Avicenna, used quarantine measures five centuries before the Venetians, but it was here that isolation began to be called quaranta giorni (quaranta forty days), which gave rise to the word "quarantine", so familiar to modern people.

Unfortunately, the isolation in "peacetime" was rather conditional: for a certain fee one could go on a forty-day "journey" around the mainland or have fun in the arms of courtesans, drinking rum, shouting songs, fighting with sailors of other crews, mutilating each other, on the small island of Poveglia (where the ships were anchored) with an area of only 0.075 km^2, located 600 metres west of the Lido Island. If you walk through the narrow streets of Venice early in the morning past the church of San Giorgio dei Grechi, the centre of the Greek community's church life, to the Riva degli Schiavoni promenade, you can see the sun rising slowly and majestically from the sea, illuminating the Lido, which seems to float out of the sea. And if you then cross the San Marco Canal by vaporetto[2] and climb the campanile of the Basilica of San Giorgio Maggiore, which rises majestically above the island of the same name, you can see the entire Lido, flooded with the light of the morning sun, famous for its beaches of fine golden sand and the oldest international film festival, held every year since 1934 on the initiative of the infamous dictator Benito Mussolini.

Perhaps it was the "catering" for the "fun" of the island of Poveglia that led to the famous Ponte delle Tette bridge, located in the very centre of Venice. The atypical arrangement of the window shutters in the houses on both sides of the bridge still reminds pilgrims of the times when local ladies used to display their breasts to remind young men of the charms of a female body (it is said that this way the "epidemic of youthful homosexuality" in the 16th century was eliminated, and very successfully). Not far from this bridge, even today a tired traveller can dine in the trattorias Alla busa (Ven. "in the hole") and Antiche Carampane (Ven. "old purse"), reminiscent of the former "profession" of the inhabitants of this place.

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[1] ابن سينا, 980–1037; the Persian physician and philosopher, author of a huge number of works, including the medical encyclopaedia Canon of Medicine, which was used in universities until 1650.

[2] River tram, the main form of public transport in Venice.

Those who were forced to quarantine on the ship, having fallen ill, most often died of starvation, and ships laden with goods and corpses were burnt. It must be realised that the "business" on Poveglia was illegal and flourished for profit. And it was this that played a cruel joke on Venice. During the plague epidemic it was from Poveglia that the disease got to the main islands of the lagoon, being transmitted both from courtesans and from gondoliers who illegally brought them to Poveglia, claiming hundreds of lives. On Poveglia itself the "fun" also died down, the sick were transferred to Lazaretto, and the island became one big funeral pyre. It is said that the souls of the dead still roam it in search of a final resting place…

Speaking of the forty-day quarantine in Venice, one cannot help but recall similar measures carried out in England in the 17'th century. Apparently, the successful experience of the Venetians, despite all the disadvantages, contributed to the spread of the "quarantine tradition". Thus, the death of someone in London was notified by a bell, which became a signal for a man of extravagant profession – a death seeker. It was his duty to find the house where the person died and examine the corpse. If plague was detected (apparently by the presence of a characteristic bubo, although this method of diagnosis cannot be considered specific because of the difficulties of differentiation, for example, with the bubonic form of tularemia, purulent lymphadenitis and even secondary syphilis, and also because of the possibility of "overlooking" the pneumonic form of plague) the house with its inhabitants was closed for forty-day quarantine, the doors were marked with a red cross with the inscription "*O Lord, have mercy on us*", and a guard was placed near the door to ensure strict observance of the quarantine. However, people are people, and the seekers of death, who were often poorly educated old ladies, for a certain amount of money could officially declare another cause of death, so that the inhabitants of the house avoided quarantine. All this resulted in what history calls the Great Plague Epidemic in London (1665-1666), which killed about 100,000 people (or 20 per cent of the city's population).

Thus, both of these stories remind us of another important law of epidemiology*: anti-epidemic measures cannot be neglected even in "peacetime"*.

However, it would be unfair to *Y. pestis* not to mention its positive impact on mankind. Thus, in view of the difficult epidemiological situa-

tion in the autumn of 1665, classes at Trinity College, Cambridge University, stopped, forcing one 23-year-old young man to go home to the Manor of Woolsthorpe (Lincolnshire, England). There, in solitude, probably out of boredom, he would decompose white colour into spectra, discover the law of universal gravitation, and derive the basic theorem of analysis. He will go down in history as the greatest mathematician and father of classical physics, Sir Isaac Newton. He would later write about his "plague" seclusion: "*...At this time I was experiencing the best period of my youth*".

A few years later, in 1679, the plague raged in the Austrian city of Vienna. Despite the fact that the city situated on the Danube was an important crossroads of trade routes between Europe and Asia, the hygienic situation in it was abnormal even for that time: stinking piles of rubbish due to the lack of public sewers and drainage systems, warehouses of fabrics and grain, in which rats successfully breed... It is not surprising that this environment was very favourable for the plague, which took in its Viennese waltz about 76 thousand inhabitants, because of which in many parts of Europe for a long time it has been entrenched the name of the "Viennese death".

The population was helped by the Brotherhood of the Holy Trinity, which built hospitals, offering treatment with vomit infusions and bloodletting. However, we will talk about the role of brotherhoods during plague epidemics in the next chapter, and here we will only point out that a really effective measure during this epidemic was the removal of corpses to the outskirts of the city, where they were piled in large pits for burning. Here, however, they remained in the open air for long periods of time, becoming easy prey for the local rats.

One of these pits is associated with a legend that became the basis for the folk song Ach, du lieber Augustin (Ger. Ah, my dear Augustin). The town singer Augustin, known for his addiction to alcohol, fell into a ditch when he was not too sober and fell asleep. A passing patrol, mistaking him for one of the victims of the plague, threw the unfortunate Augustine into one of the plague pits, where, after sleeping until morning, he managed not to get infected. Beer saved him from infection! Despite this, Augustine died of alcohol poisoning on 11 March 1685 at the age of 35, as recorded in the church book.



Burial scene during the Great Plague Epidemic in London. Fragment of one of silver mugs (1675/76), ordered as a gift to friends of Sir Edmund Berry Godfrey, who was granted a knighthood in recognition of his services to the city (inscription: "By the Grace of God and King Charles II / what was a plague for others, for him was health" / EBG 1665). Courtesy of the Metropolitan Museum of Art

Augustine's story became so famous that many drinking establishments and hotels in Vienna itself were named in his honour, and in 1770 the chimes of the Spasskaya Tower of the Moscow Kremlin rang out the melody of his song, but not for long. In the same year the plague came to Moscow. At that time the Archbishop of Moscow was Ambrose (Sertis-Kamensky), who, fearing the spread of infection due to the crowds at the Theotokos of Bogolyubovo, ordered that pilgrimages to it be forbidden (the belief that only crowded prayer services could stop the "pestilence" spread faster than the plague). This action led to the fact that after the bell ringing on 15 September 1771, several thousand "true believers" armed with clubs, axes, stones and stakes, with cries of "*The Theotokos is being robbed!*" plundered the Chudov Monastery. The next day the Donskoy monastery was also looted, where the maddened crowd, having seized the 62-year-old archbishop, already outside the walls of the monastery gave him an interrogation with passion, from which he died. Obviously, the described events only worsened the situation in the city, where already more than a thousand people were dying a day, because for almost a year the complex epidemic situation was

hushed up at all levels (from the factory where it began to the city authorities), and the podlekars and students who were thrown into liquidation, according to the memories of Daniil Samoylovich Samoylovich-Sushchinsky[1] (who was also beaten to a pulp by the mob), "*experienced terrible suffering and mostly died*".

Thus, it is important to remember that the first anti-epidemic measure should be to explain the vital necessity of their use to citizens, and ideally to create a model of primary and secondary education in which citizens will have no doubts about the need for quarantine measures during epidemics. At the same time, many people, in addition to the fear of death, are driven mad by the sense of time passing meaninglessly. As Metropolitan Dorotheus of Mitilin astutely noted in his work The History of the Council of Florence: "*The deadly bubonic plague struck Ferrara, and the city wept for the untimely departed lives; we were seized with a cowardly fear of death, and the grief of impending doom almost drove us mad*".

2 THE SCUOLA

The sun is setting, giving way to evening gloom. People in white tunics emerge from the marble Renaissance building decorated with Corinthian pilasters and statues and walk majestically across the square to the basilica called San Zanipolo (Ven. St John and St Paul's). This large red brick building, in the traditional Gothic style of the city, differs little from others of that era: two vertical pilasters divide it into three parts, where in the centre, just above the arched entrance decorated with columns, rises a large round rose window, and on the sides are symmetrically arranged smaller windows. The top of the façade is crowned by three sharp-angled vaults. And there is nothing particularly remarkable in the facade and interior, there are only the sarcophagi of those who found their last shelter in it. This basilica has had the honour of being

[1] *Даниил* Самойлович Самойлович-Сущинский, 1744-1805; the Ukrainian military physician. He proved the possibility of contact infection with plague.

the refuge of many Doges and famous citizens, and now the members of the ancient medical fraternity go there to pray and, when they leave, to do their work, perhaps never to return to it alive again.

Just as it is impossible to talk about the plague without mentioning Venice, so it is impossible to talk about Venice without mentioning this brotherhood, the Scuola Grande di San Marco (Vet. The Great Scuola of St Mark's). Being the cultural brainchild of the eastern part of the Roman Empire, Venice absorbed not only the elements of architectural style, the imprint of which still bears on many buildings, but also the best achievements of the Roma ("Byzantine") medicine, based on the works of the great physicians of the past. The concept itself, which differs significantly from the European one, assumes that in every major city and monastery there are institutions in which, regardless of nationality, sex, social status and profession, any sick person will be provided not only with medical care, but also with clothing and food. A hospital has a chief physician, his deputies, administrators and staff physicians of various specialities, and both men and women. It is worth mentioning separately the complex Shelter (370), which, in addition to the hospital, included a hotel for poor foreign citizens, a nursing home, a leprosarium, a shelter for the poor and an educational institution where, while undergoing treatment, one could learn a new profession. It is this idea, organisationally very similar to modern medicine, that was reflected in Venice. First of all, it is necessary to note the transfer of such a unique phenomenon as Scuola, which, although it "penetrated" from Byzantium to different countries, but took root and survived to this day only in Venice.

Scuola was a charitable society of citizens (brotherhood) united under the patronage of a saint. Although the members of Scuoles prayed together and attended church services, the Scuoles themselves were not part of the church apparatus and existed independently of the church. Perhaps that is why they played a major role in the social and even political life of the Republic. The Scuola Grande di San Marco was founded in 1261, being the first professional association of physicians in the

Procession of the members of the Scuola Grande di San Marco ►



world. Like all the other Grand Scuoles, it was closed by Napoleon I Bonaparte in 1807; the building was converted into an Austrian hospital in 1819 and the property, most of which consisted of paintings (the Scuoles were the main employers of many artists, among them such famous ones as Tintoretto, Bellini and Michelangelo), was transferred to galleries in France and Austria, although some paintings were returned to Venice after a while.

The Scuola Grande di San Marco interests us primarily because it played a major role during the plague epidemic (both the one already mentioned and the one a hundred years earlier). These were probably the first cases when the fight against plague was led by physicians who, in addition to effective anti-epidemic measures (which we have already mentioned), carried out surgical ones, which consisted in opening buboes (conglomerates of lymph nodes fused with the skin), while "ordinary" plague physicians only cauterised them and monitored the presence of pulse, and even then by means of a long cane to avoid contact. Of course, remote inspection allowed to reduce the risk of infection, but did not help the patient in any way, because cauterisation did not allow the contents of the buboes to escape, but contributed to their destruction inside the body. Autopsy, on the contrary, although it increased the risk of infection for the physician many times over, was apparently an effective procedure[1] that reduced the content of the pathogen in the organism, since the buboes are abundant multiplication of the pathogen and, therefore, contain a sufficient amount of it ready to break the protective function of the conglomerate and migrate into the bloodstream to spread by blood throughout the body. It is fair to note that the contents released from the opened buboes seriously increases the risk of aerogenic infection and, as a consequence, the spread of the pulmonary form of plague, given that the plague aerosol is able to "hang in the air" of the room for several hours, not to mention the possibility of infection of the physician performing the procedure, but this method greatly increased the patient's chances of recovery.

It is believed that the infected heroes found their last refuge in the underground crypt of the church of San Simeone Piccolo, where they were moved after the epidemic and the quarantine was lifted. Enclosed

[1] Thus, O. I. Bronstein at the beginning of the last century wrote about the effectiveness of this procedure.

in the crypt, they are isolated from the ground and water. At the entrance to the "plague" part of the crypt there is an allegorical image of death in the form of a skeleton and the inscriptions: "*Memorare novissima tua et in eternun non peccabis*"[1] and "*Miseremini mei miseremini mei*"[2], recalling in historical memory only one motto Memento mori[3].

They came out then to fight back, for the first time in world history, against the most formidable enemy. Of course, the merits of individual physicians, such as the already mentioned Michel de Nostradamus and Grigoris, cannot be diminished, but the struggle of the brothers of the Scuola Grande di San Marco is distinguished by the fact that it was not an exception to the rule, when one physician decided to help the "plague doctors", but the coordinated work of the first infectious disease health care system. And these people, whose names we do not even know, realised the ancient principle of aliis inserviendo consumor[4].

Another important introduction was a compulsory washing of hands with soap and disinfecting them with vinegar (especially for physicians!). Of course, it is not possible to say that hygiene was something new for Venice, since hygiene was part of the cultural heritage of the Roman Empire, but if we consider the Venetian experience in the context of the European experience (remembering at least the fact that one of the founders of asepsis was Ignaz Philipp Semmelweis[5], who lived in the mid-19th century), hygiene and asepsis also made an important contribution to defeating the plague.

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[1] Lat. "*Remember thy end, and thou shalt not sin*"; a fragment of a verse from the Book of Sirach: "*Omnibus operibus tuis memorare novissima tua, et in aeternum non peccabis*" (Lat. "*In all thy works remember thy end, and thou shalt not sin*"; Sir. 7:39).

[2] Lat. "*Have mercy on me, have mercy on me*"; a fragment of a verse from the Book of Job: "*Miseremini mei miseremini mei saltim vos amici mei quia manus Domini tetigit me*" (Lat. "*Have mercy on me, have mercy on me you my friends, for the hand of God has touched me*"; Job 19:21.).

[3] Lat. "Remember death". It is noteworthy that this motto came from Ancient Rome, where during the triumphal procession of a commander, a special slave standing behind him reminded the commander at regular intervals that he, despite all his glory and greatness, was only a mortal. This tradition was also preserved during the Eastern Roman Empire, where a specially appointed minister used to say it to the Emperor.

[4] Lat. "Serving others, I waste myself"; in Russian it has entered as "Shining light on others, I burn myself".

[5] 1818–1865; the Hungarian physician, obstetrician, professor at the University of Budapest.



Fresco depicting death in the crypt of the church of San Simeone Piccolo

Speaking about hygiene, it is impossible not to pass briefly to Bavaria of the end of XIX century, where Max von Pettenkofer[1], the president of Bavarian Academy of Sciences, estimating, however, curves not of plague, but of typhus and cholera epidemics and seeing their identity with curves of subsurface water fluctuations, put forward quite interesting theory called localistic, soil (Grundwassertheorie), according to which, along with the causative agent, physical-chemical and organic features of soil and soil water play no less, if not more, importance in the epidemic process (i.e. he opposed the theory of Robert Koch[2] about the exclusive role of the causative agent). To prove his theory, on the morning of 7 October 1892, when he was 73 years old, Max Joseph von Pettenkofer even drank cholera vibrios in front of Robert Koch and other witnesses and was lucky not to get sick! The theory lasted a long time, and, although it proved to be wrong, it had a great influence on the development of sanitation and hygiene measures, especially the improvement of the water supply and sewerage system, making Munich at that time one of the cleanest, epidemiologically safe cities in the world. Many of his students, among them Fyodor Fyodorovich (Friedrich Huldreich) Erismann[3], spread hygiene teaching throughout the world.

Here it would be fair to mention again the physician Daniil S. Samoylovich-Sushchinsky, who proved that the "plague contagion" could be transmitted through the instruments of a physician opening buboes, because "*although [I] cleaned the pus from my bistruths and lancets, but, as I needed them every minute, I always had them with me. From this it is easy to conclude that not only was I always dealing with plague poison, but that this poison was always in my pockets*". Having established that "one does not get the plague twice", he suggested the possibility of vaccina-

[1] 1818–1901; the Bavarian and German chemist and physician, hygienist, founder of the first European Institute of Hygiene (Munich).

[2] 1843–1910; the Prussian and German physician, bacteriologist. Member of the Prussian Academy of Sciences. Winner of the Nobel Prize in Physiology and Medicine (1905). He isolated and cultivated anthrax pathogen outside the organism, discovered tuberculosis pathogen. Adapted in 1877 the postulates formulated by German (Bavarian) pathologist Friedrich Gustav Jakob Henle (1809-1885), which were later called Henle-Koch Postulates.

[3] Фёдор Фёдорович Эрисман, 1842–1915; the Swiss and Russian physician and hygienist. Founder of the doctrine of school hygiene.

tion and, together with Kassian Iosifovich Yagelsky[1], used the smoke from a powder developed by the latter to fumigate dwellings, hospitals and public buildings, which played a certain role in the elimination of the plague epidemic in Moscow[2].

All this leads us to another law of epidemiology (epidemiological triad): the spread of infection stops when at least one link in the chain "pathogen – vector – susceptible organism" is destroyed (as practice shows, a major role in the emergence, for example, of nosocomial ("hospital") infections is played by neglect of hygiene rules by medical personnel who carry infections "on their hands").

After the victory over the plague epidemic in Venice in 1631, which had caused lasting damage to the population and played a major role in the decline of the economy, the already mentioned church of Santa Maria della Salute, which later became the small papal basilica, was built. Great financial assistance in the construction was given by the brothers of the Scuola, who thus honoured the memory of their fallen brethren. To this day, in it and in the building of the Scuola Chapter, one can still see the amazing paintings of Domenico Tintoretto, a member of the Scuola (since 1585) and participant in those events, who found his last refuge in the crypt of the Gothic church of Madonna dell'Orto on 17 May 1635.

[1] Кассиан Иосифович Ягельский, 1736–1774; the Ukrainian and Russian physician, one of the founders of epidemiology in the Russian Empire.

[2] It should be noted that for a long time fumigation remained the only effective means in the fight against plague. Thus, back in 1812 during the plague epidemic in the city of Odessa fumigation was carried out according to the method of French chemist Louis-Bernard Guyton-Morveau (1737-1816), including the use of "acid-salt oxidised gas" consisting of 3 parts of table salt, 1 part of black manganese (manganese oxide) and 2 parts of sulphuric acid. It is not difficult to guess that the gas given off by the reaction is chlorine.

3 STORIES

I

– What am I going to feed them with? – Emperor Justinian asked, standing on the balcony of his palace, gazing out into the Bosphorus. For 10 years now he had ruled the largest empire in the civilised world. Alamanian, Gothic, Frankish, Germanic, Ante, Lankan, Vandal, African – it was all about him. It was he who surpassed Solomon by building the most magnificent basilica in history. A warlord and a reformer. A great one. And now he just can't feed his subjects. Not so long ago, it seemed that the worst event of his life was the rebellion that became known as Nika, and now....

The years 535 and 536 were unusually cold. Procupius of Caesarea, in his fourth book of the History of Wars, writes: "*All year long the sun emitted light like the moon, without rays, as if it were losing its power, no longer shining purely and brightly as before. It is said that this may have been caused by volcanic eruptions or the fall of a meteorite, or perhaps by the cruelty with which the rebellion was put down, who knows. But there is no harvest and the people have nothing to eat*".

At the same time, on the other side of the civilised world, in what is now Yemen, the kings of Aksum and Himyar, who have just recovered from an unknown disease that caused the deaths of many of their subjects, are thinking about how to live on, when there has been no trade for a long time and the treasury is empty. But the land is fruitful and yields a bountiful harvest of wheat, which is travelling to Egypt, and it seems that life goes on. There, from the large granaries of the city of Pelusium, which the Egyptians themselves call Per Amun, the wheat is sent to the capital, the glorious Constantinople, where its starving inhabitants wait for it just as the ancient Jews in the desert waited for manna from God. But, unlike manna, this cargo will not bring joyful satiation, for while the ship with wheat is still swaying peacefully on the waves, in Pelusium the first inhabitant, bitten by a rat or a flea, dies of septicaemia, one might say, burns to death for no apparent reason. And



Bust of one of 600 (or 730) statues of the ancient Egyptian goddess Sekhmet, made for the temple of Pharaoh Amenhotep III (1400-1351 BC), as a gratitude for protection from the plague
Courtesy of the Brooklyn Museum (Acces. No. 1991.311)

this event will be the starting point for the first of three plague pandemics[1].

Between 532 and 580, the disease swept through many countries, travelling around the world in two directions: eastwards through Palestine to Asia, and westwards through Alexandria to Africa, to come again to the Arabian Peninsula and make it a land of sands and Bedouins. Due to the ongoing epidemic, it became impossible to keep the irrigation system running, resulting not only in crop failure but also the loss of much of the fertile land. Lacking sufficient food, the locals were forced to develop trade (especially since frankincense from Hadhramaut was valued above gold at the time). It was here, among caravans of camels laden with goods, that the Prophet Muhammad was born.

Poorly knowing the geography and not having worked out in detail the route of its journey, the plague followed trade routes: passing from coastal areas deep into the continents, it reached its peak on the territory of modern Greece, Italy and France (541-545) and completed its journey in England and Ireland (683), killing about 100 million people.

[1] Of course, Chinese manuscripts of that time describe a major plague epidemic that swept across China in 468, but the starting point of the "global journey" was the city of Constantinople, where the pathogen arrived from Egypt. The way, by which *Y. pestis* got to Egypt, is still a subject of numerous disputes. Some believe that it came from the Nubian desert, others – from China, Tibet or northern India. Either way, genetic studies show that there were many strains circulating during the first pandemic, so it is possible that there were several original sources.

The already mentioned Procopius of Caesarea wrote: "*There was no salvation from it, no matter where he lived - neither on an island, nor in a cave, nor on the top of a mountain ... Many houses were deserted, and it happened that many of the dead, for lack of relatives or servants, lay for several days unburnt...*" According to contemporaries, the disease was accompanied by buboes and ulcers on various parts of the body, including the head, from which we can conclude that the main vector was fleas. On the other hand, there is no mention of haemoptysis or other lung lesions, indicating the predominance of the bubonic form, or perhaps the complete absence of the pulmonary form.

When talking about the first pandemic, one cannot ignore Ancient Egypt, whose medicine had achieved considerable success for its time. In historical do-



Statue of a cat (bronze, gilded), made during the reign of the XXII Dynasty (c. 945-712 BC), when the cult of Bastet reached its peak. At that time the capital of Ancient Egypt was the city of Per-Bastet (Bubastis) dedicated to her. The statue was called to protect from infectious diseases and to help in childbirth. Courtesy of the Museum of Archaeology and Anthropology of the University of Pennsylvania (Inv. No. E14284)

cuments, such as, for example, the medical papyrus of Edwin Smith, we find references to jꜣdwt rnpt (iadet renepet), i.e. an annual epidemic that occurred in summer during the flooding of the Nile. The cause of the epidemic was thought to be the destructive power of the goddesses Bastet and Sekhmet, whose arrows and demon messengers carried the disease to the winds. The famous Cairo Calendar mentions that anyone born on the day when the goddess barque of Bastet was released would die. Only rituals to propitiate the goddesses can serve as protection and deliverance. Some researchers connect the annual epidemic with bubonic plague, which is confirmed by numerous statues of the goddess Sekhmet, made by order of Pharaoh Amenhotep III (1400-1351 BC), as well as a letter of his son Ehnaton, which clearly speaks of the plague. Another proof is the metamorphosis of the image of the goddess Bastet from a lioness into a domestic cat, which, being a formidable exterminator of rodents, itself acts as a carrier of plague (so from the goddess comes both the disease and salvation). On the other hand, to date, no mummies with pathological changes characteristic of plague or the presence of DNA fragments of *Y. pestis* have been found, so as an etiological agent iadet renepet are also considered pathogens of poliomyelitis, smallpox and malaria[1].

It should be said that another argument "against" plague is the fact that before the development of trade relations (approximately until the 7th century BC) the only natural carrier of plague in ancient Egypt was the grass mouse (*Arvicanthis niloticus*), which lived far away from human settlements. At the same time, on a number of tomb frescoes there is its image. Of greatest interest here is a fresco from the tomb of Menna (TT 69), made during the reign of Amenhotep III, in which Menna and his family are fishing and hunting. It is believed that the action takes

[1] Here it should be noted that the neighbouring Hittite kingdom after a short war with the Egyptians at about the same time began an epidemic, mentions of which have reached us in the prayers of King Mursilis II (1343-1295 BC), in which, in particular, the following words are found: "*And the land of Hatti has been sorely, greatly oppressed by the plague. Under my father (and) under my brother there was constant dying. And since I became priest of the gods, there is now constant dying under me. Behold, it is twenty years since ... will the plague never be eliminated from the land of Hatti?*" And although the Hittite word hi-in-ga-na-az is traditionally translated as "plague" and there are even reports of *Y. pestis* found in burials, by now most researchers are inclined to believe that the epidemic was caused by the tularemia microbe. All this speaks of the difficulties in determining the etiological agent of ancient epidemics.



Fragment of a fresco from the tomb of Menna
Courtesy of Dr. Roman Alexandrovich Orekhov

place in a swamp (the marshy part of the Nile River), and the killing of its inhabitants is sacred in defence of the Egyptians against evil (how can one not recall the medieval doctrine of miasmas!). Among other things on the fresco one can see a grass mouse sneaking to the nest. It is quite possible that shortly before the annual flood they moved en masse to human dwellings and migrated to their usual habitats when the water receded, which determined the seasonal nature of the epidemic (the mouse in the fresco can be seen as associated with the "swamp evil"), which theoretically could have been plague, given that ancient strains of *Y. pestis* most likely caused a relatively mild form of infection (at least the most successful descendants of ancient strains circulating to date in foci in the Eurasian region rarely cause disease in humans). Nevertheless, it is currently impossible to reliably identify the etiological agent of *iadet renepet*.

II

In the 14th century, the plague began its second journey around the world, going down in history as Atra mors (Lat. black death). Having come, in all probability, from the expanses of Mongolia, it reached, in the words of George Norbertovich Gabrichievsky[1], "*a spread and strength unparalleled in history*", claiming up to 200 million lives and exterminating "*almost half of all mankind*", as the English chronicler Thomas Walsingham would write.

Having travelled victoriously through the lands of the Golden Horde, by 1346 the plague had reached the Crimean peninsula. There, the Janibek Khan of Golden Horde, in order to conquer the Genoese fortress of Caffa, ordered catapults to throw the corpses of dead soldiers, using the latter as a biological weapon. The siege had to be lifted because of the army weakened by the disease, while the Genoese sailed

[1] Георгий Норбертович Габричевский, 1860–1907; the Russian physician of Saxon origin, bacteriologist, private associate professor at Moscow University, founder of the Moscow Scientific Bacteriological School. Since 1909, the Bacteriological Institute established by him in 1895 has borne his name (now the G. N. Gabrichevsky Moscow Research Institute of Epidemiology and Microbiology). Tragically died after being infected during an experiment on immunisation of animals for preparation of vaccine against croup pneumonia.

away safely, spreading the plague throughout the Mediterranean ports. Thus the plague found its way to Constantinople. Again.

"*Every day it is our job to carry away friends*," writes Demetrius Cydonius, a scholar and close friend of Emperor John Cantacuzene, "*and what torments us most is that people avoid each other for fear of contact with the disease... Every day the Great City is emptied. The traces of that disease were rashes on the skin ... and at the same time blood consumption*," reports historian Nicephorus Gregoras.

...In the royal chambers, under the porphyry vaults, lies a 13-year-old boy, Andronikos, the youngest son of Emperor John Cantacuzene. A few hours ago he was as vigorous and active as ever, but now, like an extinguished candle, he is fading before his eyes. He is pale, with purple patches on his skin. As soon as the sun disappeared behind the smooth surface of the Bosphorus, the young man gave out his breath, as if rushing after him. 'There is no help from anywhere,' sighed the inconsolable father. And the grief-stricken mother will keep this pain in her heart throughout her life... True, true, the Venetian poet Giacomo Ruffini called the plague *the monster of darkness* (Lat. monstra nigrantis).

As John Cantacuzene would later write, the plague "*not only travelled through Pontus, Thrace and Macedonia, but also through Hellas, Italy, all the islands, Egypt, Libya, Judea, Syria and almost the whole circle of the Ecumene*". On ships she visited Genoa, Marseilles, Alexandria, Venice, Rouen, London... Conquering coastal cities and capitals, with refugees she penetrated deep into the continent, reaching even Mesopotamia and Persia. Only reaching the Faroe Islands and Greenland by 1353 did it complete its second journey, although it made itself known as isolated outbreaks in 1545-1683 and 1710-1830. The high mortality rate was due to the incredible density of the population, forced to live behind fortress walls to escape prolonged wars, and the almost total lack of hygiene in the cities, which led to a proliferation of rats and fleas. According to chroniclers, in Constantinople alone, up to 8/9 of the city's inhabitants died, although this city was distinguished from others by its cleanliness.

The second journey was the greatest ever, leaving an indelible mark on the memory of mankind. Black flags on church belfries as a universal

symbol of disease. Plague pillars[1] as a symbol of remembrance and gratitude for salvation.

III

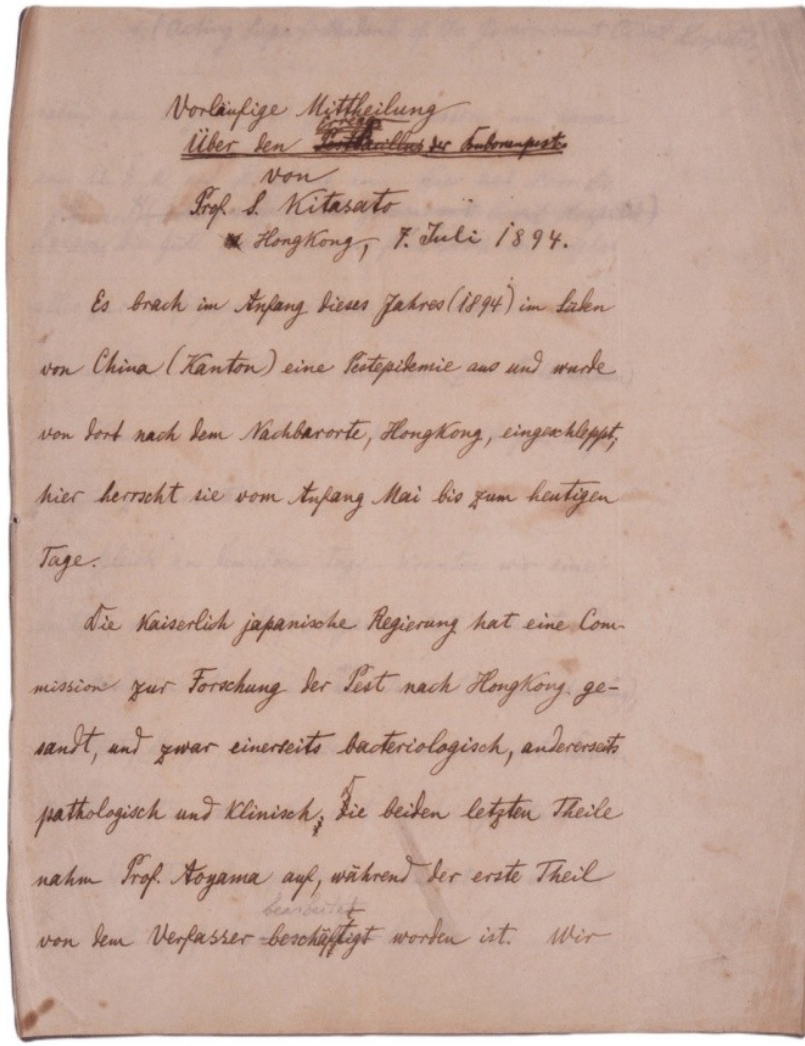
The third journey occurred in the 1850s and is not recognised by some researchers as an independent pandemic, as it is not even possible to determine the exact location of its beginning, limiting it to foci in Central and South-Eastern China (most likely the one in Yunnan province). Nevertheless, more than 15 million people became victims. The widespread spread across the world as far as the Arabian Peninsula, Africa and the Americas was made possible by high human migration amid global trade and political conflict. It was not until 1960 that the number of cases began to decline and outbreaks became sporadic (i.e. intermittent).

June 1894 was a landmark year. Two men arrive in the city of Hong Kong to investigate an outbreak of bubonic plague: Alexandre Émile Jean Yersin[2], at the request of the French government and the Pasteur Institute, and Kitasato Shibasaburō[3], at the request of the Japanese government. A representative of the Pasteur school of science and a representative of the Koch school of science. The former works in a small hut because the British deny access to hospitals and the latter works in his

[1] Plague pillars (Marienplatz), monuments with a statue of the Virgin Mary or saints (St. Roch, St. Barbara, etc.) erected on them. The first plague pillar was a monument erected in 1638 in Munich on Marienplatz.

[2] 1863–1943; the French and Swiss physician and bacteriologist. In addition to the plague pathogen, he discovered diphtheria toxin (together with Emile Roux). He worked extensively in Vietnam, where locals gave him the nickname Ông Năm (Viet. Mr Nam).

[3] 北里 柴三郎, 1853–1931; the Japanese physician, bacteriologist. Member of the Japanese Academy of Sciences (1906). Nominee for the Nobel Prize in physiology or medicine (1901). His surname 「北里」, like many Japanese surnames written in characters (kanji), traditionally had several readings. It could be read as 「キタザト」 (Kitazato) and 「キタサト」 (Kitasato), and was originally read as the first reading. However, after a scientist signed himself as "S. Kitasato", the second reading began to be used. Note that when writing East Asian names, the surname is written first and then the first name (sometimes all letters of the surname are capitalised to avoid confusion). In addition, it should be remembered that the traditional transliteration of a name into Russian is very conventional, so here and below, after the original spelling of the name, the pronunciation close to the original will be given.



Vorläufige Mittheilung
über den ~~Pestbacillus der Bubonenpest.~~
von
Prof. S. Kitasato
Hongkong, 7. Juli 1894.

Es brach im Anfang dieses Jahres (1894) im Süden von China (Kanton) eine Pestepidemie aus und wurde von dort nach dem Nachbarorte, Hongkong, eingeschleppt, hier herrscht sie vom Anfang Mai bis zum heutigen Tage.

Die Kaiserlich japanische Regierung hat eine Commission zur Forschung der Pest nach Hongkong gesandt, und zwar einerseits bacteriologisch, andererseits pathologisch und klinisch; die beiden letzten Theile nahm Prof. Aoyama auf, während der erste Theil von dem Verfasser beansprucht worden ist. Wir

Draft of an article by Kitasato Shibasaburō on the discovery of Y. pestis, written in German. The English translation was submitted to The Lancet and the original, together with samples of the culture obtained, was sent to Robert Koch in Germany
Courtesy of the 学校法人北里研究所北里柴三郎記念博物館

own laboratory. Both of these men will discover[1] the plague pathogen in June, a few days apart (**Figure 3.1**). Kitasato Shibasaburō would do so early, but the lead[2] would eventually go to Alexandre Yersin.

The genus (Lat. species; usually written "spp.") to which *Y. pestis* belongs would later be named *Yersinia* in honour of Alexandre Yersin. And Kitasato Shibasaburō, now also recognised as the discoverer of the plague pathogen, would return to Japan, where he would later establish an institute in the city of Tokyo to protest against the incorporation of the Institute of Infectious Diseases into the University of Tokyo[3].

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[1] Kitasato S. *The bacillus of bubonic plague (preliminary notice)*. The Lancet. 1894 (July 7); 1:428–430; Yersin A. *La peste bubonique a Hong-Kong*. Ann Inst Pasteur (Paris). 1894; 8:662–667.

[2] Initially, both researchers were considered to be the discoverers of the plague microbe (the existing contradictions in Kitasato Shibasaburō's report could be related to the peculiarities of the Japanese language and high workload due to caring for sick colleagues). In his article, Alexandre Yersin pointed out the impossibility of Gram staining of the plague microbe culture ("*et ne se teignant pas par la méthode de Gram*"), which was confirmed by many subsequent researchers (probably the dyes were of insufficient quality). Kitasato Shibasaburō also noted in his article that he was not ready to answer the question of staining ("*I am at present unable to say whether or no «Gram's double-staining method» can be employed*"). However, some time later, the Japanese physician Aoyama Tanemiichi (1859-1917), a colleague of Kitasato Shibasaburō, reported that the cultures isolated by the latter were in fact diplococci and stained Gram's blue (i.e. Gram-positive). The possibility of this was confirmed in 1896 by Kitasato Shibasaburō himself, claiming that the plague microbe, unlike other members of the genus, could be Gram-positive. However, already in 1899 he recognised this view as erroneous. A comparison by other researchers of the cultures of the two scientists, stored respectively at the Pasteur Institute in France and at the Institute of Infectious Diseases (now the Robert Koch Institute) in Germany, showed that both were plague, but the culture isolated by Kitasato Shibasaburō was contaminated with pneumococcus. Probably the culture obtained was not pure initially or the contamination occurred during transport. Either way, the description of the culture is the same as that of Alexandre Yersin. In scientific papers of the time, both researchers are listed as discoverers of the plague microbe, but after the publication of an article by the French physician Emile Lagrange, which falsely stated that Kitasato Shibasaburō refused to be considered the discoverer (see Lagrange E. *Concerning the discovery of the plague bacillus*. Journal of Tropical Medicine and Hygiene. 1926; 29:299-303), his name was gradually forgotten, and Kitasato Shibasaburō himself felt unworthy to seek justice. Although his name was rehabilitated after additional culture tests carried out at the end of the last century, it is still almost unknown among chumologists.

[3] This event meant the reassignment of the Institute from the Ministry of the Interior to the Ministry of Education, i.e. the actual loss of functions of the supervisory body for epidemiological welfare. In addition, while still interning in Germany with Robert Koch, Kitasato had debates with his friends Komoto Jujiro (1859-1938), Aoyama Tanemichi

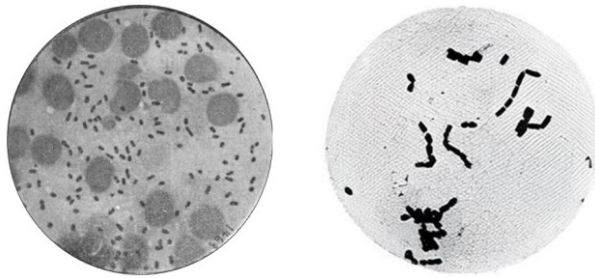


Figure 3.1. *Y. pestis cultures:*
left - isolated by Kitasato Shibasaburō from blood (by Kolle W., 1897)[1]; right - isolated by Alexandre Yersin from buboesis and after growth in broth (by Yersin A., 1894). Kitasato Shibasaburō described the isolated culture as "ovoid sticks with rounded ends and a somewhat lighter centre, covered with a capsule" and Alexandre Yersin as "short, thick sticks with rounded ends, often covered with a capsule"

The tragedy of Kitasato Shibasaburō's life is that in 1890, during his internship in Germany, together with Emil Adolf von Behring[2], he will receive an antidiphtheria serum, but the Nobel Prize for this discovery

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(1859-1917), Ogai Mori (1862-1922), and Masanori Ogata (1853-1919) about what caused beri-beri disease. The latter suggested a bacterial nature to the disease, about which he published scientific articles that Kitasato sharply criticised. Although they were the same age, Manasori was Kitasato's superior (i.e. a kind of teacher) before his internship, so criticism was perceived at the University of Tokyo primarily as disrespect for the teacher (the truth was relegated to the background). In particular, Kato Hiroyuki (1836-1916), president of the University of Tokyo, called Kitasato Shibasaburō "*one who does not understand the way of the master and the student*". Although Kitasato Shibasaburō and Masanori Ogata remained friends in their personal lives, the doors of the University of Tokyo were closed to the former, and he was perceived as an individualist separated from the medical community.

[1] The story behind this image is amazing. Kitasato Shibasaburō sent the isolated culture, along with a draft of the article, to Robert Koch in Germany. Then, since the article contained only drawings of the culture, the German physician and medical photographer Emil Zettnow (1842-1927) took photographs of the cultures and gave them to the German physician Wilhelm Kolle (1868-1935), who published them.

[2] 1854–1917; the Prussian and German military physician, bacteriologist, immunologist. Winner of the Nobel Prize in Physiology or Medicine (1901). The financial situation of his family did not allow Emil von Behring to study at a medical school, so in 1874 he entered the Military Medical School, which was free of charge (after graduation it was necessary to serve in the army for 10 years), and graduated in 1878. Signer of the letter Towards Cultural Peace (in history it became known as Manifesto of Ninety-Three; Germ. Manifest der 93), which justified actions of Germany in the First World War. Author of works on infectious diseases, founder of serotherapy.

in 1901 will be awarded only to the latter (the Nobel Committee will consider that the idea of creating serum therapy belongs to von Behring, not Kitasato). In 1894, as already mentioned, he will discover the causative agent of plague, but the palm of primacy will then go to Alexandre Yersin. However, when his students Shiga Kiyoshi[1], Endo Shigeru[2] and Hata Sahachirō[3] made their discoveries, Kitasato Shibasaburō refused to co-author the papers so that the glory would go to the students; when the still unknown Noguchi Hideyo[4] was caught stealing books from the library, he sent him to work as a quarantine officer in the port of Yoko-

[1] 志賀潔, 1871–1957; the Japanese physician, bacteriologist. He discovered and described in 1897 the causative agent of bacterial dysentery (*Shigella dysenteriae*). Rector of Keijo Imperial University (1929-1931) in present-day Seoul (South Korea). He later considered the change from research to administration as 'the road of grief' ('Looking back, I regret that I did not devote the best years of my life to scientific research and books, having given so much mental and physical strength to worldly concerns'). By the time he returned home, his teacher had passed away, so Shiga Kiyoshi dedicated the calligraphy 'Follow your teacher's spirit, not his footsteps' to him. The genus *Shigella* and the Shiga toxin produced (i.e. secreted) by some bacteria (*Sh. dysinteriae* serovar 1, *E. coli* O157:H7) are named after Shiga Kiyoshi.

[2] 遠藤滋, 1870–1937; the Japanese physician and bacteriologist. In 1904, he created a differential-diagnostic nutrient medium for the isolation of *Enterobacteriaceae*, called Endo medium. In 1906, at the peak of his career, in obedience to a centuries-old tradition, he returned to his hometown of Shizuoka to continue the family business of private medical practice (his father had been drafted into the army as a retired military physician, and his older brother had passed away). He paid much attention to tuberculosis patients, but the opening of a specialised hospital was opposed by local residents. After his father's death he returned to Tokyo, where he opened a small pulmonological office. The name of the scientist is almost unknown in his homeland.

[3] 秦 佐八郎, 1873–1938; the Japanese physician, bacteriologist. Nobel Prize nominee in chemistry (1911) and in physiology or medicine (1912, 1913). Engaged in plague research under the direction of Kitasato Shibasaburō. One of the authors of the 'Law for the Prevention of Infectious Diseases' (Japan, 1897).

[4] 野口 英世, 1876-1928; the Japanese physician, bacteriologist. Multiple nominee for the Nobel Prize in Physiology or Medicine. Detected pale treponemes (*Treponema pallidum*) in the cerebral cortex of patients with progressive paralysis. Studied yellow fever in Peru, isolating *Leptospira icteroides* as the causative agent, which was later disproved (the causative agent was the arbovirus *Viscerophilus tropicus*). It is believed that the reason for the error was inaccuracy in differential diagnosis - the disease in Peru was not yellow fever, but leptospirosis (the vaccine developed by Noguchi Hideyo is effective against this disease). Suggested the generic name *Leptospira*. The species *Leptospira noguchii* is named in honour of Noguchi Hideyo.



Alexandre Yersin
Courtesy of the Bibliothèque interuniversitaire de Santé



Kitasato Shibasaburō during an internship in Germany.
The photo was taken to commemorate the creation of anti-diphtheria serum.
The scientist is holding a device he invented for cultivating anaerobic bacteria
Courtesy of the 学校法人北里研究所北里柴三郎記念博物館

hama instead of dismissing him – is this not an expression of bushido[1] philosophy?

These three stories, while seemingly unnecessary at first glance, have a lot to offer. We see that the causative agent of plagueō *Yersinia pestis*[2]ō lives in certain places (natural foci) and "comes" to people accidentally, carried by animals migrating due to hunger, mainly rodents. Human infection is carried out through the bite of a flea, such as the *southern rat flea* (*Xenopsylla cheopis*), which parasitises the *black rat* (*Rattus rattus*) living in houses, which in turn carries the plague germ. It is worth noting that the *human flea* (*Pulex irritans*) does not parasitise rats, but is also

[1] Jap. "the way of the warrior"; a code of samurai ethics and morality that emerged in Japan during the Edo period (1603-1868) and has influenced Japanese social life to the present day. It is important to note that Kitasato's mother was from a lineage of samurai, so he himself wanted to become a samurai. However, the Meiji Restoration that began in 1868 ended the samurai era, which eventually led Kitasato into medicine

[2] *Bacterium pestis* before 1900, *Bacillus pestis* before 1920, *Pasteurella pestis* (*Pestisella pestis*) before 1947.

capable of carrying plague, only from person to person (rarely). This is how we can explain the presence of buboes not only in the groin area (bite of the southern flea in the leg), but also on other parts of the body and even on the head (bite of the human flea of sleeping people).

The predominance of the bubonic form during the first pandemic and the appearance of the pulmonary form during the second pandemic indicates the evolutionary development of the genetic structure of the bacterium, which influences intraspecific differentiation.

The first attempts at intraspecific classification date back to the beginning of the last century, when in 1928 Anna Artemyevna Bezsonova[1] divided[2] all strains of *Y. pestis* according to one biochemical property – the ability to ferment glycerol. Thus strains of *Y. pestis* were divided into glycerol-positive (i.e. able to ferment glycerol) and glycerol-negative (i.e. not able to ferment glycerol). Already 10 years later, Abram Lvovoch Berlin[3] and Alexander Kirillovich Borzenkov[4] proposed to rename the glycerol-positive strains into the continental race and the glycerol-negative strains into the oceanic race. The names of the races were not chosen by chance. The reason is that the former circulate more frequently in rodent populations in the central part of the Eurasian region ("on the continent"), while the latter are more frequently isolated from rats in seaports ("in the ocean"). In 1951, René Devignat[5] proposed[6] to differentiate *Y. pestis* by three indicators: glycerol fermentation, nitrification and denitrification. Thus he distinguished three

[1] Анна Артемьевна Безсонова, 1889–?; Russian and Soviet physician, bacteriologist. Winner of the Stalin Prize. Author of works on the microbiology of plague, tularemia and pseudotuberculosis microbes. There is a spelling of the surname «Бессонова» ("Bessonova").

[2] Безсонова А. А. *О двух разновидностях B. pestis, обнаруживаемых при росте на глицериновых средах.* Вестник микробиологии. 1928; 7(3):250–253.

[3] Абрам Львович Берлин, 1903–1939; the Russian and Soviet physician, bacteriologist, epidemiologist, developer of serological diagnostic reactions for plague, engaged in modernisation of vaccine production.

[4] Александр Кириллович Борзенков, ?-?; the Russian and Soviet physician, bacteriologist.

[5] ?-?; the Belgian physician, bacteriologist. Director of the hospital in Costermansville (now Bukavu), the School of Tropical Medicine and the School of Indigenous Medicine in Elisabethville (now Kinshasa), Belgian Congo..

[6] Devignat R. *Variétés de l'espéce Pasteurella pestis. Nouvelle hypothése.* Bull. Org. mond. Santé (Bull.World Hlth Org.). 1951; 4(2):247–263.

biovars (spelled "bv.")[1]: antiqua, medievalis, orientalis, suggesting that each of them was responsible for the occurrence of one of the pandemics. In 1957, Victor Mikhailovich Tumansky[2], using the ecological approach of Abram L. Berlin and Alexander K. Borzenkov, proposed to name these biovars marmotae, citelli, and ratti. In subsequent years, a number of other criteria for classification were proposed (including molecular genetic criteria), and the discovery of new strains also made adjustments.

The classification of René Devignat, to which two more biovars have been added: intermediate (intermediate between antiqua and orientalis) and pestoides[3], is now widely used, and the classification has taken the following form:

bv. antiqua (ANT)

bv. medievalis (MED)

bv. orientalis (ORI)

bv. intermediate (IN)

bv. pestoides (PE)

The pestoides biovar includes those ancient (ancestral) strains found in numerous natural foci in the Eurasian region[4]. They are highly virulent for their hosts (voles and pika) and laboratory mice, but are usually weakly virulent or avirulent for guinea pigs and humans, rarely causing disease in humans, and therefore of *low epidemic significance*. Nevertheless, their study is important not only for understanding the microevolution of *Y. pestis*, but also for identifying common (universal) pathogenicity and virulence factors. Intraspecific classification of these strains is usually carried out by researchers from the countries where they are isolated.

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[1] From Lat. bios and varietas – "biological variant"; a non-taxonomic systematic category for designating variants of one strain of a microorganism differing in biochemical features.

[2] Виктор Михайлович Туманский, ?-?; the Russian and Soviet physician, bacteriologist. Laureate of the USSR State Prize.

[3] The term was first coined by Russian and Soviet physician Ivan Lukyanovich Martinevsky.

[4] The natural foci from which these strains (with the exception of Angola) are isolated are located in the territories of the former USSR and neighbouring China and Mongolia.

In 1985, the USSR adopted its own "departmental" classification, which implied division into subspecies (Lat. subspecies; usually written "subsp." or "ssp."). Having been updated by the staff of the anti-plague institute "Microbe" (Saratov, Russia)[1], taking into account some SNP-typing data (more about it in Chapter 38), the current classification is as follows:

main subspecies:

subsp. *pestis* (includes ANT, MED, ORI and IN)

nonmain subspecies:

subsp. *caucasica*

subsp. *angolica*

subsp. *central asiatica*

bv. altaica

bv. hissarica

bv. talassica

bv. microtus

subsp. *tibetica*

subsp. *ulegecia*

subsp. *qinghaica*

It can be seen that four biovars are additionally distinguished within the *central asiatica* subspecies.

On paper, all this has the following form (example):

– "*Y. pestis* subsp. *pestis* bv. antique",

– "*Y. pestis* subsp. *central asiatica* bv. altaica"[2].

[1] Попов Н. В., Ерошенко Г. А., и др. *Эпидемиологическая и эпизоотическая обстановка по чуме в Российской Федерации и прогноз её развития на 2020–2025 гг.* Проблемы особо опасных инфекций. 2020; 1:43–50; DOI: 10.21055/0370-1069-2020-1-43-50.

[2] Note that species and subspecies names are written in italics, while biovar names are written in regular font, which is the tradition for all living organisms. In rare cases, the last

On the other hand, employees of the State Research Centre for Applied Microbiology and Biotechnology (Obolensk, Russia)[1] propose a division into:

main subspecies:

subsp. *pestis* (the monophyletic grouping of epidemic and pandemic strains)

nonmain subspecies:

subsp. *microti* (the paraphyletic group that unites all endemic strains).

The basis of this classification, as can be guessed, is the medical significance of the differences between groups of strains, since the allocation of other systematic ranks for a species of *Y. pestis* of other systematic ranks less than a taxonomic unit (subspecies) is not acceptable.

Both variants of intraspecific classification are based on SNP clustering, which is virtually identical and allows comparison of the results of studies conducted not only in these two organisations, but also in any laboratory where SNP typing of *Y. pestis* strains can be performed. Nevertheless, the issue of intraspecific taxonomy remains open to date, and its final resolution is possible only after the publication of the appropriate classification by the International Committee on Systematics of Prokaryotes (ICSP)[2].

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name of the author who gave the name and the year of publication may be added in parentheses. For example: *Y. pestis* (van Loghem, 1944).

[1] Кисличкина А. А., Платонов М. Е., и др. *Рациональная таксономия Yersinia pestis*. Молекулярная генетика, микробиология и вирусология. 2019; 37(2):76–82; DOI: 10.17116/molgen20193702176.

[2] The observation of Canadian researchers is very relevant here: "*The biovar system is simple in application, as it largely focuses on two traits: the ability to ferment glycerol and reduce nitrate. However, this simplicity is offset by the growing recognition of regional inconsistencies in metabolic profiles. This is further exacerbated by the sequencing of non-viable, "extinct" Y. pestis strains for which metabolic sub-typing is challenging. Researchers have responded to this uncertainty in a variety of ways, by extrapolating existing biovars and creating new pseudo-biovars (PRE). Others have foregone the biovar nomenclature altogether in favor of locally developed taxonomies. Despite extensive research, it remains unclear which metabolic traits, if any, can be used to classify Y. pestis into distinct populations on a global scale*" (see Eaton K., Featherstone L., et al *Plagued by a cryptic clock: insight and issues from the global phylogeny of Yersinia pestis*.

Palaeontological studies of ancient DNA, isolated mainly from the teeth of prehistoric humans because it is best preserved there, suggest that *Y. pestis* evolved from a common ancestor[1] and split from *Y. pseudotuberculosis* serotype I (probably *Y. pseudotuberculosis* O:1b) about 7000-20,000 years ago, during the late Pleistocene epoch, by acquisition of two plasmids and loss of chromosomal genes. It is suggested that speciation occurred in Central Asia in the Mongolian marmot population under the influence of climatic factors[2,3]. In any case, it is the acqui-

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Commun Biol. 2023; 6:23; DOI: 10.1038/s42003-022-04394-6). Also the opinion of the Soviet and Russian physician Igor Valerianovich Domaradsky (1925-2009) is of interest: *"My attitude towards such classifications [i.e., the "departmental" classification of the anti-plague institute "Microb"] was formed over 25 years ago (1971) and has not yet been changed since then. I still believe that the now known differences between the strains cannot be an argument strong enough to favour the existence of subtypes of Y. pestis. I do not see why varieties ("biovars" or "biotypes") should not be recognised, as is done, for example, in the case of Y. enterocolitica. It should also be emphasised that all the known schemes of intraspecific classification of Y. pestis strains are based on the results of the studies of the strains from natural foci of the CIS and are therefore narrow, "national". The data on the strains from foreign natural foci are not involved in their compilation at all. I can assume, if we follow the logic of the Russian researchers, in this case the number of 'subspecies' will increase several times! And one more remark. The recognition of subspecies is given such great importance in our country, because it supposedly allows to bring more clarity to the problem of studying natural plague foci and to outline the 'right ways' for their eradication. But regardless of the type of the carrier and the geographical zone of plague pathogen isolation, one should always strive to eliminate its foci (I do not mean natural foci, but foci of direct infection of people). What matters is not whether the microbe ferments glycerol and reduces nitrates, but to what extent it is capable of causing the disease"* (see Домарадский И.В. *Чума*, М: Медицина, 1998, 174 с.).

[1] In scientific literature, the common ancestor is usually referred to as MRCA (**m**ost **r**ecent **c**ommon **a**ncestor) *или* LCA (**l**ast **c**ommon **a**ncestor).

[2] On the other hand, it has been suggested that there is an origin in the Nile Valley in a population of rats.

[3] According to the scenario proposed by Soviet and Russian biologists Victor Vasilyevich Suntsov (b. 1947) and Nina Ivanovna Suntsova (b. 1952), speciation occurred during the Sartan glaciation, when the soil in present-day Mongolia froze to the depth of the wintering chambers of Mongolian marmot-tarbagans (*Marmota sibirica*). Under these conditions, due to positive thermotaxis (i.e. direction of movement of a living organism to the zone of optimal temperature), larvae of the marmot flea *Oropsylla silantiewi*, usually inhabiting the nest lining, moved into the wool cover, from where they penetrated into the oral cavity, where they broke the integrity of mucous membranes. The bite sites were infiltrated by the bacteria *Y. pseudotuberculosis*, which were present in the oral cavity together with faecal particles, as marmots use them to build a hibernation plug closing the entrance to the mink. During hibernation, the body temperature of marmosets slowly changes up to 15 times in the range of 5-37°C / 41-98.6°F, which changes the activity of the immune system, which played a leading role in the formation of transitional forms of

sition of two plasmids by *Y. pestis* acquisition of two plasmids (pFra and pPla, but about this later) and inactivation (promoter mutation) of the *pde3* gene became, from the point of view of genetics, the "point of no return" that permanently separated *Y. pestis* from *Y. pseudotuberculosis*. To be fair, it should be noted that some researchers have combined both microorganisms (*Y. pestis* and *Y. pseudotuberculosis*) into the "Y. pseudotuberculosis complex" due to the high homology (i.e., similarity) of their genomes. However, this purely biological view is not accepted by the medical community due to differences in ecology, routes of spread, infectiousness (i.e. contagiousness) and clinical presentation, clearly demonstrating the difference between biological and medical approaches when considering the same subject matter.

This point should be discussed in a little more detail. Acquisition of plasmids is a complex process that is carried out by so-called horizontal transfer, i.e. acquisition of genes by an organism not from its parents. And unlike uncontrolled acquisition from parents, which is typical for mammals and humans, gene acquisition in this case is a controlled process (specific genes are acquired), we can say, "conscious". Horizontal gene transfer is possible in three ways: *transformation* (penetration of DNA inside the bacterium through the membranes of competent cells), *transduction* (infection with a moderate bacteriophage[1]) and *conjugation* (transfer of DNA between bacterial cells in direct contact). There is speculation that *Y. pestis* acquired the pFra and pPla plasmids many years ago from other bacteria (by conjugation). In any case, having ac-

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Y. pestis / *Y. pseudotuberculosis*. In the warm season of the year, when the body temperature of the marmot is stable at 37°C 98.6°F, the microbe is suppressed by the immunity of a healthy animal, so this period the evolving microbe survives in the flea as a biofilm. At the last stage of the evolutionary process, the microbe acquired the ability to synthesise a substance (bacteriocin) capable of suppressing the growth of *Y. pseudotuberculosis*, which led to the impossibility of coexistence of the two microbes (it should be noted that not all strains are capable of synthesising bacteriocin).

[1] Bacteriophages (phages) or bacterial viruses are divided into *virulent* and *temperate*. As a result of their life cycle, the former produce new copies inside the bacterial cell and cause destruction of its wall, i.e. realise the *lytic cycle*, due to which they are also called lytic. The latter 'live' in the bacterium in the form of genetic information (more often embedding it in the genome of the bacterial cell - this state is commonly called "*prophage*"), i.e. they realise the *lysogenic cycle*, but at the onset of 'hard times' they can switch to the realisation of the *lytic cycle*.

quired them, *Y. pestis* became a kind of "white crow" of the genus *Yersinia*.

The inactivation of a number of chromosomal genes, in particular *ureD*, *rcsA*, *flhD*, *pde2* and *pde3*, and the rather rapid acquisition by the microbe of the plasmid pFra, which carries the *ymt* gene encoding the synthesis of **Yersinia murine toxine** (Ymt, "mouse" toxin), homologous to phospholipase D, made a decisive contribution to the ability of *Y. pestis* to be transmitted by fleas (both by the aforementioned southern rat flea and by fleas of the *Oropsylla* spp. parasitising mainly rodents). It got its name because of its ability, when administered as a pure preparation, to cause death of mice and rats (with no toxicity to guinea pigs, rabbits and monkeys). Interestingly, the synthesis of the toxin is carried out by the bacterium at temperatures not exceeding 28°C / 82.4°F[1], hence the toxin is not synthesised in the mammalian body, and only after ingestion with the blood of the mammal in the flea does the bacterium begin to synthesise it into the cytoplasmic membrane. The function is to protect the bacterium from the action of the bactericidal digestion product of blood erythrocytes, which provides better survival in the digestive tract. It should be noted, however, that the protective effect of the toxin depends on two factors: the rate of digestion of blood and its source. Thus, since females suck more blood than males, they digest it more slowly and, consequently, the probability of their bacterial colonisation is much higher. In addition, Ymt is required when human, mouse or black rat blood is involved, but it is not required in the case of the grey rat (*Rattus norvegicus*). These data are in favour of the fact that even before the acquisition of the murine toxin, *Y. pestis* could be transmitted by fleas from the grey rat, but the acquisition of Ymt widened the range of mammals from which flea transmission was possible, which in turn increased transmissibility and eventually led to the first plague pandemic, characterised by the predominance of the bubonic form of plague.

Another important acquisition was the pPla plasmid, which carries several important genes, among which we will single out *pla*, which is responsible for the production of the Pla protease capable of cleaving host (mammalian) plasminogen. An ancient form of Pla is sufficient for

[1] Hereinafter, where there are differences in units, SI units are given first, followed by conventional units separated by " / ".

the development of plague pneumonia, and the replacement of just one amino acid in this protein resulted in an increased ability to activate plasminogen (discussed in more detail in Chapter 6), which played a key role in the ability to cause the invasive infection associated with the bubonic form of plague. And it was this event that permanently separated the ancestral (ancient), "transitional", strains of *Y. pestis* (lineage 0)[1] from the modern strains (lineages 1, 2, 3, and 4). It is reported that strains lacking *pla* (caucasian strains) are highly virulent[2] to their main hosts, voles, but can be avirulent to humans or cause a relatively mild form of infection, which leads us to the following idea: the acquired plasmid and mutation do not play a leading role in the question of overall virulence. It is important to note that the loss of the genus-specific (i.e., common to pathogenic species of the genus *Yersinia*) pCad plasmid makes the strain avirulent (i.e., unable to cause an infectious process).

The microevolution of *Y. pestis* led to the division into two main phylogenetic branches: branch 1, which consists of the antiqua (1.ANT[3]) and orientalis (1.ORI) subbranches, and branch 2, which consists of the medievalis (2.MED) and asian antiqua (2.ANT) subbranches. We said earlier that the ANT, MED and ORI biovars are responsible for the three pandemics. However, the current distribution of strains by pandemic is as follows: first – 0.ANT2-5, 3.ANT, 4.ANT и 2.MED; second – 1.ANT, 1.IN; third – 1.ORI1.

As for the ancient strains, some variants are extinct (e.g., branches Gok2[4] and LNBA[1]), while the descendants of the most successful ones

[1] These are the ones that are categorised as pestoides or nonmain subspecies.

[2] The concepts of "virulence", "pathogenicity" (i.e., destructiveness to the organism, ability to cause disease) and "susceptibility" (i.e., ability to cause disease), as rightly noted by Igor V. Domaradsky, "*cannot be separated from each other, and if this is done, it is done only for methodological purposes*". It is worth noting that the list of virulence factors (determinants) changes with time. Thus, for example, W-antigen and pesticin, which were previously considered virulence factors, are not considered as such at present.

[3] Designation for a branch (subbranch) of a phylogenetic tree, where the digit before the dot indicates the branch number. Corresponds to SNP type.

[4] Existed 5700 years ago; DNA of the strain of the same name was found in a Neolithic burial site (Frälsegården in Gökhem parish, Falbygden, western Sweden). It is impossible to assess pathogenicity (no live bacterium), but the detection of DNA fragments of the strain in teeth indicates that its titre in blood was high.

are still circulating today. All of them belong to branch 0 and have, within the framework of the classification proposed by the staff of the Anti-plague Institute "Microbe", the following "ratios" of designations (SNP types) to non-main subspecies: 0. PE7 (subsp. *tibetica*), 0.PE2 (subsp. *caucasica*)[2], 0.PE10 (subsp. *qinhaica*), 0.PE3 (subsp. *angolica*), 0.PE4 (subsp. *central asiatica*), 0. PE4a (bv. altaica)[3], 0.PE4h (bv. hissarica), 0.PE4t (bv. talasica), 0.PE4m (bv. microtus)[4], 0.PE5 (subsp. *ulegeica*)[5]. In this case, these designations may be called *genetic variants* or *genovariants* (written "var.")[6]. In practice, however, such "linkage" causes certain difficulties due to the presence of alternative classifications, so most researchers limit themselves to specifying the SNP-type (i.e. 0.PE2, 0.PE7, etc.)[7].

Returning to the question of virulence, we note that strains of the main subspecies have high virulence for their main carriers and humans, which makes them of high epidemic significance. The subspecies of ancient strains may have different virulence, but in general they cause isolated diseases and, therefore, have low epidemic significance.

When sequencing the genome of *Y. pestis* (and compared with *Y. pseudotuberculosis*), a region encoding a prophage called **Ypfφ** (Yersinia

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[1] Existed 2500-5000 years ago; genome studies of strains of this lineage show that, like modern strains of *Y. pestis*, they could synthesise Ymt, but, exhibited urease activity (like strains of the pseudotuberculosis microbe) and probably lacked the ability to form a block in fleas.

[2] Includes genetic branches I, II and III, with branch II consisting of IIa (Prisevan), IIb (Zangezuro-Karabakh) and IIc (Zangezuro-Karabakh-Priaraksin).

[3] Includes clusters 0.PE4a-1 and 0.PE4a-2, which in turn are subdivided into subcusters 0.PE4a-1-1, 0.PE4a-1-2, 0.PE4a-2-1 and 0.PE4a-2-2.

[4] It is divided into biovars xilingolensis and qinghaiensis by some researchers.

[5] The most "late" branch of evolution among all non-main subspecies, which separated from branch 0 (about 3500 years ago). Includes genetic branches 0.PE5/1 and 0.PE5/2, with the latter subdivided into subbranches 0.PE5/2-1 and 0.PE5/2-2.

[6] Non-taxonomic category, which is understood as a microorganism that differs from the main strain genetically, but not enough to be considered a separate taxonomic unit (subspecies, species). That is, when we are talking about the fourth genetic variant of the strain *Y. pestis* subsp. *central asiatica* of the null branch, they write "var. 0.PE4".

[7] The designation PE was proposed in 2004 by Canadian and British biologist Mark Achtman (b. 1943), who used the first letters of the term pestoides, previously proposed by Russian physician Ivan L. Martinevsky.

pestis filamentous phage) was identified, representing the genome of a filamentous bacteriophage (**Figure 3.2**).

The filamentous bacteriophages belonging to the genus *Inovirus* of the family *Inoviridae* are quite widespread, but only some of them parasitise in a moderate form, i.e. "live" in the bacterial genome in the form of a prophage. What the role of the prophage Ypfφ is currently unknown. Interestingly, the protein encoded by the *zot* gene is structurally homologous to the Zot protein (zonula occludens toxin) of other bacteria. For example, in *Vibrio cholera*, this protein, encoded by the CTXφ gene, is a virulence factor because it increases the permeability of epithelial barriers in the intestine, leading to the development of gastroenteritis. The functions of the Zot protein in *Y. pestis* have not yet been elucidated. It is only known that the formation of a complex with the GspD2 protein (it is also encoded by the Ypfφ gene) facilitates the assembly and secretion of Ypfφ virions from host cells, which means that the bacterial cell has the potential for horizontal transfer of its genes, which, as a consequence, plays a role in the creation of new pathogens! At the same time, stable conservation of the Ypfφ prophage is observed only in strains of the biovar orientalis, whereas in strains of other biovars it can be detected only as an unstable extrachromosomal element (i.e. by itself inside the bacterial cell), although such conservation is ruled out by some researchers[1]. In any case, the stable preservation of the prophage only in strains of the orientalis biovar allowed a number of researchers to suggest[2] the leading role of strains of this biovar in the spread of infection during the third pandemic.

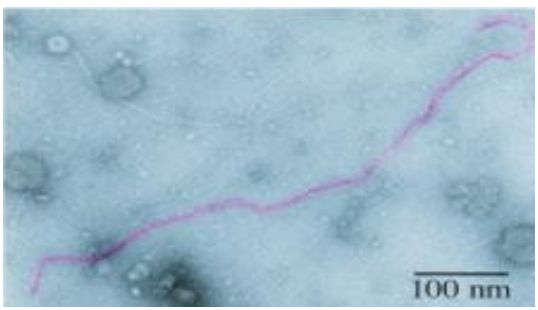


***Figure 3.2**. Bacteriophage Ypfφ (by A. Derbice, 2014)*

And here it is nec-

[1] Bonczarowska J. H., Susat J., et al. *Ancient Yersinia pestis genomes lack the virulence-associated YpfΦ prophage present in modern pandemic strains*. Proc Biol Sci. 2023; 290(2003):20230622; DOI: 10.1098/rspb.2023.0622.

[2] Derbise A., Carniel E. *YpfΦ: a filamentous phage acquired by Yersinia pestis*. Front Microbiol. 2014; 5; DOI: 10.3389/fmicb.2014.00701.

essary to specify that in genome of *Y. pestis* there are a lot of prophages. Most of them are fixed due to mutations. Thus, one of the inactive prophages present in the genome of most strains is used as a detection tool (target of primers "3a" in polymerase chain reaction). However, why bacteriophage stabilisation is possible only in ORI strains, despite the identical integration mechanism in all strains, is still an unanswered question. It is quite possible that this was made possible by mutations in genes responsible for recombination (i.e. redistribution) or repair (i.e. damage repair) processes.

It is worth digressing a little here to note that all the previously described intraspecific evolution (branching) is based on molecular methods to establish phylogeny (i.e., relatedness) and to construct the so-called phylogenetic tree (**Figure 3.3**). However, the assessment of gene changes is a complex process and is not always objective, so that it is possible to say which strain originated from which strain with a certain degree of probability (i.e., "it was probably like this"). Nevertheless, we present a phylogenetic tree to illustrate both the previously described microevolution of *Y. pestis* and intraspecific diversity.

Returning to our topic, let us not forget that the acquisition of new properties became possible not only due to the acquisition of plasmids and prophages, but also due to the reduction (simplification) of the genome by inactivation or deletion (loss of a section) of genes. And in this connection we would like to recall the remarkable words once said by Igor Valeryanovich Domaradsky[1]: "*Acquiring something new, microbes often lose old, no less important features*". And indeed, simultaneously with the acquisition of the plasmid pFra there was inactivation of a number of genes, in particular, the *ureD* gene, responsible for urease activity, that is, all members of the genus Yersinia show a fairly powerful urease

[1] Игорь Валерианович Домарадский, 1925–2009; the Soviet and Russian physician, bacteriologist. Academician of the USSR Academy of Medical Sciences (1991). Director of the Irkutsk (1957-1964) and Rostov (1964-1973) Anti-plague Institutes. As a major specialist on plague and tularemia, from the 1970s he was involved in the Soviet programme for the development of bacteriological weapons "Biopreparat" (work on genetic modification of bacteria to increase their virulence and antibiotic resistance); deputy director for science at the All-Union Scientific-Research Institute of Applied Microbiology in Obolensk (now – the State Research Center for Applied Microbiology and Biotechnology). In his autobiographical book Shifter (Rus. «Перевёртыш») he expressed remorse for this work.

activity[1], but *Y. pestis* – no (remember that strains of the LNBA line had it), which, on the other hand, can be used for differential diagnosis in the laboratory. As another example, inactivation of the *yadA* gene of the genus-specific pCad plasmid, which is responsible for the synthesis of the adhesin YadA. This adhesin plays a leading role in serum resistance and attachment of the bacterium to enterocytes with subsequent translocation through M cells of the intestinal mucosa.

Thus, the loss of the ability to produce the YadA adhesin resulted in the impossibility of infection through the intestine (as occurs in other

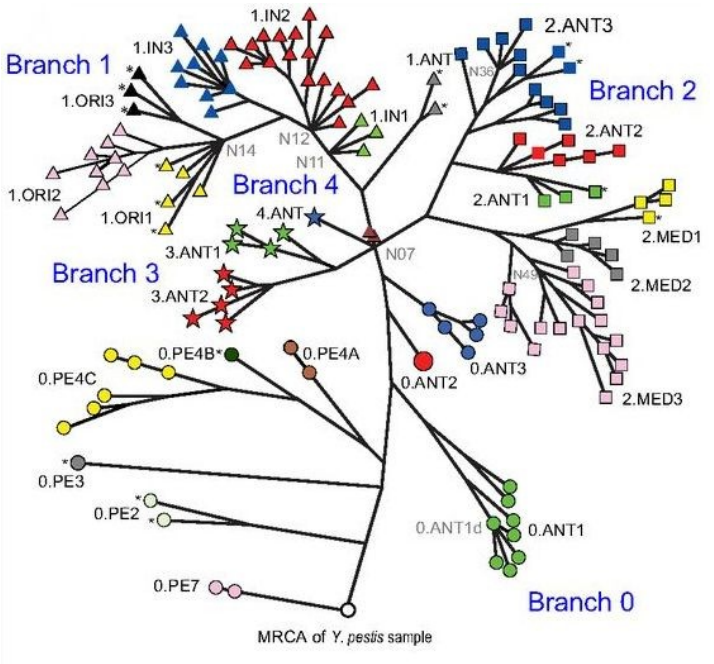


Figure 3.3. *Phylogenetic tree (by Cui Y., 2013). It should be noted that during the accumulation of actual data, the tree may undergo changes. For example, branches 0.PE7, 3.ANT and 4.ANT, first shown in the presented tree, were formed, and branch 0.PE1 was moved from the trunk to branch 0.PE4. However, the branch 0.PE5 (Ulegei strains), which is located between 0.PE4 and 0.ANT1, is still missing here*

[1] The enzyme urease catalyses the conversion of urea to ammonia and carbon dioxide.

pathogenic *Yersiniae*, in particular *Y. enterocolitica* and *Y. pseudotuberculosis*) and, therefore, significantly influenced the establishment of the mechanism of infection by *Y. pestis*. As a peculiar "cherry on the cake", we would like to point out that during the whole evolutionary development of *Y. pestis* (relative to *Y. pseudotuberculosis*) acquired only 32 chromosomal genes, not counting the two plasmids already mentioned, while it lost 317 genes and another 150 became pseudogenes (lost the ability to encode proteins), indicating that as much as 13% of *Y. pseudotuberculosis* genes no longer function in *Y. pestis*. And it is this reductive evolution, according to some authors, that is probably more important than the acquired genes, since the latter do not contribute much to virulence (remember: the loss of the genus-specific pCad plasmid makes the strain avirulent!), but it is this (gene loss) that probably allowed *Y. pestis* to emerge from a less virulent closely related progenitor.

4 NAMES

Along a narrow path winding among birches, pines and firs, a man in a black coat reaches the summit of Kuhavuori mount. A marvellous country of many small plateaus formed by islands of amphibolites and quartzites rising to the surface, piercing the cover of grass and moss, carpeted with carpets of violet-white violets, opens before his eyes. The mountain is in bloom. Fresh mountain air, soaked with subtle pine and floral aromas, fills the chest. One can breathe easily and freely here. At the foot of the mountain the light blue waters of Airanne lake spill over, on which the rays of the evening sun play, scattered with beads of light, and on the opposite side, on both sides of the Vakko River, the town of Sortavala majestically spreads out. Everything around is filled with peace and tranquillity. Somewhere in the distance you can hear the

crystal tinkling of a kantele[1]. It is probably the runesmiths singing the ancient verses of the Kalevala epic, to the collection of which Elias Lönnrot[2] was sent by the fight against cholera. Or not, Vainämöinen[3] himself is singing runes about ancient deeds. Here he sits before the cliff, either an old man with a grey beard or a husband in full bloom. He has briefly returned to Kalevala, the land of the ancient Karelians and the land of Marjatta's child[4], to indulge in sweet memories. The sun is slanting towards the horizon to be closer to him and, as before[5], to listen to his sad and beautiful song to the crystal shimmers of music. He sings about the creation of the world, about his birth, about his exploits... How he fought for Sampo, how the old woman Lowhi made the children of Lovjatar, goddess of diseases, to do his Kalevala. His song is long; we hear: "kunka ruhtosi *rutoksi*"…

The word **rutoksi** (rutto, "plague") comes from the ancient Karelian-Finnish word **ruttotauti**, which means "fast", "spreading quickly" and indicates the speed at which the infection spreads. German (die) **Pest** and Italian **peste** (**pestilenza**) come from the Latin **pestis**, which gave its name to the pathogen and means "contagion", "destruction", indicating the extent of the infection. The Avar **вабаъ** (waba'a) comes from the Arabic وَبَاء (wabā), meaning "epidemic". At the same time, the Arabic name of the disease طاعون (taeun) comes from the classical Syriac ܛܥܘܢܐ (ṭāʿōnā) → ܛܥܶܢ (ṭʿen), which means "to carry", and both in the sense of bearing the burden and ordinary carrying, indicating the presence of vectors of the disease. Mongolian **тарбагану-тахал** (tarbaganu takhal,

[1] A stringed plucked instrument of the zither type; a close relative of the Estonian kannele and Russian psaltery.

[2] 1802–1884; the Finnish physician, folklorist, collector of the Karelian-Finnish epic. Participant in the elimination of the cholera epidemic in Finland.

[3] The main character of the Karelian-Finnish epic Kalevala, the first man, the son of the goddess Ilmatar, a sorcerer and performer of runic songs (runes) on the kantele.

[4] According to Rune 50 of the Kalevala epic, Marjatta conceived a child by eating lingonberries in the forest. Vainämöinen offered to kill the baby, but the child reproached him for his cruelty. Having realised the superiority of the infant's spiritual power, Vainämöinen created a boat by magic and sailed away from Kalevala forever. It is believed that this last rune (song) testifies to the change of Karelian-Finnish paganism to Christianity.

[5] According to Rune 47 of the Kalevala epic, because of Vainämöinen's captivating play, the sun and moon descended lower to the earth to listen to her, which helped the old woman Louhi to capture them and imprison them in a rock.

"plague of tarbagans") already specifically refers to the disease of marmot tarbagans (*Marmota sibirica*), from contact with which a person can become infected. The Tibetan ཇི་རིམས (jhi rim) means "mouse infection" (jhi - mouse, rim – lit."one [person] after another"), which not only indicates the possibility of infection from mice, but also the infectious nature of the disease. Another Tibetan term གཉན་ཕྱི་དྲུག་ནག་པོ། (nyen chi thuknagpo) means "black poison-nyen coming from outside", which is roughly analogous to the medieval Latin name **Atra mors**, which appeared during the second pandemic, and indicates the septic form. The Slavic **чума** (chuma) comes from Turkish **çum**a, meaning "boil", "pimple" and indicating the characteristic symptom of the disease, the abscess or buboes. Finally, the English (the) **plague** is derived from the post-Neolatin **plaga** and means "blow", indicating a characteristic sudden rise in temperature.

All these names reflect the peculiarities of the course of the disease, which people noted, and the fear of it even led to deification. Thus, in the Lithuanian view, the plague was the goddess of death, Giltine. She was a beautiful woman who, however, was buried alive. Having got out of the grave, she takes revenge on people: extracting poison from corpses, she kills the living with it. The Bulgarians have a legend that the older sister of the plague was a cat, which often beat her for disobedience and laziness, so the plague kills cats, and in the representation of Ukrainians plague lives in the north, driven there by dogs for taking away their voices. The Greeks represented it as a blind old woman in black, holding a bat in her hand, roaming the countryside to kill everyone she meets. The Japanese imagined her as bake-kujira, a ghost (yōkai) in the form of a skeleton whale, appearing near coastal whaling villages on dark, rainy nights....

Let us return to the Arabic, Mongolian and Tibetan names. This is extremely curious, because for a long time it was believed that plague was carried only by fleas and rats. Here it will be appropriate to make a reservation and accept the terminology of Yuri Mikhailovich Rall'[1], according to which *"carriers" are understood as warm-blooded animals, sick in*

[1] Юрий Михайлович Ралль, 1907–1965; the Russian and Soviet zoologist. Author of the book Lectures on the epizootiology of plague (Russ. «Лекции по эпизоотологии чумы»), which was awarded the I.I. Mechnikov Prize of the USSR Academy of Sciences in 1960.

nature with plague, and under *"vectors" – fleas*[1]*, able to suck infected blood and transmit the pathogen to the animal-carrier*. The chain consisting of the causative agent (*Y. pestis*), carriers (rodents) and vectors (fleas) is commonly referred to as a *triad*.

So, since ancient times only mice and rats have been known as carriers. In the Bible we find that during an epidemic accompanied by boils (buboes), buboes and mice made of gold were used as a sacrifice of obedience (compare 1 Samuel 6:4). The poem Bagavata Purana, written in Sanskrit, advises leaving one's dwellings as soon as rats begin to fall from the roofs and die, and in the poem Death of Rats, written by the Chinese poet Shi Daonan a few days before he died of the plague, we find that "*a few days after the death of rats, people fall like collapsing walls*". However, no other carriers were known to the world (except the Mongols and Tibetans). Moreover, the Mongols were so well aware of the danger of encountering sick tarbagans that they developed a set of rules: if they saw sick or already dead tarbagans, they should report it so that no one touches them, but it is possible to graze herds and live in this place.

Only in the XIX century Danylo Kirillovich Zabolotny[2], working in Mongolia and China, paying attention to these rules and conducting his own research, wrote: "*Various breeds of rodents, in all probability, represent the environment on which the plague bacteria persist*" (1899). This opinion would find ardent opponents, among them Wu Lien-teh[3], who, while accepting the possibility of sporadic (i.e. accidental) infections of tarbagan marmots, denied the very possibility of their direct participation in the epidemic process, in particular, as sources of the pneumonic form of plague in Manchuria (epidemic of 1910-1911). Subsequently, he

[1] It should be stipulated that rarely other ectoparasites (more often mites) may act as vectors.

[2] Данило Кирилович Заболотный, 1866–1929; the Ukrainian and Soviet physician, bacteriologist, epidemiologist. Academician (1922) and president (1928-1929) of the All-Ukrainian Academy of Sciences. Academician of the USSR Academy of Sciences (1929). He founded the world's first chair of epidemiology (1920). Author of works on plague, cholera and other infectious diseases.

[3] 伍連德, 1879–1960; the Chinese-born Malay physician and epidemiologist. Nominee for the Nobel Prize in Physiology or Medicine (1953). First ethnic Chinese student to graduate from Cambridge University. He made a major contribution to the Manchurian plague epidemic (1910-1911).

changed his position, which was probably facilitated by the following story, which it would be a crime not to mention

Autumn 1912. The detachment of Ippolit Alexandrovich Deminsky[1] started studying hibernating gophers. Dissecting one of them (*Citellus pygmaeus*), dug out of a burrow in the vicinity of the village of Rakhinki (left bank of the Volga River, Russia), the physician was able to isolate a plague culture, having been infected with the pneumonic form of plague. Seeing in what happened only a scientific experiment, he left detailed notes on the course of the disease, and in a telegram sent to Nikolai Nikolaevich Klodnitsky[2], the Head of the Astrakhan anti-plague laboratory, he said: 'I contracted pneumonic plague from gophers. Come get the cultures. The records are all in order. The lab will tell you the rest. Open up my corpse as a case of experimental human infection from gophers. Farewell. Deminsky'. Three days later the scientist died.

The discovery of Danylo K. Zabolotny, supported by the tragic death of Ippolit A. Deminsky, was of world importance, having laid the foundation of epizootology and the theory of natural foci of plague. Without detracting from the feat of these two physicians, it is only fair to note that in 1898 Paul-Louis Simond[3], working in India, established[4] the possibility of plague of palm squirrels (*Funambulus palmarum*). However, his discovery went unnoticed.

To summarise, plague is a naturally occurring infection. We have seen that its spread can start both in the wilderness and in a harbour city. That is why all foci are usually divided into *natural foci* (also called "wild" plague foci) and *anthropurgic* (from the Greek "[related to] human activity") or *synanthropic foci*. The latter, as noted by Igor V. Domaradsky, should be considered rather ephemeral, since they occur in the case of introduction of an infected carrier or vector and are usually quickly enough detected and eliminated by humans. Therefore, when

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[1] Ипполит Александрович Деминский, 1864–1912; the Russian physician, bacteriologist, epidemiologist.

[2] Николай Николаевич Клодницкий, 1868–1939; the Belarusian and Soviet physician, bacteriologist, epidemiologist.

[3] 1858–1947; the French physician, bacteriologist, botanist. Director of the Pasteur Institute in Saigon (1898-1901). Proved the role of fleas in the transmission of bubonic plague.

[4] Simond P. *La propagation de la Peste.* Ann Inst Past (Paris). 1898; 12(10):625–687.

we speak about sanitary surveillance of plague foci, we mean first of all the foci of "wild" plague, enclosed in clear territorial boundaries. All these foci are located in a relatively large territory (the so-called "zona pestica"[1]): in the northern hemisphere they extend south of 50° N latitude, and in the southern hemisphere they reach the extreme limits of the continents, with the exception of Australia (with the island of New Zealand) and Antarctica.

In the previous chapter, we spoke of pandemics, as a result of which plague spread over large areas, but most often did not gain a foothold and "drifted" back to the boundaries of natural foci. However, the existence of foci in North America is due to the penetration of the pathogen during the third pandemic by sea with the help of rats and the subsequent formation of new (so called imported) foci, but not over the whole territory, but only west of 98° W longitude. It could be assumed that the border is determined by the habitat of the carriers (rodents), but this is not entirely true. Studies show that a distinctive feature of these foci is high aridity and salinity of soils, i.e. these two indicators are also important determinants of the existence of foci and their boundaries. Other indicators include soil texture, mineral content and pH. Interestingly, natural foci in North Africa are located less than 3 kilometres from the Mediterranean coast and the edge of chotts (salt lakes), and *Y. pestis*, in the form of L-forms (i.e. in this case bacteria with a thin cell wall; see Chapter 30 for details), is isolated from soils containing up to 70 g/L NaCl. Experiments[2] show that *Y. pestis* can survive in concentrations of up to 150 g/L NaCl!

Climate change also contributes: warming leads to an increase in the host population and, as a consequence, to the expansion of natural foci. Thus, the expansion of the range of the great sandfly (*Rhombomys opimus*) led to an increase in the area of the North Priaral desert plague foci and Kyzylkum desert plague foci (Kazakhstan) by 29.2% and 0.4%, respectively. However, the regression of the Aral Sea (Kazakhstan) and increasing industrial development of the territories - construction of the railway line Zhezkazgan–Beineu, oil and gas pipelines, etc. – also

[1] The term was proposed in 1944 by Yuri M. Rall'. In 1959 Boris Konstantinovich Fenyuk proposed to use the term "zone of natural or wild foci of plague".

[2] Malek M. A., Bitam I., et al. *Yersinia pestis halotolerance illuminates plague reservoirs*. Sci Rep. 2017; 7:40022; DOI: 10.1038/srep40022.

played an important role in this process. All this leads us to the idea of the important role of natural landscapes in the formation of foci. Back in 1930 Nikolai Akimovich Gaisky[1] wrote: "*Gophers, being spread far beyond the endemic region [i.e. natural foci], become dangerous in relation to plague in conditions of a strictly defined «geographical landscape», meaning the whole sum of conditions, i.e. geographical position of the area, climate, soils, flora and fauna*".

Natural plague foci are also characterised by repeated alternation of *epizootic processes*, which is understood as *a process of continuous interaction of the pathogen population with the populations of its carriers ("natural hosts") and the external environment, which ensures the existence of the pathogen in a given territory*. The epizootic process begins with the accumulation of the pathogen, which is accompanied by isolated cases of carrier disease (**phase 1**). Then comes an acute (intensive) epizootic, accompanied by the inclusion of a large number of carriers in the infectious process, which may lead to mass mortality of animals (≥ 90%) - the centre "burns" (**phase 2**). At this time, the infection may spread rapidly within the territory of the centre, including the formation of microfoci. Further, there is a fading due to a high immune layer among carriers with a relatively high flea population (**phase 3**). During this period, atypical (avirulent and weakly virulent) strains usually appear. Finally, there comes the inter-epizootic period (**phase 4**), accompanied by complete rest (absence not only of carrier mortality but also of pathogen detection), which may last for many years. For example, the inter-epizootic period of the outbreak in the area of Guljenga settlement (Transbaikal, Russia) lasted from 1947 to 1960, and a long-term study of various carriers (about 300,000!) during this period yielded negative results.

To date, it has not been fully established how *Y. pestis* persists during the inter-epizootic period. Some researchers suggest that it persists in a

[1] Николай Акимович Гайский, 1884–1947; the Russian and Soviet physician, bacteriologist, epizootologist, creator of the tularemia vaccine (Gaisky-Elbert), Stalin Prize laureate. In 1931, being the head of the anti-plague laboratory, he was sentenced to 5 years of imprisonment; he was rehabilitated in 1960. According to E. I. Demikhovsky, the reason for the sentence was the necessity to force the scientist (as well as many others) to develop bacteriological weapons within the walls of a secret military laboratory located in a monastery in Suzdal (Russia).

latent form in the soil ("telluric plague")[1], penetrating there from the corpses of carriers. Since *Y. pestis* does not form spores, it is certainly preserved in a non-culturable form (more on this in Chapter 30), which explains the absence of an infectious process and the lack of detection of the pathogen during examination of foci. Other scientists put forward a version of transition to a saprophytic state, which seems difficult due to the need of *Y. pestis* in certain amino acids (auxotrophy), although prototrophic strains are known to science. There is also talk of persistence (persistence) of the pathogen in plants[2] and soil protozoa, in particular, in amoebae (**Figure 4.1**)[3]. This is particularly important because amoebae, which are ubiquitous in the soils of outbreaks, are able to survive unfavourable environmental conditions and can be transported by wind and water over considerable distances, lysing at 37°C / 98.6°F, i.e. releasing their "cargo" into wounds or food in animals and humans. Cultivation experience[4] with acanthamoebae isolated from the Gorno-Altaisk high-mountain focus (the Russian part of the transboundary Sailugem focus) showed the survival of *Y. pestis* without additional nutrients for 22 months! Finally, others claim that it persists as a biofilm on the surface of nematodes or in the stomachs of fleas. It has been reported to persist in tap water for up to 16 days[5] and in bottled water for up to 100 days[6]. It is interesting to consider that there is no inter-epizootic period at all and that the epizootic process pulsates and moves

[1] There is evidence of persistence in the soil for up to a year (see Mollaret H. H. *Experimental Preservation of Plague in Soil*. Bull Soc Pathol Exot Filiales. 1963; 56:1168–1182).

[2] Some Mongolian peoples believed that the plague pathogen lived in the rhizomes of the Khun-ubusun (Mong. хун-убусун, literally "man-grass") plant. It should be noted that the possibility of preserving Y. pseudotuberculosis has been experimentally demonstrated in stem and leaf tissues of potato, into which the pathogen penetrated through the root system (see Маркова Ю. А., Романенко А. С., и др. *Взаимодействие Yersinia pseudotuberculosis с пробирочными растениями картофеля*. J Sress Physiol Biochem. 2006; 2(1):21–27).

[3] This is particularly interesting because amoebic structures are synonymous with human macrophages.

[4] Макашова М. А., Оглодин Е. Г., и др. *Длительное сохранение Yersinia pestis в ассоциации с Acanthamoeba castellanii в эксперименте*. Проблемы особо опасных инфекций. 2022; 4:82–89; DOI: 10.21055/0370-1069-2022-4-82-89.

[5] Wilm *Hygeien Rundschau*. 1897; 7:285–300.

[6] Torosian S. D., Regan P. M., et al. *Detection of Yersinia pestis over time in seeded bottled water samples by cultivation on heart infusion agar*. Can J Microbiol. 2009; 55(9):1125–1129; DOI: 10.1139/w09-061.

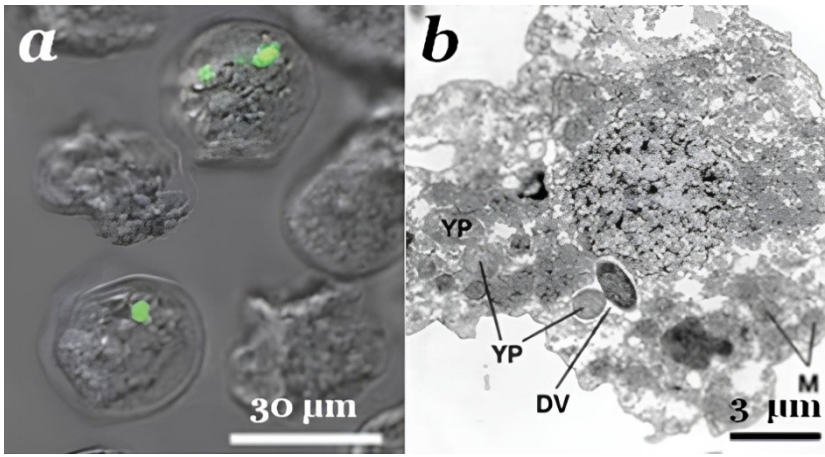


Figure 4.1. *Y. pestis in Acanthamoeba castellanii (by Markman D. W., 2018): a - fluorescence confocal microscopy (Y. pestis fluoresces green); b - transmission electron microscopy (24h co-cultivation; YP – Y. pestis, DV – digestive vacuole, M – mitochondria)*

in space. In any case, all this inevitably leads us to the realisation that the processes occurring in foci are much more complex than a simple *carrier – vector – carrier* relationship.

Each particular foci is characterised by the presence of its "own" carrier (see Supplementary Materials). If there is only one such carrier, the locality, according to the terminology of Evgeny Nikanorovich Pavlovsky[1], is called a *monogostal foci*. For example, the main carrier of the Balkhash (Pre-Balkhash) desert focus (Kazakhstan) is the great gerbil (*Rhombomys opimus*), but the long-eared hedgehog (*Hemiechinus auritus*), which often inhabits its burrows, does not play a role in the epizootic (although isolated cases of infection do occur). Therefore, monostable foci are also referred to by their host: marmot foci, gopher foci, vole foci, and so on. Each such centre is characterised by its own peculiarities. For example, an important feature of the Tian Shan high-

[1] Евгений Никанорович Павловский, 1884–1965; the Russian and Soviet physician, parasitologist, zoologist, entomologist, founder of the Soviet school of parasitology, introduced the concept of 'natural foci'. Academician of the USSR Academy of Sciences and the USSR Academy of Medical Sciences. Hero of Socialist Labour.



The Pallas's pika



Yellow-bellied marmots

land focus (Kyrgyzstan) is the presence of so-called is the presence of so-called elementary foci[1] located in deep warm burrows of the main carrier – the grey marmot (*Marmota baibacina*), whose high activity in summer is accompanied by visits to a large number of burrows. Infection of marmots occurs exclusively in them, because in the cold climate of high mountains it is deep burrows that keep constant temperature and high humidity, which determines the ability of marmot ectoparasites (fleas: *Oropsylla silantiewi* and *Rhadinopsylla li ventricosa*) to retain infectivity for a long time. In addition, low temperatures contribute to the persistence of *Y. pestis* in the marmot organism by cooling it (the organism) during winter hibernation

Along with monogostal foci, there are also *polygostal foci* with several (as a rule, two or three) main carriers. For example, the main carriers of the Volga-Ural sandy focus (Russia) are the tamarisk jird (*Meriones tamariscinus*) and the midday jird (*Meriones meridianus*).

It is important to realise that a species that plays a major role in the epizootic of one focus may play a minor role in the epizootic of another. As an example, the long-tailed ground squirrel (*Citellus undulatus*) is the main carrier for the Tuvinian mountainous focus (Russia), but a minor carrier for the Yaru-Bogdyn Foci (Mongolia).

According to the epizootological division proposed by Boris Konstantinovich Fenyuk[2], all carriers are subdivided into *main carriers* and s*econdary* (*nonmain) carriers*. The latter, in turn, are subdivided into *occasional (temporary) carriers* and *facultative (additional) carriers*.

The characteristic feature of the main carriers is their high and relatively stable abundance. They belong to desert-steppe forms and are widely distributed in areas of dry steppes and deserts. Their burrows are deep and are excellent breeding grounds for fleas. A great number of species have been identified as the main carriers of plague, among which we should mention the little ground squirrel (*Spermophilus pygmaeus*), the great gerbil (*Rhombomys opimus*), the Palla's pika

[1] That is, places of direct presence of infected carriers and/or vectors (burrow, cave with infested fleas, etc.) in the natural centre of the outbreak.

[2] Борис Константинович Фенюк, 1902–1969; the Russian and Soviet zoologist. Winner of the Stalin Prize (he gave part of the money to his wife Vera Alexandrovna Artisevich for the construction of the Saratov University library).

(*Ochotona pallasi*), the midday jird (*Meriones meridianus*), the Libyan jird (*Meriones lybicus*), the grey marmot (*Marmota baibacina*), the tarbagan marmot (*Marmota sibirica*), rats (*Rattus* spp.), possums (*Didelphis* spp.), guinea pigs (*Cavia* spp.), rabbits (*Sylvilagus* spp.).

A characteristic feature of the secondary carriers is their highly unstable numbers (from outbreaks to near extinction). Their burrows are shallow and their fleas are short-lived. However, during an outbreak they may become involved in an epizootic, posing a huge threat to humans, but they are unable to sustain it without the primary carriers. As representatives of secondary carriers, we note the house mouse (*Mus musculus*), the yellow-bellied marmot (*Marmota flaviventris*) and the four-striped grass mouse (*Rhabdomys pumilio*).

The yellow ground squirrel (*Spermophilus fulvus*) is a classic example of a facultative secondary carrier because, when it is involved in an epizootic along with the main carrier, the little ground squirrel (*Spermophilus pygmaeus*), it is not able to sustain the epizootic permanently due to its small numbers and spatial sparseness. On the other hand, in Africa and the Arabian Peninsula, where natural outbreaks often border human settlements, carriers may include pigs, cows and even camels, which act as occasional secondary carriers, i.e. those that can become infected and ill but play no role in the epizootic.

Some of the secondary carriers may be main carriers for anthropurgic foci. Recall that the primary carrier in the first and second pandemics was the rat (an occasional carrier in a natural outbreak). On the other hand, any farm and domestic animal can be a carrier in an anthropurgic centre if they are susceptible to plague. Camels, saigas, sheep, particularly the Tibetan sheep (*Ovis aries*), may well be carriers, and their meat and milk, if not properly heat-treated, as well as the slaughter process itself, may pose a risk to humans. On the other hand, such large animals as horses, elephants, buffaloes and deer have sufficient immunity to plague and can only be infected by artificial "pumping" with high doses of the pathogen.

Interestingly, species and individuals resistant to *Y. pestis* are also found among rodents. The second is of particular interest: the midday jird (*Meriones meridianus*) living on the left bank of the Volga River is immune to plague, which is not the case with their highly susceptible

relatives living, for example, on the right bank. Researches[1] have shown that the reason for this is the difference in the content of 2,3-diphosphoglyceric acid and phosphorus in erythrocytes, as well as Ca^{2+} in serum and blood plasma: in the former it is significantly lower, which determines their resistance (recall that noon gerbils are considered to be the main carriers).

It is worth talking separately about domestic animals. Dogs themselves are not susceptible to plague (they have a mild, more often latent form of the disease), but they pose an epidemiological danger to humans in the sense that they can carry infected fleas (however, it is necessary to remember about rare cases of the primary pulmonary form, which will be discussed in detail in Chapter 8).

Cats, those much-loved furry rumbling lumps of happiness, are, on the contrary, highly susceptible to plague (remember the Bulgarian belief that plague takes revenge on cats) and can infect humans, e.g. through bites, faeces and exhaled aerosol (10% of cases). The bubonic form of plague in cats occurs either through a flea bite (distal lymph nodes are affected) or through wounds in the oral cavity and interdental fissures during ingestion of infected meat (submandibular and cervical lymph nodes as well as tonsils are affected). As a result of further entry into the bloodstream and spread of infection, the lungs may be infected and a secondary pulmonary form may develop. Direct inhalation of the pathogen from the oral cavity or inhalation of plague aerosol (from a patient with the pulmonary form) may lead to the development of the primary pulmonary form. In addition, cats can realise an aerogenic mechanism of transmission (as can humans). It is likely that cats, as the

[1] Смирнова Е. Б., Аванян Л. А. *Содержание 2,3-дифосфоглицериновой кислоты в эритроцитах чувствительных и резистентных к чуме полуденных песчанок*, в сборн. *Патологическая физиология особо опасных инфекций (Труды противочумных учреждений)*. Саратов: Всесоюзный ордена Трудового Красного Знамени научно-исследовательский противочумный институт «Микроб», 1981, 184 с.; Чурикова Н. В., Семененко Р. Д., и др. *Содержание Ca^{2+} в сыворотке и плазме крови полуденных песчанок с различной резистентностью к микробу чумы*, в сборн. *Микробиология и биохимия особо опасных инфекций (Труды противочумных учреждений)*. Саратов: Всесоюзный ордена Трудового Красного Знамени научно-исследовательский противочумный институт «Микроб», 1988, 104 с.

main rodent controllers during plague epidemics[1], have made an important contribution to its spread.

5 BLEEP OF CHEOPS

Wearing gold and a coat my Flea lived,
And complete freedom in the palace it was given…
Johann Wolfgang von Goethe. Faust

In the long debate about what came before, the hen or the egg, in this case we can unequivocally answer: the egg. And this egg, which did not know love of mother, because, being only one of several dozens of laid eggs, fell into a small crevice, where it hatched and existed as a larva, eating dead skin cells and excrement of relatives. But here came the moment of metamorphosis, when the unsightly larva turned... No, not into a butterfly - into a flea. And the flea lived a regular flea life. For breakfast, lunch and dinner, it feasted on the blood of small mammals, mostly rodents. And everything would be routine and monotonous, if one day somewhere in the Sudan it was not found by Nathaniel Charles Rothschild[2], a banker and part-time genius entomologist that, having

[1] In the Middle Ages, there was a reward for killing a rat, but it was so small that it did not justify the risk of infection. Interestingly, many years later, the unofficial motto of the so-called tunnel rats (units of the armed forces of the USA, Australia and New Zealand operating during the Vietnam War in underground tunnels dug by Vietnamese guerrillas) was "Non Gratum Anus Rodentum" (Not Worth a Rat's Ass). On the other hand, at the beginning of the 20th century in Japan, through the efforts of Kitasato Shibasaburō, a reward of 5 sen was offered for one rat, while lunch (a plate of soba) cost only 2 sen. In general, the sums allocated for the elimination of one outbreak were enormous – from 14600 to 352500 yen (i.e. about 17 million plates of soba), but it played an important role in the complete elimination of plague in Japan by 1929.

[2] 1877–1923; the English banker and entomologist; amassed a large entomological collection. Suffering from encephalitis, he committed suicide at the age of 46.

discovered in her a new species[1], for the first time in her flea life treated her respectfully, giving her the name *Pulex cheopis*[2] in honour of the famous pyramid. And did Pharaoh Khufu (Cheops) know that so many years later his greatest pyramid and a small flea would be equally important in the eyes of mankind?



A preparation of the flea X. cheopis (Rothschild's preparation)

In the previous chapters, we have talked quite a lot about the role of fleas in both the epizootic and spread of *Y. pestis* through infection of mammals, including humans. Although each flea has its own host (this is not strictly specific)[3] and habitat, the mechanism of transmission of *Y. pestis* from one host to another is generally similar, so let us dwell on it in more detail. The process of blood sucking lasts from one minute to several hours and varies from species to species. Thus, *Xenopsylla cheopis* sucks blood in excess, while *Ctenocephalides felis* sucks a little but often. However, the mechanism of suction in all is reduced to the operation of two pumps (cibarial and pharyngeal), which contract one after the other to push a portion of blood through the oesophagus to the proventriculus and on to the stomach, called the midgut (**Figure 5.1**). Between portions of blood, the proventriculus remains tightly closed by a valve. However, because it is constantly opening and closing during suction, some blood from the midgut may regurgitate into the pre-gastric cavity. There, under the action of the "mouse" toxin, which we discussed earlier, and the prod-

[1] Rothschild N. C. *New species of Siphonaptera from Egypt and the Sudan (with two plates).* The Entomologist's monthly magazine. 1903; 39(2):83–87.

[2] Subsequently renamed *Xenopsylla cheopis* (Glinkiewicz, 1907) with inclusion in the appropriate genus

[3] It has already been mentioned that, for example, *Pulex irritans* is parasitic on humans and *Xenopsylla cheopis* on rodents. The flea *Ctenocephalides felis* on cats and *Cediopsylla spillmannii* on American rabbits of the *Sylvilagus* spp. (see Supplementary materials for information on vectors in natural foci).

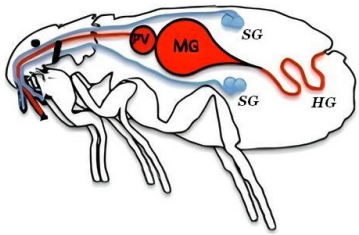


Figure 5.1. *Anatomy of the flea: PV - proventriculus, MG - midgut, SG - salivary glands, HG – hindgut*

ucts of chromosomal **hemin storage genes** (hms[1]), hemin accumulation proteins, what is commonly called *blockade* is realised.

The mechanism of flea proventriculus blockade was first described[2] in 1914 by Arthur William Bacot[3] and Sir Charles James Martin[4]. The scientists found that *Y. pestis* grew as large clusters in the midgut of infected fleas. In addition, some fleas also developed clusters in the proventriculus lumen, which they believed was critical for continued efficient transmission of the bacterium to the host (**Figure 5.2** and **Figure 5.3**).

The so-called *coagulase model*, which assigned a central role to the **plasminogen activator** encoded by the pPla plasmid, dominated for quite a long time in the question of the blocking mechanism. Although rapid plasminogen activation leads to the destruction of fibrin clots at 37°C / 98.6°F, at the lower temperature that is characteristic of the flea gut, on the contrary, thrombopoiesis and plasmacoagulase activities are observed, so that *Y. pestis*, enveloped and proliferating within the fibrin clot, lodges among the pre-gastric spicules, growing to a complete block. While this model seems logical, it contradicts, for example, the fact that strains that do not synthesise plasminogen activator (i.e. Pla-

[1] Present in all Yersiniae on the so-called chromosomal pigmentation region (*pgm*-region; distinguished by single nucleotide substitutions). Named for their ability to sorb acidic dyes and hemin.

[2] Bacot A. W., Martin C. J. *LXVII. Observations on the mechanism of the transmission of plague by fleas.* J Hygiene (Lond). 1914; 13:423–439; Bacot A. W. *Further notes on the mechanism of the transmission of plague by fleas*. J Hygiene (Lond). 1915; 14:774–776.

[3] 1866–1922; the British entomologist who studied the pathogenesis of plague in fleas. He studied lice and their relationship to trench fever (causative agent: *Bartonella quintana*, formerly *Rickettsia quintana*) and typhus (causative agent: *Rickettsia provazekii*). Died after contracting the latter.

[4] 1866–1955; the British physician, physiologist, member of the Royal Society of London. Director of the J. Lister Institute of Preventive Medicine (1903-1930). Director of the Lister Institute of Preventive Medicine (1903-1930). Author of works on snake toxins, epidemiology.

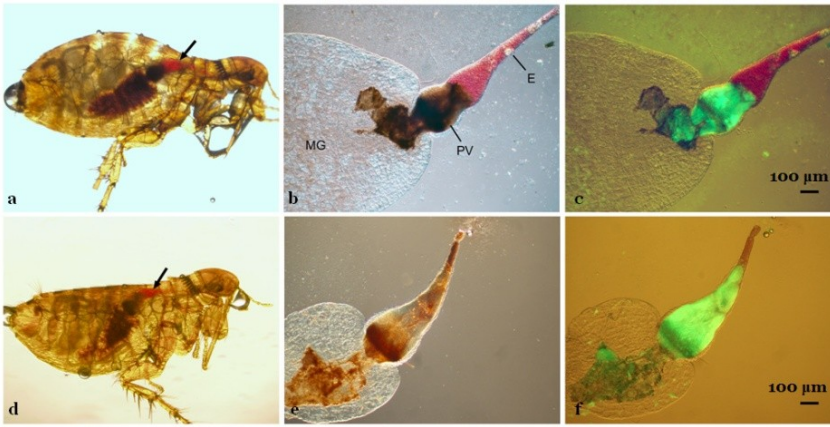


***Figure* 5.2.** *Proventriculus blockade of the flea Oropsylla montana (by Hinnebusch B. J., 2017): a and d – blocked flea, female (a) and male (d) individuals (arrow indicates fresh blood that cannot enter the stomach); b and e - dissection of the digestive tract (differential interference contrast microscopy; E - fresh blood, PV - stomach with blocked proventriculus, MG - midgut); c and f - combination (b and e) with fluorescence microscopy*

negative strains; spelled "Pla-")[1] are also able to block the flea *Xenopsylla cheopis*. Therefore, this model has been replaced by a new model called *the biofilm model*. As discussed earlier, murine toxin promotes the survival of the bacterium in the midgut from the bactericidal breakdown products of red blood cells. At the same time, temperature reduction triggers the production of the transcription regulator RovM, which stimulates the synthesis of *hms* gene products (HmsH and HmsF proteins in the outer membrane and HmsR, HmsS, HmsT and HmsP proteins in the inner membrane), i.e. the formation of an extracellular matrix, which in turn surrounds a dense microcolony of bacteria as they grow, forming a biofilm. In addition, OmpF porin synthesised in response to the acidic gut environment contributes to biofilm formation. The biofilm formed enhances bacterial aggregation on the surface of the proventriculus spicules (**Figure 5.4**), leading to its blockage.

Further, fleas die rather quickly (after 5-10 days), caused by starvation and disruption of the function of the organs cramped by the swol-

[1] In the reverse situation, the strain is called "Pla-positive" or "Pla+".

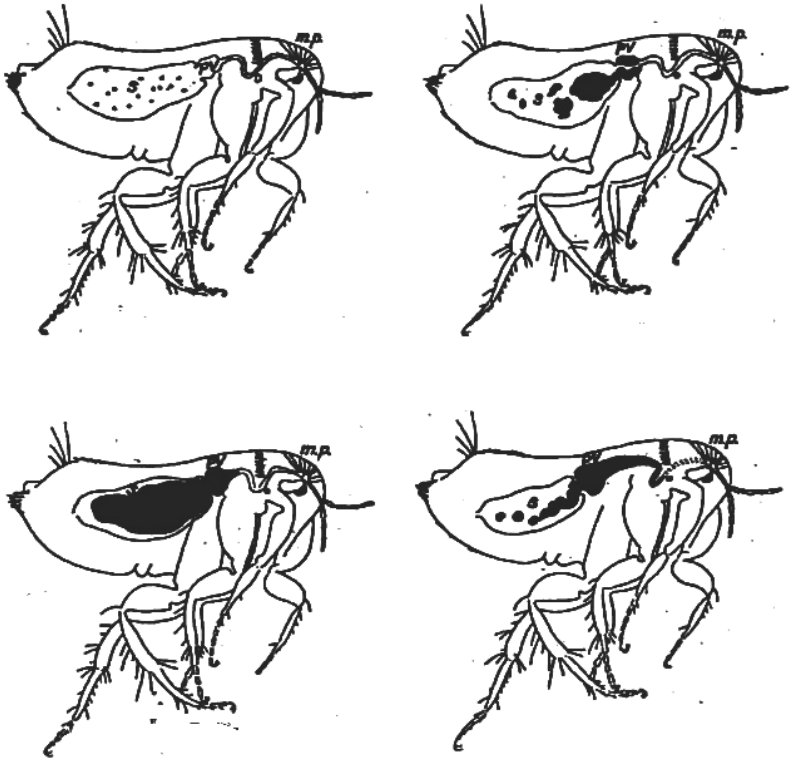


Figure 5.3. *Classical drawing of the process of block formation in the proventriculus of fleas (by Bacot A. W., Martin C. J., 1914; published by permission of The Cambridge University Press)*

len proventriculus. At this time the flea acts as an active vector[1], as each incoming blood portion is regurgitated back into the mammal, carrying *Y. pestis*. However, since the sucked portion is 0.1-0.3 μl, remember that infection of the flea requires *a bacteraemia of at least 10^6 CFU/ml in the carrier*.

Interestingly, *Y. pseudotuberculosis* (as well as all members of the genus *Yersinia*) also has *hms* genes, but experiments show that they do not give the bacterium the ability to form a biofilm and use it to block fleas. But this process is not uniform in *Y. pestis* either. For example, *Y. pestis* strain KIM6+ (2.MED) shows colonisation not only of the proventriculus

[1] A "passive" vector would be a flea eaten by the carrier.



***Figure 5.4**. Bacterial colonisation (shown in yellow) of flea proventriculus spicules, scanning electron microscopy (colour image created by the National Institute of Allergy and Infectious Diseases)*

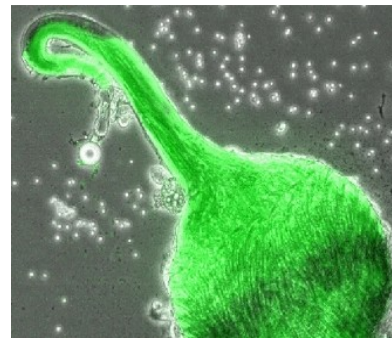
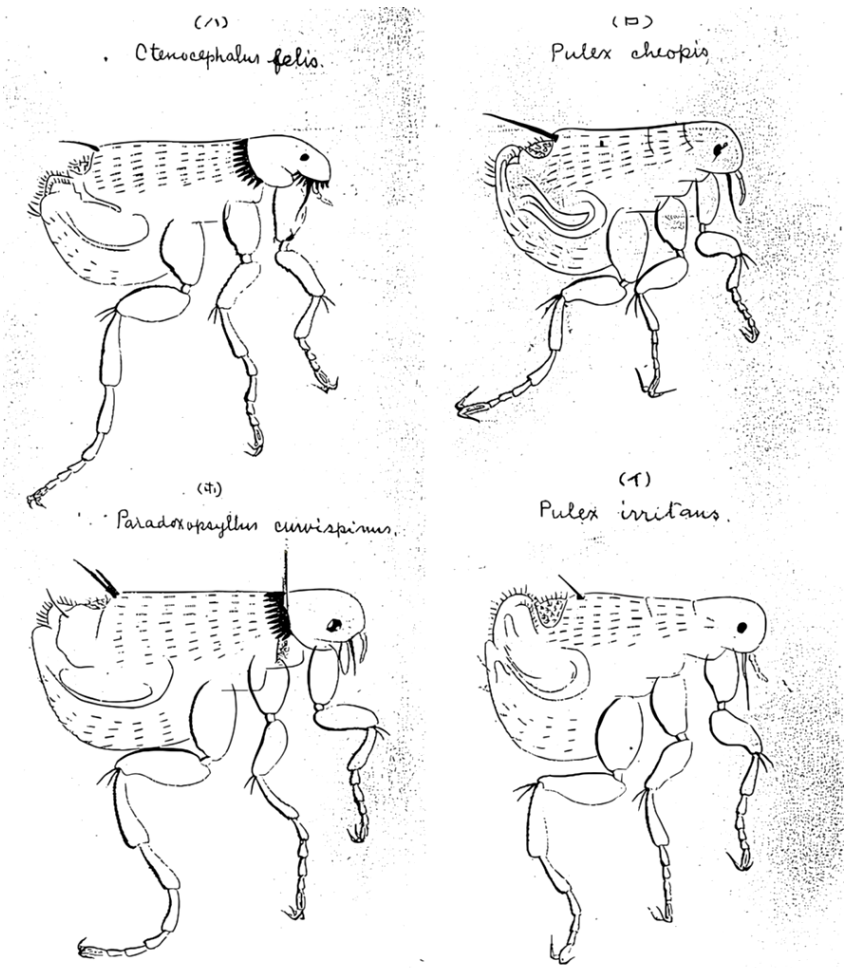


***Figure 5.5.** Colonisation of the entire length of the oesophagus by Y. pestis strain KIM6+ (by Lemon A., 2020)*

but of the entire length of the oesophagus (**Figure 5.5**)! However, the proximity to the oral parts of the flea in this case does not lead to a higher efficiency of regurgitative transmission.

In contrast, an alternative mechanism is possible, as not all fleas are blocked, and in some fleas blocking is partially realised. It is reported that block occurs in *Xenopsylla cheopis* only in 60% of cases, in *Oropsylla montana* in 25% of cases, and in *Frontopsylla semura* fleas parasitising mountain gophers only in 3% of cases! Nevertheless, already members of the Indian Plague Research Commission in 1907 showed[1] the possibility of infecting mammals with such fleas, calling it *the early phase of transmission*, which confirms Arthur William Bacot's assumption that the formation of a plague block (*the late phase of transmission*) is not the only way of transmission of bacteria by fleas. Certainly, one unblocked flea, in which almost the whole blood portion has got into the stomach and middle intestine with almost complete absence of regurgitation (the closed valve of the stomach interferes), is not able to infect a mammal through a bite, but for 10-25 fleas the rate of transmission will already be about 70%. Since this mode of transmission requires the participation

[1] *Indian Plague Research Commission. Further observations on the transmission of plague by fleas, with special reference to the fate of the plague bacillus in the body of the rat flea (P. cheopis)*. J Hygiene (Lond). 1907; 7:395–360.



Drawing of fleas captured during a plague epidemic on Awaji Island, Japan, by Kitasato Shibasaburō (by 北里柴三郎, 1909)

of many fleas, it is also called *mass transmission*. In addition, the realisation of this type of transmission depends on temperature, stopping at 10°C / 50°F, which indicates some protective function of the block, since blocked fleas transmit in the whole temperature range (at 27-30°C/ 80.6-86°F it is somewhat reduced). Of course, early transmission is less effective, but it too plays an important role in the epizootic process,

since block formation takes time and also contributes to the death of up to half of all infected fleas. It is conceivable that mammals may also become infected from dead fleas through alimentary transmission by eating them.

To sum up, it is worth talking about epidemiology. **Figure 5.6** summarises the main transmission routes and mechanisms. As mentioned earlier, plague "lives" in natural foci and exists in them within the epizootic process (black dotted circle), but it is possible to "escape" from it, for example, when rodents[1] and fleas parasitising them migrate during famine, as was the case during the first pandemic. It is also possible that an infected flea may be spread to domestic and farm animals and subsequently infect them if the outbreak is close to residential buildings. This route is mainly realised in the countries of North and South America and Africa. To preserve the outbreak within natural boundaries, a set of measures is implemented, led in some countries by the so-called anti-plague service. Among the measures we should mention the monitoring of epizootic manifestations of plague (including capture of animals and environmental sampling for microbiological indication of the pathogen), as well as preventive and anti-epidemic measures, including reduction (extermination) of carriers and fleas (remember: destruction of at least one link of the epidemiological chain is necessary to stop the spread of infection).

The main mode of spread (**red bold arrows**) is by *vector-borne mechanism*, where a flea, after sucking blood from an infected host, bites another host or a person and the infected blood from the flea enters the blood of the bitten person. It can also be realised directly by biting an infected host, such as a rodent or cat (red dotted arrows).

Other mechanisms should be considered as secondary.

The *contact mechanism* (black solid arrows) realises the transmission of infection through contaminated items such as the patient's clothing and medical instruments (recall the words of Daniil S. Samoylovich-Sushchinsky: "*although [I] cleaned pus from my bistruths and lancets... not only was I always dealing with plague poison, but... the poison was always in my pockets*").

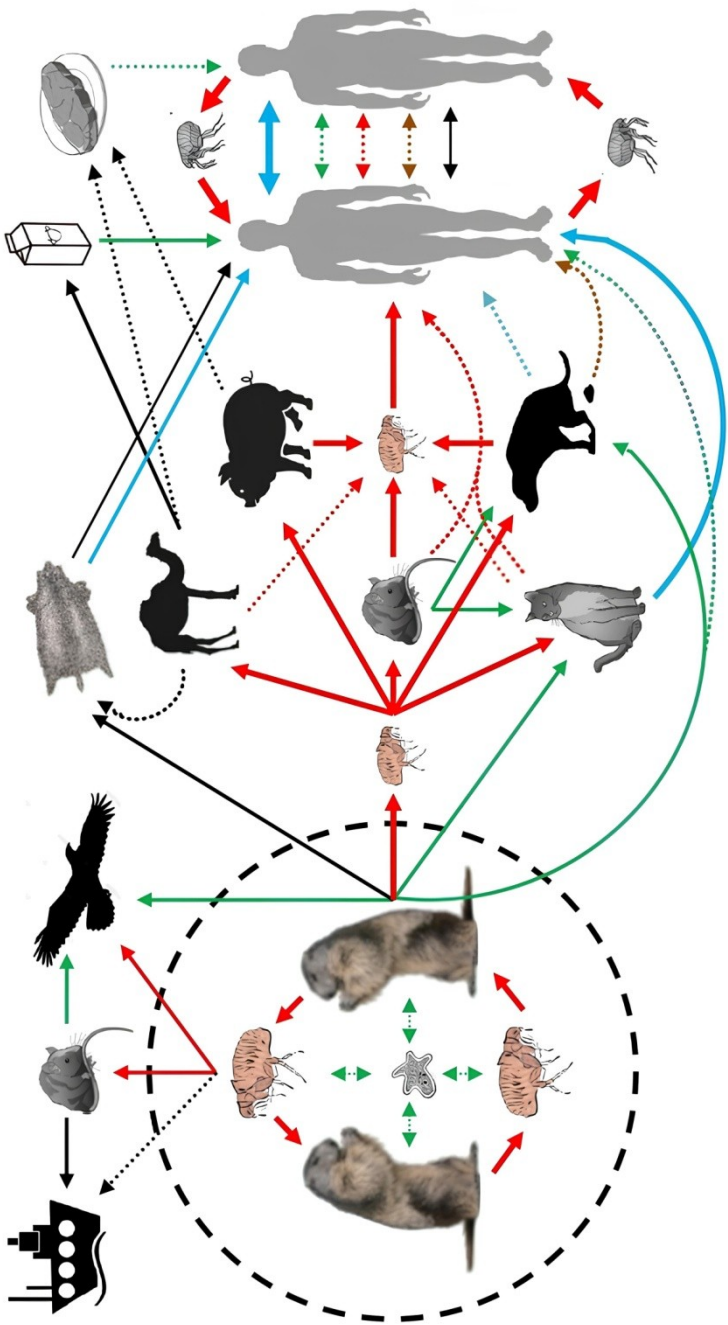
[1] The great jerboa (*Allactaga major*) can reach speeds of up to 50 km/h!

The *aerogenic mechanism* (blue arrows) realises transmission through contact with a patient with pneumonic plague (*airborne transmission*), as well as through cut carcasses of infected animals (*air-dust transmission*).

The *faecal-oral mechanism* (brown arrows) involves transmission through human contact with faeces and urine of an infected animal, most commonly a domestic animal (dog or cat). It is rarely realised. The *alimentary transmission* of this mechanism (green arrows) transmits the infection through eating infected foods that have not been heat-treated (*Y. pestis* dies at 50°C / 122°F in 30-40 minutes, boiling - in a minute).

In addition, there are additional transmissions that are realised by a combination of mechanisms. Thus, a cat infected by flea bite or having consumed a rodent carrier, in addition to transmission by vectorial mechanism via flea, in case of pulmonary form of the disease, can transmit the infection by aerogenic mechanism (airborne transmission). When cutting carcasses of infected animals (gopher, marmot, hare, camel), in addition to the contact mechanism and alimentary transmission, aerogenic mechanism (air-dust transmission through inhalation of infected aerosol by a person engaged in carcass cutting) is possible. In the same way, infection is possible when dissecting a gopher (remember how Hippolyte A. Deminsky died). Poultry can be infected through a flea bite (e.g. *Ceratophyllus borealis*, *Frontopsylla frontalis*) or by eating a rodent carrier. Infection can then be carried outside the centre of the outbreak, where it can be spread through pellet (i.e. compacted undigested food debris), bird carcasses and fleas. Infection in the laboratory may occur through accidents during handling of the culture or through contamination of laboratory animals. In this case, both vector-borne (skin damage) and aerogenic (airborne) mechanisms are possible. Finally, a carrier or a vector can be transported via transport (e.g. ship, aircraft, car) to any corner of the planet, as, for example, the rat that started the first plague pandemic. Of course, when the carrier is travelling alone, it is 'necessary' that a susceptible organism, such as a flea or a person who will eat it, should meet it on arrival, although human infection directly from a rodent through a bite is also possible but rare.

Figure 5.6. *Scheme of transmission routes and mechanisms of plague spread* ►
Dotted lines indicate mechanisms that rarery occur



It is important to realise that the division of transmission into primary, secondary and additional transmission is solely related to the probability of their realisation, but even the most unlikely route, such as eating the pellet of infected birds, can start a new epidemic, since the natural susceptibility of humans to plague is extremely high and does not depend on sex and age. This is probably why the hadith[1] narrated by Aisha bint Abi Bakr (7th century) says that any epidemic (and especially plague) is sent by the Almighty as a punishment and a mercy for man, because suffering from it is equal to the suffering of martyrs. Therefore, "*If you hear of an outbreak of plague in a land, do not enter it; but if the plague breaks out in a place while you are in it, do not leave that place*".

6 THE INNER WORLD

Up to now we have talked a lot about the causative agent of plague, but we still haven't got to know it. Now is the time to remedy this.

Yersinia pestis, belonging to the genus *Yersinia* of the family *Yersiniaceae* of the order *Enterobacterales*, is a Gram-negative small (1–3 x 0.5–0.7 μm) bacillus with polymorphism (long bacilli and cocci may occur). But let us leave the microbiological acquaintance for the corresponding chapter, focusing on the inner world of a bacterium - its genetic structure.

As we have already mentioned earlier, *Y. pestis* genome consists of a chromosome and three plasmids, of which one is genus-specific (i.e., common to all pathogenic bacteria of the genus Yersinia) and two are

[1] Arab. "discourse"; In Islam, a report of the words or actions of the Prophet Muhammad that has been transmitted by reliable transmitters whose spirituality and morality are authentic and beyond question. Every hadith has an unbroken chain of transmitters (insad), which is proof of its authenticity. Collection and study for reliability is carried out by muhaddis, among whom the most authoritative is Imam Muḥammad ibn Ismāʿīl al-Bukhārī (810-870), who for his collection Al-Jāmiʿ al-Ṣaḥīḥ (The Authentic Collection) studied about 700 thousand known hadiths, attributing to reliable about 1%. This hadith is cited by Ṣaḥīḥ al-Bukhārī as 3474, 5728, and 6619.

species-specific ("proprietary"). All three plasmids are non-conjugative (non-transmissible), i.e. unable to be transferred from one bacterial cell to another in the process of conjugation[1]. The first discovery and proof of their role in virulence belongs to the group of scientists from the USSR (diploma for the discovery "Plasma" No. 001 from 27 June 1983; priority from 27 December 1977)[2,3], whose research priority has been unknown to the world science for a long time, because the conducted researches and discoveries were a state secret[4]. Here is how this event is described by Ilya Vladimirovich Darmov[5]: "*Autumn 1976, laboratory in Building 21, I open the rotor of the preparative ultracentrifuge "Beckman", take out a tube with Y. pestis DNA in a gradient of cesium chloride solution with fluorescent dye, illuminate it with ultraviolet light and see two strips: the upper is chromosomal Y. pestis DNA, and the lower is the long-awaited extrachromosomal Y. pestis DNA. Excited, I look for Eugene Viktorovich Smirnov, and together with have been standing for a long time in the bluish semi-darkness of the laboratory and fascinated to look at these glowing orange*

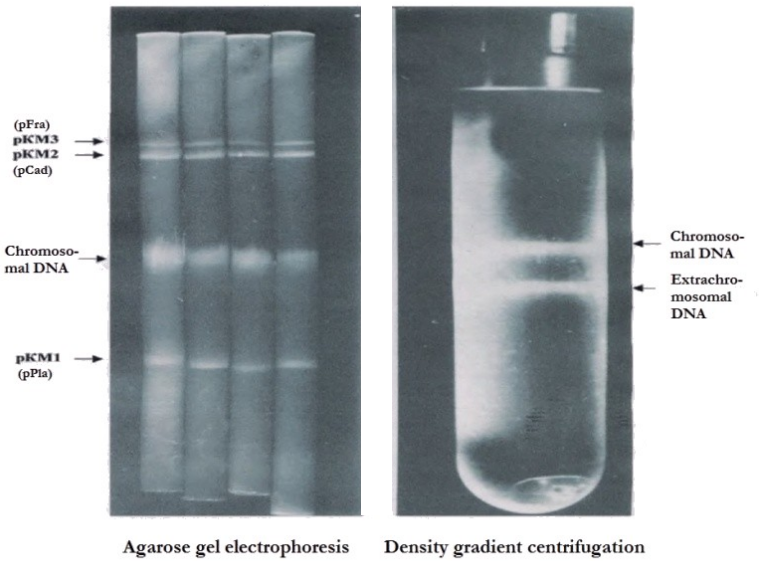
[1] Transmission is only possible by mobilisation by heterologous conjugative plasmids or transduction.

[2] The study was carried out between 1976 and 1978 on the basis of the All-Union Scientific-Research Institute of Applied Microbiology (now – the State Research Center for Applied Microbiology and Biotechnology) in Obolensk and the Scientific-Research Institute of Epidemiology and Hygiene of the Red Army (now – Branch Office of 48th Central Scientific Research Institute of the Ministry of Defense of the Russian Federation) in Kirov. It is known that Igor V. Domaradsky and his colleagues took part in the study from the first organization (de facto conducted research at the All-Union Scientific-Research Institute of Biosynthesis of Protein Substances; only he is listed in the Diploma), and Soviet and Russian military physicians Timeryan Gabdrakhmanovich Abdullin (Тимерьян Габдрахманович Абдуллин; 1926–2022), Ilya Vladimirovich Darmov (Илья Владимирович Дармов; b. 1952) and Eugene Viktorovich Smirnov (Евгений Викторович Смирнов; ?-?) took part in the study from the second organisation. Igor V. Domaradsky writes: "*My laboratory worked with vaccine strains EV, 1 and 17, while SRIEH [i.e. the Scientific-Research Institute of Epidemiology and Hygiene of the Red Army] also used virulent strains, and worked with the DNA*" (see Домарадский И. В.. *Чума*, М.: Медицина, 1998, 174 с.).

[3] Indirect indications of the presence of *Y. pestis* plasmids (specifically "pesticinogenicity plasmids") were obtained by Soviet physicians G.Yu. Pak (1969) and E. G. Koltsova (1970).

[4] In the United States, the first report of *Y. pestis* plasmids did not appear until 1981 (see Ferber D. M., Brubaker R.R. *Plasmids in Yersinia pestis*. Infect Immun. 1981; 31(2):839-841; DOI: 10.1128/iai.31.2.839-841.1981)

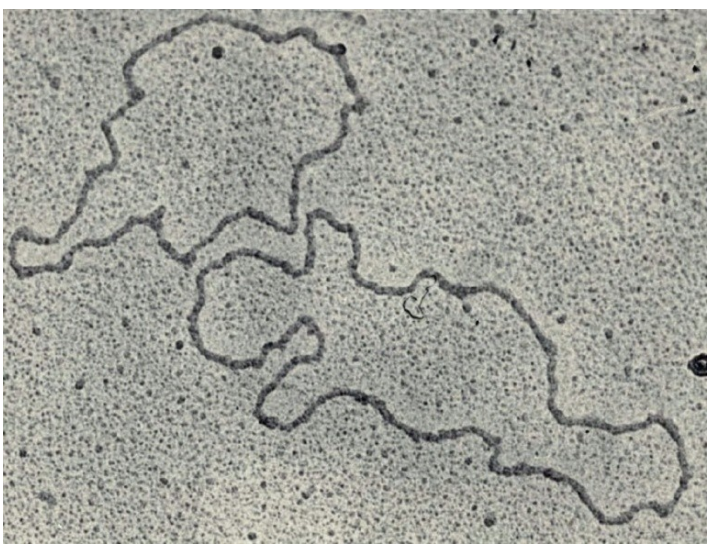
[5] Илья Владимирович Дармов; b. 1952; the Soviet and Russian military physician, bacteriologist. Head of the 48th Central Scientific Research Institute of the Ministry of Defense of the Russian Federation (2010-2012).



Results of experiments on the detection of extrachromosomal Y. pestis DNA (1976). Courtesy of Prof. Ilya Vladimirovich Darmov

▲ *Agarose gel electrophoresis (more in Chapter 36) and density gradient centrifugation (modern plasmid names are stated by the author for convenience)*

▼ *Electron micrographs of plasmid pPla (left) and plasmid ColE (right) taken as a standard for contour length determination*



stripes, two fractions, which concentrated all the hereditary information of the plague pathogen. Now the genome of the plague pathogen, as well as the genomes of other pathogens of especially dangerous infections is studied in detail and fully sequenced, but at that time all this was covered by the darkness of the unknown and aroused great interest"[1].

The discovered plasmids were named pKM1, pKM2 and pKM3 (Kirov-Moscow plasmids), but these names are not used because the discovery was kept secret even after it was made by specialists in other countries. Now pKM1 is called pPla (other names for pYP, pPst и pPCP1); pKM2 is called pCad (other names for pYV, pVW, pLcr and pCD1); pKM3 is called pFra (other names for pYT and pMT1). The genome size varies depending on the strain: for chromosome it is 4.5–4.78 Mb[2], for pCad plasmid it is 68.1–71.5 Kb[3], for pFra plasmid it is 96.2–137 Kb[4] and for pPla plasmid it is 9.6–10.7 Kb[5].

The genome is extremely rich in insertion (i.e. mobile) sequences obtained as a result of horizontal gene transfer (mainly from other bacteria), and shows anomalies of guanine-cytosine base shifts, when they are abundant or scarce in a certain region of the nucleic acid, indicating frequent intragenomic recombination. In addition, as mentioned earlier, the genome contains 150 pseudogenes, many of which are remnants of the "former youth" – enteropathogenic (i.e. intestinal) lifestyle of *Y. pseudotuberculosis*.

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[1] Дармов И. В., Янов С. Н., и др. *История открытия собственных плазмид чумного микроба.* Вестник войск РХБ защиты. 2024; 8(2):185–198; DOI: 10.35825/2587-5728-2024-8-2-195-198

[2] Since DNA is a double-stranded molecule made up of nitrogenous nucleotide bases, the length of the entire molecule is usually expressed in the number of nucleotide pairs (np) or base pairs (bp), which is English for base pair, so a thousand bp is expressed in kilobases (Kb), a million bp in megabases (Mb), and a billion bp in gigabases (Gb).

[3] Molecular mass of the subsp. *pestis* – 46 MDa (ANT, foci No. 37, 41), 47 MDa (ANT, MED, ORI) and 48 MDa (ANT, focus No. 35); Talas strains – 46 MDa; Ulegei and Hissar strains – 47 MDa; Caucasian strains – 50 MDa.

[4] Molecular mass of the subsp. *pestis* – 64 MDa (ORI) and 67 (ANT, MED); ulegeica strains – 67 MDa; hissarica and talassica strains – 70 MDa; altaica strains – 72 MDa; caucasica strains – 82 MDa.

[5] Molecular mass of the subsp. *pestis* – 6.4 MDa; Altai strains – 6.4 MDa (focus No. 36; 10% of isolated strains may lack the plasmid) and 6.8 MDa; Ulegei, Hissar and Talas – 6.4 MDa. Caucasian strains lack the plasmid.

The chromosome

The chromosome contains the pigmentation *pgm*-region, which includes the *hms* locus (remember: it encodes hemin accumulation proteins, which play a leading role in the creation of biofilm and block in fleas) and the **high pathogenicity island** (HPI) 35–45 Kb in size, on which the genes of the ybt region are localised, participating in the biosynthesis of the siderophore (Greek "iron carrier") **Yersiniobactin** (Ybt), which is a type of metallophore (i.e. metal carrier). The Ybt molecule forms a stable complex with iron (Ybt-Fe^{3+}) due to five "active centres" (benzene ring, two thiazolidine rings and two thiazoline rings). This complex is recognised by the appropriate receptor on the bacterial outer membrane and translocates into the cytosol, where the iron is released and used by the bacterium. That is, it "hijacks" the iron bound to host proteins (e.g. haemoglobin), which enables the bacterium to survive in iron-depleted environments and multiply in mammalian blood. Accordingly, loss of HPI renders the strain avirulent[1]. Another recently discovered metallophore is **Yersinopin** (one "active centre" – imidazole ring), which is homologous to opines synthesised by some filamentous fungi, mosses and bacteria (e.g. *S. aureus*, *P. aeruginosa*) to "capture" zinc, iron and nickel ions.

Next to the *pgm*-region are genes responsible for the secretion of porins – outer membrane proteins that act as pores, responsible for the transport of metabolites across the outer membrane and the release of ions to maintain osmotic pressure. In particular, the porins OmpF, OmpA and TolC ensure the survival of the microbe as L-forms in sodium chloride medium. Interestingly, many highly virulent strains carry the *ompY* gene (responsible for secretion of the OmpY porin) inactivated by the IS100 element, which can lead to elimination (disappearance) of the *pgm*-region (and hence HPI, rendering the strain avirulent). This ability seems to be a peculiar regulator of the number of highly virulent strains characterised by the lightning-fast infectious process. Selective

[1] Some strains of SNP-type 0.PE4 (Central Asian) have HPI but are avirulent for humans. Comparative analysis of *ybt* genes showed that in the *ybtS* gene of these strains, at position 661, there is a substitution of cytosine for thymine (write: "C→T"), which leads to the synthesis of the amino acid serine (instead of proline) in the YbtS protein. It is likely that this leads to the inability to synthesise yersiniobactin and is therefore one of the reasons for avirulence.

elimination of the *pgm*-region ensures stable epizootics with dominant participation of strains with selective virulence, the infectious process of which is slower, but which do not have an IS100 element (strains of non-main subspecies). This is logical, since the absence of such a "regulator" would quickly lead to the death of all carriers and, consequently, to the end of epizootics in the centre. This idea is also confirmed by the fact that introduction of the IS100-element into the porin gene in *Y. pseudotuberculosis* does not lead to elimination of the *pgm*-region.

Also on the chromosome is the *psa*-operon[1], which encodes the synthesis of **pH6-antigen** (pH6-Ag; Psa; PsaA) at 37°C / 98.6°F and pH ≤ 6 (acidic medium), which is a fimbriae (pili), i.e. filamentous protein structures located on the surface of the bacterial cell. This antigen ensures binding of the bacterium to the cells of the alveolar epithelium by binding to phosphatidylcholine located on the surface of the epithelial cell membrane. It also provides inhibition (i.e. reduction, slowing down) of macrophage phagocytosis. Previously, it was thought that this was achieved by adhesion (i.e. attachment) of bacteria by pili (pH6-antigen) followed by delivery of Yops (discussed below), the synthesis of which is encoded by the pCad plasmid. However, experiments[2] using strains synthesising and not synthesising pH6-antigen showed that there is no difference in attachment to macrophages, but there is a significant difference between the distribution of cells within the macrophage. For example, the strain synthesising the pH6-antigen (Psa-positive)[3] showed approximately twice the number of microbial cells captured (**Figure 6.1**). Thus, inhibition of phagocytosis in this case is provided not by adhesion and delivery of Yops, but by prevention of bacterial cell capture by macrophage (i.e. bacteria are attached to macrophage, but due to pH6-antigen they are not absorbed by it). It should be reminded here that pH6-antigen is synthesised at pH≤6 (in the phagolysosome of macrophage), so it provides protection only against reabsorption by macrophages, when the infectious process from the intracellular phase has

[1] Functional unit of the genome in prokaryotes.

[2] Huang X.-Z., Lindler L. E. *The pH6 antigen is an antiphagocytic factor produced by Yersinia pestis independent of Yersinia outer proteins and capsule antigen.* Infect Immun. 2004; 72(12):7212–7219; DOI: 10.1128/IAI.72.12.7212-7219.2004.

[3] It is customary to write "Psa$^+$" or "PsaA$^+$" to distinguish them from strains that do not synthesise the pH6 antigen or are Psa-negative (written "Psa-" or "PsaA-"). This entry indicates the absence (or presence) of a factor in the strain.

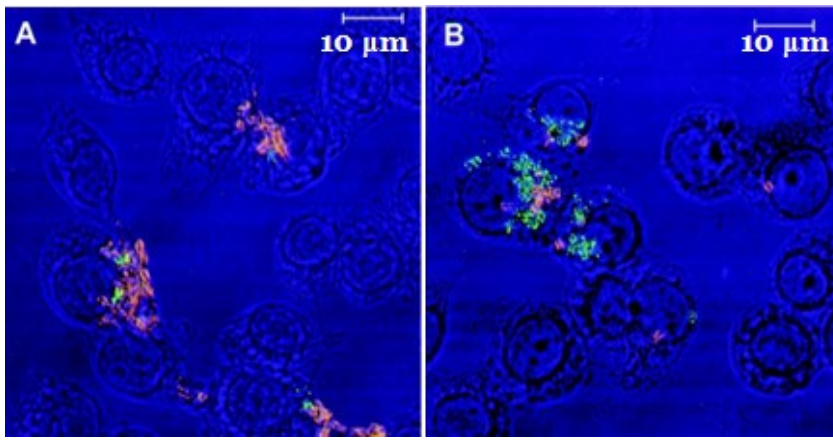


Figure 6.1. *Confocal fluorescence microscopy of RAW264.7 macrophages infected with strains of Y. pestis. Bacterial cells inside macrophages are labelled in green, outside macrophages in orange (Huang X.-Z., 2004; published under licence from The American Society of Microbiology, permission granted through Copyright Clearance Center, Inc):*
A - Y. pestis strain KIM5-3001 (PsaA+); B, Y. pestis strain KIM5-3001.1 (PsaA-)

moved to the extracellular phase. In addition, pH6-antigen agglutinates erythrocytes, which releases iron for its subsequent capture by yersiniobactin, and also binds to plasma lipoproteins containing apolipoprotein B (apoB), which prevents the pathogen from being recognised by the immune system of body (while antibodies to apoB do not prevent the bacterium from binding to lipoproteins).

In addition, the chromosome contains the ail gene, which encodes the synthesis of the **Ail protein** (attachment invasion locus [protein]) located on the outer membrane. This protein has several functions. Firstly, it provides serum resistance by preventing bacterial lysis over the entire temperature range (from 6°C / 42.8°F during hibernation to 37°C / 98.6°F in the mammalian body), which is especially important in the absence of YadA adhesin synthesis (recall: the *yadA* gene in *Y. pestis* is inactivated). Secondly, Ail ensures binding of the bacterium to the mammalian cell, including through interaction with components of the extracellular matrix, which increases the rate of delivery of Yops that destroy the mammalian cell from the inside (cytotoxic action). The de-

livery of Yops is most often to macrophages, which act as host cells. Thirdly, Ail provides invasion of the bacterium into non-professional phagocytes (fibroblasts, epithelial cells, etc.) and promotes autoaggregation, i.e. formation of clusters (aggregates), which plays an important role both in the formation of buboesis (formation of conglomerates in lymph node macrophages) and the development of the pulmonary form of the disease

The sections of the chromosome that are prophages – genomes of incorporated temperate bacteriophages - deserve special attention. The first temperate bacteriophage of *Y. pestis*, called **phage H**, was isolated[1] in 1965 by Nikolai Nikolaevich Novoseltsev[2] from the organs of a guinea pig killed by plague. The following year he also isolated the second temperate bacteriophage H-I from a culture of *Y. pestis* strain EV line NIIEG (Russ. НИИЭГ, see Chapter 26 for more details on this strain).

In addition to the previously discussed bacteriophage Ypfφ, the filamentous bacteriophage CUS-2, whose genome is also embedded in the chromosome of *Y. pestis*, deserves special attention. This prophage inactivates the *rovA* gene encoding the RovA transcription regulator, which is produced at 37°C / 98.6°F and activates the *psa*-operon. In other pathogenic *Yersiniae*, however, RovA is involved only in invasion, i.e. integration of the prophage has altered its function. In the previous chapters, we mentioned that the synthesis of extracellular matrix for biofilm formation is carried out by the products of hms genes, the synthesis of which is stimulated by the production of the transcription regulator RovM. Thus, we can conclude that the genes responsible for the synthesis of the RovM and RovA regulators are switched on alternately depending on temperature, and all the complex processes leading to flea blockage and resistance to phagocytosis (in fact, survival both inside a cold-blooded vector and a warm-blooded host) became possible due to the successful integration of the genome of the bacteriophage CUS-2 into the *Y. pestis* genome.

[1] Новосельцев Н. Н. *Умеренный фаг чумного микроба*. Автореферат дис. … доктора медицинских наук, Саратов, 1973, 44 с.

[2] Николай Николаевич Новосельцев, ?-?; the Soviet physician, bacteriologist.

pCad plasmid (pKM2, pYV, pVW, pLcr and pCD1)

A genus-specific plasmid, named *calcium-dependence plasmid* because of its ability to determine the thermoregulatory dependence of cells on calcium ions. It is also referred to as *a virulence plasmid*. First of all, it is interesting because it carries the so-called LCR genes (low-Ca^{2+}-response), which at 37°C / 98.6°F in response to low Ca2+ content provide the synthesis of the virulence protein **LcrV**, discovered[1] in 1956 as a V-antigen[2]. In case of contact with a mammalian cell, it plays a role in the realisation of the type III secretion system (T3SS)[3] – penetration into the mammalian cell. An alternative mechanism is also possible (**Figure 6.2**), when LcrV penetrates from a bacterial cell into a mammalian cell with the help of a LcrV-transporting contact-activated translocator (VCAT). Once in the cytoplasm of a mammalian cell by one route, LcrV probably plays an immunosuppressive role (suppression of TNFα and IFNγ). On the other hand, LcrV synthesis stimulates the secretion of **Yersinia outer proteins** (Yops), whose genes are scattered throughout the plasmid. LcrV interacts with LcrG, which allows the proteins (primarily YopB, YopD and YscF) to organise a structure that is commonly referred to as a molecular needle (the "free" LcrG blocks its construction, so LcrV "pushes back" LcrG as if it were a gate), and then aims the needle to contact the mammalian cell (target cell), allowing the effector Yops (YopE, YopH, YopJ/YopP, YopM, YpkA/YopO) to penetrate into the cytoplasm of the mammalian cell (**Figure 6.2**). These proteins work together to disrupt the mammalian cell cytoskeleton and disrupt the regulation of signalling pathways. For example, YopE mimics GTPase-activating proteins, resulting in suppression of Rho GTPase signalling proteins that play important roles in a multitude of common cellular functions. YopT cleaves and releases membrane-bound Rho GTPases. YopJ is an acetyltransferase that blocks the signalling of both mitogen–

[1] Burrows T.W., Bacon G.A. *The basis of virulence in Pasteurella pestis: an antigen determining virulence*. Br J Exp Pathol. 1956; 37(5):481–493.

[2] Discovered together with surface lipoprotein W-antigen (function unknown) and for some time they were combined into VW-antigen due to the difficulty of separation (см. Burrows T.W., Bacon G.A. *V and W Antigens in Strains of Pasteurella pseudotuberculosis*. Br J Exp Pathol. 1960; 41(1): 38-44). The name "V-antigen" is still found in the scientific literature.

[3] A type of bacterial secretion system by which proteins from the bacterial cell penetrate the host cell (mammalian cell). Some bacteria use this system to modify the structure of the host cell wall in order to subsequently penetrate the cell.

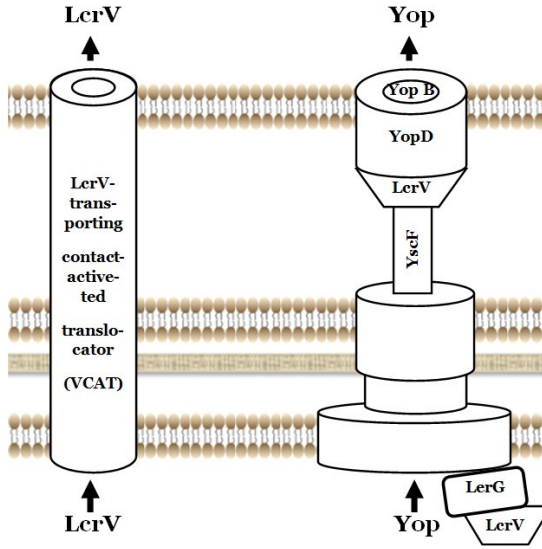


***Figure 6.2**. Schematic (not to scale) of penetration of LcrV and Yops into mammalian cell cytoplasm by VCAT and T3SS*

activated protein kinase and transcription factor-κB (read "kappa-bi"), thereby inhibiting the production of pro-inflammatory cytokines. Interestingly, YopH, which catalyses the dephosphorylation of specific macrophage proteins, leading to, among other things, reduced interleukin production (and consequently reduced immune system function), while probably of eukaryotic origin

To some extent, effector Yops can be considered as the bacterial equivalent of viral oncoproteins, as they mimic or capture cellular signals more efficiently than their eukaryotic counterparts, causing dysregulation of normal cellular functioning by excessive activation or inhibition of the signalling pathway, which leads to the indicated consequences. At the same time, unlike viral oncoproteins, Yops remain dormant in the bacterial cell, becoming "active" only in target cells (in response to changes in temperature and calcium content).

It is important to emphasise that under acidic conditions (pH ≤ 6), the expression of YopE, YopN and YopD is significantly reduced, so the pH6-antigen discussed earlier acts as an independent factor in the resistance of *Y. pestis* to phagocytosis.

pFra plasmid (pKM3, pYT and pMT1)

Y. pestis probably acquired this plasmid from *Salmonella enterica* subsp. *enterica* ser[1]. typhy by horizontal transfer, as it is 97% identical to the plasmid pHMC2 of this species. The pFra plasmid contains a *caf*-operon (capsular antigen fraction)[2], which encodes the synthesis of **capsular antigen** or **fraction I** (Fra[3], F1 and FI), discovered by Alexandre Yersin but isolated[4] in pure form by Edgar E. Baker[5] (and colleagues) in 1952 (Baker's method[6]). Capsule antigen is a multifunctional protein existing in three isomorphs[7]:

Caf1$_{NT1}$ (global type)

Caf1$_{NT2}$ (found in foci No. 4–7)

Caf1$_{NT3}$ (found in focus No. 39)

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[1] It denotes a serovariant, i.e. a group of microorganisms of the same species united by a common antigenic structure determined by serological methods.

[2] The gene bank in *E. coli* that Soviet and British chemist Andrei Vladimirovich Karlyshev (Андрей Владимирович Карлышев; b. 1953), with his a coll., constructed from *Y. pestis* EV DNA fractionated into chromosomal and plasmid components allowed the production of an *Escherichia coli* clone synthesising capsule antigen. Further studies showed that capsule formation is controlled by *caf*-operon genes. It is noteworthy that the results of these studies, published in 1990-1992, refuted the then existing assumption that the genes responsible for the synthesis of capsular antigen are located on the chromosome. All this became possible thanks to the cosmid vector pHC79, which made it possible to clone (introduce into *E. coli*) large fragments of the genome, which is very critical, since the synthesis of capsule antigen requires the presence and expression of a whole cluster of genes.

[3] Hence the main name of the plasmid ("p" – **p**lasmid + "Fra" – **fra**ction).

[4] Baker E. E., Sommer H., et al. *Studies on Immunization Against Plague: I. The Isolation and Characterization of the Soluble Antigen of Pasteurella Pestis*. J Immunol. 1952; 68(2):131–145; DOI: 10.4049/jimmunol.68.2.131.

[5] ?-?; the American physician, bacteriologist.

[6] *Y. pestis* culture grown for three days on a dense nutrient medium at 37°C / 98.6°F is washed with 0.9% sodium chloride solution and dehydrated twice with cooled acetone. Then fraction I is extracted with 2.5% sodium chloride solution at room temperature for 24 hours, after which it is isolated from the salt solution with saturated ammonium sulfate solution (pH 7,5).

[7] Have single nucleotide substitutions: Caf1$_{NT2}$ has serine instead of alanine at position 48, Caf1$_{NT3}$ has valine instead of phenylalanine at position 117 (see Kopylov P. K., Platonov M. E., et al. *Yersinia pestis Caf1 Protein: Effect of Sequence Polymorphism on Intrinsic Disorder Propensity, Serological Cross-Reactivity and Cross-Protectivity of Isoforms*. PLoS One. 2016; 11(9):e0162308; DOI: 10.1371/journal.pone.0162308)

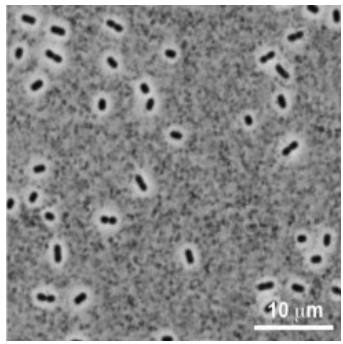


Figure 6.3. *Capsule production by Y. pestis strain VTnF1 grown at 37°C / 98.6°F (by Derbice A., 2015)*

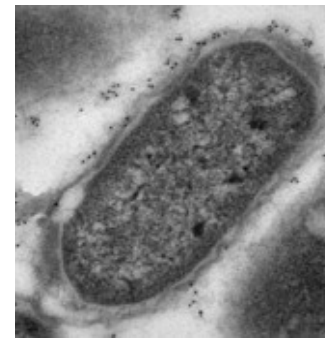


Figure 6.4. *Capsule covering the surface of a bacterium*

Capsular antigen (fraction I) is synthesised at 37°C / 98.6°F (at 28°C / 82.4°F it is synthesised 1000 times less!) and forms a large gel-like polymer shell (capsule) covering the entire outer surface of the bacterium (**Figure 6.3** and **Figure 6.4**). Visually (light microscopy) it is an amorphous substance, which at high resolution (electron microscopy) presents a structure of individual fimbrile-like strands up to 200 nm long, diverging in different directions from the bacterial surface. A number of structural elements of capsular antigen are homologous to some interleukins (e.g. IL-1β), which may cause the former to compete for binding to receptors on lymphoid cells, preventing the development of an adequate immune response. It is important to understand that, unlike the T3SS, the mechanism of resistance to phagocytosis is provided not by the introduction of proteins into the phagocyte with subsequent cytotoxic action, but by blocking the possibility of bacterial uptake by the phagocyte. Implementation of this mechanism is rather delayed in time (up to 5 hours), but it reduces the probability of phagocytosis from 30 to 5%. At the same time, there are strains that, although not capable of synthesising capsular antigen (fractionless strains), are also capable of high virulence.

In addition, the pFra plasmid contains the *ymt* gene that triggers at 28°C / 82.4°F the synthesis of Ymt (in analogy to capsular antigen, this is sometimes referred to as Fraction II or FII) which was discovered[1] in 1955

[1] Ajl S. J., Reedal J. S., et al. *Studies on plague. I. Purification and properties of the toxin of Pasteurella pestis*. J Bacteriol. 1955; 70(2):158–169; DOI: 10.1128/jb.70.2.158-169.1955.

and about which we have discussed quite a lot earlier. Recall that the toxin is essential for the survival of *Y. pestis* in the intestine of the flea to form the plague block with the participation of the *pgm*-region.

pPla plasmid (pKM1, pYP, pPst and pPCP1)

This plasmid appears to have been acquired by *Y. pestis* as a result of horizontal gene transfer from the same Salmonella species, but of a different serovar, *Salmonella enterica* subsp. *enterica* ser. typhimurium. It contains the *pla*, *pst*, and *pim* genes, which encode the synthesis of plasminogen activator, bacteriocin (pesticin), and pesticin immunity protein, respectively.

The **plasminogen activatior** (Pla) is a surface protease (i.e. an enzyme that cleaves peptide bonds between amino acids in proteins), and since it has high homology with four surface proteases of other *Enterobacteriaceae* (PgtE – *Salmonella enterica,* SopA – *Shigella flexneri,* OmpT and OmpP – *Escherichia coli*), all of them are grouped into one family of omptin proteases (OmpT; note: not to be confused with porin OmpY!), which cleave the peptide bond with the help of a water molecule. Interestingly, at different temperatures, plasminogen activator is able to exhibit both *fibrinolytic activity* (37°C / 98.6°F) and *plasma coagulase activity* (28°C / 82.4°F). The former was shown by R. R. Madison[1] in 1936[2], the second by Ernest Jawetz[3] and Karl Friedrich Meyer[4] in 1944[5], but the relationship between these properties was proved by the already mentioned Igor V. Domaradsky (with colleagues) only in 1963[6]. Plasminogen activator plays an important role in the initial stages of the pathogenetic process in the bubonic form of plague. Through plasmocoagulase activity, it cleaves and inactivates tissue factor path-

[1] ?-?; the American physician, bacteriologist.

[2] Madison R. R. *Fibrinolytic specificity of B. pestis.* Proceeding of the Society for the Experimental Biology and Medicine. 1936; 34(3):301–302; DOI: 10.3181/00379727-34-8597G.

[3] 1916–?; the Polish-born Austrian and American physician and bacteriologist. Emigrated to the United States in 1939 to escape the Nazis.

[4] 1884–1974; the Swiss and American veterinarian, bacteriologist, pathologist. A major researcher of many infectious diseases, called "Pasteur of the 20th century".

[5] Jawetz E., Meyer K. F. *Studies on plague immunity in experimental animals. II. Some factors of the immunity mechanism in bubonic plague.* J Immun. 1944; 49(1):15–30.

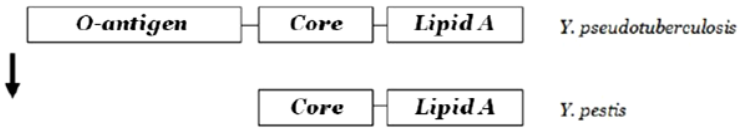
[6] Домарадский И. В., Яромюк Г. А., и др. *О коагуляции плазмы чумным и псевдотуберкулёзным микробами.* Бюллетень экспериментальной биологии и медицины. 1963; 7:79–82.

way inhibitor (TFPI), allowing blood coagulation at the site of entry into the mammal. Then, due to fibrinolytic activity arising under the action of body temperature, it provides activation of plasminogen (hence its name), which in its active form (plasmin) causes destruction of fibrin clots, allowing at the bite site to form a large bacterial focus with a small number of inflammatory cells that do not interfere with the dissemination of the pathogen to the lymph nodes. In the reverse situation, when plasminogen activator is not synthesised, a multitude of inflammatory cells quickly block the pathogen cells, significantly limiting their dissemination, which is the case in strains lacking the pPla plasmid (remember: Caucasian strains). It participates in resistance to the complement system (i.e. the cascade of enzymes that facilitate the fight against the infectious agent) by destroying the C3 component, and also exhibits proteolytic activity (i.e. the ability to break down proteins). In the case of the primary pulmonary form of plague, plasminogen activator is not required for dissemination but provides an abrupt and rapid multiplication, leading to the development of lightning pneumonia and pulmonary oedema, i.e. the fulminant pulmonary form that we have already mentioned in Chapter 3. The role of plasminogen activator in the development of the primary septic form (when the microbe enters the bloodstream directly from outside) has not been recognised.

It is important to note that plasminogen activator is active only by binding to the endotoxin **lipopolysaccharide** (LPS), discovered[1] in 1956. Lipopolysaccharide, the synthesis of which is encoded by chromosomal genes, is inherent in all Gram-negative bacteria and, being "anchored" in the bacterial membrane, provides structural integrity of the bacterium, protecting its membrane from the effects of chemical agents, such as antibiotics. It consists of *lipid A*, a "moored" disaccharide, and polysaccharide chains of *O-antigen* (O-side chains), which are connected to each other by an oligosaccharide chain, i.e. the so-called *oligosaccharide* or *core* (S-form of LPS). Lipid A is responsible for its high toxicity because, when released into the bloodstream, it is capable of inducing a systemic immune response accompanied by fever up to septic shock. As part of the LPS, it is shielded by the other structures, just as a Christmas tree shields the presents placed under it from children's eyes with its luxuriant branches. LPS of *Y. pestis* is characterised by the absence of the

[1] Davies D. A. L. *A specific polysaccharide of Pasteurella pestis*. J Biochem. 1956; 63:105–116.

O-antigen (R-form of LPS) due to five acquired mutations in the genes synthesising LPS, but this allows the bark part to bind to the plasminogen activator and influence the stereometry (spatial structure) of its molecule.



LPS structure is temperature-dependent and varies at 37°C / 98.6°F (mammalian), 27°C / 80.6°F (environmental/flea) and 6°C / 42.8°F (mammalian overwintering).

Core is quite conserved. It consists of three residues of L-*glycero*-*α*-D-*manno*-heptose (LD-Hep) and two residues of 3-deoxy-*α*-D-manno-oct-2-ulosonic acid (Kdo)[1]. Its inner region acts as a receptor for most lytic bacteriophages used in diagnostics, such as φA1122. As temperature changes, stereometric changes occur in the part closest to lipid A: replacement of the side residue of 3-deoxy-*α*-D-manno-oct-2-ulosonic acid (Kdo) by a residue of D-glycero-*α*-D-talo-oct-2-ulosonic acid (Ko). At 37°C / 98.6°F it is Kdo, at 27°C / 80.6°F it is Kdo + Ko, and at 6°C / 42.8°F it is Ko. However, these changes do not affect the fibrinolytic and plasma coagulase activities, especially since in some subspecies (e.g., Caucasian strains) no substitution occurs at all. On the other hand, loss of the part of the cortex most distant from lipid A (its shortening) leads to a complete loss of fibrinolytic and plasma coagulase activity.

Lipid A, in contrast, is not conserved (**Figure 6.5**). At 27°C / 80.6°F it is most often 6-acyl (i.e. it contains six fatty acid residues[2]), and at 6°C / 42.8°F and 37°C / 98.6°F it is 4-acyl[1], which is not recognised by the Toll-like receptors (TLRs)[2] of the immune system, in particular TLR-4,

[1] Interestingly, the core of LPS of Caucasian, Altai, and Hissar strains differs from the core of LPS of strains of the main subspecies and Ulegei strains by the absence of a D-glycero-D-manno-heptose (DD-Hep) residue in the heptose residue furthest from lipid A (LD-HepIII).

[2] Four residues of 3-hydroxymyristic acid, one residue of lauric acid and one residue of palmitoleic acid. The synthesis of 3-acyl, 4-acyl, 5-acyl forms is also possible at this temperature.

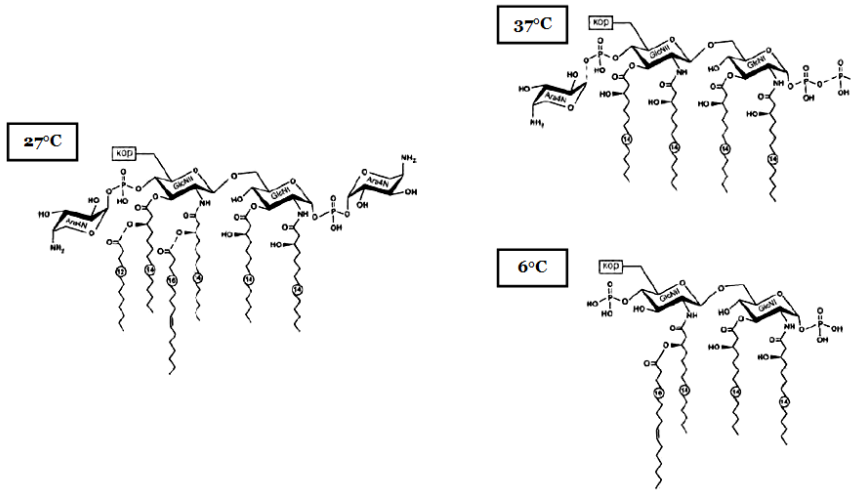


Figure 6.5. *Structures of lipid A at different temperatures (by Knirel Yu. A., 2012)*

but more on this later. Now it is important to understand that the presence of 4-acyl LPS at 6°C / 42.8°F is one of the mechanisms of survival ("evasion" from the immune system) of *Y. pestis* in the host body during winter hibernation, when its body is cooled to approximately this temperature. It should also be noted that at the same synthesis of plasminogen activator its activity at 37°C / 98.6°F is higher, hence, 4-acyl lipid A in the LPS composition has a better effect on the stereometry of plasminogen activator of *Y. pestis*. This idea is confirmed by a number of authors who point[3] out that the dependence of fibrinolytic and plasma coagulase "activity"of plasminogen activator on temperature is only

[1] Four residues of 3-hydroxymyristic acid. Synthesis of 3-acyl and 6-acyl forms is sometimes encountered at this temperature.

[2] Germ. "cool"; named after the gene of the same name in Drosophila flies, which in turn is named after the line "Das war ja toll!" (Germ. "What a class!") said by German biologist Christiane Nüsslein-Volhard (b. 1942), said at the time of the discovery of mutant Drosophila larvae with an underdeveloped ventral body part. The first mammalian receptor, TLR-4, was discovered in 1997 by the Soviet (Uzbek) and American immunologist Ruslan Maksutovich Metzhitov (Руслан Максутович Меджитов; b. 1966) with his collegues.

[3] Korhonen T. K., Haiko J., et al. *Fibrinolytic and coagulative activities of Yersinia pestis*. Front CellInfect Microbiol. 2013; 3:35; DOI: 10.3389/fcimb.2013.00035.

indirect, because in fact the "activity" of plasminogen activator depends solely on the structure of LPS (which, in turn, already depends on temperature). In this connection, it is also worth noting that LPS of *Y. pseudotuberculosis* containing O-antigen does not "activate" the plasminogen activator at all, because the side chains of O-antigen create stereometric obstacles (shield the plasminogen activator).

In 1966, by subjecting *Y. pestis* to extraction with trichloroacetic acid at 4°C / 39.2°F (André Boivin[1] method), A. A. Popov and Vladimir Vladimirovich Akimovich[2] isolated[3] a substance identified as the **basic somatic antigen** (BSA, Russ. основной соматический антиген) or O-antigen[4]. However, earlier we said that as a result of evolution *Y. pestis* has lost it. The fact is that the inactivation of the genes encoding the synthesis of the O-antigen was proven almost half a century later. The antigen obtained in 1966 was indeed not similar to the typical O-antigen of Gram-negative bacteria in chemical structure and turned out to be completely non-toxic to white mice and guinea pigs. Closer analysis revealed that BSA is a cyclic tetramer of repeating trisaccharide units consisting of acylated mannosaminuronic acid, deoxygalactose and glucose, i.e., it is chemically an **Enterobacterial Common Antigen** (ECA) characteristic of most Enterobacteriaceae. The synthesis of ECA, which many scientists still refer to as BSA, is carried out by chromosomal genes and is closely related to the synthesis of LPS. In fact, it can be considered as a "defective" O-antigen, which is not toxic but is an active inducer of antibodies.

Pesticin (P1, Psn) is a bacteriocin, i.e. a substance of protein nature that inhibits the growth of sensitive bacteria of the same genus or species. In this case, pesticin causes lysis of the cell wall of some strains of

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[1] 1895–1949; the French physician, bacteriologist. Working at the Institute of Human Bacteriology and Immunology (now Institutul Național de Cercetare-Dezvoltare Medico-Militară "Cantacuzino"), together with Lidia and Ion Mesrobianu, he biochemically characterised endotoxins using the trichloroacetic acid extraction method.

[2] Владимир Владимирович Акимович, 1912–1968; the Russian and Soviet physician.

[3] Попов А. А., Акимович В. В. *Производство бакпрепаратов для профилактики и диагностики особо опасных инфекций*. Саратов, 1966, С. 140–143.

[4] Isolation was possible only from strains that had lost pFra: strains 358/12P-III, EV-2 and later from vaccine strains EV and 7 (see Тараненко Т.М., Дальвадянц С.М., и др. *Особенности структуры и функции основного соматического антигена чумного микроба*. Проблемы особо опасных инфекций. 2005; 89:51-54).

Y. pestis and *Y. pseudotuberculosis* I serovar strains containing 3,6-dideoxy-B-ribohexoses (acting as a receptor for P1) due to hydrolysis of the bond between N-acetylglucosamine and N-acetylmuramic acid in murein (cell wall lipoprotein). To protect against pesticin, the bacterium synthesises a **pesticin immunity protein**, the genes for which are located on the pPla plasmid, so pesticin synthesis is targeted at bacteria lacking the immunity protein – strains lacking or defective in the pPla plasmid and *Y. pseudotuberculosis* I serovar strains.

New plasmids

Earlier we said that strains lacking pPla or pFra but capable of high virulence are found in nature. It is difficult to say what factors ensure this, but there is evidence that *Y. pestis* may contain *conjugative* (i.e. transmissible) plasmids[1]. This was reported in the 1980s[2], but their functions have not yet been fully understood. For example, the strain *Y. pestis* strain Java-9 (bv. orientalis), isolated on Java Island, Indonesia, in 1957 from a dead Malaysian house rat (*Rattus rattus diardi*), lacks the pFra plasmid but has new plasmids pJARS35 (35.044 Kb) and pJARS36 (36.085 Kb). An important feature of the strain is resistance to arsenic and arsenite, for which, according to genomic analysis[3], is responsible the *Tn2503* transposon[4], which is found not only on the new plasmids pJARS35 and pJARS36, but also on the plasmids pCD1 and pPla. It is important to note that the new plasmids also carry genes responsible for the implementation of the *type IV secretion system*, which can carry out the transfer of proteins and DNA into eukaryotic or prokaryotic cells, i.e., in our case, carry out conjugative transfer of plasmids[5]! The detected common plasmid loci with *Aeromonas culicicola* suggest that the strain

[1] It should be noted that the pFra plasmid of caucasian strains contains an incomplete *tra*-operon, which previously could function for its intended purpose, i.e. to ensure the process of conjugation (transfer of genetic information).

[2] Tsukano H., Wake A., et al. *Plasmid-like properties of the four virulence-associated factors of Yersinia pestis*. Microbiol Immunol. 1986; 30(9):837–848; DOI: 10.1111/j.1348-0421.1986.tb03011.x.

[3] Eppinger M., Radnedge L., et al. *Novel plasmids and resistance phenotypes in Yersinia pestis: unique plasmid inventory of strain Java 9 mediates high levels of arsenic resistance*. PLoS ONE. 2012; 7(3):e32911; DOI: 10.1371/journal.pone.0032911.

[4] I.e., a section of DNA capable of transposition (movement) within the genome.

[5] The experiments demonstrated the possibility of plasmid transfer to *Y. enterocolitica* strains.

acquired plasmids from an unrelated bacterium. This is important because it was long thought that gene transfer *Y. pestis* was only possible in the laboratory. It also supports the important idea that the gut of blood-sucking insects (particularly fleas) can act as a potential site of genetic exchange. It can be said that the acquisition of Ymt *Y. pestis* not only contributed to the expansion of mammals (which increased transmissibility), as we said earlier, but also became the starting point of the evolutionary pathway for the acquisition of new genes (including antibiotic resistance genes).

On the other hand, the presence of additional plasmids (called *cryptic plasmids*[1]), while retaining the "own" plasmids (pCad, pFra and pPla), usually does not lead to a decrease in virulence. Thus, the presence of the cryptic plasmid pTR33 (34 Kb)[2] in *Y. pestis* strain KM932, isolated in 1987 in the Tuva Mountain Foci (Russia), does not affect virulence, and its function is currently unknown. However, *Y. pestis* strain C-267, isolated[3] in 1986 in the Central Caucasus Highland Foci (Russia), containing the cryptic plasmid pSKF (5.4 Kb), has reduced virulence and proline requirement. In any case, these plasmids are currently used as genetic markers of strains from these outbreaks.

Strains from China are known. These are the avirulent *Y. pestis* strain 91001 containing an additional cryptic plasmid pCRY (22 Kb) and unnamed strains of *Y. pestis* containing an additional cryptic plasmid pYCR (5.919 Kb). The first strain was isolated in 2004 from the Brandt's vole (*Lasiopodomys brandtii*) [4], and the latter strains were isolated in 2000 in regions of Yunnan province[5].

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[1] The presence of these plasmids is not reflected in the phenotype.

[2] Structural and functional analysis of this plasmid revealed it to be the ring gene of a bacteriophage.

[3] Оглодин Е. Г., Ерошенко Г. А., и др. *Структурно-функциональный анализ криптических плазмид штаммов Yersinia pestis из двух природных очагов чумы России.* Проблемы особо опасных инфекций. 2015; 4:82–85; DOI: 10.21055/0370-1069-2015-4-82-85.

[4] Song Y., Tong Z., et al. *Complete genome sequence of Yersinia pestis strain 91001, an isolate avirulent to humans.* DNA Res. 2004; 11(3):179–197; DOI: 10.1093/dnares/11.3.179.

[5] Dong X. Q., Lindler L. E., et al. *Complete DNA sequence and analysis of an emerging cryptic plasmid isolated from Yersinia pestis.* Plasmid. 2000; 43(2):144–148; DOI: 10.1006/plas.1999.1432.

Non-coding RNA

Non-coding RNAs (ncRNAs, formerly small RNAs, smRNA) are short RNA molecules that are important components of many signalling pathways in bacteria from which the ribosome does not translate protein synthesis. Temperate bacteriophages can be a source of ncRNAs. Consider the functions of some of the ncRNAs of *Y. pestis*: ncRNA, which is an RNA thermosensor (RNA thermometer), in response to a temperature increase to 37°C / 98.6°F, triggers the *lcrF* gene, which encodes the synthesis of the LcrF protein, which in turn triggers the T3SS; ncRNA Ysr141 regulates YopJ synthesis; ncRNA Ysr170, affecting the genes of plasmid pCad, increases the survival of the bacterium in macrophages; ncRNA HmsB enhances the activation of biofilm formation, probably affecting the hms genes (recall that *hms* function is also affected by the genome of bacteriophage CUS-2). In general, the functions of many of the already identified ncRNAs have not yet been determined, but their important role in the bacterial life cycle is evident.

CRISPR

Clustered regularly interspaced short palindromic repeats (CRISPR) are small repetitive sequences 21-37 bp in length that are separated by so-called spacers (unique non-coding sequences) similar to the way some parts of a sentence are separated by commas. CRISPR were discovered in 1987 by a group of researchers led by Ishino Yoshizumi[1], and for a long time their biological function was unknown. Studies continued by the group of Francisco Mojica[2], allowed to detect CRISPR in many bacteria and archaea, as well as *cas* genes encoding cas-proteins. Only in 2005 they proved that spacers ("commas") are much more important than "words" because they are sequences from the genomes of bacteriophages, giving bacteria resistance to them[3]. That is, in essence, a mechanism of adaptive immunity, a bacterial analogue of mammalian immunity, where the bacterium "collects" fragments of foreign genomes it has encountered (much like people keep photos in a photo album). In

[1] 石野 良純, b. 1959; the Japanese molecular biologist. In addition to CRISPR, he discovered the *iap* gene in *E.coli*, subsequently sequencing the entire genome.

[2] b. 1963; the Spanish microbiologist, author of the term CRISPR.

[3] Mojica F. J. M., Díez-Villaseñor C. D., et al. *Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements.* J Mol Evol. 2005; 60(2):174–182; DOI: 10.1007/s00239-004-0046-3.

the genome of *Y. pestis* three CRISPR elements were detected in the genome of *Y. pestis*: YP1, YP2 and YP3, the spacers of which are prophage genes (they are not mentioned in the relevant section).

Subsequent CRISPR research and the discovery of the Cas9 protein led to the creation of the genome editing technology (CRISPR/Cas9) for which Emmanuelle Charpentier[1] and Jennifer Anne Doudna[2] received the 2020 Nobel Prize in Chemistry. The use of this system allows humans to effectively suppress the chromosomal genes of *Y. pestis*, in particular *hmsH* and *ail*, thereby significantly reducing its virulence.

* * *

Perhaps all of the above seems complex and incomprehensible at this stage, but this will be remedied in the next Act when we consider pathogenesis in detail. For now, three things should be understood: firstly, the complexity and diversity of the mechanisms that ensure the survival of the bacterium in a mammalian organism, since, as can be seen, most of the "mechanisms" are triggered only at 37°C / 98.6°F; secondly, many of the "mechanisms" encoded by different genes located on different genetic structures (be it a chromosome or plasmids) complement each other, in fact fulfilling one common task; and, finally, thirdly, the "mechanisms" are quite variable among different subspecies and may be generally different from one another. Perhaps, in order to better understand and structure the information obtained (to create a semantic link), this chapter should be read again, since we will inevitably return to it later, when talking about pathogenesis. In the meantime, think of your loved ones and spend time with them, because at this stage everyone should, like the translator of the Acts of the Council of Ferrara-Florence, admit to oneself, say: "Fessus sum"[3].

[1] b. 1968; the French physician, bacteriologist, member of the French Academy of Sciences.

[2] b. 1964; the American physician, biochemist, geneticist, member of the U.S. National Academy of Sciences.

[3] Lat. "I am exhausted"; the original Acts has the entry: *Interpres. Fessus sum* (Lat. "Interpres. I am axhausted").

AKT II

Vita





The garden of Kōmyō-in Temple in Kyoto

7 USHIJIMA

A heavenly floating bridge that floats over the waters of the primordial world, separating it from Takama-no-Hara, the Plain of the High Heaven, just as the bridge at Ise separates the world of kami[1] from the world of mortals. Upon it stand two, Izanagi-no-Mikoto and Izanami-no-Mikoto, a man and a woman, seventh-generation heavenly kami. They take the heavenly jeweled spear and, plunging it into the water, take it out. And the drop that fell from the sharp end of the blade became firm ground[2].

***

In the pavilion of Kōmyō-in Temple[3], a man sits with his straw hat beside him, gazing deep into the garden called the Rainbow Moss Temple. In this place, among the rocks and mosses that slowly float on an ocean of white sand, against a backdrop of red and green maples, sun-drenched bushes of flowering rhododendrons, he feels complete peace. It would be worth spending eternity here, but his thoughts take him to the bank of the Sumida River, to the Ushijima-jinja Shrine, built at the behest of kami Susanoo-no-mikoto, brother of kami Amaterasu Ōmikami, who subdued the demon of plague that once visited the shrine in the form of a bull[4].

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[1] Jap. 神 – in Shinto, a spiritual entity, deity, spirit. In contrast to the yōkai (i.e., supernatural force, ghost or monster-like creature), they existed originally.

[2] Author's retelling of a Japanese legend about the creation of the earth; chapters 1-3 of the first scroll of the first book of the "Kojiki" (Jap. Records of Ancient Matters), a monument of literature of the 8th century. Bridge in Ise (absent in the legend) - a reference to the real wooden Uji Bridge, which is the entrance to the world of kami, i.e. to the territory of the inner shrine (Naikū) Ise Jingū, the main Shinto shrine of Japan, dedicated to the supreme kami Amaterasu Ōmikami, from whom, according to the belief, the family of emperors of Japan is descended. Ise-jingu houses national treasures, including the sacred mirror of Amaterasu Ōmikami, which is an Imperial Regalia (Jap. Sanshu no jingi).

[3] The Buddhist temple in Kyoto (1397) is subordinate to Tōfuku-ji Temple. The main garden was created in 1939 by landscape designer Shigemori Mirei (重森三玲; 1896-1975), who, before creating his first garden, made a detailed research of existing gardens, resulting in the publication of a 26-volume Illustrated Book on the History of the Japanese Garden.

[4] There is also a sculpture of a cow, which, according to beliefs, brings health. It is interesting that in the legends of the Ryūkyū Islands (Japan, an independent Kingdom until

As we said earlier, bulls, like many farm animals, are less susceptible to plague than rodents, but the pathological processes occurring in the infected organism are similar in all carriers and are summarised by the following mechanism[1]. Most often in the body of the animal, the pathogen penetrates through the skin (flea bite) or mucous membranes (food, including eaten rodent). Then with the lymph flow it migrates (within macrophages and less often independently) to the regional lymph nodes nearest to the place of "entrance" without manifestations of lymphangitis (i.e. lesions of peripheral lymphatic vessels). This process takes 10 to 60 minutes. In the case of a bite, these are usually the prescapular and axillary lymph nodes, and in the case of food, the submandibular and cervical lymph nodes, as well as the tonsils. It is worth noting that since in the host body the bacteria are warmed to a temperature sufficient for synthesis of many pathogenicity factors (in contrast to the "cold" flea), once in the mouth of a predator (in fact, the second host) with food, they are more ready for immune evasion (survival) than those bacteria that penetrated the skin as a result of a bite, so the infectious process in foodborne infection proceeds faster.

Skin penetration may rarely result in a cutaneous form characterised by a petechial rash (**Figure 7.1**), swelling or a specific reaction at the site of the bite ("*entry gate*"), the so-called *primary affect* – pustule with haemorrhagic contents, ulcer or carbuncle (**Figure 7.2**).

In the skin, bacteria are taken up by phagocytes such as macrophages and neutrophils (there is little interaction with dendritic cells). Most of the bacteria captured by neutrophils are phagocytised (i.e. killed), whereas in macrophages, including those that have migrated to lymph nodes, a significant proportion survive and multiply, stimulating the synthesis of pro-inflammatory cytokines (TNFα, IL-1, IL-6 and IL-8) and fibrin. As a consequence, an acute inflammatory reaction develops, which is accompanied by lymphadenitis (i.e. enlargement of lymph nodes) characterised by vascular thrombosis and necrotic changes in the

1879), a talking cow acts as a protector against the demon of plague, as well as any other infectious disease arriving on the island in a boat (an image of incoming infection). On the other hand, from the imperial chronicle Nihon Sandai Jitsuroku (Jap.日本三代実録) it is known that in the second half of the ninth century, plague epidemics repeatedly broke out in the capital city of Kyoto, which led to the emergence of a purification festival (goryo-e) Gion matsuri (Jap. 祇園祭), held at the Gion-jinja shrine (now Yasaka-jinja).

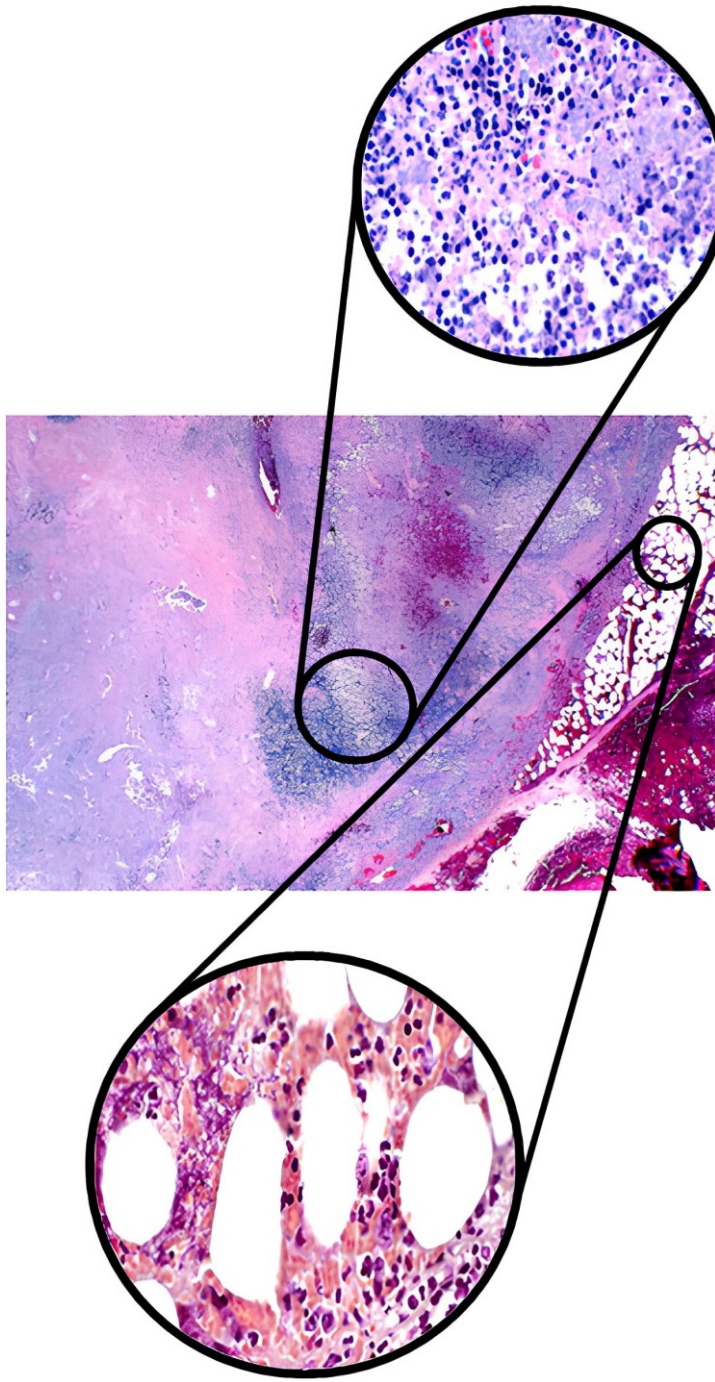
[1] We will consider the pathogenesis in detail on the example of a human being.

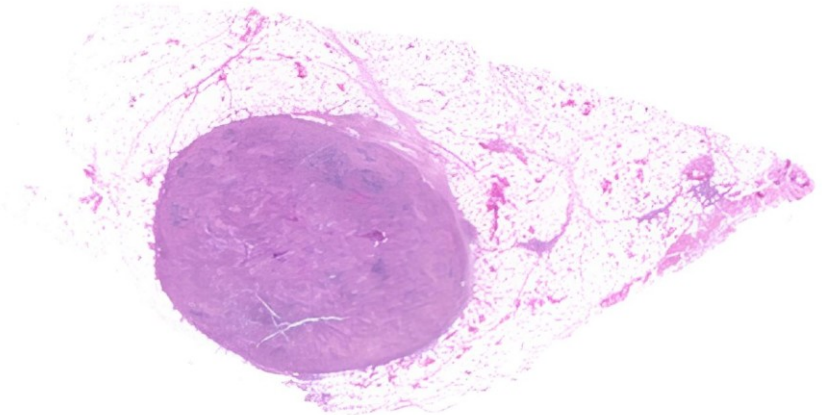


Figure 7.1. *Petechial rash in the rock squirrel (Otospermophilus variegatus) and primary affect (marked with arrow)*



Figure 7.2. *Limbs of a Maine Coon cat found dead in the home of a hospitalised person. The forefoot and right forearm are about twice as swollen as normal, and there are open wounds on the cranial (i.e., closer to the head) side of the right wrist with dried exudate (i.e., fluid released from small blood vessels of a tissue or organ during inflammation) on the fur adjacent to the wounds (WSC Case ID 141001, 2014; published with permission from The Joint Pathology Center)*





▲ ***Figure 7.3.*** *Lymph node and perinodal (i.e. surrounding) adipose tissue of a female white-tailed prairie dog (Cynomys leucurus), H&E. There is a general loss of lymphocytes with loss and replacement of the normal architecture of the lymph node (capsule, cortex, paracortex and medulla) by necrotic debris, oedema, fibrin, minor haemorrhages, numerous neutrophils (both degenerative and viable) and fewer macrophages. Vascular lesions characterised by discontinuous endothelium, middle sheath expansion with fibrin, and swelling of the adventitial (i.e. outer) sheath are suggestive of fibrinoid vasculitis. There are numerous perivascular (i.e. around vessels) and to a lesser extent intravascular large colonies of coccobacilli measuring 1 x 2 µm. The indicated is disseminated into and partially replaces perinodal adipose tissue (VSPO Case ID 764 (H-B05), JPC 2131320, 2021; published with permission from The Joint Pathology Center)*

◄ ***Figure 7.4*** *Tissue of the pharyngeal lymph node and perinodal adipose tissue (Brown & Brehn, 2x; highlighted areas 40x) of a female hybrid of Siberian polecat and black-footed ferret (Mustela eversmanii x Mustela nigripes). Extensive necrosis and inflammation with foci of hemorrhage and multiple large reddish-blue bacterial colonies multifocally within areas of suppuration and necrosis are observed (WSC Case ID 12 (93W 9960-4), AFIP 2642052, 1998; The Wyoming State Veterinary Laboratory, published with permission of The Joint Pathology Center; adaptation of author)*

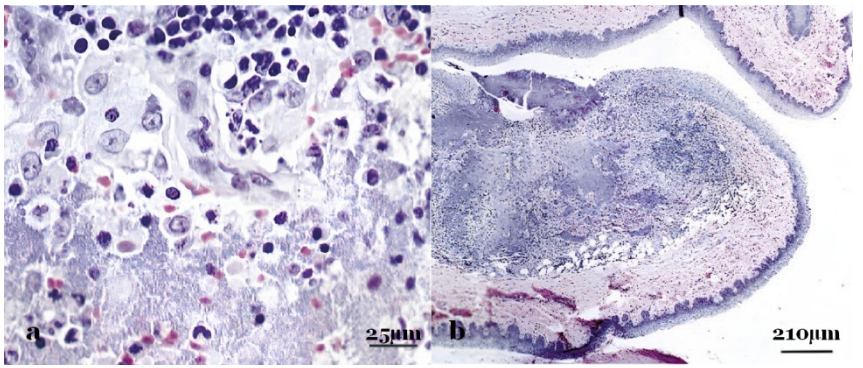


Figure 7.5 . *Tissue of lymph node (a) and pharyngeal tonsil (b) of a cat, H&E (by Watson R.P., 2001; published under licence from SAGE Publications Inc.): a – acute necrotizing inflammation, haemorrhage, swelling, massive bacterial colonies with an admixture of macrophages and degenerate-necrotic neutrophils are observed; b – necrosis of lymphoid tissue and rupture of the overlying epithelium of tonsils, massive bacterial colonies are observed. Paratonsillar (i.e., near the tonsil) soft tissues are enlarged due to oedema, fibrin and haemorrhage, and bacteria are present in the fascia of the adjacent bundles of facial skeletal muscles (not shown)*

node tissue involving the subcutaneous tissue (**Figure 7.3 – Figure 7.5**), leading to their fusion into a single conglomerate, i.e. formation of a *bubo* (bubonic form). Soreness and adhesion to the skin are important features in differential diagnosis because, for example, they are absent in tularemia, which is also characterised by buboes (the buboes do not hurt on palpation and are free to "walk" under the skin).

As a result of disruption of the barrier function of the lymph node by the breakdown products of macrophages and multiplication of the pathogen, the latter penetrates into blood – *bacteraemia* occurs (**Figure 7.6**). Fever begins, and the body temperature of the animal rises to an average of 41-42°C / 105-107 °F [38-40°C / 100-104 °F][1].

At the initial stage, bacteria from blood are "filtered" by the spleen and liver, with no inflammatory reaction observed (no infiltration of phagocytes, reduced synthesis of pro-inflammatory cytokines), which

[1] Hereinafter, reference values are given in square brackets and values from articles are in bold. Reference ranges are given in square brackets where appropriate. The value "μL" corresponds to "mm^3".

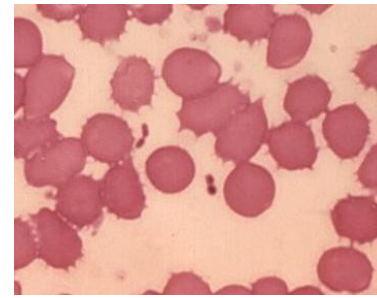


Figure 7.6. *Microphotograph of a blood smear containing Y. pestis*

allows the bacteria to multiply rapidly, as the cells of these organs literally serve as its nutrient medium! In the liver (**Figure 7.7**-a), hepatocyte swelling, vacuolar hepatopathy (i.e., abundant glycogen accumulation in hepatocytes), dilatation and hyperaemia (i.e., pathological dilation accompanied by increased blood flow) of the central vein of the hepatic lobules and slight congestion in the hepatic sinus, as well as capsular neoplasms and necrotic nodules, may be observed. In the spleen (**Figure 7.7**-a), there may be a decrease in the white pulp and swelling of the splenic thrusts of the red pulp.

A huge amount of bacteria from the destroyed liver and spleen rushes into the blood, which marks the beginning of the *secondary septicemic form* (the primary form is realised by direct entry of the pathogen into the blood, bypassing the lymph nodes). Massive septicaemia develops (up to 10^8 microbial cells per ml of blood), with the bacteria spreading throughout the organs, and sometimes secondary buboes are formed. It is at this stage that the concentration of the pathogen in the blood is sufficient to ensure transmission through the flea (remember, 10^6 microbial cells per ml of blood are sufficient to infect a flea!).

As a special type of septicemic plague, the *pulmonary form* is distinguished, in which capillaries are abundantly filled with bacteria (rarely with leucocytes) and necrotic alveoli can be found near large bronchi and vessels. The subpleural alveoli show accumulation of serous exudate and bacteria, while the other alveoli are usually clear. The alveolar epithelium is swollen and necrotic. Bronchioles have acute inflammatory changes and haemorrhages with bacterial accumulations.

During septicaemia, the lungs are the most commonly affected organ (*secondary pneumonic form*), which is manifested by inflammation of the vascular tissue of the lung (vasculitis) or the lung tissue itself (pneumonia). In the latter case, bacteria avoid interaction with alveolar macrophages, so the first day there is abundant multiplication in the absence of inflammatory response (pre-inflammatory phase). Then the proin-

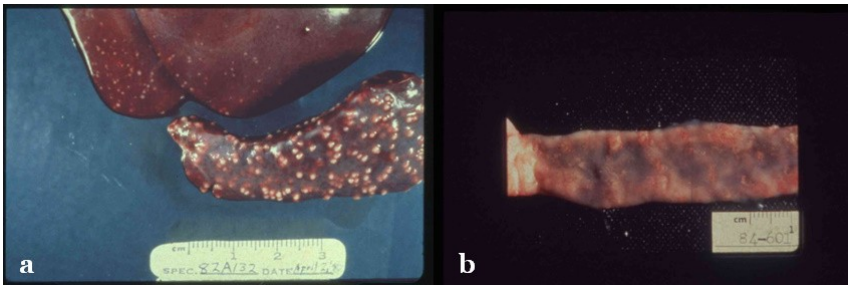


Figure 7.7. *Organ lesions in a female laboratory animal (images VP03104 and VP03257; published with permission from The Joint Pathology Center):*
a – liver fragment (top) and spleen; capsular neoplasms and necrotic nodules observed;
b – Intestinal fragment; necrotic nodules and minor haemorrhages observed

flammatory phase begins, accompanied by an abundant influx of neutrophils and cytokine storm (activity of T cells, NK cells and macrophages), leading to the development of severe purulent multifocal pneumonia and, as a consequence, significant damage to lung tissue (up to whole lung lobes; **Figure 7.8**). There may be subtle involvement of the lung lymph nodes. Untreated, this form has an almost 100% mortality rate. Secondary pneumonic form occurs predominantly in cats[1] (**Figure 7.10** and **Figure 7.11**), as only isolated cases of pneumonia have been described in dogs, camels (*Camelus bactrianus*, *Camelus dromedaris*) and mule deer (*Odocoileus hemionus*).

The kidneys (capsular effusion, interstitial oedema[2] and vascular swelling), the pia mater of the brain and upper part of the spinal cord (meningitis; bacteria are more commonly found in the liquor) and the intestine (necrotic nodules, capsules; **Figure 7.8**-b) are rarely affected. Pathological changes of the heart are usually not observed.

At postmortem, bacteria are found in affected lymph nodes, kidneys, spleen, blood clot in the heart, brain and bone marrow (located in the femur).

[1] Shortly before death, they usually retreat to a quiet, peaceful place where they curl up and die. The last hours are accompanied by copious expectoration of bloody sputum, which can often be seen dried on the corpse (**Figure 7.9**).

[2] Characterised by acute swelling of the stroma with expansion of the interstitial space (i.e. extracellular space) without simultaneous enlargement of the surrounding cells or extracellular matrix.

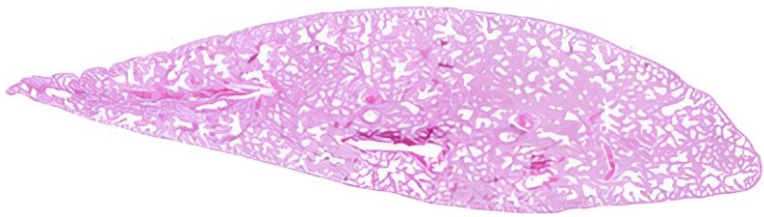
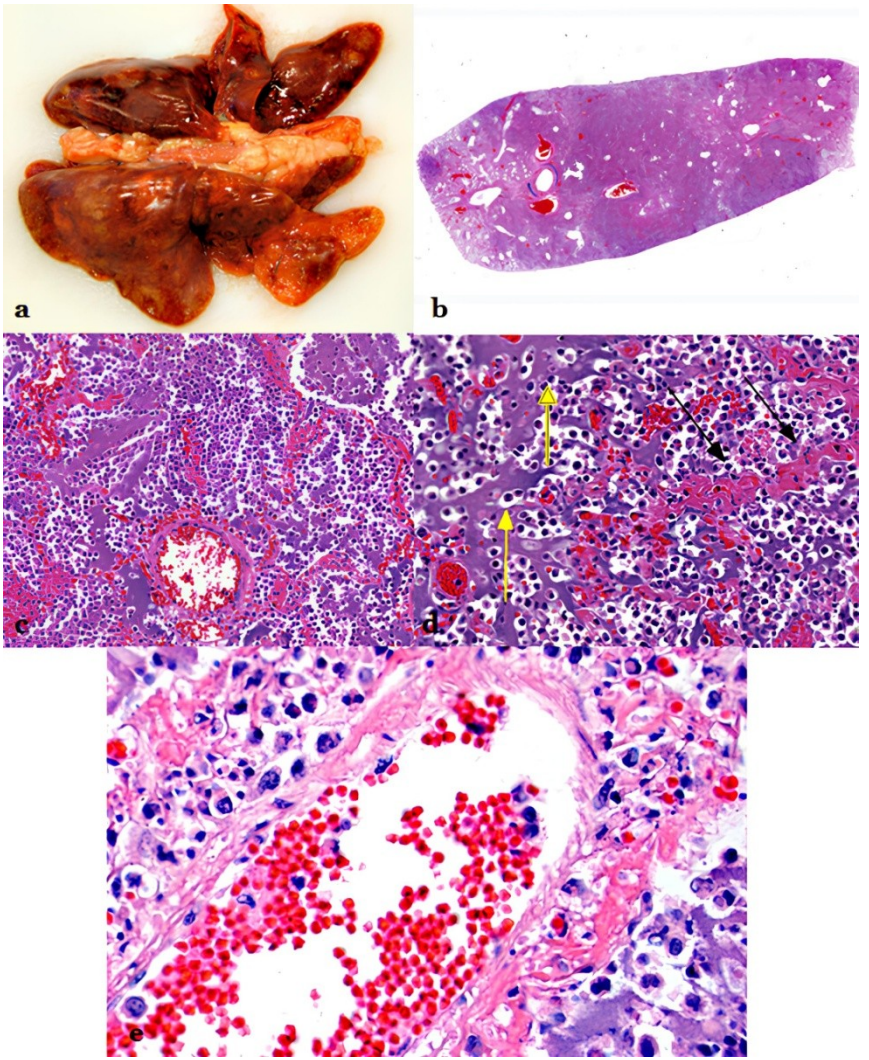


Figure 7.8. *Lung of a female white-tailed prairie dog (Cynomys leucurus), H&E. Diffuse fibrinoid vasculitis is observed, characterised by rupture and dilation of the middle membrane with fibrin, haemorrhages, oedema, neutrophils and a small number of macrophages. The perivascular connective tissue is expanded due to oedema, fibrin and haemorrhages. The alveolar septa are hyperaemic (i.e. full of blood) and dilated up to 35 µm. The alveolar lumen contains small numbers of alveolar macrophages. Bronchioles contain small amounts of cellular debris (i.e., cell remnant surrounded by a plasma membrane), red blood cells, and detached respiratory epithelium (VSPO Case ID 764 (H-B05), JPC 2131320, 2021; published with permission from The Joint Pathology Center)*

It should be noted that in rodents infected with a highly virulent strain, after the appearance of buboes (buboes), splenomegaly (i.e., enlargement of the spleen) and liver damage, death occurs most often (3-5 days of infection) without signs of systemic damage to other organs. Experiments[1] on infection of a mouse with a *Y. pestis* bioluminescent strain CO90(pLux)[2] has shown (**Figure 7.12**) that secondary lymphoid organs (which include the lymph node, spleen and liver) are affected, followed by septicaemia expressed by bioluminescence throughout the animal, including peripheral areas (ears and tail). Interestingly, between the lesion of secondary lymphoid organs (stage E) and septicaemia (stage F), no bioluminescence was detected in anatomical sites corresponding to the lungs, as well as the kidneys and intestine. Biolumines-

[1] Nham T., Filali S., et al. *Imaging of bubonic plague dynamics by in vivo tracking of bioluminescent Yersinia pestis*. PLoS One. 2012; 7(4):e34714; DOI: 10.1371/journal.pone.0034714.

[2] It was obtained by introducing into *Y. pestis* strain CO92 pGEN-*luxCDABE* plasmid containing the *luxCDABE* operon from *Photorhabdus luminescens* under the control of the *Pem7* promoter.


a
b
c
d
e



▲ ***Figure 7.9*** *Domestic cat that died of a pneumonic form of plague. You can see dried sputum with blood that she excreted before she died*

◄ ***Figure 7.10*** *Lung (a) and lung tissue (b-e) of a Maine Coon cat, H&E (WSC Case ID 141001, 2014; published with permission of The Joint Pathology Center):*
a – multiple randomly arranged solid microfoci are observed;
b - 6.3x; 75% of alveolar spaces are observed to be filled with cellular exudate;
c – 168x; alveoli containing numerous viable and degenerate neutrophils and bacterial colonies are observed;
d – 168x; multifocal septal necrosis is observed in the areas of infiltration: with septal rupture and loss (yellow arrows), with significant dilation and replacement by fibrin and cellular detritus (i.e., destroyed cells; black arrows);
e – 360x; dilatation of the pulmonary vein wall with neutrophils, cellular detritus and oedema (vasculitis) is observed

cence of these organs is also not detected at post-mortem examination (autopsy).

In the case of infection with a less virulent strain, severe lesions of the spleen and minor lesions of the liver and lungs in the form of necrotic nodules are found, and death occurs in about a week. In dogs the disease is most often accompanied by fever, but without systemic organ

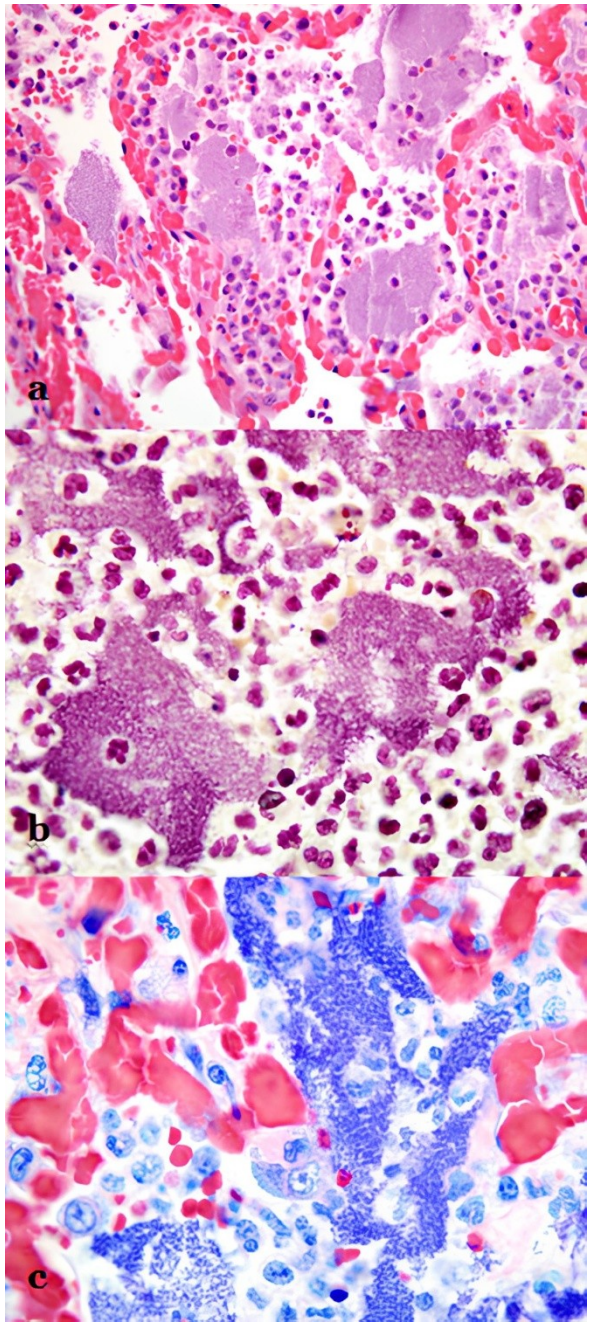


Figure 7.11 *Lung tissue of a 7-year-old neutered domestic shorthair cat (Felis catus). Alveoli containing numerous neutrophils (viable and degenerate) and fewer macrophages, with large bacterial colonies 1 x 3 µm in size, are observed (WSC Case ID 090303, 2009; published with permission of The Joint Pathology Center):*
a – 1000x, H&E;
b – 1000x, Brown-Hopps Gram (BH);
c – 1000x, Giemsa stain

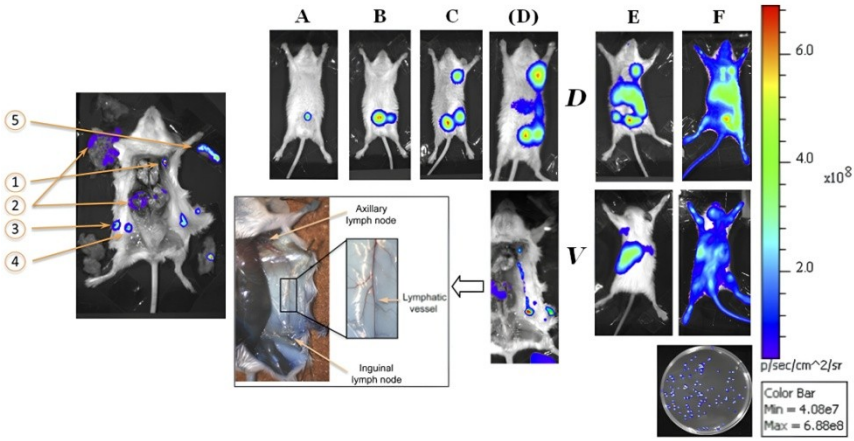


Figure 7.12. *Stages of the infection process in vivo in a mouse during experimental infection with Y. pestis bioluminescent strain CO92(pLux); mages were obtained on the IVIS100 device. At the initial stage (stage A), bioluminescence is restricted to the injection site. It then reaches the inguinal lymph node (stage B), on one or both sides, before being visualised in the ipsilateral (i.e. located on the same side) axillary lymph node (stage C). Sometimes the luminescence is found as a thin line between the lymph nodes (additional stage (D), top image), which corresponds to a connecting lymph vessel (additional stage (D), bottom image) through which the bacteria migrate before bacteraemia. The boxed image shows this lymphatic vessel detectable at post-mortem examination. The bioluminescence then spreads to the subdiaphragmatic area (position D), anatomically corresponding to the liver, to the dorsal area (position V), anatomically corresponding to the spleen (stage E) and finally to the whole body (stage F). Positions: D - dorsal, V - ventral. A postmortem post-mortem examination is shown on the left, showing bioluminescence of axillary (1) and inguinal (4) lymph nodes, liver (2; one lobe removed and presented next to each other), spleen (5; removed and presented next to each other), and the injection site (3) divided into two parts during post-mortem examination. The intestine removed and presented next to the right hind leg. The peritoneum, including part of the focus of infection (bioluminescence visible), has been removed and is presented next to the left hind leg. The scale of bioluminescence intensity encoded on the images from indigo (4.1 x 10^7 photon/s.cm2.steradian) to red (6.9 x 10^8 photon/s.cm2.steradian) is shown on the right. The minimum bioluminescence value for lymph node is 1.5 x 10^4 CFU/ml, for liver is 1.3 x 10^5 CFU, and for spleen is 8.3 x 10^5 CFU/ml. Bioluminescence of the injected bacterial culture is shown on the bottom right for clarity (by Nham T., 2012; adaptation of author)*

damage, whereas in cats, against a background of fever and lethargy, lymphadenopathy (i.e. palpatorily perceptible enlargement of lymph nodes), abscesses, lesions of the spleen, liver and lungs are always found, and the latter may be accompanied by purulent pneumonia. It should be reminded that cats are the main mammals[1] capable, along with humans, of aerogenic transmission (infecting others and infecting themselves). Moreover, infection can occur both by inhalation of «plague aerosol» and, unlike humans, in the process of feeding, when bacteria caught in the mouth with infected meat are carried into the lungs by the flow of inhaled air. In both cases it is the primary pneumonic form, which proceeds similarly to the secondary form, with subsequent development of bacteraemia (destruction of the liver and spleen) and septicaemia, rarely bronchial lymph nodes may be affected.

8 PRIMARY PNEUMONIC PLAGUE IN DOGS

Earlier we said that dogs are characterised by bubonic and septicemic forms of plague and do not get the pneumonic form, posing a danger to humans only as a carrier of plague fleas, but there are exceptions to every rule.

On 20 July 2009, a 34-year-old herdsman living in Xinghai County[2] (Hainan Tibet Autonomous Region, Qinghai Province, China) was looking for a lost dog. After finding it panting, he brought it home. The dog had a severe cough with bloody sputum and died two days later. The herdsman buried it by carrying it on his shoulders down a slope about half a kilometre from the house. The next day his second dog died

[1] Primary pneumonic plague has been reported in dogs (see below). In addition, primary pneumonic plague is possible in susceptible animals such as guinea pig, gopher, rabbit and mouse, as evidenced by laboratory studies in these animals. However, primary pneumonic plague does not occur in nature in these animals (nor does secondary plague) because death occurs before it has time to develop.

[2] The plague foci confirmed in August 1956 (the first human infection occurred in August 1962).

with similar symptoms. As early as 24 July in the afternoon, the herdsman felt sick, and the next day he had a fever and cough, so despite the care of his wife and two sons, his younger brother took him by motorbike to the village clinic on 26 June. He was seen by two physicians who were not wearing personal respiratory protection. The first physician examined the throat of patient and the second physician administered a drip. They described the symptoms of patient as fever, cough with bloody sputum, pallor of the face and chest pain. Physicians advised to take the patient to the hospital, which was carried out by the two elder brothers and the younger one. On the way, the patient developed sudden dysphoria (i.e., a profound state of anxiety) and vomiting. He died on 26 July at 5 pm (4 days after the death of the first dog). On the same day the corpse was taken home, where the two younger and two older brothers already mentioned, as well as the father-in-law and nephew, performed the necessary rites according to Tibetan tradition. The next day early in the morning he was buried. After 2-4 days, all 12 participants in this story (a friend visited the patient while he was in the hospital) suddenly developed a fever accompanied by chest pain and cough with bloody sputum (the physician who administered the drip remained healthy). The friend and father-in-law died.

Y. pestis (bv. antiqua) was detected in lung samples from both dead dogs and a herdsman, in sputum samples from a doctor and a friend, and in sputum samples and a throat swab from one of the older brothers. It was not possible to isolate the plague microbe from samples from other participants in this story, as all were initially receiving prophylactic antibiotic therapy. Only capsular antigen was isolated from the sputum samples (the physician had a protracted illness, so capsular antigen was isolated from the sputum until the twentieth day of illness).

The epidemiological investigation determined[1] that the dog was most likely infected from the meat of a dead marmot (antibodies to capsular antigen have been found in marmots in the area). It had developed primary pneumonic plague (by introducing bacteria from its mouth into the lungs with inhaled air) and was able to realise an aerogenic mechanism of transmission, which resulted in all participants in this story also

[1] Wang H., Cui Y. *A dog-associated primary pneumonic plague in Qinghai Province, China.* Clin Infect Dis. 2011; 52(2):185–190; DOI: 10.1093/cid/ciq107.

developing primary pneumonic plague. In addition, the patient had a further 61 direct and 256 indirect contacts, but all of these people were not infected

The other two case took place in the state of Colorado (USA)[1].

*In June 2014 (case 1), a two-year-old neutered male domestic mixed breed dog was brought to the veterinary clinic for evaluation of jaw rigidity, salivation, pallor of mucous membranes, and lameness of the right forelimb. On examination, the dog was febrile (41.1°C / **106°F**) with mild leucopenia (white blood cell: 5.32 × 10^9/L [6–17 × 10^9/L] / **5320 cells/µL** [6,000–17,000 cells/µL]) and thrombocytopenia (platelets: 56 × 10^9/L [200–500 × 10^9/L] / **56,000 cells/µL** [200,000-500,000 cells/µL]). In December 2017 (case 2), a 3-year-old mixed breed dog was brought to the veterinary clinic for lethargy for the past 8 hours, anorexia, and tremors preceded by a mild 2-day cough. On examination, the dog had fever (39.5°C / **103.1°F**) with leucocytosis (white blood cell: 23.4 × 10^9/L [5–16 × 10^6/L] / **23,400 cells/µL** [5,050–16,760 cells/µL]) and neutrophilia (neutrophils: 18.9 × 10^9/L [2.6–11 × 10^9/L] / **18,920 cells/µL** [2.6–11.0 × 10^3 cells/µL]). In both cases, the dogs were euthanised. They had necrohaemorrhagic and suppurative pneumonias caused by Y. pestis (primary pneumonic plague), determined only on pathological examination (**Figure 8.1** and **Figure 8.2**), whereas the lifetime diagnoses were pulmonary contusion due to trauma or diphacinone toxicosis (case 1) and foreign body/aspiration pneumonia (case 2). Computed tomography (**Figure 8.3**) and radiographs (**Figure 8.4**) taken during life were also uninformative and did not help in making a correct diagnosis*

One of the owners of the first dog was hospitalised 5 days after contacting the veterinary clinic (a total of 4 cases confirmed), in the case of the second dog there was no human infection. The owners of both dogs later reported seeing dead meadow dogs and chest radiographs were taken in the second case, but the rarity of this form and the inter-epizootic period were an obstacle to a correct diagnosis.

[1] Schaffer P. A., Hershkowitz C. S., et al. *Delayed diagnosis of fatal pneumonic canine plague: clinical and pathologic features in two naturally infected Colorado dogs*. BMC Vet Res. 2020; 16:160; DOI: 10.1186/s12917-020-02361-z.

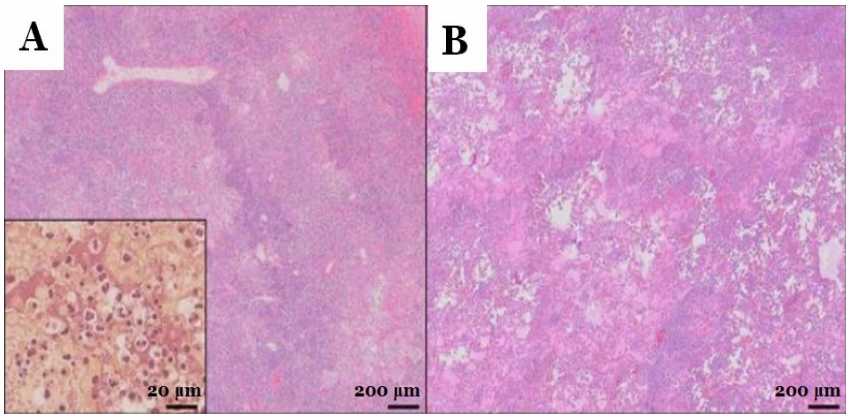


Figure 8.1. *Lung tissues of a case 1 dog (A) with diffuse severe necrohaemorrhagic pneumonia and a large number of bacteria within the lesion, and a case 2 dog (B) with similar pathology but without bacteria within the lesion, H&E, inset Gram stain (by Schaffer P. A., 2020)*

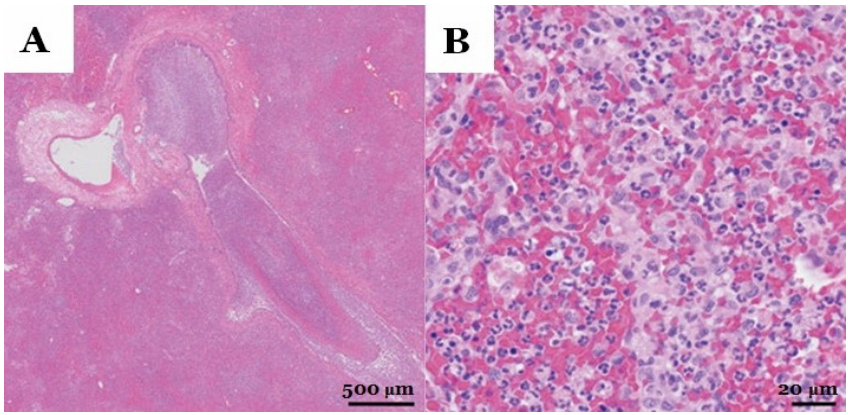


Figure 8.2 *Tissues of the accessory lobe of the lung (case 2), H&E. Diffusely flattened parenchyma (A) and alveolus (B), concealed by necrosis, haemorrhage and purulent inflammation, but without bacteria inside the lesion focus, are observed (by Schaffer P.A., 2019)*



Figure 8.3 *Transverse CT scan of a dog (case 2) on the second day of hospitalisation. Consolidation of the accessory lobe of the lung is observed (by Schaffer P. A., 2019)*

The presented cases are isolated, but clearly show that very rarely dogs can not only get the primary pneumonic form of plague, but also realise the aerogenic mechanism of transmission, posing a serious threat to human health, and perhaps even greater than cats, due to the extreme rarity of this phenomenon. It is also noteworthy that in the 2009 case, one dog was able to transmit the infection by airborne transmission to another dog, revealing the susceptibility of these animals to aerogenic infection.

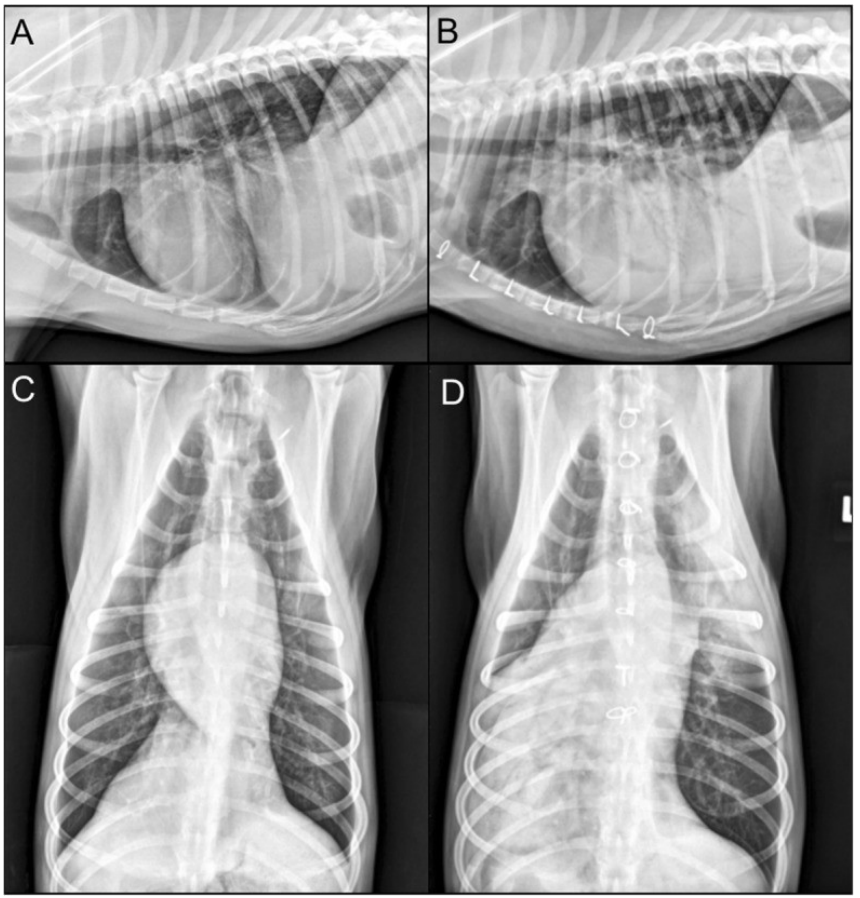


Figure 8.4 *Chest radiographs of a dog (case 2). Left lateral (A) and ventral-dorsal (C) chest imaging (A) taken on admission demonstrate ill-defined alveolar patterns in the right accessory lobe of the lung, whereas radiographs in the same positions (B and D, respectively) taken after 5 days of hospitalisation show multiple lobes of the lung with a patchy lobular alveolar pattern (by Schaffer P. A., 2020)*

9 PESTIS CAMELORUM

In 1907, Nikolai N. Klodnitsky (the same person to whom Ippolit A. Deminsky, who contracted the plague, wrote a telegram) recorded a case of camel plague in Astrakhan province, where 11 people died from the meat of a sick camel[1]. In 1976[2], a sick camel was slaughtered in a remote Libyan village and all five people involved died within four days from plague. In 1994[3], cases of severe lymphadenitis were reported in people eating raw camel liver in north-western Saudi Arabia. However, *Y. pestis* was isolated not only from camel bone marrow but also from jirds (*Meriones libycus*) caught in the enclosure of camel. In February 1997[4], cases of plague infection from eating camel meat (reportedly after cooking in one case) were also identified in the Jordanian village of Azraq ad-Druze. All these, and many other examples, have allowed *camel plague* (*Pestis camelorum*) to be recognised as an independent disease. And this is particularly interesting because other large farm animals (horses, elephants, buffaloes) are not very susceptible to plague.

A distinctive feature of the pathogenesis of camel plague is the involvement of not only internal but also external lymph nodes. Submandibular lymph nodes are particularly affected in case of aerogenic infection. Lymph node involvement in the limbs leads to lameness, making it difficult for the animal to rise and lie down. The buboes are large, reaching the size of an adult human fist (**Figure 9.1**). Sometimes they collapse completely, forming masses of necrotic tissue, fibrin and pus, which at post-mortem examination are defined by a crumbly white or grey mass. At the same time, lesions of internal organs are superficial and characterised by small haemorrhages in the mucous membranes, as well as minor consolidations in the lungs, which are sometimes filled with

[1] Interestingly, this information has been questioned for some time, as it was not possible to infect camels under laboratory conditions.

[2] Christie A. B., Chen T. H., et al. *Plague in camels and goats: their role in human epidemics.* J Infect Dis. 1980; 141(6):724–726; DOI: 10.1093/infdis/141.6.724.

[3] Bin Saeed A. A., Al-Hamdan N. A., et al. *Plague from eating raw camel liver.* Emerg Infect Dis. 2005; 11(9):1456–1457; DOI: 10.3201/eid1109.050081.

[4] Arbaji A., Kharabsheh S., et al. *A 12-case outbreak of pharyngeal plague following the consumption of camel meat, in north-eastern Jordan.* Ann Trop Med Parasitol. 2005; 99(8):789–793; DOI: 10.1179/136485905X65161.

bloody frothy fluid. It is extremely rare to find foci of necrosis in the adrenal tissue.

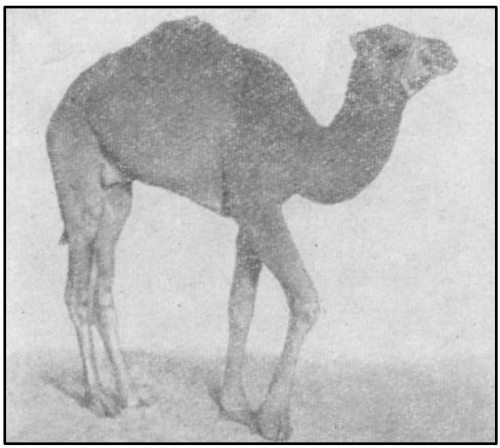


Figure 9.1. *Plague bubo under the camel's right shoulder blade (by Орлов Ф. М., 1973)*

The disease, as in the case of other carriers, is accompanied by an increase in body temperature, general oppression, lethargy, weakness of the limbs. Rarely, tremors in the muscles of the trunk can be observed. Appetite decreases and eventually disappears altogether, leading to exhaustion. Spontaneous abortions (miscarriages) may occur. If the lungs are affected, pneumonia, dyspnoea and coughing may develop, but bacteria are found only in nasal secretions, faeces, urine and milk (the latter can be eaten after boiling). The posture of the animal is characteristic of death: it lies on its side, makes smooth movements with its limbs, and its head is thrown back. It dies in this position, and only by the characteristic position of the skeleton can the cause of death be determined.

10 OCULAR PLAGUE

On 8 August 1987, an adult female black-tailed deer (*Odocoileus hemionus columbianus*)[1] was transported to Fort Hunter Liggett (Califor-

[1] Jessup D. A., Murphy Ch. J., et al. *Ocular Lesions of Plague (Yersinia pestis) in a Black-Tailed Deer (Odocoileus hemionus columbianus).* Journal of Zoo and Wildlife Medicine. 1989; 20(3):360–363.

nia, USA) after having died en route. The animal was externally healthy, with no lesions or external signs of trauma. The only feature was exophthalmos (i.e. bulging of the eyeball), with both eyeballs respectively enlarged: 5.5 and 6 cm in diameter and weighing 22 and 24 g, about twice as much as normal (**Figure 10.1**). Separated eyeballs and fluid aspirates from the posterior segment of the eye were submitted to the Wildlife Investigations Laboratory of the California Department of Fish and Game and the Veterinary Medical Teaching Hospital of the University of California at Davis for identification. Bacteriological cultures were positive for *Y. pestis* (*Enterobacter aerogenes* and *Enterobacter cloacae* were detected in conjunctival swabs). The pathological changes (**Figure 10.2**) in both eyeballs are identical and suggest panophthalmitis (i.e. acute purulent inflammation of the eye). Necrotic cellular debris and degenerating inflammatory cells (mainly neutrophils) are observed in the anterior and posterior segments of the eye. The vasculature and retina are markedly infiltrated with neutrophils, and the retinal mononuclear retina is detached and degenerated in many areas. The inflammatory process has spread anteriorly and surrounds the lens, but the cornea and conjunctiva are minimally infected. The inflammatory process most likely originated from the vasculature and grew most in the posterior segment. Necrotising vasculitis is expressed in the small arteries supplying blood to the retina.

Plague in mule deer (*Odocoileus hemionus*) has only been reported[1] once before, but that case was limited to fibrinous pleurisy (i.e., inflammation of the pleura frequently accompanied by serous and sometimes sanguineous effusion in the pleural cavity and ending up with fibrosis of the pleura through organization of the inflammatory exudates), rare pulmonary abscesses in the ventral lobes of the lungs, and marked thoracic lymphadenopathy. Infection of mule deer with plague is even rarer than that of camelids - no ocular plague was reported before 1987, and after that only 3 cases[2] are known from Wyoming and Oregon (D2-D4, **Table 10.1**), whose territory, as we said earlier, is located in the plague focal zone (**Figure 10.3**).

[1] Thorne E. T., Quan T. J., et al. *Plague in a free-ranging mule deer of Wyoming.* J Wildl Dis. 1987; 23(1):155–159; DOI: 10.7589/0090-3558-23.1.155.

[2] Edmunds D. R., Williams E. S., et al. *Ocular plague (Yersinia pestis) in Mule Deer (Odocoileus hemionus) from Wyoming and Oregon.* J Wild Dis. 2008; 44(4):983–987; DOI: 10.7589/0090-3558-44.4.983.



Figure 10.1. *Head of a female black-tailed deer with bilateral panophthalmitis (by Jessup D. A., 1989; published with permission of the American Association of Zoo Veterinarians)*

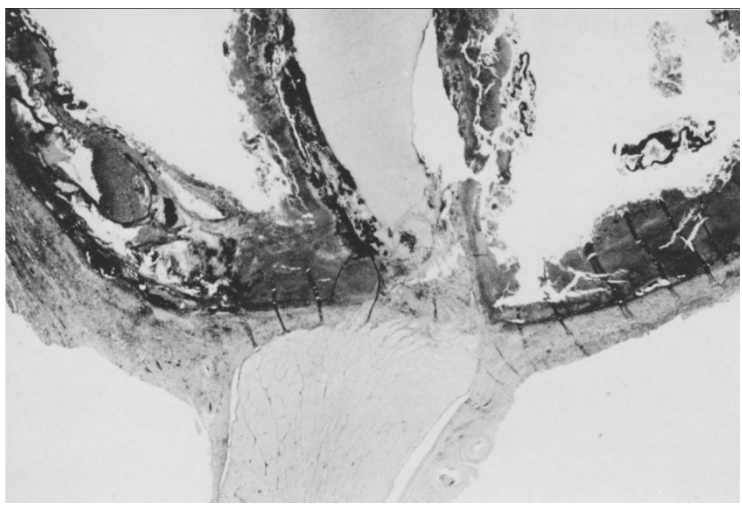


Figure 10.2 *Posterior aspects of a black-tailed deer eye (20x), Brown & Brehn, H&E, PAS (by Jessup D. A., 1989; published with permission of the American Association of Zoo Veterinarians)*



Figure 10.3 *Map of locations where mule deer with ocular plague (red line symbolises the boundary of plague foci, which coincides with the natural habitat of the species)*

Table 10.1. *Cases of ocular plague in mule deer*

Case	Place of discovery		Gender	Diagnosis
	coordinates	region		
D1		Fort Hunter Liggett, State of California	female	Panophthalmitis
D2	41.1531°N 105.2977°W	Albany County, State of Wyoming	male	Keratoconjunctivitis on the background of multifocal necrotising and fibrinous purulent pneumonia, disseminated necrotic lymphadenitis
D3	44.4161°N 118.9519°W	Grant County, State of Oregon	male	Bilateral exophthalmos with associated keratoconjunctivitis and corneal ulceration (Figure 10.4) in mild non-purulent encephalitis[1]
D4		Grant County, State of Oregon	female	Postmortem retinal detachment with probable panophthalmitis

[1] Probably not of plague genesis (i.e. origin).

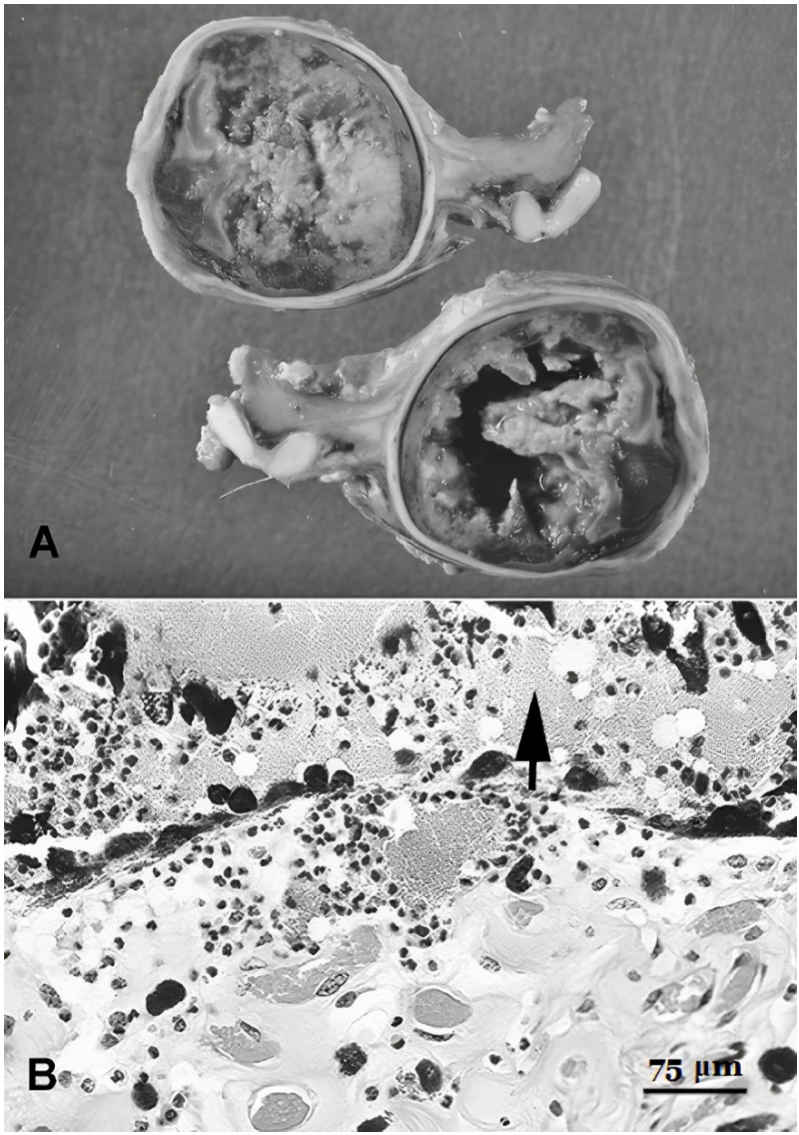


Figure 10.4. *Eye from a mule deer (case D3), H&E (by Edmunds D.R., 2008; published with permission of The Journal of Wildlife Diseases, The Wildlife Disease Association):*
A - sagittal section of the eye with pronounced fibrotic-purulent inflammation and necrosis of the inner membranes;
B - tissue of the vasculature (above) and sclera (below) with purulent inflammation and numerous intralesional coccobacilli (arrow)

Of the three cases, two were keratoconjunctivitis (i.e. combined inflammation of the cornea and conjunctiva) combined with lymphadenitis and pneumonia typical of plague, suggesting that the bacteria were introduced into the eyes by the blood stream during bacteraemia. However, on the one hand, as it has been often said before, large ungulates are rarely infected with *Y. pestis*, but on the other hand, eye lesions are absent in all animals, even in highly susceptible rodents and humans, indicating the individual ability of the plague microbe to affect the eyes of mule deer (especially a black-tailed deer).

11 DIGESTIVE PLAGUE

Alexandre Yersin in his famous article[1] of 1894 reports that if you feed rats and mice fragments of spleen and liver of animals that died of plague, they will also be fatally infected. Of course, in nature rodents are mostly non-carnivorous (except for the very rare consumption of stillborn littermates in burrows), which is not the case with predators such as coyotes, dogs and cats, which, as stated earlier, when eating infected carriers (rodents) become infected themselves. Most often they are infected with the bubonic form of plague, in which submandibular and cervical lymph nodes and tonsils are mainly affected, and less often with the pneumonic form, in which pneumonia develops and bronchial lymph nodes may be affected. However, it has been shown[2] experimentally in mice (*Mus musculus*), rats (*Rattus rattus*) and hairy-tailed bolo mouse (*Zygodontomys pixuna*[3]) that foodborne infection can be fatal without the development of bubonic and pneumonic forms. Postmor-

[1] Yersin A. *La peste bubonique a Hong-Kong.* Ann Inst Pasteur (Paris). 1894; 8:662–667.

[2] Butler T., Fu Y. S., et al. *Experimental Yersinia pestis infection in rodents after intragastric inoculation and ingestion of bacteria.* Infect Immun. 1982; 36(3):1160–1167; DOI: 10.1128/iai.36.3.1160-1167.1982.

[3] Currently regarded as a junior synonym of *Necromys lasiurus*.

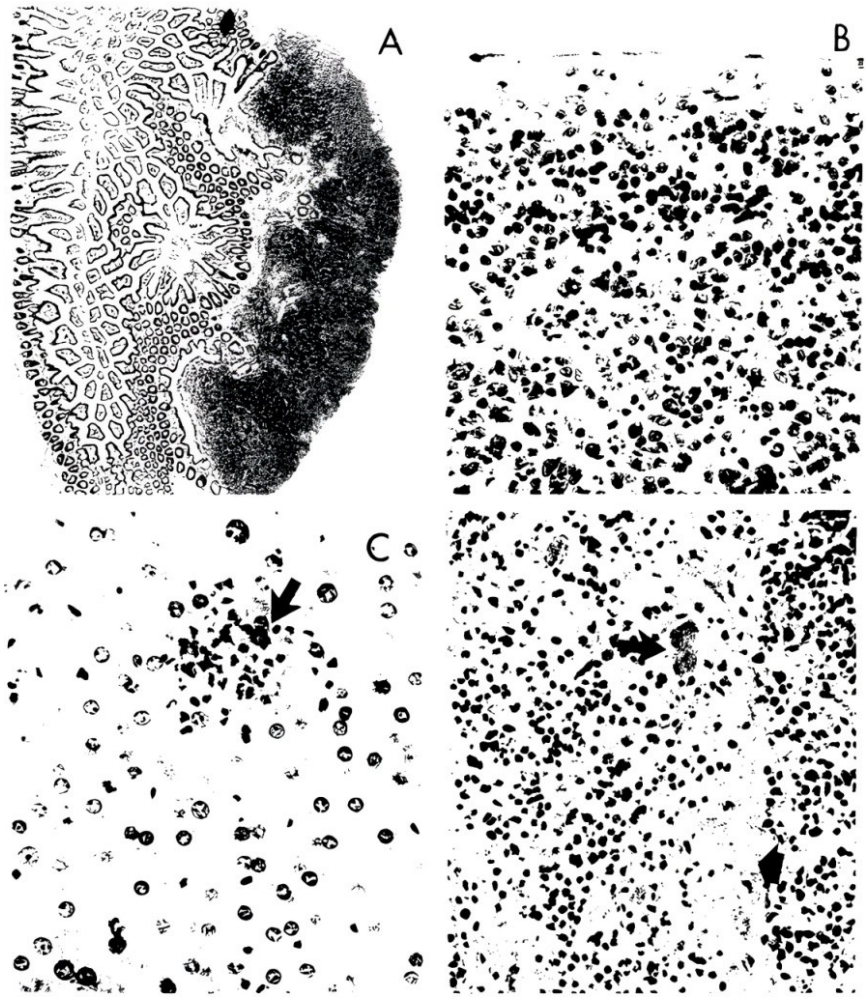


Figure 11.1. *Pathological changes in mice, Brown & Brehn, H&E (by Butler T., 1982; published under licence from The American Society for Microbiology, permission conveyed through Copyright Clearance Center, Inc.):*
A - 31x; fragment of the terminal ileum. Lymphoid hyperplasia of the peyer's patch is observed. The mucous membrane covering the peyer's patch and villi is intact;
B - 320x; mesenteric lymph node tissue. There is an increase in the number of mononuclear cells;
C - 400x; liver tissue. There are microabscesses with accumulation of neutrophils (marked by arrow);
D - 320x; red pulp tissue of the spleen. Extensive necrosis with neutrophil infiltration, presence of macrophages (marked by a large arrow) and a large number of extracellular bacteria (marked by a small arrow) is observed

tem examination shows absence of pathology in the lungs[1], pancreas and kidneys, but reveals (**Figure 11.1**) spleen and liver pathology characteristic of plague, as well as minor changes in the gastrointestinal tract, although the intestinal mucosa is intact (i.e. unchanged) throughout. The mesenteric lymph nodes and peyer's patch are hyperplastic (i.e. have an altered structure with an increased number of constituent cells), with increased numbers of mononuclear cells in the sinuses (some show bacteria even after 72 hours). There is no enteritis or abscess in the small intestine, and excretion of bacteria with faeces is extremely rare

In contrast to other members of the genus *Yersinia* (in particular, *Y. enterocolitica*), which are characterised by acute abscesses of the peyer's patch and mesenteric lymph nodes, oral infection *with Y. pestis* results in minimal intestinal pathology, indicating a generalised systemic infection (i.e. spread of the pathogen mainly by the lymphohaematogenic route) rather than an intestinal form. The site of "transition", i.e., the specific anatomical site where the bacteria cross the surface of the gastrointestinal epithelium, remains unknown. It is most likely to be the mucosa of the abdominal oesophagus or proximal stomach, as the bacteria cannot resist the action of gastric juice (*Y. pestis* dies at pH < 3).

Thus, the infectious process with the implementation of the alimentary mechanism of infection can proceed without the development of bubonic or secondary pneumonic forms, passing from the organs of the gastrointestinal tract to generalised infection, revealing lesions of the spleen and liver characteristic of the secondary septic form, as well as minor lesions of lymphoid structures of the small intestine (as a rule, its lower part).

[1] In this case, this rules out inhalation or aspiration of bacteria, i.e. it proves that the animals were infected via the gastrointestinal tract.

12 PLAGUE IN PRIMATES

Primates are not naturally infected with plague, but they are used as model animals, along with guinea pigs (reference) and mice, in experiments for preclinical evaluation of candidate vaccines and new treatments[1], especially when modelling primary pneumonic (inhalation) plague, because, unlike bubonic and septicemic plague, it is asymptomatic for quite a long time. Adult female dwarf vervet monkeys (*Chlorocebus pygerythrus*), crab-eating macaques (*Macaca fascicularis*) and rhesus macaques (*Macaca mulatta*) are used as primates, which are subjected to inoculation (bubonic model with subsequent development of secondary septicemic and secondary pneumonic forms) or aerogenic (primary pneumonic model with subsequent development of secondary septicemic form) infection as the main routes of human infection. In this

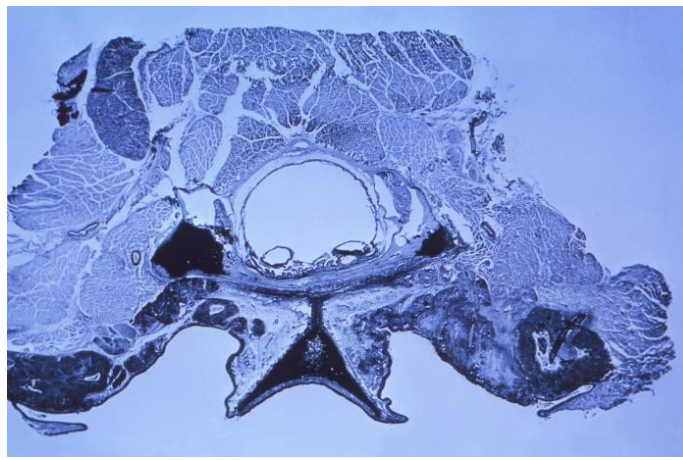
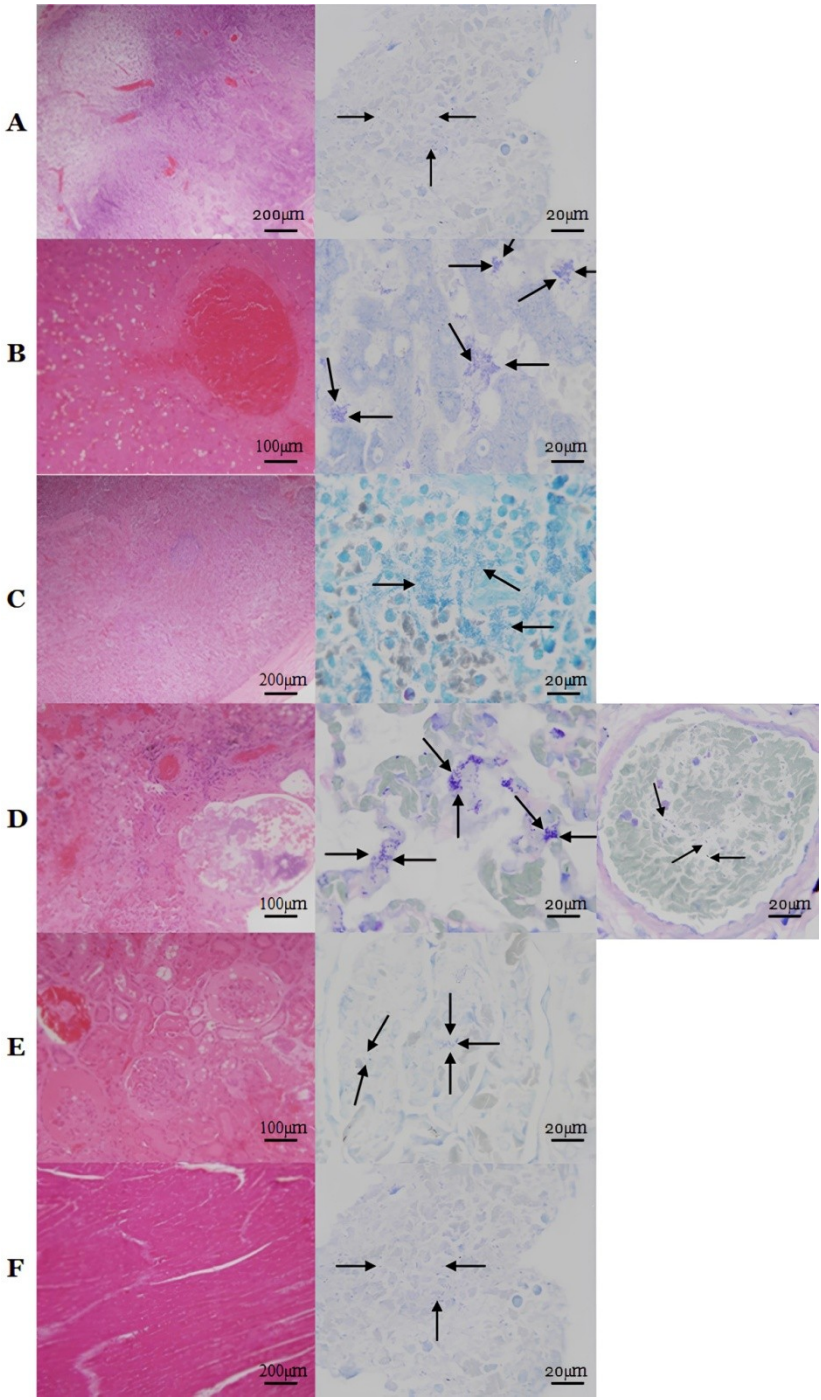


Figure 12.1. *Transaxial section of the primate neck through the level of the nasopharynx. Inflammation of the lymphatic pharyngeal ring and bacterial colonies are observed*

[1] For example, Gepotidacin is a first-in-class triazaacenaphthylene antibacterial agent that selectively inhibits bacterial DNA gyrase and topoisomerase IV through a unique binding mode, was tested in a nonhuman primate model of pneumonic plague (see Jakielaszek C., Hossain M., et al. *Gepotidacin is efficacious in a nonhuman primate model of pneumonic plague*. Sci Transl Med. 2022; 14(647):eabg1787; DOI: 10.1126/scitranslmed.abg1787).


A
200μm
20μm
B
100μm
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C
200μm
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D
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20μm
20μm
E
100μm
20μm
F
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◄ ***Figure 12.2.*** *Tissues of lymph node (A), liver (B), spleen (C), lung (D), kidney (E) and heart (D) of a rhesus macaque injected with Y. pestis strain 141; death occurred on day 5, H&E (left column) and Giemsa stain (right column), arrows indicate bacterial clusters (by Tian G., 2011):*
A – there is a change in architecture, severe swelling and oedema, decreased lymphocyte count, medullary strands (i.e. brain matter) are damaged and surrounded by a large number of bacteria;
B – there is a hepatocyte oedema, dilatation and hyperaemia of the central vein of the hepatic lobules, slight congestion in the hepatic sinus, in which several large clusters of bacteria are observed;
C – there is a decrease in the white pulp, swelling of the splenic thrusts of the red pulp and many large clusters of bacteria, the number of lymphocytes is reduced;
D – there is a haemorrhage, effusion and oedema, inflammatory cell infiltration and necrosis; bacterial accumulations are found in the alveolar septum (first right column image) and within blood vessels (second right column image);
E – there is a capsular effusion, interstitial oedema and vascular swelling, small clusters of bacteria are found inside the tubule;
F – no pathological changes are observed, bacteria are detected in the blood of the heart

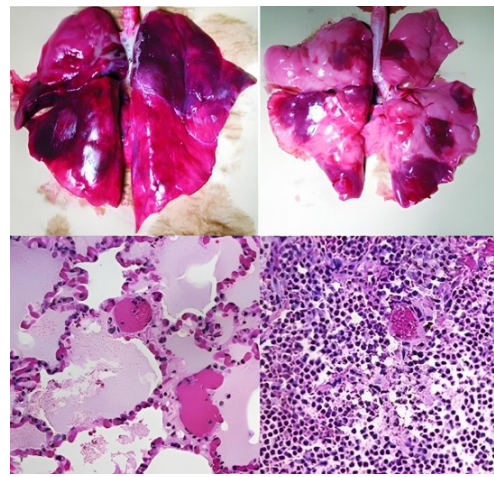


Figure 12.3. *Lungs (top row) and lung tissue (bottom row; 200x, H&E) of crab-eating macaques (Macaca fascicularis) aerogenically infected with Y. pestis strain C092. Multiple areas of severe stasis and haemorrhage are observed. Slices revealed multifocal acute bronchopneumonia, neutrophils, macrophages and monocytes, minor necrotic debris and large accumulations of bacteria in interstitial spaces within the alveoli are present (by Cornelius C. A., 2008; published under licence from the American Society for Microbiology, permission conveyed through Copyright Clearance Center, Inc.)*

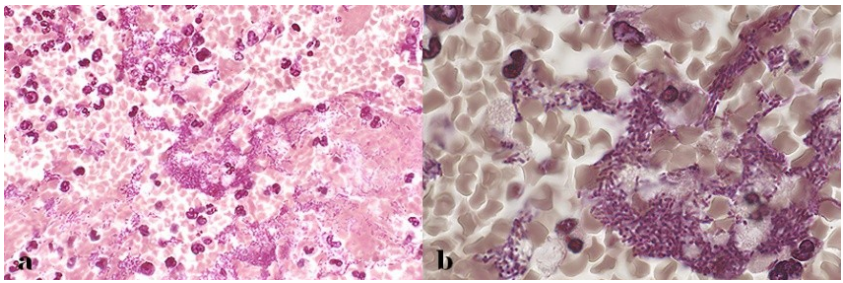


***Figure 12.4**. Lung tissue from an adult female pygmy green monkey aerogenically infected with Y. pestis strain Java 9; death on day 5 (WSC Case ID 2 (941200, AFIP, AFIP6615, USAMRIID, 1995; published with permission of The Joint Pathology Center): a - 40x, H&E; b - 100x, Brown-Hopps Gram (BH)*

case, the infectious process is not very different from that described previously **(Figure 12.1 – Figure 12.5**), except for inflammation in the pharyngeal lymphoid ring (Waldeyer's tonsillar ring; **Figure 12.1**). Interestingly, even when a strain lacking the pFra plasmid, i.e. unable to synthesise the capsule (*Y. pestis* strain Java 9), is used, a severe multilobar pneumonia with serous haemorrhagic pleural effusion (i.e. fluid accumulation in the pleural cavity) and minor fibrinous pleuritis[1] develops (**Figure 12.4**). Multilobar (i.e., bilateral) pneumonia is characterised by red, heavy, oedematous lobes that do not subside. Spherical or disc-shaped necrohaemorrhagic foci (usu-

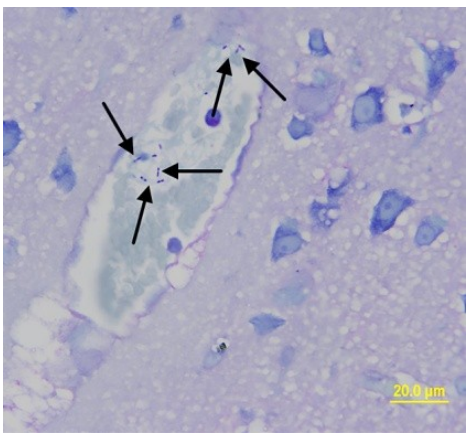


***Figure 12.5.** Brain tissue of rhesus macaque monkeys injected with Y. pestis strain 141; death on day 5, Giemsa stain. Arrows indicate small clusters of the bacterium found in blood vessels of the brain (by Tian G., 2011)*

[1] This is important because, for example, when assessing vaccine efficacy, antibody titre to the capsule antigen (capsule) is measured, i.e. fractionless strains, which, however, are capable of causing primary pneumonic plague, are not taken into account.

ally up to 2 cm in diameter) are present in the affected lung lobes (**Figure 12.2**-D and **Figure 12.3**). Rare pulmonary fibrin microthrombi are present, which are characteristic of disseminated intravascular coagulation (DIC) resulting from bacterial endotoxin (LPS)[1].

13 SEVEN HUNDRED AND THIRTY-ONE

…From all this we may learn that there are two races of men in this world, but only these two – the "race" of the decent man and the "race" of the indecent man. Both are found everywhere; they penetrate into all groups of society. No group consists entirely of decent or indecent people... Man is that being who invented the gas chambers of Auschwitz; however, he is also that being who entered those chambers upright, with the Lord's Prayer or the Shema Yisrael on his lips.

Viktor Frankl. Man's Search for Meaning

A faint breeze wafts across the training area at Anda Railway Station in Manchuria. Many people, tied to poles and suffering from the heat, wait quietly for their fate. They are nameless "logs", as they are disparagingly called. In a little while, porcelain bombs filled with plague fleas will rain from the sky. Everything happens under the supervision and direction of the military of the Epidemic Prevention and Water Purification Department, the infamous Japanese unit 731, commanded by Ishii

[1] The mechanism of DIC is not fully understood, but it is believed that LPS activates macrophages, lymphocytes and the complement system, localises on/in endothelial cells (causing vasculitis), and activates clotting factor XII (Hageman factor), which leads to thromboembolism and ultimately to the development of DIC.

Shirō[1] during the Second World War. Not far from them stands a man we know. He would be glad not to be here: to contemplate the play of the sun on the stones of the garden instead of watching it play on the stone faces of the victims. But history cannot be erased or forgotten, nor can the rotten fruits that a nation, afflicted by the spirit of militarism and the belief in its superiority, can spew forth. They may be forgotten, denied or justified by a higher purpose, but history, that inexorable witness, preserves everything in its memory, because every event is imprinted in the gaze of the eye.

In the first division, the Takahashi group is researching plague, while the Okamoto and Ishikawa groups are researching pathogenesis. In the second division, the Yakenari group is engaged in the production of ceramic bombs, and in the fourth division, the Karasawa group is building up the bacterial mass. During the years of plague research alone (from 1938 to 1945), about 3.5 thousand people died (about a third of all dead "logs"), and several outbreaks were caused in China. Infection of people occurred both in the field and in the laboratory. Doses and conditions were selected. Sometimes infected people were dissected alive to better understand pathological changes in their organs, and the artist used a fine bamboo brush to transfer what he saw to paper in great detail. Everything was done for the forthcoming invasion of neighbouring countries, which was justified by the sole desire to "improve" the lives of their inhabitants by "liberating" them from Western influence through "compelled" annexation. War for the sake of "peace"[2]. But at the same time, these horrific experiments shed light on the pathogenesis of plague and other infections under research. While it is impossible to justify even one life ruined in this way, it would be hypocrit-

[1] 石井 四郎, 1892–1959; the Japanese physician, bacteriologist, lieutenant general, developer of bacteriological weapons, war criminal, leader of Unit 731. After World War II, he received immunity from prosecution in exchange for the results of his research (almost all documents of the unit were destroyed during the retreat). There are reports that he took consultative part in the work of the American programme for the development of biological weapons (there are also reports that some members of the unit took part in a similar programme of the USSR).

[2] It should be remembered that in previous centuries the Japanese had been subjected to racial discrimination by European countries and the United States on several occasions. In 1919, the Japanese government submitted a proposal to eliminate racial discrimination to the Paris Peace Conference, which was supported by the majority of delegates but blocked by Thomas Woodrow Wilson, US President (1856-1924) for violating voting rules.

ical not to recognise that we have used the knowledge gained, that we have accepted it and based new, albeit humane, research on it. We should therefore remember that the history of bacteriology is written not only by heroes who sacrifice their own lives for others, but also by criminals who make bloody sacrifices on the altar of war[1].

A flea bites the unfortunate man and he is returned to prison, but the plague bacteria have already entered the wound ("entry gate"), marking the beginning of the first stage of the infection process. This stage is rather poorly understood as it is extremely difficult to model. It should be noted that fleas introduce bacteria into the skin tissue rather than subcutaneously, as the length of their oral apparatus does not allow them to penetrate deeper than the dermis (**Figure 13.1**). Flea saliva contains the anticoagulant apyrase, which slightly blocks platelet and neutrophil aggregation, which, however, does not contribute significantly to the infection process. At the site of the entry gate, an inflammatory focus (*cutaneous form*) may subsequently form, accompanied by an area of redness (rarely a petechial rash) and then a so-called *primary affect* (ulcer, flectena, carbuncle). Only in 5% of cases the inflammatory focus is sufficient for visual detection

At this stage, each bacterium faces two seemingly opposite tasks: on the one hand, it must ensure evasion from the action of innate immunity factors (survival), and on the other hand, it must penetrate macrophages, an important cellular factor of innate immunity, to multiply *(intracellular phase of the infectious process*).

Cationic antimicrobial peptides are probably the first obstacle that the plague microbe encounters on its way. LPS (or rather, the presence of N-acetylglucosamine in the cortical part) and modification of phosphate groups by 4-aminoarabinose, for which the protein glucose-1-phosphaturidylyltransferase (GalU) is responsible, are responsible for reducing the binding of the bacterium to positively charged peptides. Further, the iron content of tissues is drastically reduced, which should

[1] Although the victorious countries condemned the Japanese experiments at the Tokyo (1946-1948) and Khabarovsk (1949) trials, quite quickly they (particularly the USA and the USSR) set up their own biological weapons programmes, which existed right up to the 21st century, and probably still exist today.

Я знаю, что в мае-июле 1940 года в Центральном Китае, в районе Нимбо генерал ИСИИ, в то время полковник, руководил операцией боевого применения бактерий чумы против китайских войск.

Об этой операции я лично видел в отряде № 731 кинофильм заснятый непосредственно в районе боевых действий. На экране я увидел следующие кадры: к самолету прикрепляют специальные сосуды, причем в пояснении говорилось, что в этих сосудах помещены блохи, зараженные чумой, в самолет садятся люди, лиц их не видно, так как они обращены к самолету. Самолет отрывается от земли, а в отдалении стоят люди участники экспедиции и напутственно машут руками. Самолет снят в воздухе над расположением противника, на земле видно какое-то движение китайских войск и населенный пункт, сосуды под крыльями самолета открываются, из них вылетают блохи ввиде небольшого облачка. Самолет возвращается на аэродром. Надпись "операция закончена". Из самолета выходят полковник ИСИИ и майор ИКАРИ. Следует надпись: "Результат". На экране показана китайская газета и японский перевод ее. В китайской газете написано, что в районе Нимбо внезапно вспыхнула сильная эпидемия чумы. Предполагалось, что эта эпидемия возникла по вине японских войск, так как очевидцы видели японский самолет, летевший на небольшой высоте и выпускающий облачко, так как было доказано, что в данном случае источником заразы были не грызуны. Делался вывод, что эпидемию чумы вызвали японцы искусственно. Далее, на экране показан фотоснимок из китайской газеты: китайский противоэпидемический отряд в сопровождении полицейских дезинфецирует местность. Потом показан групповой фотоснимок китайского противоэпидемического отряда, участвовавшего в этой работе.

inhibit bacterial multiplication. However, the yersiniabactin{chromosome}[1] siderophore synthesised by *Y. pestis* binds the available iron (Ybt-Fe^{3+} complex) and delivers it to the bacterium, providing it in sufficient qu-

[1] Hereinafter in Chapters 13-15, the genetic structures on which the genes encoding the synthesis of the indicated factor are present will be indicated in curly brackets.

◀ *Fragment (pp. 7 and 8) of the Protocol of the interrogation of the prisoner of war Lieutenant Colonel of the Medical Service of the Japanese Army Nishi Shuney (Toshihide) from 15-17 January 1947. Courtesy of the ФКУ «Государственный архив Российской Федерации» (Ф. Р-9492. Оп. 10с. Д. 147. Л. 405–417):*
"I know that in May-July 1940 in Central China, in the Nimbo area [probably, Níngbō], General Ishii, then a colonel, led an operation of combat use of plague bacteria against Chinese troops. About this operation, I personally saw in Unit 731 a film shot directly in the combat area. On the screen I saw the following shots: special vessels were attached to the plane, and the explanation said that these vessels contained fleas infected with plague, people got into the plane, their faces were not visible, as they were facing the plane. The plane is lifted off the ground, and in the distance stand people - members of the expedition and waving their hands in farewell. The aeroplane is filmed in the air above the enemy position, some movement of Chinese troops and a settlement are seen on the ground; the receptacles under the wings of the aeroplane open, fleas fly out of them in the form of a small cloud. The aeroplane returns to the aerodrome. The inscription "the operation is over." Colonel Ishii and Major Ikari come out of the aircraft. The caption "result" follows. The screen shows a Chinese newspaper and a Japanese translation of it. The Chinese newspaper says that a severe plague epidemic suddenly broke out in the Nimbo area. It was assumed that this epidemic was caused by Japanese troops, as eyewitnesses saw a Japanese plane flying at a low altitude and releasing a cloud, since it was proved that in this case the source of the plague was not rodents, it is concluded that the plague epidemic was caused artificially by the Japanese. Next, a Chinese newspaper photo is shown on the screen: a Chinese anti-epidemic squad accompanied by police officers disinfecting the area. Then a group photo of a Chinese anti-epidemic squad involved in this work is shown"

antities[1]. In addition, the bacterium takes up zinc (with the help of yersiniabactin{chromosome}) and copper (probably with the help of yersinopin{chromosome}).

[1] The use of siderophores is not an "invention" of *Y. pestis*. Many Enterobacteriaceae use them for iron binding, but they are readily inactivated by lipocalin-2, so the "trick" of the plague microbe is that it synthesises yersiniabactin, which, unlike other siderophores, is not inactivated by lipocalin-2.

The bacterium is then exposed to lysozyme, synthesising in response to it the inhibitor of vertebrate lysozymes{chromosome} (ivy), which binds to lysozyme and inhibits it[1]. It also contributes to survival in contact with polymorphonuclear leukocytes, primarily neutrophils.

Plasminogen activator{pPla} (most likely together with the Ail{chromosome}) provides resistance to the complement system, which is the next factor in innate immunity: plasminogen activator{pPla} degrades the C3 component, and Ail{chromosome} binds factor H and the protein that binds the C4 component. By binding to LPS (still 6-acyl), plasminogen activator{pPla} cleaves and inactivates tissue factor pathway inhibitor (TFPI), which is a major inhibitor of coagulation (plasma coagulase activity[2]), ensuring blood clotting at the entry gate site (a kind of sealing of the infection). However, as the bacteria move deeper into the dermis, the 6-acyl LPS begins to transform into 4-acyl LPS, resulting in a change from the plasma coagulase activity of plasminogen activator{pPla} to fibrinolytic activity. Plasminogen is transformed into its active form (plasmin), which causes the breakdown of immune defence-boosting fibrin clots, allowing a large bacterial focus to form at the bite site with few inflammatory cells, promoting proliferation that will attract dendritic cells, neutrophils and tissue-resident macrophages quite rapidly (**Figure 13.2** and **Figure 13.3**). The bacterium is recognised by the Toll-like receptor TLR-9 located on dendritic cells, which leads to an increase in interferon synthesis by them. However, the uptake by dendritic cells, which is characteristic of most bacteria, practically does not occur. The plague microbe seems to avoid them. The reasons for this phenomenon are not yet fully understood, but there is evidence that the YopJ{plasmid pCad} provides inactivation of dendritic cells. On the other hand, dendritic cells can capture *Y. pestis* with the mannose receptor CD209 (DC-SIGN, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-in-

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the plague microbe is that it synthesises yersiniabactin, which, unlike other siderophores, is not inactivated by lipocalin-2.

[1] Interestingly, *Y. pseudotuberculosis* uses LPS rather than ivy to inhibit lysozyme. It was previously thought that because *Y. pestis* lost a number of genes during evolutionary separation from *Y. pseudotuberculosis*, the virulence genes retained by *Y. pestis* are also required by *Y. pseudotuberculosis* as its ancestor, but the fact that LPS is utilised to inhibit lysozyme makes this theory questionable.

[2] Remind: plasma coagulase activity was previously thought to provide block formation in fleas.

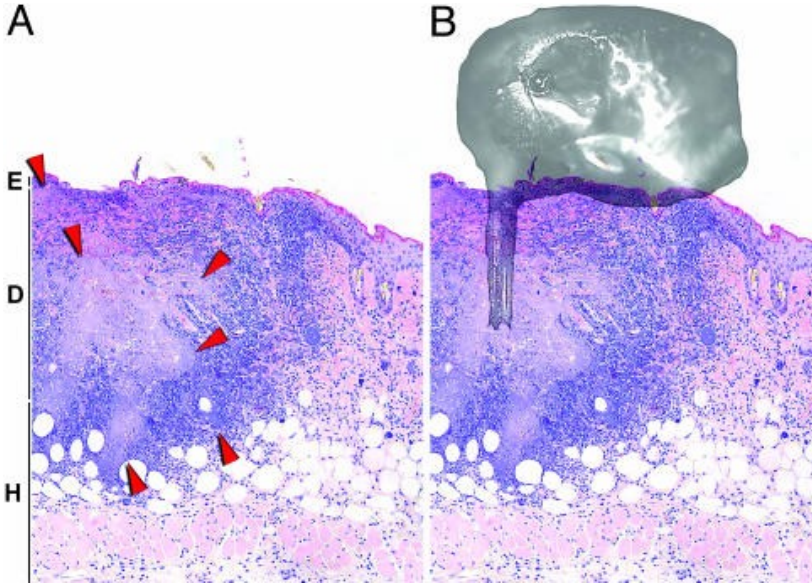


Figure 13.1. *Skin section 3 days after a bite by an infected flea (mouse model, Y. pestis strain 195/P), H&E. A large bacterial mass in the form of a light purple area (surrounded by red arrows) extending from the middle of the dermis (D) to the epidermis (E) and hypodermis (H) is observed (A, 100x), which corresponds to (B, 100x) the location of the mouth apparatus of the flea (by Sebbane F., 2006; Copyright (2006) The National Academy of Science, U.S.A., published with permission of the copyright holder)*

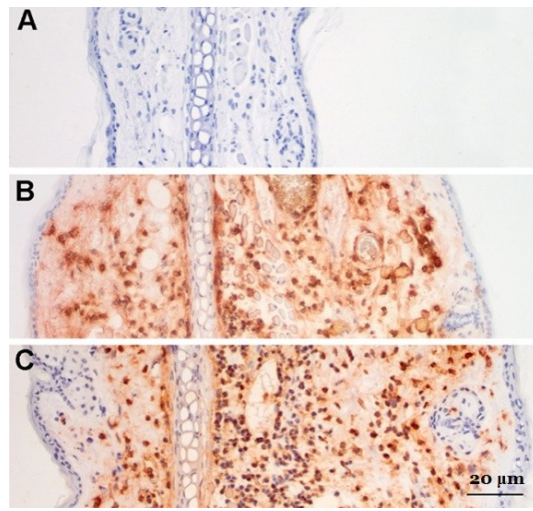


Figure 13.2. *Inflammatory reaction at the site of subcutaneous injection after 48 hours (mouse model), H&E, immunehistochemical staining. Polymorphonuclear leukocytes (mainly neutrophils) of brown-orange colour are observed (by Guinet F., 2008): A – control (saline); B – Y. pestis strainCO92; C – Y. pseudotuberculosis strain IP32953 (comparison)*

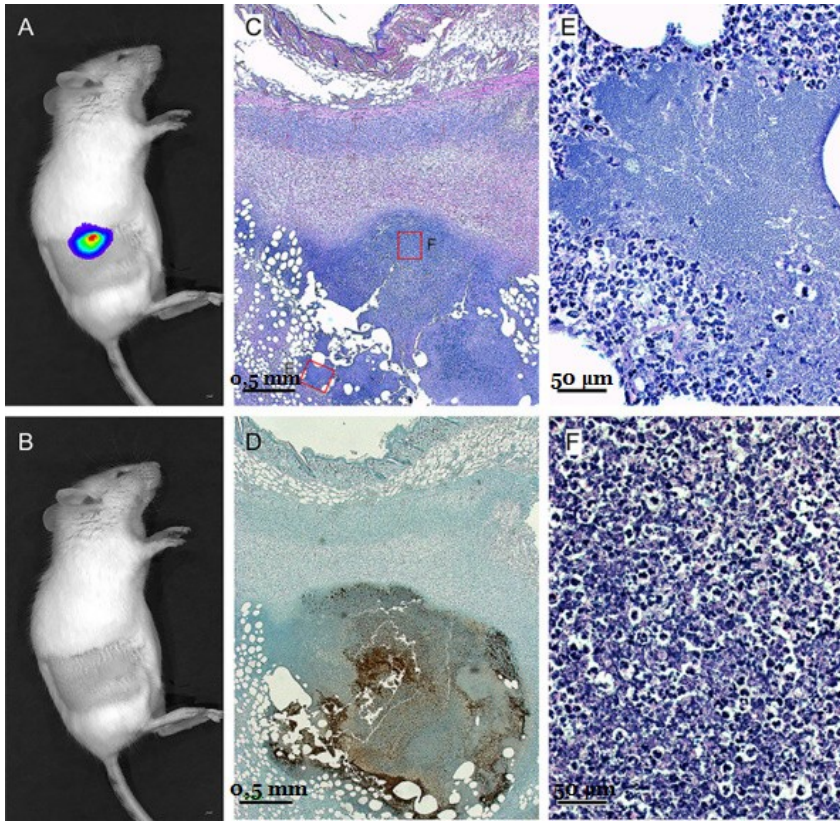


Figure 13.3. *Inflammatory response at the site of flea bite (mouse model, Y. pestis bioluminescent strain pGEN-luxCDABE). Mouse photographed (B) with bioluminescence (A) marking the bite site (red) and inflammation. Skin sections taken from this site (C, H&E; D, immunohistochemical staining) reveal ulcerative and necrotising dermatitis and a large accumulation of bacteria (D, brown). The areas highlighted by red rectangles (in C) show (E) large clusters of bacteria surrounded by neutrophils and (F) an area of viable and degenerate neutrophils and macrophages (necrosis) mixed with bacteria under magnification (by Bosio C. F., 2020)*

tegrin) present on the surface of dendritic cells and able to bind to 6-acyl LPS (which is still present[1]) for subsequent entrapment in lymph nodes. Within the dendritic cell, bacterial killing occurs with subsequent cleavage to effect major histocompatibility complex (MHC) class II loading. After loading, MHC with a peptide (in this case - a piece of cleaved bacteria (bacterial fragment), such a peculiar trophy[2]) goes to the cell surface, where it interacts with T-cell receptors located on the surface of CD4-positive lymphocytes (i.e. T-helpers)[3], which ultimately leads to the release of cytokines[4] and activation of B-lymphocytes (antibody synthesis), cytotoxic T-lymphocytes (T-killers) and NK cells[5]. This is when acquired immunity comes into play.

Bacteria captured by neutrophils (**Figure 13.4**) will undergo phagocytosis[6], which only 10-15% will survive[7]. The main role in this will be played by the presence of the capsule{pFra}, the previously mentioned LPS modifications that reduce the action of cationic antimicrobial peptides, and the Yops{plasmid pCad} (YopE, YopH, YopJ and YpkA), which, acting together, block the release of primary granules (containing bactericidal enzymes) and disrupt the neutrophil cytoskeleton. In addition, the YitA{chromosome} and YipA{chromosome}[8] proteins which were synthesised

[1] We said in Chapter 6 that a small fraction of 6-acyl LPS is synthesised at 37°C / 98.6°F.

[2] I.e. antigen – **anti**body **gen**erator..

[3] Since this process is carried out in lymph nodes, we can assume that binding of plasminogen activator{pPla} to CD205 is a strategy for the bacterium to deliver to the lymph node.

[4] Interestingly, in the case of some animals, such as dogs, the bacteria have little or no resistance to the process, so the disease runs more easily.

[5] It should be noted that cytotoxic T-lymphocytes are activated few (about 10%) due to the presentation of MHC class II antigen (there is no information about the possibility of presentation of MHC class I antigen, which activates the majority of cytotoxic T-lymphocytes), and NK-cells are found only in the lymph node and then in small numbers.

[6] Here it is important to specify that a part of infected neutrophils will be taken up by macrophages in the process of efferocytosis ("scavenging"), leading to the secretion of cytokine 1L-1RA, which blocks inflammation and increases the efficiency of the T3SS (synthesis of Yops).

[7] There are reports that some of the captured bacteria are even able to multiply in neutrophils!

[8] Both proteins belong to the insecticidal-like toxin complex (Tc) family of proteins and, being synthesised at low temperature (in the flea), persist at 37°C / 98.6°F for several hours. Consequently, if the source of infection is a warm-blooded host, survival in neu-

in the flea and localised on the outer membrane, increase the resistance of bacteria to phagocytosis. However, it is currently unclear to what extent microbe survival in neutrophils is important for pathogenesis, since virtually all released bacteria will remain at the site of primary affect.

A little later than neutrophils arrive macrophages, which are a so-called *replicative niche* for *Y. pestis*, so they tend to attach to them, with some macrophages carrying them to regional lymph nodes (some of the bacteria will reach them independently via lymphatic vessels). Attachment to the macrophage is facilitated by Ail{chromosome} and plasminogen activator{pPla}. The former probably carries out the attachment of the bacterium to the β-integrins of the macrophage membrane. The latter modifies the YapE protein so that it acquires adhesive properties[1] and also binds to the mannose receptor CD205 (DEC205; LY75) on the macrophage surface, which is responsible for bacterial uptake by the macrophage. In addition, like dendritic cells, macrophages have the mannose receptor CD209 on their surface, which can also capture *Y. pestis* by binding to the 6-acyl LPS (of course, in those bacteria that still have it).

The bacteria are then absorbed by the macrophage into the phagosome (**Figure 13.5**), formed by closing the cell membrane around the bacteria, which then fuses with the lysosome (i.e. a small organelle that ensures the degradation of cellular elements) to form the phagolysosome. It is difficult to say what will happen to the unabsorbed bacteria (whether they will migrate to the lymph node or remain in the skin), but those who are in the macrophage now face an important task – to survive, because it is in the phagolysosome that optimal conditions for bacteriolysis (i.e., destruction of the bacterial envelope) and subsequent cleavage in order to load class II MNS and, consequently, to perform presentation (i.e., presentation) to the T helper and release of cytokines in the scenario described above. Given that antibodies are most

trophils is greatly reduced. The role of other proteins, members of this family, is not fully understood, although they are known to be active against murine fibroblasts but not against human intestinal epithelial cells, indicating host selectivity. In addition, there are reports that these proteins are activated in macrophages and utilise a molecular needle (T3SS) to enter the macrophage cytoplasm.

[1] This process is called protein post-translational modification, i.e. chemical modification (change) of the protein after synthesis on the ribosome, which is usually the last step in protein biosynthesis.

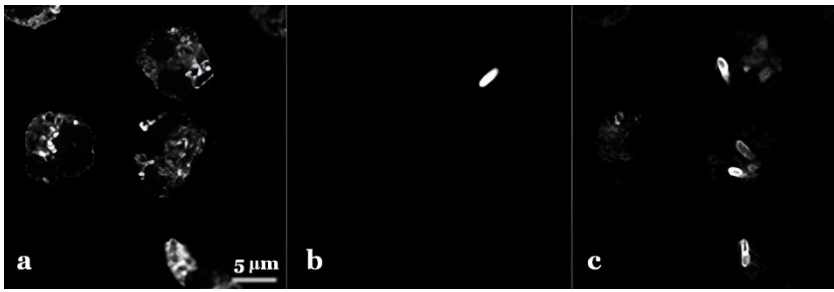


Figure 13.4. *Confocal microscopy of neutrophil phagosomes containing live and dead cells of Y. pestis KIM6+ strain, immunohistochemical staining (by Dudte S. C.; 2017): a - neutrophil phagosomes; b - live bacteria; c - dead bacteria*

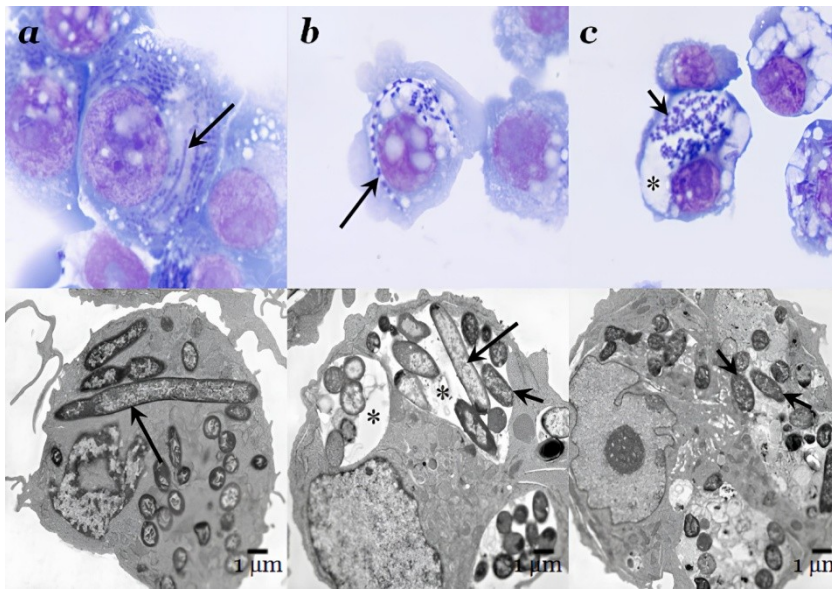


Figure 13.5. *Light microscopy (top row, Wright-Giemsa stain, 1000x) and transmission electron microscopy (bottom row, glutaric aldehyde fixation, 6000x) of RAW 264.7 macrophages 2.5 (a), 7.5 (b) and 27.5 (c) hours after capture of Y. pestis strain KIM6+. Long and short arrows mark filamentous bacteria and coccobacilli, respectively, and an asterisk marks extensive vacuolar expansion of phagolysosomes (by Ponnusamy D., 2012)*

often produced to the capsule antigen{pFra}, which is synthesised specifically in the macrophage, it should be assumed that presentation of bacterial fragments of class II MNS by macrophages is more efficient than presentation by dendritic cells.

Entry into the phagolysosome of a macrophage marks the beginning of the second stage of the infectious process (2-3 days after bacterial entry into the organism)[1], during which the bacterium is exposed to the action of reactive nitrogen and oxygen compounds. Protection from the former is provided by the RipA{chromosome} protein and butyryl-CoA-transferase, and from the latter by yersiniabactin{chromosome}[2]. A distinctive feature of the phagolysosome is the acidity of the medium (i.e. pH<6), at which the bacterium begins to synthesise pH6-antigen{chromosome}, i.e. the fimbriae required for attachment to the phagolysosome wall. Another feature of phagolysosomes is a low calcium ion content, at which the bacterium begins to synthesise LcrV{pCad}, which stimulates the secretion of Yops{pCad}, i.e. activation of the T3SS. LcrV{pCad} organises and targets molecular needles (YopB{pCad} and YopD{pCad}), allowing Yops{pCad} to enter the cytoplasm, where they disrupt signalling systems (YopE, YopH, YopJ, YopM, YopT, YpkA), disrupt the cytoskeleton (YopE, YopH, YopT, YpkA) and cytokine-inducing activity (YM, YopJ) of the macrophage. The YopH{pCad} enables mitophagy, that is, it damages mitochondria, preventing them from releasing mitochondrial reactive oxygen species (mROS). The YopJ{pCad} induces apoptosis. It should be noted that when synthesised for long periods of time (more than 36 hours) inside macrophages, capsular antigen{pFra} is also able to exert cytopathic effects through the formation of pores in their outer membrane.

At this point, the bacteria move from the intracellular phase to the *extracellular phase of the infection process*. Having multiplied in macrophages, they are well prepared to resist the action of immunity. Together with the apo-B-lipoprotein with which the pH6 antigen{chromosome} covers the surface of the bacterium, the capsular antigen{pFra} (remind: its

[1] Synthesis of all factors that ensure survival at this stage is carried out during the first four hours in the macrophage.

[2] In addition, yersiniabactin{chromosome} provides copper ion binding by exhibiting superoxide dismutase activity, transforming reactive oxygen species into less toxic compounds.

synthesis begins while still in the macrophage) screens the action of plasminogen activator{pPla} and Ail{chromosome} (remind: which were previously involved in attachment to macrophages). Of course, as we said earlier, capsular antigen{pFra} may not be synthesised, which would only affect the speed of the infection process (slow it down). Also, the pH6 antigen{chromosome} prevents new uptake of the bacterium by the macrophage (see Chapter 6, Figure 6.1).

The presence of bacteria in the lymph nodes (**Figure 13.6**) stimulates the synthesis of pro-inflammatory cytokines (some bacteria, as we have already mentioned, are phagocytised by antigen-presenting cells[1]) and fibrin. The leading role in this belongs to the still present 6-acyl LPS. The fact is that 6-acyl LPS is recognised by the TLR-4 receptor, whose activation induces the synthesis of pro-inflammatory cytokines TNFα, IL-1, IL-6 and IL-8. As a consequence, an acute inflammatory reaction develops, which is accompanied by lymphodenitis characterised by vascular thrombosis and necrotic changes in the node tissues involving the subcutaneous tissue, leading to their fusion into a single conglomerate, i.e. formation of a buboon (*bubonic form*). In most cases, the appearance of buboes coincides with fever.

Despite the inflammatory process, few neutrophils are present in the resulting buboes, and their migration is inhibited by the YopJ{pCad}, which inhibits IL-8 synthesis (which in turn promotes neutrophil recruitment and activation), as well as by LcrV{pCad} and Ail{chromosome} (the mechanisms are not understood). NK cells, which are few in lymph

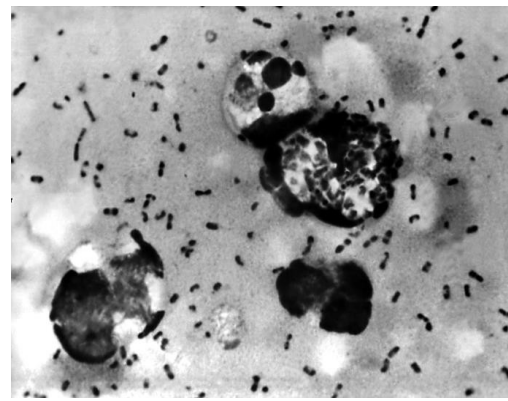


Figure 13.6. *Lymph smear from buboesis with cellular debris and multiple bacteria (photograph from the collection of Dr. Karl Friedrich Meyer, 1965). These are exactly the cells seen by Alexandre Yersin, compare with* ***Figure 3.1***

[1] This is the name given to cells that present antigen to T-helper cells (in this case, dendritic cells and macrophages).

nodes, are able to destroy bacteria (they do not need antigenic determinants), but they are killed by the YopM{pCad}, which leads to decreased production of INFγ (which activates macrophages).

An equally important role is played by plasminogen activator{pPla}, which, exhibiting proteolytic activity, provides the bacteria with nutrition (creates an optimal environment for growth), or protects the bacteria from the bactericidal action of the immune system. Either way, it has been shown[1] experimentally (with Pla-negative strains) that plasminogen activator{pPla} ensures good bacterial survival in the lymph node and, consequently, its high bacterial load (**Figure 13.7**).

Bursting of the buboesis and penetration of bacteria into the blood[2] (**Figure 13.8**) marks the beginning of the third stage of the infectious process and, as can be guessed, is accompanied by bacteraemia[3]. A major role at this stage is played by 4-acyl LPS, which has a low ability to bind to the TLR4/MD2 receptor complex (this complex, by binding to LPS, activates the immune response). Capsular antigen{pFra} (capsule) depletes the complement system by selective activation of C2 and C4 components (remind: earlier this function was performed by plasminogen activator{pPla} and Ail{chromosome}, but they are shielded by capsular antigen{pFra} to prevent binding to phagocytes) and evades capture by phagocytes (literally "eludes" them).

The bacteria that have entered the bloodstream are quickly enough filtered by the spleen and liver. No inflammatory reaction is observed in these organs: firstly, due to the action of 4-acyl LPS, and secondly, due to the action of YopM{pCad}, which suppresses the migration of dendritic cells into the spleen and, by activating the cysteine protease caspase 3 (CASP 3), causes apoptosis of neutrophils and macrophages in the liver.

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[1] Guinet F., Avé P., et al. *Dissociation of Tissue Destruction and Bacterial Expansion during Bubonic Plague*. PLoS Pathog. 2015; 11(10):e1005222; DOI: 10.1371/journal.ppat.1005222.

[2] It should be noted that it is not always the appearance of some amount of pathogen in the blood that indicates a lymph node rupture.

[3] At the same time, the bacterium can be transported through the lymphatic vessels to other lymph nodes, causing inflammation in these nodes that develops into buboes (similar to the second stage of the infection process described earlier). Such buboes are commonly referred to as *second-order buboes*, but their formation is rare (we could see a second-order buboes in **Figure 7.12**-(D)/frame).

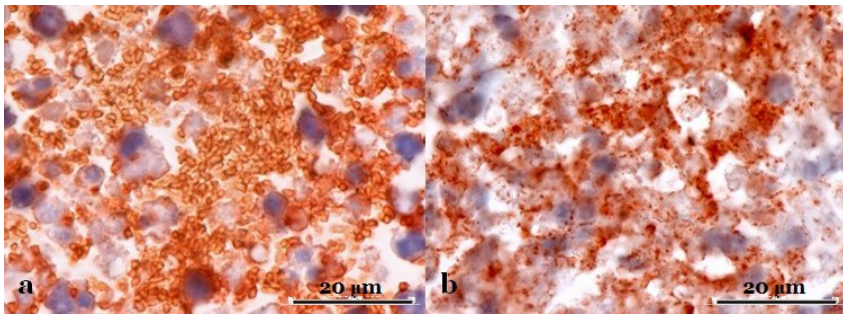


Figure 13.7. *Lymph node tissue 48 hours (mouse model) after intradermal injection of Y. pestis strain 6/9, bv. orientalis: Pla+ (a) and Pla- (b), immunohistochemical staining. The orange-brown colour of the staining shows intact cells of the wild-type strain against dot staining of dead cells (probably cell fragments) of the Pla- strain (by Guinet F., 2015)*

In addition, by inactivating caspase-1 together with YopJ{pCad}, it inhibits the synthesis of IL-1β and IL-18[1].

The absence of an inflammatory response leads to uncontrolled bacterial multiplication and, as a consequence, destruction of the liver and spleen with the entry of large quantities of bacteria into the blood, which marks the beginning of the fourth (terminal) stage of the infectious process, accompanied by massive septicaemia (*secondary septicemic form*)[2]. At this stage, on the one hand, there is haematogenous dissemination (entry of the pathogen into various organs), and on the other hand - a generalised inflammatory reaction accompanied by high concentrations of pro-inflammatory cytokines. The formation of secondary buboes is possible.

Earlier we said that 4-acyl LPS has low cytokine-inducing activity, but under the influence of glycolipids present in liver, spleen and erythrocytes (remind: pH6-antigen{chromosome} binds lipids and also destroys erythrocytes), LPS conformational changes (i.e. changes in the spatial

[1] Caspase-1 inactivation prevents the maturation of the inflammosome, which is responsible for the activation of the inflammatory response and, in particular, the secretion of IL-1β and IL-18.

[2] To date, there is no clear boundary between the third (bacteraemia) and fourth (septicaemia) stages of the infectious process, so their separation is for methodological purposes only.

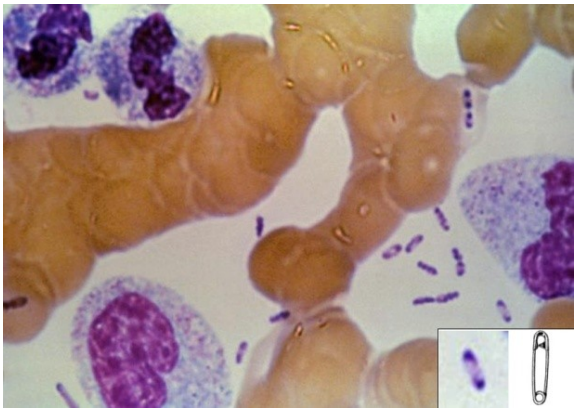


Figure 13.8. *Microphotograph of a blood smear containing Y. pestis, Wright's stain. You can see bipolarly stained ends like an safety pin (the lower right corner compares the stained bacterium with a pin for clarity). This is exactly the kind of cells seen by Kitasato Shibasaburō, compare it with* ***Figure 3.1***

structure of the molecule) occur. LPS, which, on the one hand, together with Brown's lipoprotein (Lpp)[1] induces production of TNFα, IFNγ, IL-1β, IL-6 and development of septic shock, and on the other hand, activates blood coagulation factor XII, which leads to thromboembolism and DIC. Due to the combined action of pH6-antigen$^{\{chromosome\}}$ and yersiniabactin$^{\{chromosome\}}$ (destruction of erythrocytes and capture of iron), anaemia develops. Georgiy Pavlovich Rudnev[2] reports[3] a red blood cells to **3-3.5 x 10^{12}/L** [male: 4.0-5.9 x 10^{12}/L, female: 3.8-5.2 x 10^{12}/L] / 3-3.5 million/μL [male: 4.0-5.9 million/μL, female: 3.8-5.2 million/μL] and a decrease in haemoglobin to 50-60%. In severe forms, he notes the rare appearance of normoblasts (i.e., cells - precursors of red blood cells), polychromasia[4] and anisocytosis (i.e., changes in the shape, size and colour of red blood cells). The number of white blood cells increases to **20-30 x 10^9/L** [4.5-11 x 10^9/L] / 20,000-30,000 cell/μL [4500 – 11,000 cells/μL].

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[1] The bacterial outer membrane protein is recognised by TLR-2.

[2] Георгий Павлович Руднев,1899–1970; the Ukrainian and Soviet physician, infectious disease specialist, member of the USSR Academy of Medical Sciences. Author of classifications of clinical forms of plague, tularemia and brucellosis.

[3] Руднев Г. П. *Клиника чумы, под ред. д-ра Ф. Р. Чушинского,* Ростовский государственный противочумный институт, кафедра инфекционных болезней Ростовского медицинского института, Ростов-на-Дону: Ростиздат, Тип. им. Коминтерна, 1938, 268 с.

[4] The ability of cells (in this case erythrocytes) to stain simultaneously with acid and basic dyes in a smoky grey-violet colour when according to Romanowsky-Giemsa stain, which indicates insufficient maturity of erythrocytes. This phenomenon was first discovered by Prussian (German) physician Paul Ehrlich (1854–1915).

There are widespread changes in small vessels and capillaries. A characteristic indicator is a decrease in the resistance of skin capillaries, and a rash may appear. Disruption of blood flow in the microcirculatory bed of the extremities leads to the development of acral necrosis. Of the large and medium vessels are affected mainly veins. In this case, haemorrhages in the walls of veins are often combined with their dissection and necrosis, capturing the middle and outer sheath of the vessel and sometimes perivascular connective tissue. Ivan Ivanovich Shirokogorov[1] reports changes in the aorta manifested by haemorrhage into the surrounding fibre and inflammatory cellular infiltration around the vessels feeding the aorta (i.e. vasa vasorum).

As a result of haematogenous dissemination, the bacteria enter various organs and, first of all, the lungs, which marks the beginning of the *secondary pneumonic form* (secondary pneumonic plague), which develops in about 10% of patients (usually as a result of untimely treatment). This form is particularly dangerous because of the possibility of airborne transmission of the pathogen from person to person. However, it should be noted that infection occurs only during coughing (the plague aerosol spreads several metres away!) and sputum discharge, in which the pathogen can persist for several days, though in the absence of light and heating (and who would think of heating sputum?). It is safe for the patient to breathe. In addition, the pneumonic form is characterised by the 80:20 rule of thumb, according to which *only 20% of the host population contributes at least 80% of the transmission potential measured by the basic reproduction number* R_0, i.e. only 20% of patients are responsible for 80% of transmission cases[2].

The infectious process in the lungs proceeds in two phases. In the *pre-inflammatory phase*, the pH6-antigen$^{\{chromosome\}}$ ensures the binding of bacteria to alveolar epithelial cells for multiplication (remind: by binding to phosphatidylcholine located on the surface of the epithelial cell membrane). Thanks to 4-acyl LPS and Yops$^{\{pCad\}}$, the bacteria inhibit

[1] Иван Иванович Широкогоров, 1869–1946; the Russian and Soviet physician, pathologist, member of the USSR Academy of Medical Sciences (1944), member of the Academy of Sciences of the Azerbaijan SSR (1945). Author of many publications on pathological anatomy of plague.

[2] Woolhouse M.E., Dye C., et al. *Heterogeneities in the transmission of infectious agents: implications for the design of control programs*. PNAS. 1997; 94(1):338–342; DOI: 10.1073/pnas.94.1.338.

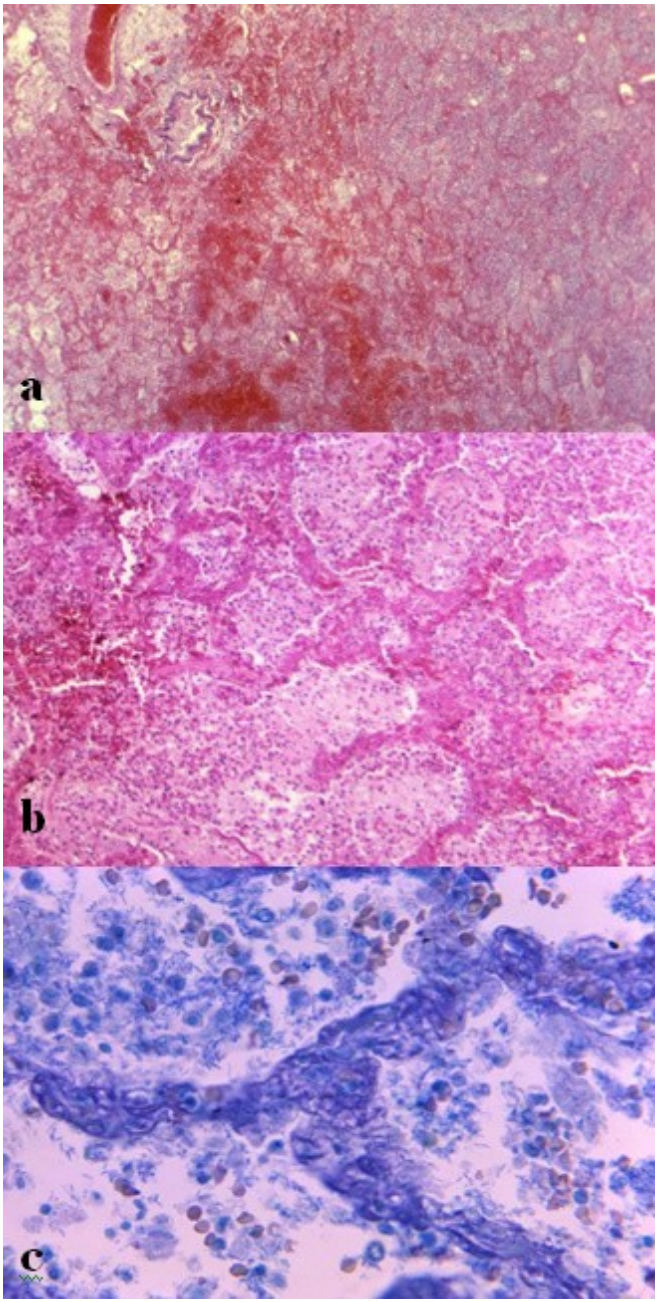


Figure 13.9.
Lung tissue taken from a patient with secondary pulmonary form of plague:
a - solitary pulmonary nodule , 30x, H&E; bright red zone of peripheral haemorrhage with consolidation zone (i.e. consolidation due to filling of alveoli with blood, exudate, etc.) on the right side is observed;
b - fragment of consolidation zone of solitary pulmonary nodule, 125x, H&E; alveoli filled with serous exudate, granulocytes and necrotic alveolar epithelial cells are observed;
c - lung tissue, 500x, Giemsa stain; numerous bacteria are observed

phagocytosis by alveolar macrophages and neutrophils (the influx of the latter into the lungs is reduced due to zinc binding by yersiniabactin{chromosome}[1], which also favours reproduction. which also favours bacterial multiplication), and their breakdown products (granule contents and active nitrogen forms) are responsible for lung tissue damage. In this case, IL-1β seems to be the only response to infection, which, however, is blocked by the antagonist 1L-1RA (as we remember, it is secreted during efferocytosis ("scavenging") by macrophages of infected neutrophils present in small numbers).

The *proinflammatory phase* does not begin until 36-48 hours later and is accompanied by activation of the proinflammatory cytokines IL-6, IL-17, TNFα I, IFNγ, and the chemokines CXCL1 and CCL2, which promotes a massive influx of neutrophils into the alveolar spaces (and some will again undergo efferocytosis), resulting in the formation of densely packed foci of inflammation that enlarge due to the constant influx of neutrophils. As a consequence, there is considerable destruction of the alveoli, accompanied by oedema, haemorrhage and finally necrosis (**Figure 13.9**). Against the background of massive pneumonia accompanied by pleural effusion, tracheobronchial lymph nodes may be affected (tracheobronchial lymphadenitis).

In 6% of cases, haematogenous entry into the brain and spinal cord membranes is possible with the development of the *meningeal form* (plague meningitis), which probably occurs through lymphatic vessels due to sufficient synthesis of the capsule (capsular antigen{pFra}) against the background of insufficient immune response at the fourth stage of the infectious process (septicemic form). Most often meningitis develops after the bubonic form, usually against the background of insufficient (inadequate) antibiotic therapy (day 9-15). It is accompanied by fever, rigidity of the occipital muscles and headache. Some patients have focal neurological disorders such as cranial nerve anomalies.

Ocular damage occurs in about 4.3% of infected people. According to Konstantin Alexandrovich Yudin[2], the mortality rate is 53.8%. Second-

[1] *Pgm*-negative strains unable to synthesise yersiniabactin are unable to cause pneumonia, even when heavily colonised. However, this does not affect mortality.

[2] Константин Александрович Юдин, 1874–1933; the Russian and Soviet physician, ophthalmologist, the first head of the Department of Eye Diseases at the Imperial Nikolaev University (now Saratov State University).

ary eye pathologies such as conjunctivitis, conjunctival haemorrhages, pustules, keratitis, corneal ulcers, keratoiritis (i.e. inflammation of the cornea and iris), keratoiridocyclitis (i.e. inflammation of the cornea and iris with involvement of the trigeminal nerve) and panophthalmitis[1] occur as a result of haematogenous spread of the pathogen. As a result of haemorrhage into the conjunctiva, enanthema (i.e. spotting) may appear on the third day of the disease. It should be remembered that in plague there may be changes in the eyes that are not accompanied by their lesions. These include dilation of venous vessels and capillaries, minor haemorrhages in the vasculature.

Very rarely, bacteria can enter the nasolacrimal duct, which leads to infection of the lacrimal sac and, consequently, its inflammation (dacryocystitis). Although rare, it is more common in children than in adults (**Figure 13.10**).



Figure 13.10. *Bilateral dacryocystitis and inflamed left axillary lymph node in a boy*

[1] The pathologies listed are similar to those in mule deer discussed in Chapter 10.

* * *

The first thing to be detected at the post-mortem examination is the primary buboesis (if it has not healed). It is usually a tumour-like mass, poorly contoured due to periadenitis (i.e. inflammation extending into the skin). On section, the lymph nodes that make up its centre are fused, so it is difficult to determine the boundaries of each individual lymph node. The lymph nodes themselves, as well as the surrounding retina are abundantly saturated with blood or serous-bloody fluid. There may be areas of necrosis in the form of grey (sometimes grey-yellow) fields. If necrosis occurs early in the disease (an important morphological feature), the central part of the buboes will be necrotic, usually grey (sometimes grey-yellow) in colour, and there will be areas of serous haemorrhagic impregnation around the buboes (**Figure 13.11**). Blood vessels and capillaries are dilated and filled with blood. There are abundant bacterial masses, especially in the subcapsular sinuses of lymph nodes. Volodymyr Konstyantinovich Vysokovych (Vladimir Konstantinovich Vysokovich)[1] wrote that in places there is such a mass of bacteria that they appear as if a sea, in which individual cells lie as islands. It is possible to find a fistula, thanks to which pus is separated, if death occurs after the bubonic rupture.

It is extremely rare to find primary affects (primary ulcer) in the form of spasmodic papules and fleckenes (**Figure 13.12**).

The spleen is always enlarged (1-5 times), dense, and may be flabby (usually in cases of mixed infection). The colour varies from red to grey-red. The capsule often shows haemorrhages, rarely follicular hyperplasia and foci of necrosis may occur. The sinuses are sharply dilated and abundantly filled with cellular elements (with erythrocytes predominating). The capsule and trabecula (i.e. the structure forming the backbone of the organ) always show oedema and homogenisation. Arteries are thickened and in some places saturated with serous fluid (**Figure 13.13**). Bacteria are rarely found.

[1] Володимир Констянтинович Висoкович, Владимир Константинович Высокович, 1854–1912; an Ukrainian physician, pathologist, bacteriologist, epidemiologist. Organiser of many expeditions to fight cholera and plague epidemics. In the lists of graduates of the Imperial Kharkiv University (now V. N. Karazin Kharkiv National University) he is recorded as «Антон Высокович» (Anton Vysokovych).



View of the body of a person who died of plague at a post-mortem examination

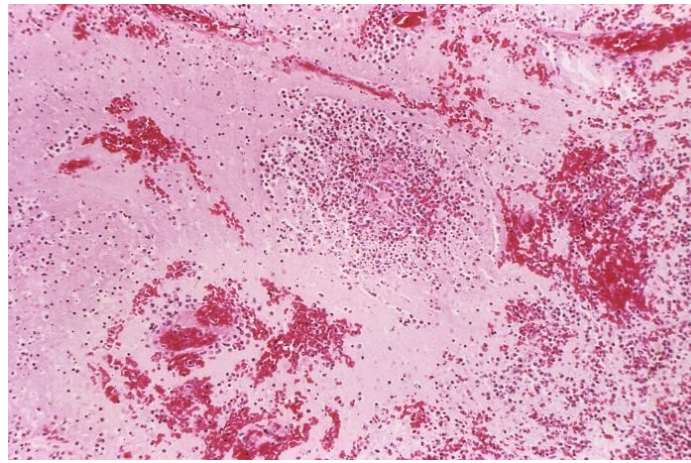


Figure 13.11. *Tissue of the buboesis, obtained at post-mortem examination (96x, H&E). Medullary necrosis is observed*

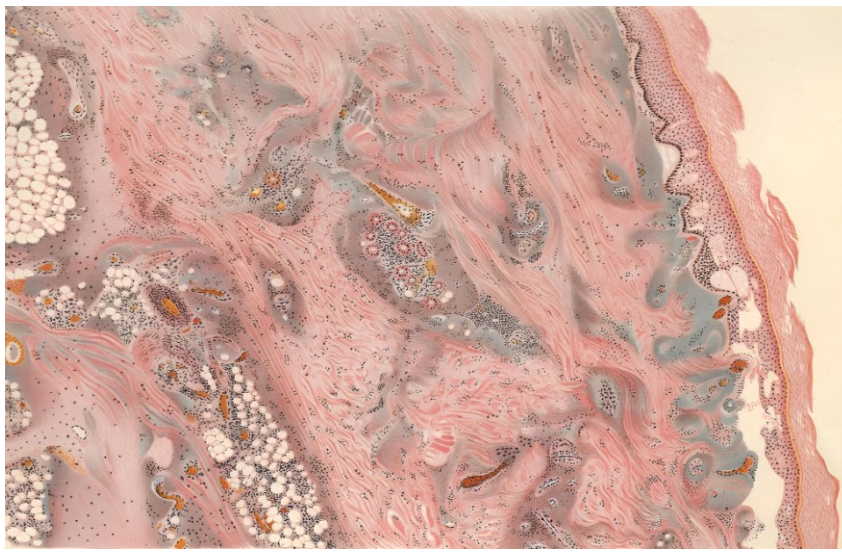


Figure 13.12. *A skin section of the inner surface of the right tibia with a flectena («primary affect») from a patient who died on the seventh day of illness during an epidemic in the city of Bombay in 1897; H&E (4 hours after death). Large bacterial clusters (blue to red-purple) can be seen, which partially replace tissue and densely surround the ducts of the sweat glands (by Albrecht H., 1898)*

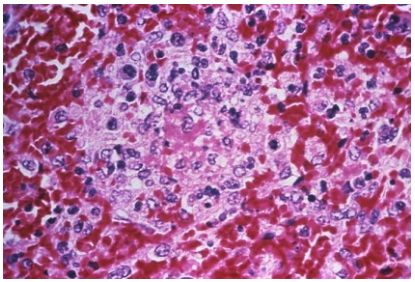


Figure 13.13. *Spleen tissue, obtained at post-mortem examination (400x; H&E). General inflammation of arterioles with haemorrhages is observed*

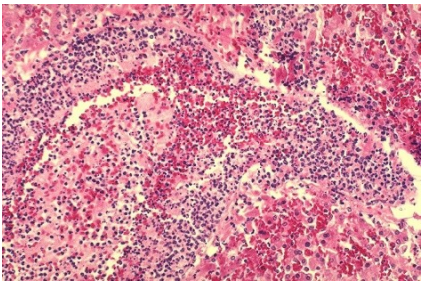


Figure 13.14. *Liver tissue, obtained at post-mortem examination (200x; H&E). Necrosis and thrombosis of the portal vein are observed*

The liver usually shows varying degrees of dystrophic changes in the form of granular dystrophy or obesity. Often observed small or large haemorrhages, possible foci of necrosis[1]. Characteristic changes in the intra-valvular capillaries, inter-valvular arteries and veins. Often found hyperplasia of capillary endothelium with peeling and penetration of cells into the lumen of capillaries, and between capillaries – accumulations of serous fluid. Small and large inflammatory infiltrates (macrophages, lymphoid cells) are observed in arteries and veins, as well as in the perivascular connective tissue, and thrombosis is possible (**Figure 13.14**).

Small foci are found in the lungs, and if death occurs in the late stages of the infectious process - large foci. They are located in all lobes of the lungs, both superficially (under the pleura) and in depth. The colour of the foci varies from grey to grey-yellow, grey-red and red. Numerous haemorrhages are observed both close to and distant from the foci. The surface of the incision is often smooth. In the pleural cavity there may be limited areas of fibrinous or fibrinous-haemorrhagic exudate with numerous haemorrhages. Alveoli are filled with exudate containing serous fluid, red blood cells, desquamated (i.e. detached) alveolar epithelium, white blood cells and significant accumulations of bacteria. Blood vessels are often swollen and necrotised (**Figure 13.15**). Accor-

[1] Volodymyr K. Vysokovych reports that in one case he found granulation tissue around the necrosis focus.

ding to Czeslaw Chęcinski[1], the mucous membrane of the bronchi has abundant mucous plaque containing polymorphonuclear leucocytes, blood, desquamated epithelium and large numbers of bacteria.

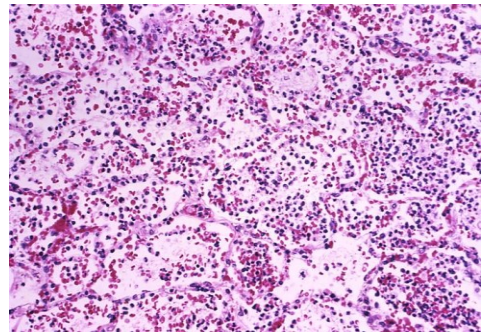


Figure 13.15. *Lung tissue, obtained at post-mortem examination (160x; H&E). Purulent pneumonia (with a large number of polymorphonuclear leucocytes), capillary swelling and intraalveolar debris are observed*

The cerebral cortex may also appear oedematous. In sympathetic ganglia there are thrombi in small vessels and capillaries, swelling of interstitial tissue, haemorrhages, necroses and accumulations of bacteria, and in ganglion cells there is vacuolisation of the protoplasm, karyolysis (i.e. dissolution of the nucleus in the cytoplasm of the cell) up to complete disintegration of the cells, which, according to Ivan I. Shirokogorov, may be the cause of severe vasomotor disorders. Dmitry Pavlovich Kishensky[2] (with colleagues) reports[3] two fatal cases of meningeal form developed against the background of bubonic form of plague. In the first case, post-mortem examination revealed an abscess in the left cerebellar hemisphere (15 x 20 mm in size) with haemorrhage. Endothelial overgrowth and leukocyte infiltration of the intima were detected in cerebellar vessels near the abscess. The vascular plexuses of the lateral ventricles were abundantly infiltrated with white blood cells. In the second case, post-mortem examination revealed haemorrhages in the walls of the lateral ventricles. In the vascular plex

[1] 1851–1916; the Polish physician, pathologist, bacteriologist, epidemiologist. Participated in the elimination of the plague and cholera epidemic in Odessa (1910); in Russia he was known as «Чеслав Иванович» (Czeslaw Ivanowicz). Developed the method of double staining of blood cells and blood parasites (in particular, malarial plasmodium).

[2] Дмитрий Павлович Кишенский, 1858–1933; the Ukrainian physician, pathologist, participant in the elimination of the plague and cholera epidemic in Odessa (1910). Rector of the Imperial Novorossiysk University (1913-1917); died in emigration.

[3] Кишенский Д. П., Тизенгаузен М. М., и др. *Патологическая анатомия чумы*, в сборн. *Чума и холера в Одессе в 1910 году: патолого-анатомическая часть*, Одесса: Изд. Одес. гор. обществ. упр., Тип. Л. Нитче, 1911, 220 с.

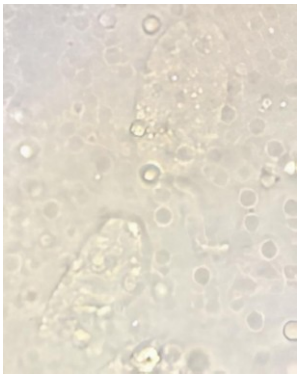


Figure 13.16. *Hyaline cylinders in the urine*

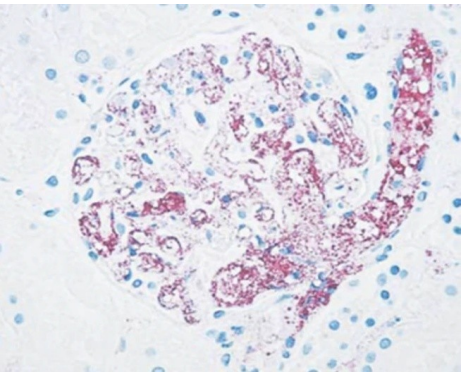


Figure 13.17. *Kidney tissue (100x), immunohistochemical staining. The presence of capsular antigen delineating blood vessels in the tubules can be seen (by Hama H. O., 2022)*

uses and on the walls of the ventricles there was a greenish-yellow mass, and in the lumen of the ventricles there was a turbid liquid of greenish colour. The third and fourth ventricles and the Sylvian aqueduct contained pus. In both cases the pus contained *Y. pestis*.

In the heart, haemorrhages in the pericardium and endocardium, granular dystrophy in the myocardium are most commonly observed.

Renal lesions are highly variable and lack specificity. Most researchers have noted enlargement and flabbiness. Note the enlargement and swelling of the tubules, which are visible on section with the naked eye. There may be haemorrhages in the mucous membrane of the renal pelvis, as well as pinpoint haemorrhages in the area of the cortical layer. There is significant granular and fatty dystrophy of the tubular epithelium, which may be on the verge of severe nephrosis (i.e. renal tubule damage). In some places on the epithelium of the tubules is necrosis, which may be so pronounced that we should already speak of necrotising nephrosis. Hyaline cylinders may sometimes be found in the lumen of the tubules (cylindruria may occur during life, **Figure 13.16**). As a result, erythrocytes are found in the urine, as well as renal epithelial cells and protein, while cylinders may not be detected in the case of albuminuria. Bacteriuria is common (**Figure 13.17**) and progresses to stage 4 (secondary septicemia form), probably due to bacterial multipli-

cation in the spleen and liver. The development of bacteriuria is associated with urinary haemorrhage.

In the adrenal glands there may be haemorrhages, necrobiosis, hemorrhage, endothelial sloughing in the capillaries of the cortical layer and sometimes small cell infiltration. According to V.V. Donskoy[1], adrenal changes are one of the main factors leading to death.

As for the digestive organs, pathological examination reveals multiple haemorrhages with ulcers, mainly in the stomach and large intestine, usually localised in the mucosa and submucosa, which explains the presence of blood in vomit and faeces. Rarely, haemorrhages may be found on the serous membrane. The mesenteric lymph nodes are usually inflamed (secondary buboes), very rarely forming buboes (of the type of so-called primary buboes). There are changes in the lymphatic apparatus of the intestinal wall in the form of hyperplasia of peyer's patch and solitary follicles[2]. Peritonitis (i.e., inflammation of the peritoneal sheets) is extremely rare. Gastroenteritis (i.e. inflammation of the mucosa of the stomach, small and large intestine) has been described.

Pathological changes are rarely found in the pancreas (**Figure 13.18**). Thus, Dmitry P. Kishensky (with colleagues) indicates the presence of hyperaemia, oedema and round cell infiltrates in the interstitial tissue, as well as dystrophy of the glandular epithelium. He reports one case of haemorrhage and bacterial accumulation in the head of the pancreas. He also reports hyperaemia and oedema of the interstitial tis-

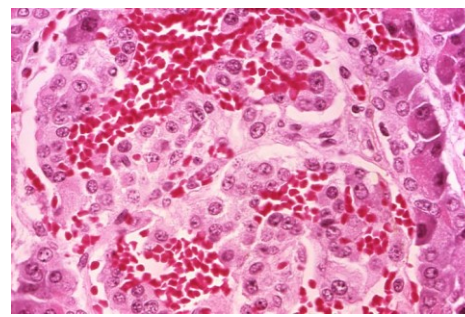


Figure 13.18. *Pancreatic tissue, obtained at post-mortem examination (400x; H&E). Focal haemorrhages in Langerhans cells are observed*

[1] Донсков В. В. *Поражения надпочечников как один из основных танатогенных факторов при чуме.* Медицинский бюллетень (Иркутск). 1939; 1:23. About this physician we know only that he was an employee of the Irkutsk Antiplague Research Institute of Siberia and Far East.

[2] These changes are similar to those found in animals with per os infection (see Chapter 11).

sue of the thyroid gland as well as surrounding fibre. In the lumen of vessels, accumulations of leucocytes and bacteria are found, and in the walls – dystrophic and necrotic changes. Inflammatory infiltrates and swelling of connective tissue, hyperaemia and accumulations of white blood cells in vessels were found in the thymus (in one corpse of a child).

The gallbladder is enlarged and covered with multiple small haemorrhages, bile flow is increased. The bile is dark green in colour and often contains bacteria. Eugenia Nikitichna Alyoshina[1] proved in an experiment[2] on mice that plague microbes can be eliminated (i.e. excreted) with bile: after subcutaneous infection of white mice *Y. pestis* is isolated from bile in 10 minutes.

In skeletal muscles, the most significant changes are found in the muscles located near the buboes. There is hyperaemia, oedema, necrosis (up to complete disintegration of muscle fibres), as well as accumulation of a significant number of polymorphonuclear leukocytes and bacteria, which in some places clog the lumen of capillaries. In the intermuscular connective tissue there are haemorrhages and necroses with accumulation of white blood cells. Muscles distant from the buboes have varying degrees of coagulation necrosis and damage to the sarcolemma (i.e., the cell membrane of the muscle fibre).

No particularly characteristic genital changes were found. Haemorrhages, swellings and inflammatory infiltrates are rarely found. Bacteria may be present.

A bone marrow may show marked hyperaemia, haemorrhages and changes in cellular composition (increased number of eosinophilic myelocytes and megakaryocytes), as well as sharp hemorrhage. In the lumen of blood vessels along with erythrocytes may be found accumu-

[1] Евгения Никитична Алёшина, ?-?; the Russian and Soviet physician, bacteriologist, participant in the elimination of the epidemiological danger on the right bank of the Volga River (1944), engaged in the training of scientific personnel during the Second World War.

[2] Алёшина Е. Н. *Элиминация чумного микроба желчью и мочой при подкожном методе заражения белых мышей.* Труды Ростовского-на-Дону научно-исследовательского противочумного института. 1945; 4:12–14.

lations of white blood cells and bacteria. At the same time, according[1] to Maria Bal'tazarovna Stanishevskaya[2], hyperaemia and haemorrhages are less pronounced in prolonged course.

14 PRIMARY SEPTICEMIC PLAGUE

On 10 September 2009[3] in Chicago (Illinois, USA), a 60-year-old male researcher with insulin-dependent diabetes, hypertension and hyperlipidaemia went to the clinic because he had been experiencing fever, chills, shortness of breath, dry cough and weakness for the past three days. Suspecting influenza or another respiratory viral infection, the doctor referred him to the emergency department, but the patient did not listen. Already on 13 September he was taken to the hospital by ambulance with a saturation level of 92% against a background of worsening symptoms. On arrival at the hospital, respiratory murmurs, abdominal bloating, peripheral cyanosis, and trace swellings of the feet were detected. There was no lymphadenopathy, rash, or jaundice. The chest radiograph was normal, but the patient continued to have difficulty breathing and required supplemental oxygen. Renal failure, initial acidosis (ie. acid-base shift), elevated liver enzymes (aspartate aminotransferase 794 IU/L [<35 IU/L]; alanine aminotransaminase 160 IU/L [7-56 UI/L]) and severe leukocytosis (white blood cell: 79.2 x 10^9/L

[1] Станишевская М. Б. *Патологическая анатомия костного мозга при чуме*, в сборн. *Чума и холера в Одессе в 1910 году: патолого-анатомическая часть*, Одесса: Изд. Одес. гор. обществ. упр., Тип. Л. Нитче, 1911, 220 с.

[2] Мария Бальтазаровна Станишевская, 1871–1937; the Ukrainian physician, infectious disease specialist, the first woman teacher (Privatdozentin) at the Medical Faculty of the Imperial Novorossiysk University (now the Odesa I. I. Mechnykov National University) and the first woman to defend her thesis there. Participant in the elimination of plague and cholera in Odessa (1910).

[3] Frank K. M., Schneewind O., et al. *Investigation of a researcher's death due to septicemic plague.* N Engl J Med. 2011; 364(26):2563–2564; DOI: 10.1056/NEJMc1010939; Ritger K., Black S., et al. *Fatal Laboratory-Acquired Infection with an Attenuated Yersinia pestis Strain (Chicago, Illinois)*, Morb Mortal Wkly Rep. 2011; 60(7):201–205.

*[4.5-11 x 10⁹/L] / **79200 cells/μL** [4500-10,000 cells/μL]) with a left shift (22% band form)*[1]*. The blood smear showed bacteria.*

The patient was initially treated with diuretics, as congestive heart failure was suspected, and then with antibiotics (vancomycin and piperacillin/tazobactam intravenously), suspecting infection. About 12 hours after admission, the respiratory of patient failure worsened and he was intubated. Another hour later, despite resuscitative measures, he died of cardiac arrest. On 18 September, the bacteria from the blood were identified as Y. pestis, and it was an attenuated (i.e. artificially attenuated) pigmentation-negative Y. pestis strain UC91309[2]*, which was found to contain antibiotic resistance genes and was not found in nature. It was a strain artificially created by the researcher as part of a research paper at the University of Chicago. He last worked in the lab on 4 September and had already reported the disease to management on 10 September.*

Earlier we said that the pigmentation region of *pgm* includes the *hms* locus and the high pathogenicity island (HPI), where the *ybt* genes involved in the synthesis of yersiniabactin, which is responsible for iron capture by the bacterium, are localised. The absence of *pgm* makes the strain non-pigmenting (written: pgm^- or Δ*pgm*), that is, unable to capture iron, which is necessary for the development of the infectious process. Such strains are attenuated and are often used as vaccine strains.

However, post-mortem examination revealed a high iron content in the liver without cirrhosis[3] (**Figure 14.1**-A) and genetic testing revealed a C282Y mutation, that is, an undiagnosed hereditary haemochromatosis in which there is a significant accumulation of iron

[1] A leftward shift of the leucocytic formula is understood as a strong increase in the production of neutrophils in response to infection, when their immature paloconuclear forms are released into the peripheral bloodstream (in this case, they are 22% with a reference range of 1-5%).

[2] Interestingly, the Centers for Disease Control and Prevention (CDC) report another pigmentation-negative attenuated strain of *Y. pestis* strain KIM D27.

[3] Postmortem examination of blood samples revealed a total serum iron of 96.88 μmol/L [11-28 μmol/L] / **541 mcg/dL** [40-160 mcg/dL], iron saturation of 83.5% [14–50%] and total iron binding capacity of 116.04 μmol/L [45.3-77.1 μmol/L] / **648 mcg/dL** [230-430 mcg/dL].

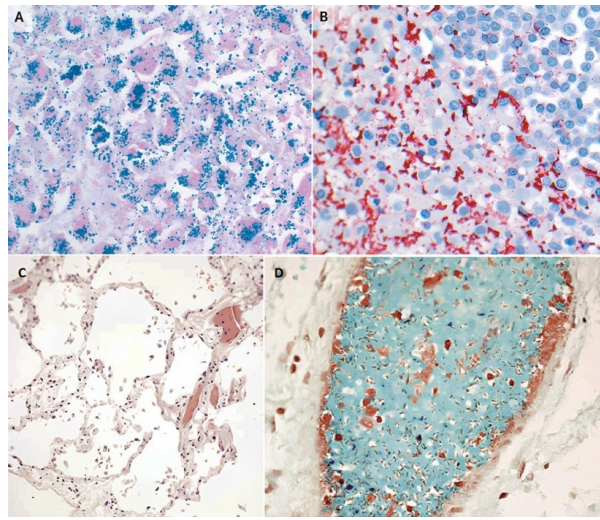


Figure 14.1. *Tissues of liver (A), spleen (B) and lung (C and D), histochemical and immunohistochemical staining. Hepatocytes of the liver (A) show significant iron deposits (stained blue), which are not present in pancreatic and cardiac tissues (not shown). Numerous bacteria are observed in the spleen (B) (coloured red). The lung tissue (C) retains an alveolar structure (open air spaces) without inflammatory infiltrates. Bacterial accumulations are found in the lumen of the pulmonary vessel (D) (A and B by Frank K. M., 2011; Copyright © 2011 The Massachusetts Medical Society, published with the authorisation of the copyright holder; C and D by Ritger K., 2011)*

in the body and thus the bacterium can fulfil its needs without yersiniabactin[1].

The mode of infection was not established, but post-mortem examination revealed open air spaces and preserved alveolar structure in the lungs (**Figure 14.1**-C), indicating that the deceased did not have lung processes characteristic of bubonic or primary pneumonic forms of plague (compare with **Figure 15.1**). All this suggests direct penetration of the pathogen into the blood (primary septicemic form), which could have occurred, for example, as a result of skin damage during laborato-

[1] Experiments on mice show that hereditary hemochromatosis restores the virulence of plague vaccine strains. It should be noted that the effect of iron on the growth and virulence of pigmention-negative strains has been known since the mid-50s of the last century, so iron preparations, such as complex of $Fe(OH)_3$ and dextran or chondroitin sulfate iron colloid (inorganic iron compounds in pure form are very toxic) are used to increase *residual virulence* (so-called "conditional virulence") in animal models without increasing the risk to the researcher.

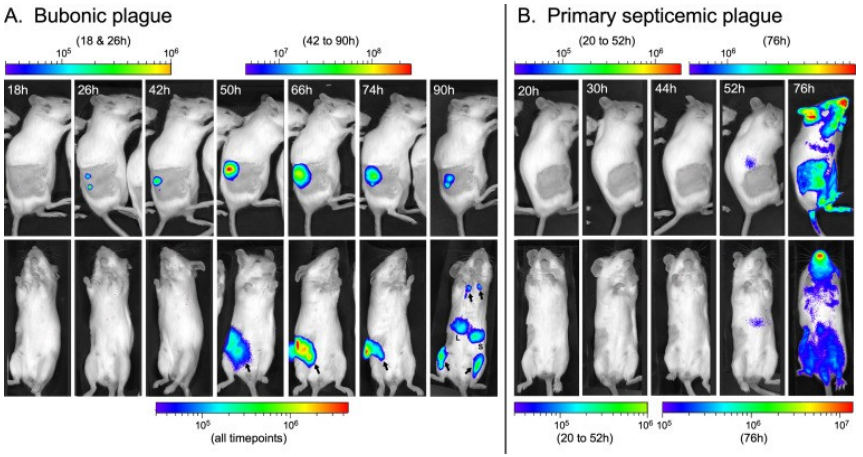


Figure 14.2. *Development of the infectious process in bubonic (A) and primary septicemic (B) forms of plague within four days after flea bite (mouse model, Y. pestis bioluminescent strain pGEN-luxCDABE). In the bubonic form of plague, bioluminescence from the dermis is detected at the bite site within two hours after infection (top row), increasing in intensity in the following days, indicating bacterial multiplication. Dissemination of bacteria to the draining lymph node (marked by arrows) is detected within three days (bottom row), and systemic dissemination with multiplication of the pathogen in the liver (L) and spleen (S) on the fourth day. In the primary septicemic form of plague, no bacteria are found at the bite site and in the draining lymph node (no bioluminescence), and the bioluminescent signal is first detected on the third day in the periphery, rapidly progressing to a generalised process on the fourth day already (by Bosio C. F., 2020)*

ry infection of bioassay animals (simply put, the researcher did not realise he had injected himself[1]).

The *primary septicemic form* is realised by laboratory infection (as shown above) or, less commonly, by a bite from an infected flea directly into the bloodstream (vein). Infection may also occur from a bite by an infected animal with bacteria in its mouth (e.g., blood from a recently eaten infected gopher), but no documented cases are known. In this form, bacteraemia occurs without lymph node involvement or bubonic formation (**Figure 14.2** and **Figure 14.3**), and the mechanism is generally similar to that of the secondary septicemic form. However, because the-

[1] It is for this reason that after removing the protective suit and washing your hands, you should treat them with alcohol (if the skin is damaged, it will sting). For more information on how to safely remove the protective suit, see Chapter 28.

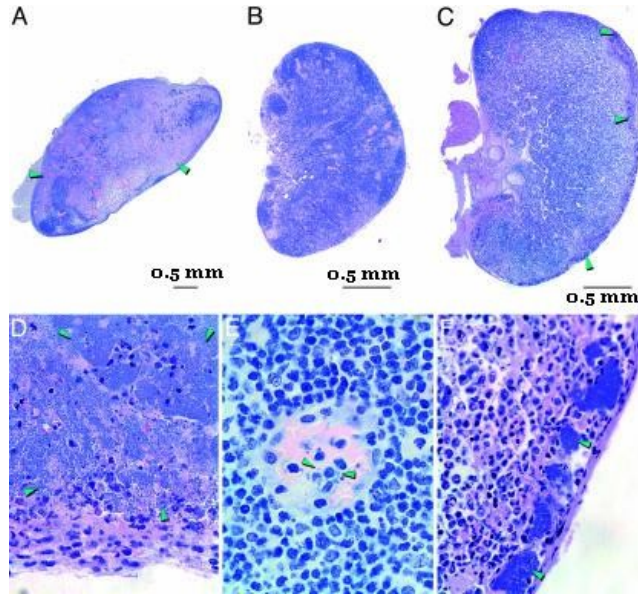


Figure 14.3. *Lymph nodes proximal to the bite site (mouse model, flea infestation) infected with Pla+ and Pla- Y. pestis strain 195/P. In the bubonic form, a severe lymphadenopathy characterised by destruction of normal tissue and its replacement by multiple bacteria (large clusters are marked with green arrows) is found in the case of the Pla+- strain (A and D, eq. 600x). In the primary septicemic form, Pla+ strain (B and E, 1000x) and Pla- strain (C and F, 600x) show lymph nodes without pathological changes, and extracellular bacteria (green arrows) are either intravascular (E) or localised in small peripheral clusters (F) associated with neutrophilic inflammation (by Sebbane F., 2006; Copyright (2006) The National Academy of Science, U.S.A., published with the authorisation of the copyright holder)*

re is no capsule (capsular antigen{pPla}) but 6-acyl LPS (which binds to the TLR4/MD2 receptor complex), unshielded plasminogen activator{pPla} and Ail{chromosome}, a significant immune response occurs quite rapidly (bacteria are readily picked up by phagocytes, primarily monocytes (these guys, circulating in the bloodstream for 1-2 days, will settle in the tissues and become resident macrophages), which explains the insignificant lymphadenopathy (without the formation of buboes), the frequent presence of bacteria within vessels and in the periphery, as well as fulminant course, leading to DIC, tissue necrosis and death. The latter usually occurs after liver and spleen involvement (**Figure 14.4**) but before dissemination to other organs (although lung involvement with secondary pneumonic form is also possible, **Figure 14.5**).

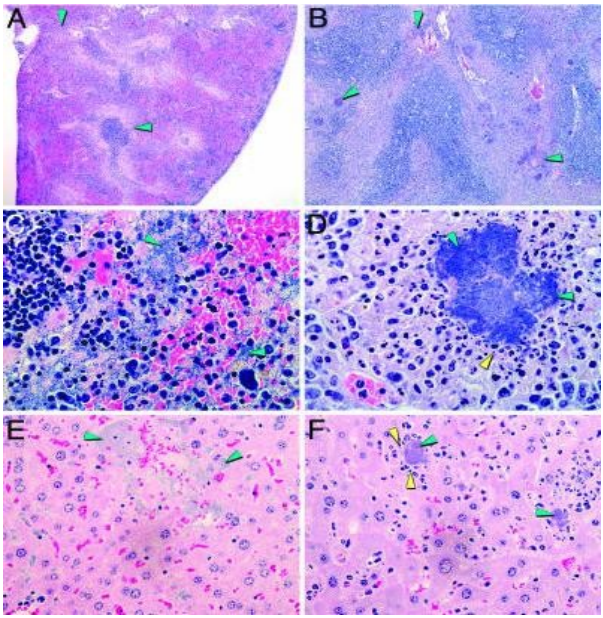


Figure 14.4. *Spleen (A-D) and liver (E and F) tissues in primary septicemic form (mouse model, flea infection) caused by Pla+ (left column) and Pla- (right column) Y. pestis strains 195/P. Extracellular bacteria (green arrows) are widespread in the case of the Pla+ strain and much less common in the case of the Pla- strain, but in the latter case the bacteria are more frequently contained in granulomatous lesions (yellow arrows) surrounded by degenerating host cells; A and B, 4x; C, 600x; D-F, 400x (by Sebbane F., 2006; Copyright (2006) The National Academy of Science, U.S.A., published with the authorisation of the copyright holder)*

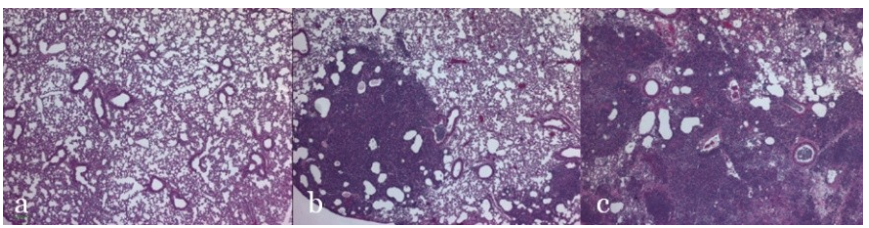


Figure 14.5. *Lung tissue in primary septicemic form (mouse model, intravenous infection with Y. pestis strain CO92), H&E. After 24 hours (a) no pathological changes are observed, whereas after 48 hours (b) large inflammatory foci densely filled with neutrophils are observed. After 72 hours (c), there is an increase in the size and number of foci, indicating hyperinflammation (courtesy of Prof. William E. Goldman, published for the first time)*

It is important to note that the development of this form occurs predominantly in Pla-negative strains[1], as they are rarely able to realise the first stage of the infectious process (due to the absence of plasminogen activator[pPla]).

* * *

The post-mortem examination shows, on the one hand, the absence of buboes, and on the other hand, particularly numerous and extensive haemorrhages in the skin, serous membranes and internal organs, although very rarely the changes may be reduced to the presence of a small haemorrhagic rash. That is, in general, the classic picture of sepsis is revealed.

Lesions of the spleen (which can be increased), liver and lungs (and in some cases, the kidneys) due to the rapidity of the infectious process are generally insignificant and, we can say, superficial. Thus, **Figure 14.1**-C and **Figure 14.5** show that the lung tissue is inflamed, but the alveolar structure is preserved, which is not seen in the secondary septicemic form (**Figure 13.14**).

15 PRIMARY PNEUMONIC PLAGUE

December 1939. Simon Zelikovich Gorelik[2], a physician on duty and part-time associate professor of the Department of Internal Medicine of the First Moscow Medical Institute, was receiving patients at the Novo-Yekatereninskaya Hospital at the Petrovsky Gate[3]. A graduate of the

[1] In mouse models, using Pla-negative strains, all mice develop primary septicemic form, whereas using Pla-positive strains, only 25% develop primary septicemic form.

[2] Симон Зеликович Горелик, 1885–1939; the Belarusian and Soviet physician, therapist.

[3] Building No. 15/29 on the corner of Petrovka Street and Strastnoy Boulevard (formerly the estate of Princes Gagarin) in Moscow (Russia). At that time the clinical base of the First Moscow Medical Institute (now I.M. Sechenov First Moscow State Medical University – Sechenov University), and now one of the buildings of the Moscow City Duma.

medical faculties of the Sorbonne and the University of Geneva, a fine family man and an educated intellectual, he was working his last day before moving to a new place where he was to discover himself as a leader. The suitcases were already packed. The last visitor was a 36-year-old man, who had been diagnosed a little earlier by another doctor as having lobar pneumonia (pneumonia obliterans, i.e. inflammation of several lobes of the lung caused by pneumococcus). This visitor was Abram L. Berlin (he had already met us during the discussion of taxonomy), formerly director of the Sanitary and Bacteriological Institute in Ulan Bator (Mongolia), organiser of the epidemiological service in the Mongolian People's Republic, and now deputy director of the antiplague institute "Microbe"[1]. He was urgently summoned to Moscow to report to the People's Commissariat for Health, so he did not have time to undergo the required six-day observation, although de jure he was thoroughly examined before departure. The diagnosis was obvious: primary pneumonic plague[2]. After locking himself in with the patient, Simon Gorelik reported the diagnosis to the management…

The physician Yakov L'vovich Rapoport[3], who performed the post-mortem examination, would later write: "*Knowing of the inevitable fatal outcome (there was no escape from the pneumonic plague), with the signs of the disease already coming, Gorelik until the last hour provided possible help to the dying Berlin, trying to alleviate his suffering. Then, in his self-isolation, he wrote letters to his relatives and to Comrade Stalin. Standing on the edge of the grave, Gorelik appealed to Stalin for his arrested brother[4], who shared the fate of*

[1] State Regional Institute of Microbiology and Epidemiology of South-East Russia – the "Microbe" Institute of the People's Commissariat of Health of the RSFSR. Currently – the Russian Research Anti-Plague Institute "Microbe". Traditionally, it is considered to be the head institution of the Russian anti-plague system.

[2] There is evidence that the contamination was due to a breach of biosafety regulations (discussed in detail in Chapters 27 and 28), namely being in a "contagious" zone without personal respiratory protection equipment. The division of laboratories into "clean" zone (Russ. «чистая» зона) and "contagious" zone (Russ. «заразная» зона) was adopted in the USSR and is now used in the CIS countries. In fact, the "contagious" zone of the laboratory is understood as the laboratory premises where direct work with infectious agents is carried out.

[3] Яков Львович Рапопорт, 1898–1996; the Ukrainian, Soviet and Russian physician, pathologist, military physician, Honoured Scientist of the RSFSR (1970).

[4] This is about Abram Zakharovich Lezhnyov (Абрам Захарович Лежнёв) whose real name Abram Zelikovich Gorelik (Абрам Зеликович Горелик; 1893-1938), a literary

many victims of the lawlessness of lawlessness of Stalin. These letters were burnt together with Gorelik". His endurance and professional heroism were not properly appreciated either during his lifetime or after his death. Therefore, the least we can do is to honour the memory of Simon Gorelik at least on the pages of this book.

The identification and quarantine of contact persons[1], carried out jointly with the staff of the Moscow City Observation Station[2], prevented the spread of infection, limiting it to a small number of deaths. The events of that winter remained a state secret for many years.

The *primary pneumonic form* (formerly Pneumonia pestica), which develops during aerogenic infection (e.g., while caring for a patient with secondary pneumonic plague), like the primary septicemic form, is characterised by a fulminant course and absence of buboes, since only tracheobronchial lymph nodes are affected against the background of already progressive pneumonia.

The infectious process in the lung proceeds in two phases. In the *pre-inflammatory phase*, plasminogen activator$^{\{pPla\}}$ ensures binding of the bacterium to alveolar epithelial cells for multiplication and suppresses the early influx of neutrophils into the lungs (through blocking the secretion of IL-6 and IL-8)[3]. Interestingly, although bone marrow cells respond quite rapidly to infection by releasing neutrophils into the circulation immediately after infection, neutrophils cannot enter the infected lung immediately (the bacteria themselves do not enter the bone marrow until 48 hours later; **Figure 15.1**). Next, plasminogen activator$^{\{pPla\}}$ together with Yops$^{\{pCad\}}$ (and possibly 4-acyl LPS) inhibit phagocytosis, which could be carried out by alveolar macrophages as well as neutrophils, which after some time enter the lung, and their breakdown products (granule contents and reactive nitrogen compaunds) are respo-

critic who was shot a year earlier. Rehabilitated by the Military Collegium of the Supreme Court of the USSR on 11 August 1956.

[1] They included, among others, train passengers, hotel staff, a hairdresser, and Michael Rossels, the first physician who is said to have been in a hurry for his birthday and was not infected.

[2] Structural subdivision of the Central Institute of Epidemiology and Microbiology of the People's Commissariat of Health of the RSFSR; nowadays – the Anti-Plague Center of Rospotrebnadzor.

[3] Remind: in the case of secondary pneumonic form, attachment is provided by pH6-antigen$^{\{chromosome\}}$ and yersiniabactin$^{\{chromosome\}}$.

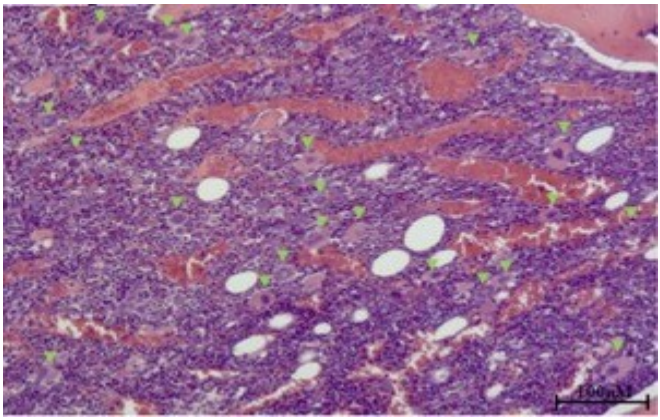


Figure 15.1. *Bone marrow tissue on a section of femur bone after 48 hours (mouse model, intranasal infection with Y. pestis KIM53 strain), H&E. Significant accumulation of blood vessels along the bone marrow is observed (red spot spots resembling oedema); green arrows indicate a significant number of megakaryocytes (by Vagima Y., 2012)*

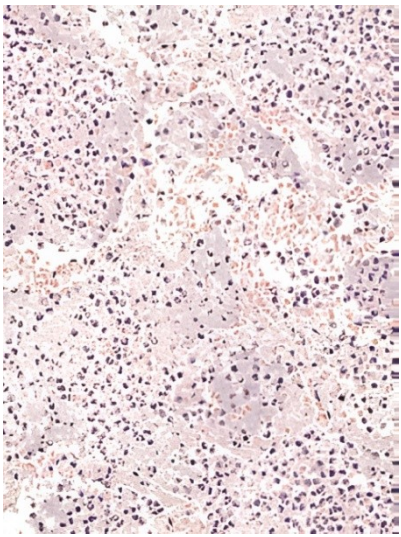


Figure 15.2. *Lung tissue in the primary pulmonary form. There is extensive inflammation, polymorphonuclear leucocytes and multiple bacteria. Note that, unlike lesions in the primary septicemic form (**Figure 14.1**), there are no open air spaces*

nsible for lung tissue damage. In this case, IL-1β seems to be the only response to infection (remind: blocked by 1L-1RA). It is difficult to say why in this case the capsule does not screen plasminogen activator{pPla}, but we can assume that it is due to the tenuous connection with the bacterium: the capsule is depleted by fluid droplets in which bacteria are transported from one infected person to another.

The *proinflammatory phase* begins only after 36-48 hours and is accompanied by activation of the proinflammatory cytokines IL-6, IL-17, TNFα I, IFNγ, and the chemokines CXCL1 and CCL2, which promotes massive influx of neutrophils into the alveolar spaces, resulting in the formation of

densely packed foci of inflammation that enlarge due to the constant influx of neutrophils (**Figure 15.2**). As a consequence, significant destruction of alveoli occurs, accompanied by oedema, haemorrhage and, finally, necrosis. Against the background of massive pneumonia accompanied by pleural effusion, tracheobronchial lymph nodes may be affected (tracheobronchial lymphadenitis resembling secondary buboes). In the absence of treatment, death usually occurs within 72 hours of infection.

It should be stipulated here that in the case of aerogenic infection with not "warmed" bacteria, e.g., artificial (intentional) aerogenic contamination or cutting up the carcass of a killed host, the bacterium, as in the case of primary septicemic form, will have a 6-acyl LPS (which binds to the TLR4/MD2 receptor complex) and unshielded plasminogen activator$^{\{pPla\}}$ and Ail$^{\{chromosome\}}$ (in the absence of a capsule), leading to a strong immune response in the pro-inflammatory phase, since, as mentioned earlier, in the pre-inflammatory phase plasminogen activator$^{\{pPla\}}$ ensures suppression of neutrophil influx and avoidance of phagocytosis by them. Unfortunately, precise data on the mechanism of the infectious process in this scenario are not available, but it is estimated[1] that 50 kg of bacterial culture released as an aerosol in a city of 5 million people could infect 150,000 people, of whom 80,000-100,000 would be hospitalised and 36,000 would die.

It is extremely rare to develop the so-called *pulmonary type* of primary pneumonic form, characterised by sudden hyperaemia and pulmonary oedema, in the absence of foci of thickening in the lungs.

The development of the primary pulmonary form is followed by further penetration of the pathogen into the blood (secondary septicemic form, discussed earlier) and dissemination to organs with the development of multi-organ failure. Interestingly, this process depends on the infecting dose. Thus, when a small volume of the pathogen is ingested, the upper respiratory tract is mainly affected, after which a secondary septicemic form develops with multiple foci of inflammation in various organs (in the spleen the pathogen is detected after 24 hours), from which death occurs. Presumably, penetration into the blood is through

[1] World Health Organization; Geneva, Switzerland: 1970. Health aspects of chemical and biological weapons.

the vessels of the nasal sinuses (*pharyngeal form*[1]) and less through the tracheobronchial lymph nodes. In contrast, when a large volume of the pathogen is ingested, the lower respiratory tract is mainly affected and severe pneumonia develops, rarely progressing to secondary septicemic form (few foci of inflammation are present).

* * *

On pathological post-mortem examination in the lungs single or multiple foci of lesions of various sizes, localised in different lobes, but more often in the root parts, are found. Georgiy Stepanovich Kulesha[2] describes the changes seen in corpses during the plague epidemic in Manchuria (1910-1911) as follows: "*From outside the affected lobes in all cases were more or less bloody and covered with delicate fibrinous plaque... The cut through the hepatitised*[3] *part was always homogeneous, then dark red, greyish-red or pinkish-grey and, finally, yellowish-grey. Often some lobes were in a state of grey hepatisation, others – red; or the centre of hepatization appeared more grey, pinkish- or yellowish-grey, the periphery was dark red and swollen, which spoke for eccentric expansion of inflammation. The surface of the incision through the affected lobe never had that granularity, which is characteristic of lobar pneumonia, but was more or less smooth or shagreen; from the surface of the incision flowed often in abundant quantities of turbid, bloody or pus-like, somewhat mucous fluid*".

The areas of necrosis present are more often microscopic, as death occurs before the foci of decay reach a significant size. When compression occurs, bloody, frothy fluid forms on the cut surface. In the pleural cavity there is a small accumulation of serous or serous-haemorrhagic fluid, as well as fibrinous deposits (after antibiotic therapy), and multiple haemorrhages are found under the pleura. The lymph nodes of the lung roots and mediastinum may show signs of inflammation.

The pharyngeal tonsils are usually significantly enlarged, the tissue on section is juicy, full of blood, rarely with foci of haemorrhage. The

[1] An extremely rare form that results from swallowing or inhaling plague bacteria. The tonsils and cervical lymph nodes are usually affected. Swelling of the parotid region may occur. The pathogen is usually excreted from the sinuses and throat.

[2] Георгий Степанович Кулеша, 1866–1930; the Russian and Soviet physician, pathologist, bacteriologist. Participant in the elimination of the plague epidemic in Manchuria (1910-1911) and outbreaks in the Caspian Sea (1911-1912).

[3] I.e., thickened, liver-like consistency.

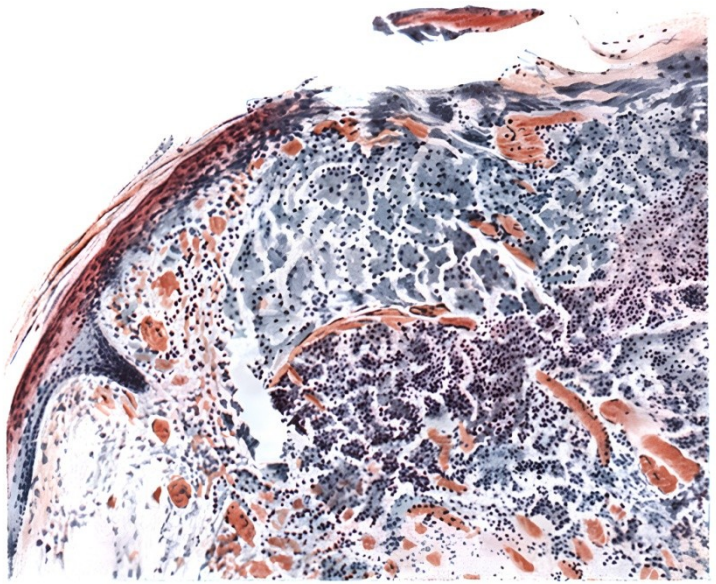


Figure 15.3. *Tissue of pharyngeal tonsil (12x), Eosin - methylene blue stain. Necrosis and detachment of multilayer squamous epithelium of crypt are observed in one place. The detached fragment and intact areas of multilayer squamous epithelium are abundantly impregnated with bacteria (blue masses). The lymphoid tissue below is almost completely displaced or filled with bacteria forming a homogeneous layer in it (light blue colour). No acute reaction is observed in the neighbouring tissues. Blood-rich vessels, running in places of bacterial accumulation, are surrounded by their dense clusters of bacteria (by Kulescha S. G., 1925; published under licence from The Springer Nature)*[1]

covering epithelium (or, more correctly, the multilayered squamous epithelium) of the crypt[2] may be stratified and sometimes necrotised (**Figure 15.3**). It may contain cavities containing pus and bacterial masses (**Figure 15.4**), and beneath it there are accumulations of leucocytes, lymphocytes and macrophages. The trachea (**Figure 15.5**) and bronchi

[1] The history of the images (**Figure 15.3 – Figure 15.5**) is quite surprising. Here is how Georgiy S. Kulesha himself writes about it: *"I was working on the [sectional] material, that I had collected, in Petrograd, but due to the war and the revolution, everything that was not related to these events was pushed aside, so the results of my observations remained unpublished"*. He was only able to publish this in 1925.

[2] A crypt is a cavity formed by the folds of the multilayered squamous epithelium of the tonsil.

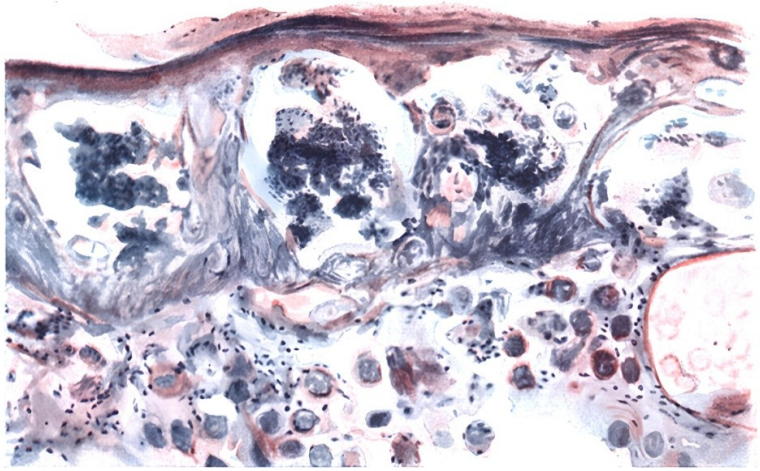


Figure 15.4. *Tissue of pharyngeal tonsil (16x), Eosin - methylene blue stain. There are three cavities containing pus and bacterial masses in the epithelial layer of the crypt. In the subepithelial layer and around the blood vessel, there are numerous bacterial clusters, causing the bacteria to enter the blood through the blood vessel wall (by Kulescha S. G., 1925; published under licence from The Springer Nature)*

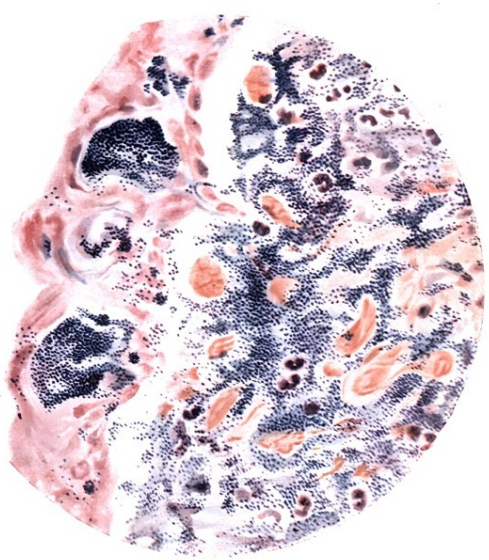
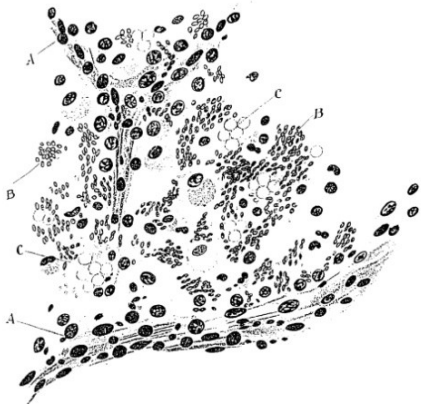


Figure 15.5. *Tissue of trachea (12x), Eosin – methylene blue stain. Partial necrosis of cylindrical epithelium cells is observed (cells are poorly stained, shrunken and wrinkled; nuclei of cells of the basal layer are also poorly stained). Bacteria of oval and irregular shapes (blue colour) are found inside separate bocular cells of the cylindrical epithelium. Massive deposits of bacteria have almost completely displaced the submucosal base and overgrow blood vessels. In bacterial masses the number of polymorphonuclear leukocytes is insignificant (by Kulescha S. G., 1925; published under licence from The Springer Nature)*



Lung tissue sample from a patient who died during the plague epidemic in the cities of Kobe and Osaka in 1899; Haematoxylin stain:
A - lung tissue;
B - bacterial clusters;
C - blood cells.
Image from the 「神戸市大阪市ペスト病調査報告」*("Investigation report on plague in Kobe and Osaka cities")*

usually show no significant pathology. A greyish bloody frothy fluid is noted on the surface of the mucosa, and haemorrhages are sometimes found directly in the mucosa. In the epithelial layer of the trachea and large bronchial branches there are significant accumulations of bacteria in the form of egg-shaped bodies in the lateral cells, which are the entrance gates. Bacterial overgrowths between epithelial cells towards submucosa are possible. In small vessels, the walls are filled and blood is mixed with bacteria. Moreover, bacteria are found only in those vessels that pass through foci of inflammation, i.e. bacteria enter the vessels from the affected tissues (**Figure 15.3 – Figure 15.5**). The described pathomorphological picture[1], called "*pestangina*" by Georgiy S. Kulesha, is, in his opinion, a factual proof of the possibility of haematogenous origin of plague pneumonia, when the pneumonic form of plague develops when plague aerosol hits the mucosa of the mouth and larynx (*tonsillar* and *pharyngeal*[2] *forms*)[3]. In addition, he hypothesised that this is

[1] Kulescha S.G. *Beitrag zur pathologischen Anatomie der Lungenpest.* Virchows Archiv Für Pathologische Anatomie Und Physiologie Und Für Klinische Medizin. 1925; 254(1):174–183; DOI: 10.1007/bf02112658.

[2] It is extremely rare that this form can be realised through contact of oral mucosa with infected hands.

[3] The history of research on pneumonic plague in children deserves special attention. Georgiy S. Kulesha writes: "*...based on the experience in Manchuria, the general impression was that children were less susceptible to pneumonic plague. This was further reinforced by the fact that of the more than 100 autopsies of plague corpses that I conducted in Manchuria, there had never been a child's corpse among them. This view was erroneous because of the considerable number of*





plague cases and deaths among Kyrgyz children [during the 1913 outbreak in the Urals], which proved that the plague apparently cared little for the age of the diseased".



▲ *Corpses in the street awaiting post-mortem examination and*
◄ *post-mortem examinations on a child (top photo;*
Georgiy S. Kulesha probably did not know about this corpse)
and an adult (bottom photo) in Harbin (South Manchuria)
in November 1910 during the pneumonic plague epidemic (1910-1911).
This was the last major plague epidemic, killing more than 60,000 people. Note that some of the medical staff of the Russian mission (bottom photo) did not use personal respiratory protection equipment, as the latter was just coming into use (mainly due to the efforts of Wu Lien-teh). As a result, the losses among medical personnel (Russians, Chinese, Japanese and French) were enormous. One of those infected, Russian medical student Ilya Vladimirovich Mamontov (Илья Владимирович Мамонтов; 1889-1911), wrote in the letter to his mother:
"It seemed to me that there was nothing better than life. But out of a desire to preserve it, I could not run away from the danger to which everyone is exposed... I must believe that all this is not in vain and that people will achieve, even if through much suffering, a real human existence on Earth...".
Photographs from the 『明治四十三四年南滿州「ペスト」流行誌附録寫眞帖』
("An account on the Plague in South Manchuria, 1910-'11")

how Abram L. Berlin was infected, and it is likely that some animals (e.g. camels) are also infected.

Pathological changes in other organs are similar to those in the secondary septicemic form.

It seems most appropriate to end this chapter with a description of the autopsy of corpse of Abram L. Berlin, conducted by Yakov L. Rapoport: "*Chest cavity... There is an insignificant amount of fluid in the right pleura. Both sheets of the left pleura are fused by adhesions that are difficult to tear. The right lung is free, enlarged in volume, does not shrink, its tissue is tense. The upper lobe is dark red in colour, the middle lobe is normal, the lower lobe is brown-red. On the surface of the upper lobe fresh haemorrhages. It is compacted, at incision the edges diverge, and bloody-foamy fluid protrudes from the incision. The lower lobe is considerably denser, with turbid-bloody fluid protruding from its incision... The tissue of the left lung is dark cherry-coloured. On the cut there is a bloody-foamy fluid. Peribronchial lymph node are significantly enlarged, fused in a package, edematous, hemorrhagic, surrounding the trachea... In the pericardial cavity about 50 cm2 of serous fluid with jaundiced colour. There is a considerable amount of clotted blood in the right ventricle, less in the left. The muscle of the heart is flabby, on the cut the muscle of the left ventricle is brown-red with a waxy sheen. Abdominal cavity. Spleen moderately enlarged, flabby, pulp marginal, easily scraped, dark cherry-coloured. The liver is not enlarged, dense, dirty-brown in colour. On cultures from organs – cultures of plague bacillus. In smears from the lung there is a huge amount of typical plague bacilli*".

16 TRANSMISSION AND MECHANISMS

In Chapter 6, we considered the mechanisms of spread of infection, distinguishing between vector-borne, contact, aerogenic and faecal-oral. It is clear from the previous chapters that the vector-borne mechanism leads to the bubonic and primary septicemic forms of plague, while the

contact and aerogenic (airborne) mechanisms lead to the primary pneumonic form of plague.

The faecal-oral mechanism of transmission is probably the rarest, as it is possible to occur when handling faeces from infected animals. Earlier we said that when infected food is consumed, the animal develops a generalised infection, with spleen and liver lesions characteristic of the secondary septicemic form, as well as minor lesions in the lymphoid structures of the small intestine (usually in the lower intestine). In this case, with faeces the pathogen is excreted very rarely (in domestic animals (dogs and cats), caught in the digestive tract of the pathogen is excreted somewhat more often). Then, cleaning up after their pets and not observing the basic rules of hygiene (hand washing) a person can bring the infection to the mucous membranes of the eyes or mouth. On the other hand, the Mongolian marmot, before going into hibernation, arranges the so-called wintering plugs, which are used to lock the mink from within. These plugs are prepared from mixture of stones, fine-grained soil, crushed stone rolled in faeces, which accumulate in special latrines. And since burrows are often inhabited by infected fleas, the pathogen may be present in the faeces, from which it enters the marmot's mouth, as the latter delivers "building material" to the exit from the burrow in its teeth (remind the ecological hypothesis of the origin of the plague microbe). Groundhogs dug up by humans during winter hibernation are usually stained with faeces, which allows the pathogen to infect humans by air-dust (through breathing) or contact (through hands) transmission.

The air-dust transmission is most often realised through the cutting of infected animal carcasses and can lead to various forms of disease. It is important to realise that since the infecting object is most often an infected carrier, the bacterium enters the person already "warmed". Let us consider specific cases.

> *On 11 July 2014[1], in north-central Gansu Province, China, a 38-year-old shepherd while herding sheep took from his dog a Himalayan marmot (Marmota himalayana) that his oldest brother had been dis-*

[1] Ge P., Xi J., et al. *Primary case of human pneumonic plague occurring in a Himalayan marmot natural focus area Gansu Province, China*. Int J Infect Dis. 2015; 33:67–70; DOI: 10.1016/j.ijid.2014.12.044.

membered and fed to five dogs (including the one he found). Two days later, the man developed a fever, and on 15 June, when his condition worsened sharply, he went to the village clinic (on 14 June he was in Yumen City at his sister-in-law's house, where he also spoke to neighbours). From the outpatient clinic, he was transferred to the local hospital, where he was tentatively diagnosed with an upper respiratory tract infection. Despite treatment (antipyretics, anti-inflammatory drugs and clindamycin) his condition worsened, so he was transferred to Yumen People's Hospital. Examination showed pathology of both lungs, as well as partially dilated intestine, pleural effusion in the left lung, and a pericardial effusion (i.e. fluid accumulation in the pericardial cavity), and severe pneumonia was diagnosed. He was given anti-inflammatories, fluid replacement, and cefoperazone sodium. At 11:30 pm, Gram-negative bacilli with rounded dark ends were found in blood and sputum samples, and plague was diagnosed. On 16 June at 00:10 am, the patient's blood pressure decreased, dyspnoea appeared and red frothy sputum began to be secreted. At 5 am, against the background of antibiotic therapy (streptomycin intramuscularly and gentamicin intravenously), cardiac arrest occurred. Resuscitative measures were unsuccessful, and death occurred at 5:57 am. Postmortem diagnosis: primary pneumonic plague. Culture was identified two days later, antibodies to capsular antigen were detected in vivo (also detected in sister-in-law and two patients receiving blood transfusions in the same ward, all without clinical manifestations). Polymerase Chain Reaction (PCR) for Y. pestis fra and pla was positive. The patient had 151 contacts, including 63 close contacts. All received prophylactic antibiotic therapy; there were no fatalities. 5 dogs were also found to have antibodies to FI (capsule antigen) without clinical manifestations, all were euthanised.

Another case also occurred in China, in the Qinghai-Tibet plague focus area.

On 10 December 2017[1]*, a 45-year-old shepherd had a pain in his shoulder, so he took painkillers. The next day, the pain became so unbearable that the shepherd went to the hospital (it took about 7 hours to*

[1] Xi J., Duan R., et al. *First case report of human plague caused by excavation, skinning, and eating of a Hibernating marmot (Marmota himalayana).* Front Public Health. 2022; 10:910872; DOI: 10.3389/fpubh.2022.910872.

*travel), where examination revealed high fever (**39.5°C** / 103.1°F), mild cyanosis (i.e., lividity of the skin), swelling of axillary lymph nodes, and more than 5 wounds 2-8 mm long on the right arm. Respiratory symptoms were absent. He was suspected to have plague: the bubo and blood were tested (PCR (caf1 and pla) detection on 12 December, Y. pestis isolated on 13 December at 07:00 am). Antibiotic therapy (levofloxacin) was started. On 12 December at 07:20, the patient developed symptoms of septic shock, such as confusion and increasing purpura (i.e., small purple-coloured rashes on the skin and mucous membranes due to capillary haemorrhages), which progressed to a secondary septic form of plague. Shock occurred at 11:00 am and death occurred at 02:48 pm. Data from the epidemiological investigation established that the patient had skinned and consumed a Himalayan marmot (Marmota himalayana) in winter hibernation. The infection most likely occurred through contact of the wounds with contaminated marmot fluid and tissues, as the meat was carefully prepared. This case became the first for China, when a person was infected by a marmot in winter hibernation. Remind that during winter hibernation, the host's body cools down, and although the bacterium is not "warmed" in this case, it does, as we recall, have 4-acyl LPS (as in the body of a live host), which helps it avoid the human body's immunity.*

Notably, of the 10 close contacts, one (also a shepherd) was found to have antibodies to capsular antigen, and because he had no clinical manifestations, he was diagnosed as having an asymptomatic form of plague. He had long denied eating marmot, but when antibodies were detected in his dogs (which were fed the entrails), he confessed for fear of prosecution.

Another case occurred in the city of Albuquerque (New Mexico, USA).

*On Monday, 11 November 1974[1], a 62-year-old woman awoke with a severe headache, soon joined by chills, fever and nausea. The next day there was pain in the shoulder and, some time later, in the back. Examination at the hospital revealed a high fever (**39.8°C** / 103.6°F), an en-*

[1] Von Reyn C. F., Barnes A. M., et al. *Bubonic plague from exposure to a rabbit: a documented case, and a review of rabbit-associated plague cases in the United States.* Am J Epidemiol. 1976; 104(1):81–87; DOI: 10.1093/oxfordjournals.aje.a112276.

larged (3 x 3 cm) and painful epitrochlear (i.e. ulnar) lymph node on the right arm, with clear lungs and no pain at the rib-vertebral angle. No insect bites, cuts or abrasions were found on the hands. As the patient had a history of recurrent pyelonephritis (i.e., inflammatory kidney disease of infectious origin) complicated by septicaemia, gram-negative sepsis was made as a provisional diagnosis. Blood samples were taken and antibiotic therapy (ampicillin intravenously) was started. By 14 November the fever had subsided, but headache, malaise and lymph node tenderness persisted. On 15 November, a Gram-negative bacillus was isolated from a blood sample and identified as Y. pestis on 17 November. After changing the treatment trajectory (streptomycin intramuscularly and tetracycline orally), the patient improved and recovered without complications

Shortly before becoming ill, between 8 and 10 November, the patient (and 6 other people) were camping in Carson National Forest, Rio Arriba County (New Mexico, USA). Her companions were hunting deer and rabbits, so on 9 and 10 November she skinned two captured Nuttall's cottontail (Sylvilagus nuttallii) without gloves with a kitchen knife, saving the meat in the freezer (the rabbits were covered with fleas, but there were no human bites). Analysis of bone marrow from the lower limbs of rabbits removed from the freezer on 18 November (after the patient had recovered) was positive for Y. pestis. It is noteworthy that the hunting participants who directly caught the rabbits but did not cut the carcasses were not infected.

This case is not the only one, but it (and others like it; **Table 16.1**, **Figure 16.1**) differs significantly from those presented earlier. Firstly, the source of infection was an animal that was a secondary carrier (according to classification of Boris K. Fenyuk). At the same time, its own fleas turned out to be infected as well. Secondly, the infectious process affects exclusively the lymph nodes draining the upper limbs without affecting the lungs. Thirdly, all known cases occurred during the cold season (mainly winter), i.e. during the inter-epizootic period when the main carriers are in hibernation[1]. All this indicates the epidemiological

[1] It also implies that during epizootics this animal is not involved in the epizootic process.

Table 16.1. *Cases of realisation of the contact mechanism in plague infection*

Case	Date, place Gender, age Source	Incub. period (days)	Affected lymph node	Outcome
1	January 1950 (Lea). Male, 27 years old. Shot and skinned six rabbits.	3	axillary	Alive
2	January 1951 (Lea). Male, 51 years old. Shot and skinned eight rabbits.	4	axillary	lethal
3	February 1960 (Cha). Male, 23 years old. Male, 24 years old. Shot and skinned nine (ten) prairie rabbits (*Sylvilagus audubonii*)*.	6 4	axillary	alive
4	December 1963 (Apa). Male, 28 years old. Shot and skinned a rabbit, fed it to the dogs.	2–3	axillary	lethal
5	November 1968 (Lem). Male, 32 years old. Killed an snowshoe hare (*Lepus americanus*) with a stone and skinned it.	3	axillary	lethal
6	November 1970 (Wall). Male, 49 years old. Shot a hare, skinned it, gutted it and ate it.	2	axillary	lethal
7	November 1974 (Rio). Female, 62 years old. Freshened and gutted 2 Nuttall's cottontail (*Sylvilagus nuttallii*).	1–2	epitrochlear	alive

Примечание:
Lea – Lea County, New Mexico (USA),
Cha – Chaves County, New Mexico (USA),
Apa – Apache County, Arizona (USA),
Lem – Lemhi County, Idaho (USA),
Wall – Wallowa County, Oregon (USA),
Rio – Rio Arriba County, New Mexico (USA).
* the patient had multiple open abrasions on the arms



Figure 16.1. *Map of contact contamination sites of human (red line indicates the boundary of natural foci)*

importance of representatives of the family Leporidae as "out-of-focal" carriers of plague.

The alimentary transmission is realised by eating unheat-treated infected meat, although there are reports of infection by eating cooked meat. As in the case of the contact mechanism, it is important to realise that since the infectious object is most often an infected host, the bacterium enters the person already "warmed". At the same time, as we remember, the plague microbe has lost the ability to synthesise the adhesin YadA, which ensures the attachment of bacteria to enterocytes and their translocation through M-cells of the intestinal mucosa; therefore, despite the lesions of the digestive organs, which in some cases may be quite significant, we cannot speak of the existence of an «intestinal» form of plague. As Vladimir Nikolaevich Lobanov[1] correctly points out, "*most researchers admit that in the presence of sharply expressed clinical*

[1] Владимир Николаевич Лобанов, 1907–1991; the Russian and Soviet physician, bacteriologist, pathologist, honoured scientist of the RSFSR. One of the leading Soviet specialists in the field of pathology and pathological anatomy of plague.

symptoms of the intestinal-gastrointestinal tract and anatomical changes in it we are talking about generalised plague"[1].

Available reports of contamination with infected meat indicate a variety of clinical forms. For example, pharyngitis (12 persons from the village of Azraq ad-Druze, Jordan, February 1997[2]), severe pharyngitis with submandibular lymphadenitis (4 persons from the town of Goriat, Saudi Arabia, February 1994[3]) and gastroenteritis (83 persons from Nimroz Province, Afghanistan, December 2007[4]) have been reported with camel meat. Similar manifestations are often found with the consumption of groundhog meat. In this regard, the following case will be worthy of further consideration.

> *On 28 April 2019[5], a 38-year-old Kazakh man living in western Mongolia's Bayan-Ölgii aimag (province) went to the ambulance with complaints of fever, abdominal pain and bloody vomiting. Shortly after the call, before the ambulance arrived, he died. Post-mortem examination revealed enlargement of the stomach, oesophagus, liver, kidneys and lungs, their filling with blood and signs of inflammation without enlargement of lymph nodes. The Y. pestis strain was found in the blood, liver, spleen, lungs, kidneys, stomach, brain and bone marrow. It is known that on 22 and 25 April he hunted marmots and during this period, together with his 37-year-old wife, consumed their raw organs (kidneys, stomach and gall bladder). One year ago he was vaccinated with a live dry vaccine based on the vaccine strain Y. pestis strain EV76. The wife was visiting friends during this time, but already between 26 and 28 April she visited a doctor for fever, diarrhoea, abdominal pain, vomiting and headache. She was treated on an outpatient basis (erythromycin and anti-inflammatory drugs) because she refused*

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[1] Лобанов В. Н. *Патологическая анатомия и патогенез чумы у человека*, М.: Гос. изд. мед. лит. «Медгиз», 1956, 176 с.

[2] Arbaji A., Kharabsheh S., et al. *A 12-case outbreak of pharyngeal plague following the consumption of camel meat, in north-eastern Jordan.* Ann Trop Med Parasitol. 2005; 99(8):789–793; DOI: 10.1197/13648590X65161.

[3] Bin Saeed A. A., Al-Hamdan N. A., et al. *Plague from eating raw camel liver.* Emerg Infect Dis. 2005; 11(9):1456–1457; DOI: 10.3201/eid1109.050081.

[4] Leslie T., Whitehouse C. A., et al. *Outbreak of gastroenteritis caused by Yersinia pestis in Afghanistan.* Epidemiol Infect. 2011; 139(5):728–735; DOI: 10.1017/S0950268810001792.

[5] Kehrmann J., Popp W., et al. *Two fatal cases of plague after consumption of raw marmot organs*. Emerg Microbes Infect. 2020; 9(1):1878–1880; DOI: 10.1080/22221751.2020.1807412.

to be hospitalised. After the death of her husband, she was hospitalised with pharyngeal inflammation and cervical lymphadenitis (suspected plague). Despite treatment (intravenous gentamicin and ceftriaxone), she died on 1 May. The plague microbe was detected in cervical lymph node, blood and intestinal samples, and pharyngeal swabs. No cases of infection were reported among the 124 contacts (all received emergency antibiotic therapy). The couple had four children aged 9 months to 12 years…

The slope of Mount Arakura offers a marvellous view of the valley of cherry trees, which for only a few days a year are clothed in delicate pink flowers. Like pure souls, they give their eternal beauty to the world for a moment, and then, at the faintest whiff of wind, soar to the clouds, above which the incomparable peak of Mount Fuji[1] can be seen in the distance. Sadly enchanted by the vision[2], a man stands on the same slope. He is looking either at Mt Fuji, wishing to see the unearthly beauty of its mistress, into whose tender hands every soaring petal aspires to fall, or at the nearby pagoda, built so that there would be no more wars in the world. Immersed in his thoughts, he did not notice how a petal slowly descended on his shoulder, making a couple of graceful turns…

View from Mount Arakura ►

[1] A mountain (stratovolcano) in Japan, revered as a shintai (deity symbol) of Kami Konohanasakuya-hime, a symbol of the transience of life (her symbol is the sakura flower). It is located at the contact point of the Eurasian, Philippine and Okhotsk lithospheric plates. According to one version, the modern Japanese name Fujisan (Jap. 富士山) is derived from the Jap. 不二 ("incomparable").

[2] A reference to the aesthetic principle of mono no aware (lit. "the pathos of things"), meaning "sad fascination with things". It is a deep emotional feeling that expresses a longing for the infinite through a melancholic awareness of the horror of things, a wistful contemplation of them, an awareness of the impermanence and transience of the world, yet a life-affirming and luminous sensitivity to the ephemeral. Mono no aware is the happiness of contemplating cherry blossom (sakura) and the sadness of understanding its imminent withering. Each petal of its flower is a symbol of the transience of life.



17 THE CLINICAL PICTURE

In the previous chapters we have looked in detail at what happens to the human body when infected with the plague microbe. This, you could say, was an inside view. Now it is time to talk about what happens on the outside, i.e. the clinical picture.

The incubation period lasts 2-3 days, rarely lasting 6 days[1]. In the primary pneumonic form, as mentioned above, the incubation period is only 1-2 days, but in the case of high doses of aerosol contamination (e.g., in an accident while working with the pathogen) it can be reduced to a few hours[2].

In vector-borne infection, the onset of the disease is always acute, although it may be preceded by a prodromal period (i.e. the period between incubation and the disease itself) with "flu-like" symptoms. Among them, we will single out chills, which may last for half an hour or an hour, disappearing and reappearing, as it is a harbinger of a severe course of the disease. There may be pain in the sacral region, muscles and joints, as well as anxiety, nervousness and even depression.

The temperature rises rapidly (**Figure 17.1**). An important clinical manifestation of plague, regardless of the form, is the *intoxication syndrome*, which determines the severity of the course of the disease and its outcome. Alexandre Yersin wrote: "*The plague toxin exists!*"

The intoxication syndrome naturally affects the appearance of the patient: at first there is hyperaemia, puffiness of the face, changes in the eyes (they are "bloodshot", there may be lacrimation and even photo-

[1] It is for this reason that the observation period for contacts, as well as for those who have worked with the plague pathogen, is 6 days. It should be borne in mind that it is extremely rare that the incubation period can last longer (e.g. for vaccinated persons it can be 10 days), so even at the beginning of the last century Kitasato Shibasaburō insisted that the observation period should last at least 10 days.

[2] The Russian and Soviet physician Evgeny Sergeevich Kastorsky (Евгений Сергеевич Касторский; 1870-1938) reported the peculiarities of the clinical picture during the epidemic in Manchuria (1920-1921), consisting in the rapidity of the course and the lack of clearly expressed localisation of the process, which led to fatal outcomes already on the first day of the disease, although listening to the patient did not confirm any pathological processes in the lungs, which were indicated only by "rusty" sputum.

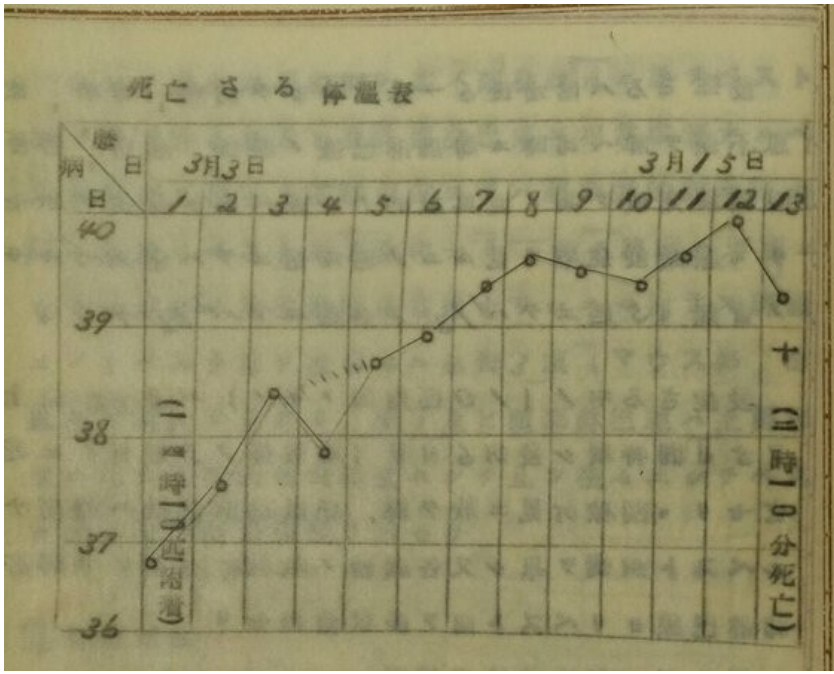


Figure 17.1. *Graph of plague-infected person's body temperature changes, made by the member of Unit 731 (shows a sharp rise in temperature on the second day after infection)*

phobia). There may be an expression of suffering or horror on the face. It is swollen, mask-like, reddish in colour. Emotions are absent. The skin is dry and hot. The lips are dry. The tongue is often also dry, but thickened and covered with white thick plaque (so-called "chalky tongue"), which persists for some time, and in the severe course of the disease darkens to dark brown. Speech becomes slurred, hearing is impaired, the eyes become glassy, and in some cases there is dizziness, which gives the impression of alcohol or drug intoxication. It is reported[1] that on one occasion a doctor refused to admit a patient to hospital, thinking that the patient had abused alcohol. The man was later found dead on a bench near the hospital, and a post-mortem examination confirmed the terrible diagnosis.

[1] Nikiforov V. V., Gao H., et al. *Plague: clinics, diagnosis and treatment*. Adv Exp Med Biol. 2016; 918:293–312; DOI: 10.1007/978-94-024-0890-4_11.



Figure 17.2. *Patient with a carbuncle visible on the leg (photograph from the collection of Dr. Karl Friedrich Meyer, 1937)*

In addition to the above, there is a severe headache (coinciding with the onset of fever), often accompanied by a *head disease*, expressed by psychomotor agitation. Patients are restless, overactive, waving their arms, trying to run, but their gait is shaky. Possible mental confusion, hallucinations and delirium. There have been cases of delirious patients leaving the bed and death catching them in a variety of positions and postures. Atilio Macchiavello[1] reports[2] that delirium and stupor are characteristic of patients in India and China, whereas this symptom is absent in patients in Africa and South America. All of this led to the fact that early in the last century some physician's manuals suggested that the "form of delirium" should be used to make a diagnosis.

Sometimes patients experience burning pain in the upper and lower abdomen, which is not relieved by drinking plenty of cold water (patients are thirsty). The pain may spread to the whole body.

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[1] 1902–1996; the Italian-born Chilean physician, hygienist, epidemiologist. He described a large number of new carriers and vectors of plague, reported the first mixed case of plague and pseudotuberculosis, and developed a method of staining chlamydia and rickettsiae. He was the first to propose the use of the insecticide DDT (1,1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) to control plague vectors.

[2] Macchiavello A. *Plague*, in *Clinical tropical medicine (Gradwohl R. B. H., Benitez S. L., et al.)*, St. Louis, 1951. P. 41.

Tachycardia, arrhythmias and decreased blood pressure are noted in the cardiovascular system. Tachypnoea (i.e. rapid shallow breathing), oliguria (i.e. decreased urine output) or anuria (i.e. complete absence of urine or its reduction to 100 ml per day) may occur. Despite all of the above, the appearance of patients resembles that of typhoid fever, so plague can be suspected at this stage only on the basis of *epidemiological history*.

As the disease progresses, there may be skin lesions expressed by a primary affect at the site of the bite and sometimes a small rash in the immediate vicinity, indicating a *cutaneous form* of the disease. The presence of cutaneous manifestations at the bite site, usually minor, is an important diagnostic sign. At first, a small, painful crimson spot appears on the skin, more like an insect bite. Over the next 24 hours, the skin hardens and the affected area increases in size, rising slightly above the skin (papule). In the centre of the papule, a small bubble with haemorrhagic contents (flectena) develops, which is accompanied by increased pain. Further, the shell of the fractena ruptures (ulcer), its edges swell, changing from pink-red to a bluish colour. The ulcer penetrates deeply into the skin and is covered with a dark scab on top (carbuncle). The carbuncle (**Figure 17.2**) usually appears before the second day of illness and may be 2-5 cm in diameter. New vesicles may form along its edges, and plague carbuncles may be confused with anthrax carbuncles (tissues adjacent to anthrax carbuncles are usually pasty (i.e. pale and inelastic), whereas those adjacent to plague carbuncles are dense).

Due to haematogenous dissemination of the causative agent, carbuncles may appear at a considerable distance from the entry gate (secondary carbuncles), but somewhat later than the primary carbuncle. However, the presence of multiple primary carbuncles is indicative of multiple consecutive bites.

Healing of carbuncles is slow and begins with the appearance of a scab and a clear line of demarcation (i.e., a restriction line) along which pus is discharged. When the scab is removed, rough scars are left on the skin.



Figure 17.3. *Right inguinal bubo in a Vietnamese girl (1965)*



Figure 17.4. *Left axillary bubo in a Vietnamese girl (1965)*



Figure 17.5. *Right submandibular tubercle and tumour in the right cheek area in a Vietnamese boy (1965)*



Figure 17.6. *Right submandibular bubo in a male with a "rupture" site from which pus oozes out (1993)*

As a rule, the carbuncle appears slightly before or at the same time as the buboesis[1]. The *bubonic form* of the disease is marked by painful sensitivity of the area of the skin under which the lymph nodes nearest to the site of the entrance gate are located (usually inguinal, axillary, cervical or submandibular[2]), so it is detected already in the first hours of the disease. The pain can be so severe that patients find it difficult to move in this area, so they often adopt unnatural postures (raised head, withdrawn arm or leg, etc.). After a while, one or two lymph nodes become inflamed (lymphadenitis; palpated as lumps) and form a conglomerate. The inflammation involves the skin (periadenitis) and the conglomerate fuses with it (buboes, **Figsure 17.3 – Figure 17.6**). This is accompanied by severe pain and it is no longer possible to palpate the buboes (**Figure 17.7**), which, at its peak, may be as large as a hen's egg. It is for this reason that Kitasato Shibasaburō refused to isolate culture from the buboes, and some specialists note that the process of collection may be difficult because of the reflex movements of the patient. In the vast majority of cases, a single bubo is formed, but there are exceptions, the rarest of which is the sim-

[1] It should be noted that the *cutaneous form* is a carbuncle without a bubo, the *bubonic form* is a bubo without a carbuncle, and the *cutaneous-bubonic form* is a carbuncle and a bubo.

[2] The inguinal lymph nodes are affected in 50% of cases, axillary lymph nodes in 20%, and cervical lymph nodes in 5%. Submandibular lymph nodes are rarely affected, hamstrings and elbows even less frequently. In isolated cases, lymph nodes of other localisation may be affected. The high incidence of inguinal lymph node involvement is due, firstly, to the high likelihood of legs bites and, secondly, to the large amount of lymph (compared to other draining lymph nodes) that collects in these nodes due to the large surface area of the skin.

ultaneous formation of buboes in the groin, axilla, and neck. The general condition of the patient depends on the localisation of the buboes: it progressively worsens as the buboes approach the head.

Swelling may extend a considerable distance from the buboes, which can be clearly detected by pressure (after removing the hand, the indentation remains). Localisation of the buboesis in the axilla in female may lead to spread of the swelling to the breast area (palpation reveals some trembling) and scapulae.

After a short time (about day 5-6), the disease peaks. Fever persists at 40°C / 104°F (**Figure 17.1** shows that the peak occurred on day 8). Symptoms of intoxication reach their peak. The face becomes distorted, with possible expressions of anxiety and terror. Blood pressure decreases, heart tones are muffled, pronounced tachycardia is detected. The liver and spleen are enlarged, indicating bubonic «rupture» and bacteraemia.

The buboes do not disappear without a trace: they scar or sclerotise. In the first case, the skin over the buboes becomes red and bluish-brown, thinning and necrotising. The upper layer may peel off (**Figure 17.8**). Pain gradually subsides, and by day 8-10, holes are formed in one or more places (sometimes the bubo bursts), from which masses of yellow-green pus are discharged (**Figure 17.6**), sometimes with an admixture of blood, but without a pungent odour. The bottom and edges of the resulting pus cavity are gradually filled with young connective tissue (granulation), and after about 3-4 weeks the buboesis becomes a rough and deep scar. In the second case, the bubo slowly shrinks, but in its place there is still palpated thickening for a long time.

Secondary buboes, resulting from the introduction of bacteria into other lymph nodes (both at the time of the bite through lymphatic vessels and on the background of bacteraemia), are much smaller in size, less painful and pass without suppuration. In isolated cases, peripheral lymphatic vessels may be affected (lymphangitis).

Exit of the pathogen from the liver and spleen leads to septicaemia and generalisation of the infectious process. The concentration of hepatic enzymes increases significantly, the number of erythrocytes decreases (as a consequence, the haemoglobin content decreases). Vomiting, less frequently nausea and diarrhoea (sometimes with mucus) appear. Gas-

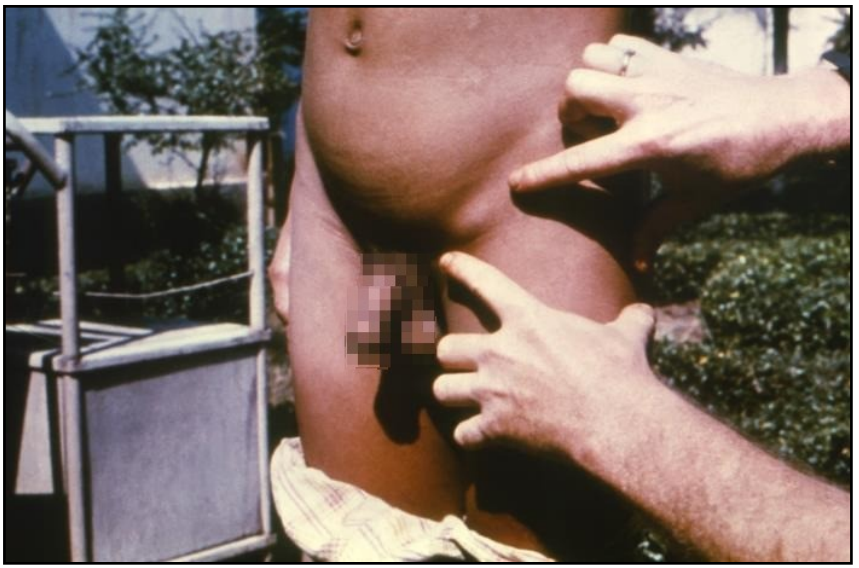


Figure 17.7. *Left inguinal bubo in a Vietnamese man (1965). The physician palpates the borders of the bubo, which are not visually defined due to periadenitis. Performing such manipulation without gloves is now a serious breach of biosafety regulations, but was the norm in the middle of the last century*

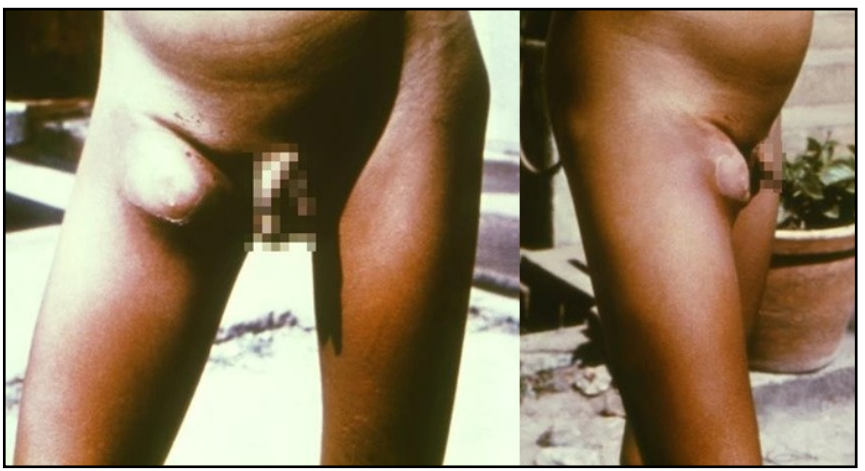


Figure 17.8. *Large right inguinal bubo in a Vietnamese boy (1965), anterior and lateral view. Note how the skin covering the bubo has detached due to severe swelling*

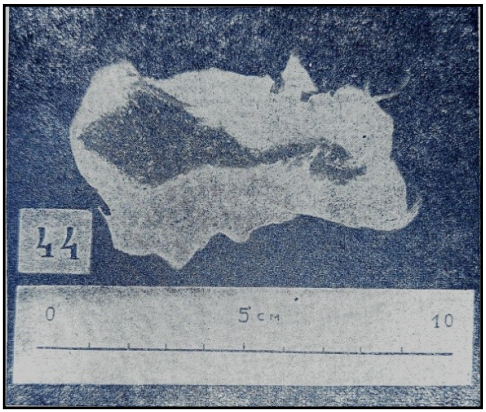


Figure 17.9. *Shwartzman phenomenon (rabbit model) expressed by necrosis spreading along the course of a large vein. In this experiment (No. 44), a rabbit was injected with a culture of Y. pestis subcutaneously and intravenously at a distance of 8 cm. One hour later, a haemorrhagic spot formed at the site of subcutaneous injection and the vein became severely swollen. After 3 hours, haemorrhage began to develop around the vein, which rapidly involved the subcutaneous injection site. After 2 days, the entire area engulfed by the haemorrhage began to necrotise, which after 6 days led to destruction of the surrounding fibre and exposure of the vein. After another 2 days, the vein finally necrotised and fell off together with the whole area, leaving behind a scar (by Жуков-Вережников Н. Н., 1940)*

trointestinal symptoms occur in half of patients and in some cases may become the dominant symptoms (this is why intestinal forms are sometimes distinguished, which is a mistake, as "intestinal" symptoms are never independent). It is not uncommon for plague germs to be isolated from faeces.

Increasing intoxication develops into infectious toxic shock, which plays a decisive role in the development of the infectious process. In response to the action of toxins, it is possible to develop para-allergic processes – a generalised reaction called the Shwartzman phenomenon (**Figure 17.9**). This phenomenon consists in the occurrence of localised or generalised (as in our case) haemorrhagic inflammation due to an excessive immune response to a toxin, which leads to thrombosis of small vessels and then necrosis of organ tissues. In fact, we are talking about a special case of systemic inflammatory response (DIC).

Tachycardia and tachypnoea increase, but blood pressure decreases (in case of recovery, it comes to normal values slowly). Anuria is not uncommon. Nasal and gastric bleeding is possible. Multiple dark-blue haemorrhages (bruises) develop on the skin and mucosa, passing into necrosis. Multiorgan failure develops.

Involvement of the lungs in the infectious process marks the beginning of the *secondary pneumonic form* of the disease, which develops in 8-10% of cases. Cough and pain in the chest when breathing (pleurisy develops). Pain in the side may appear. Sometimes the body temperature rises again to 39-40°C / 102-104°F. It is important to note that chest pain and headache are important symptoms, so their absence makes a diagnosis of plague unlikely. Rarely, a dry cough may be observed. At first, scanty vitreous sputum will separate. After a few days it becomes bloody and the amount increases. Examination reveals lobar pneumonia[1] with localisation of foci mainly in the middle lobe of the right lung and the upper lobes of both lungs. Absence of foci in the lower lobes serves as a peculiar marker indicating haematogenous penetration of the pathogen, characteristic of plague.

Shortly before death, patient faces take on a characteristic bluish colour with an expression of suffering called *facie pestica* (Lat. "plague face"). The facial features become harsh, with profuse drops of sweat ("death dew") on the forehead. Many people try to escape home, where they die curled up in a cot.

It is important to realise that the severity of the infectious process varies. Although the probability of death is quite high in the absence of treatment, in some cases clinical symptoms may be absent (*asymptomatic form*) or relatively mild (*ambulatory form*).

18 CLINIC OF PRIMARY SEPTICEMIC PLAGUE

In 2008[2], a 79-year-old woman living in a rural mountainous area of California (USA) was admitted to a local hospital with mental confusion after being found by a neighbour. She was known to have suffered

[1] It will be remeined that physician, who examining Abram L. Berlin, mistakenly diagnosed lobar pneumonia (the real diagnosis was primary pneumonic plague).

[2] Margolis D. A., Burns J., et al. *Case Report: Septicemic Plague in a Community Hospital in California*. Am J Trop Med Hyg. 2008; 78(6):868–871.

from severe lower back pain in recent months due to osteochondrosis and was unable to get out of bed or keep her house clean, which was covered with faeces from deer mouse (Peromyscus maniculatus) and wood rat (Neotoma spp.)[1].

On admission to the emergency department, an elderly woman had a temperature of ***38.2°C*** */ 100.4°F, a pulse rate 110 beats/min, a blood pressure 121/60 mmHg, an oxygen saturation 80% (breathing independently). The patient is excitable but only person-centred. Auscultation revealed slight rales in the lower lungs on the left side. No inflammation of axillary and inguinal lymph nodes was detected. The abdomen is flat and soft, painless, stools are normal. She had leukocytosis (white blood cell: 46.8 x 10⁹/L [4.5-11 x 10⁹/L] /* ***46,800 cells/µL*** *[4500-11,000 cells/µL])*[2]*, renal dysfunction (serum creatinine 168 µmol/L [53-97.2 µmol/L] /* ***1.9 mg/dL*** *[0.6-1.1 mg/dL]) and elevated transaminases (aspartate aminotransferase 336 IU/L [<35 IU/L]; alanine aminotransaminase 59 units/L [7-56 IU/L]). Chest X-ray showed atelectasis (i.e., lung tissue collapse with loss of lung volume) of the lower part of the left lung.*

The patient was diagnosed of community-acquired pneumonia and started antibiotic therapy (a dose of ceftriaxone followed by scheduled ampicillin/sulbactam and levofloxacin). Fever decreased and cognitive function improved. However, 48 hours after admission, renal failure worsened (serum creatinine 371.3 µmol/L [53-97.2 µmol/L] / ***4.2 mg/dL*** *[0.6-1.1 mg/dL]), acidosis and thrombocytopenia (platelets: 54 x 10⁹/L [150-400 x 10⁹/L] /* ***54,000 cells/µL*** *[150,000-400,000 cells/µL]) developed. On the third day, she was transferred to the Hospital (the San Diego School of Medicine of the University of California), where she soon required intubation and haemodynamic support. Blood cultures on admission were negative. Lactate dehydrogenase concentration was elevated (3296 IU/L [13-220 IU/L]). A blood smear showed a moderate number of schistocytes (i.e., red blood cell fragments), the prothrombin time*[3] *was*

[1] Typical carriers for a given plague-endemic region.

[2] An important marker of plague infection, as marked leucocytosis is not characteristic of infectious pathologies.

[3] This is the time it takes for a blood clot to form after tissue factor (i.e. clotting factor III) has been added to the plasma. On the basis of this indicator, the international normal-

1.2 times control, and the partial thromboplastin time[1] *was 1.3 times control. Based on the clinical picture and laboratory data, a new diagnosis of thrombotic thrombocytopenic purpura*[2] *was entertained, for which plasmapheresis and plasma exchange were initiated*[3].

*Several areas of necrosis with surrounding erythema were found on the chest and upper extremities (**Figure 18.1**-A), and gangrene was found on the toes (**Figure 18.1**-B). Histological examination (**Figure 18.1**-C,D) showed skin necrosis, acute inflammation and thrombosis of small vessels. Computed tomography of the abdomen (**Figure 18.1**-F) showed bilateral necrosis of the cortical layer of the kidney, indicating the Shwartzman phenomenon (remind: in this case, a generalised reaction of the organism to endotoxins causing thrombosis and, consequently, necrosis of the affected tissues - suspected bacterial infection). At this time, the previous hospital reported that a urine culture showed Pseudomonas aeruginosa (>10^5 CFU/mL), a Gram-negative motile bacillus, so the antibiotic regimen was changed to meropenem and gentamicin*

The next day, a report came back that a blood smear showed a "gram-positive rods". The inconsistency in the result forced the attending physicians at the academic hospital to send an enquiry to the previous hospital, which was answered two days later when the responsible officer went to work. The result by them was changed to "gram-negative rod". There was no indication of the specific species (or at least genus), as one automated analyser (Vitek) identified the culture as Y. pestis and another (API) as E. coli or Proteus spp. The isolated culture was not transferred to the laboratory of the academic hospital until day 7 from the day of admission (to the first institution). They were slow-growing

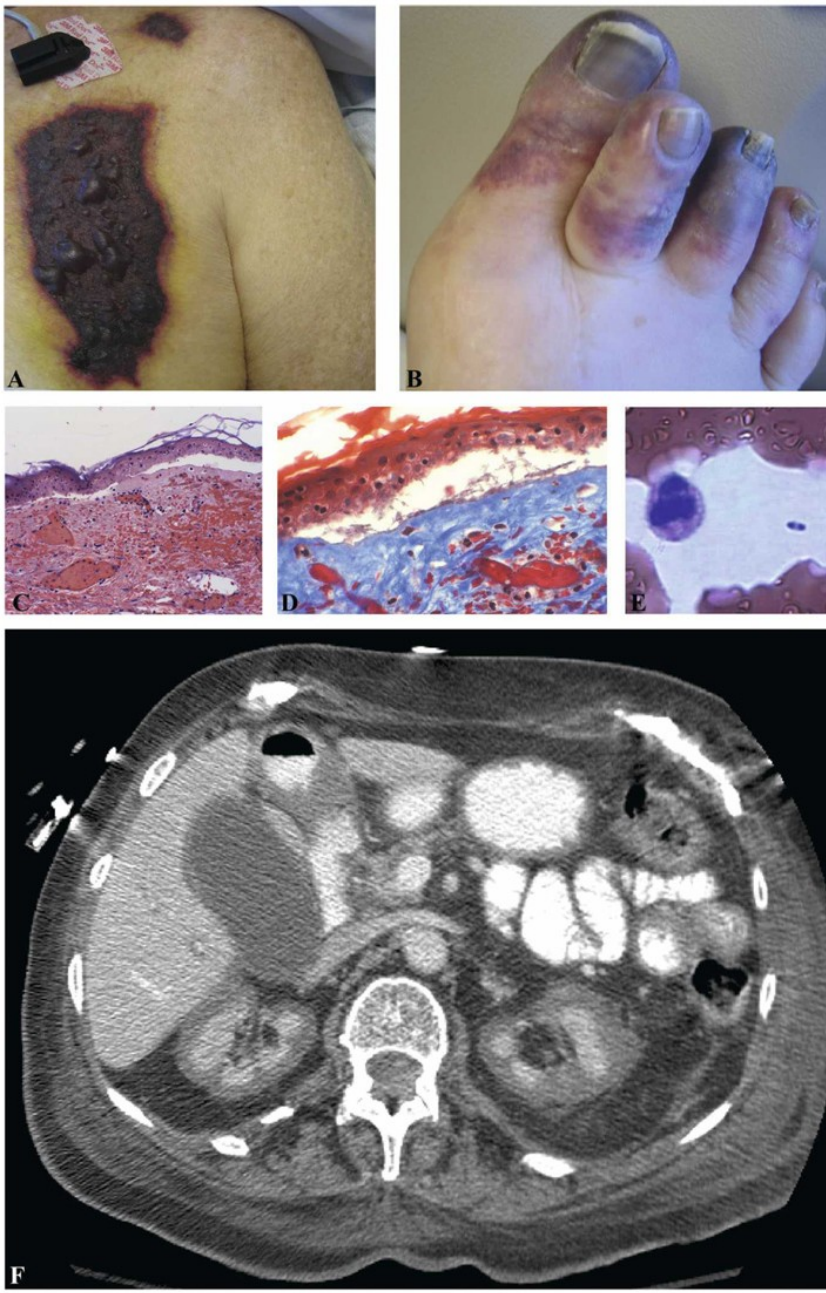
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ised ratio is determined, which is the ratio of the prothrombin time results of the test sample to the normal one, raised to a degree (international sensitivity index).

[1] This is the time it takes for a blood clot to form after chemical reagents (e.g. calcium chloride) have attached to the plasma. Reflects the operation of the intrinsic pathway and the overall cascade of the human blood coagulation system.

[2] An acute transient disease characterised by non-immunological thrombocytopenia and microangiopathic haemolytic anaemia. Platelets and fibrin accumulating in small vessels damage passing platelets and red blood cells, causing significant mechanical thrombocytopenia and anaemia, and also damage the vascular endothelium.

[3] A method in which a volume of plasma is taken from a patient and replaced with plasma substitutes and clotting factors.


A
B
C
D
E
F

*small colonies; in the smear Gram-negative bacilli, bipolar staining, immobile, not lactose fermenting. Examination of the initial blood smear, also transferred from the previous hospital, revealed a small number of bacteria with bipolar staining (**Figure 18.1**-E). Two days later, the culture obtained was identified as Y. pestis (result confirmed by the Reference Laboratory, The San Diego County Public Health Laboratory). The diagnosis was primary septicemic plague. That same evening, the nurse thought (probably against the background of all these events) that she saw live fleas in the patient's bed, which in fact turned out to be fruit flies.*

The patient was effectively treated for primary septicemic plague, but anuria, which developed in the background of the underlying disease, remained. She refused renal replacement therapy and died of uremia shortly afterwards.

The *primary septicemic form* of plague begins suddenly, accompanied by generalised weakness, myalgia (i.e. pain in muscles, ligaments, joints) and arthralgia (i.e. pain in joints). Severe headache, dizziness, chills and fever occur. After some time, lethargy and even delirium (i.e., blackout) may follow. As we have seen, these are the symptoms the patient had. Vomiting is also possible. Blood pressure is low and falling. Respiration is rapid. An important feature of the primary septicemic form is the absence of buboes (remind: septicaemia develops, bypassing the lymph nodes). After 12-40 hours, renal failure develops, leading to decreased diuresis up to anuria.

◄ ***Figure 18.1.*** *A case of primary septicemic plague (by Margolis D. A., 2008; published with permission of the American Journal of Tropical Medicine and Hygiene):*
A – several areas of skin necrosis with surrounding erythema on the patient's breast;
B – gangrene of the toes of the right foot;
C – 200x, puncture biopsy of the lesion (A); there is a subepidermal cleft over the necrotic dermis with marked extravasation (i.e. infiltration of red blood cells from the vessel into the perinadal tissues) and dermal purpura;
D – 400x, puncture biopsy (C), trichrome stain; fibrin clots observed in damaged vessels (no bacteria);
E – 40x, blood smear; a single bipolarly stained bacterium is observed;
F – abdominal computed tomography; bilateral renal cortical necrosis is observed



Figure 18.2. *The patient's leg is characteristically pale*

Infectious toxic shock occurs, which plays a crucial role in the development of the infectious process. Tachycardia and tachypnoea increase, blood pressure decreases and anuria often develops. Nasal and gastric bleeding is possible. DIC and small vessel thrombosis develop, which may result in pallor of the distal extremities (**Figure 18.2**). Multiple haemorrhages on the skin and mucosa then develop, progressing from light red (**Figure 18.3**) to dark blue (**Figure 18.1**-A,B), and on to necrosis. The size of the "spots", referred to as "death spots", can vary, reaching large sizes (**Figure 18.4**). It is believed that it was because of these "spots" that the plague was called the Black Death in the Middle Ages.

It is important to note that, in contrast to the secondary septicemic form, systemic pathological processes are more rapid in this form, while the lungs are practically not involved. Death usually occurs within two days.



Figure 18.3. *Multiple haemorrhages on the right leg*



Figure 18.4. *Hand lesions in a 59-year-old man who contracted septicemic plague after contact with a cat of neighbour and the mouse it had torn apart*

19 CLINIC OF PRIMARY PNEUMONIC PLAGUE

On 25 October 2019[1], a 43-year-old man from Xilinguole League Prefecture (Inner Mongolia Autonomous Region, China), a cattle farmer, went to Sunite ZuoQi Hospital with complaints of sudden onset of fever, chills, shortness of breath and bloody sputum. He reported that he had been tilling soil on a farm the previous day. He was diagnosed with lobar pneumonia[2] on the basis of a CT scan. Despite antibiotic therapy, there was no improvement after 2 days. Re-examination of CT scanning revealed that his pneumonia aggravated. Suspected of influenza pneumonia, he was then transferred to Xilingol League Hospital by ambulance and was admitted in the intensive care unit.

The 46-year-old wife of patient accompanied him and kindly nursed him by handled his vomitus in the spittoon. On 31 October, she also developed cough, fever, chills, fatigue, chest pain and bloody sputum. Her temperature rose to ***38°C*** */ 100.4°F. A CT scan revealed pneumonia, so she was also hospitalised.*

The couple stayed in the hospital until 3 November, and as their condition worsened (both had negative nasopharyngeal swabs for influenza), they were transferred to the Emergency Department of Beijing Chao-yang Hospital, Capital Medical University. On admission, the male patient had a temperature of ***36.3°C*** */ 96.8°F, a pulse rate 74 beats/min, a respiratory rate 20 times/min, and a blood pressure 126/85 mmHg. The patient was conscious with a bad mood. Auscultation revealed stiff breathing with moist rales, heart rhythm is correct. Inflammation of axillary and inguinal lymph nodes was not revealed. The abdomen is flat and soft, painless.*

He had leukocytosis (white blood cell: ***28.6 x 10^9/L*** *[4.5-11 x 10^9/L] / 28,600 cells/μL [4500-11,000 cells/μL]), neutrophilia (neutrophils:* ***26.53 x 10^9/L*** *[1.5-8 x 10^9/L] / 26,530 cells/μL [1,500-8,00 cells/μL]). Biochemical blood analysis revealed liver function disorders: increased albumin con-*

[1] Zhou H., Guo S. *Two cases of imported pneumonic plague in Beijing, China.* Medicine (Baltimore). 2020; 99(44):e22932; DOI: 10.1097/MD.0000000000022932.

[2] Let us again remind the story of Abram L. Berlin.

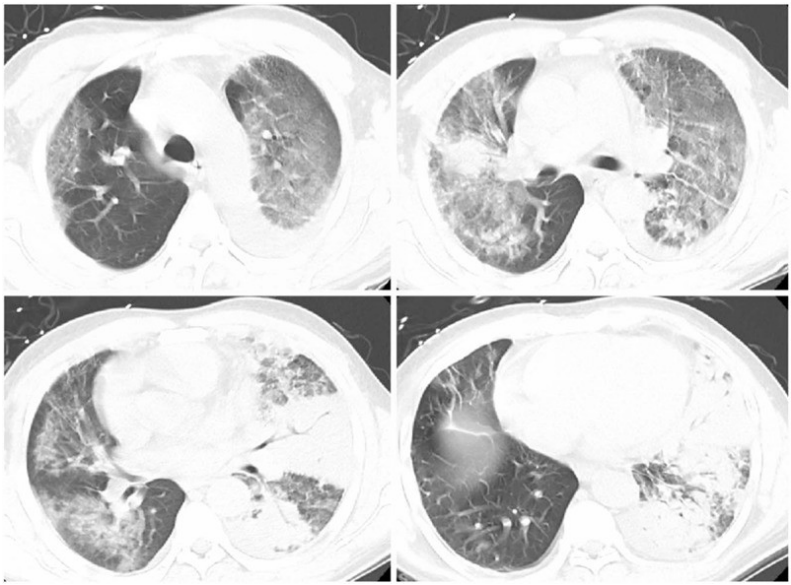
*centration (**29.1 g/L** [35-53 g/L] / 2.91 g/dL [3.5-5.5 g/dL]), alanine aminotransferase (80 IU/L [7-56 IU/L]), total bilirubin (**22.6 µmol/L** [1.71-20.5 µmol/L] / 1.7 mg/dL [0.1-1.2 mg/dL]), as well as a slight decrease in the concentration of creatinine (**44.5 µmol/L** [61.9-114.9 µmol/L] / 0.5 mg/dL [0.7-1.3 mg/dL]) and sodium (**135.7 mmol/L** [136-145 mmol/L] / 135 mEq/L [136-145 mEq/L]). Blood gas analysis revealed pO2 69.1 mmHg [75-100 mmHg], pCO2 27.2 mmHg [35-45 mmHg], $HCO3^-$ **21 mmol/L** [23-26 mmol/L] / 21 mEq/L [23-26 mEq/L] and PaO2/FiO2 330.4 on oxygen mask (saturation 100%, 10 L/min). In addition, a slight increase in fibrinogen concentration (4.6 g/L [2-4 g/L]) and erythrocyte sedimentation rate of 17 mm/h [2-15 mm/h] was found. Brain natriuretic peptide concentration (165 pg/mL [<125 pg/mL]) indicates heart failure with normal electrocardiogram (sinus rhythm, a pulse rate 75 beats/min). C-reactive protein concentration (11.30 ng/dL [<0.8 ng/dL]) indicates acute infection. CD3 T cell was 71.7%, CD4+ T cell was 41.5% and CD8+ T cell was 24.6%.*

*Computed tomography (**Figure 19.1**) revealed multiple patchy consolidations and ground glass opacity in bilateral lungs.*

*On admission, the woman had a body temperature of **37.3°C** / 98.6°F, a pulse rate 101 beats/min, a respiratory rate 22 times/min, a blood pressure 108/69 mmHg. Auscultation revealed stiff breathing with moist rales, heart rhythm was correct. Inflammation of axillary and inguinal lymph nodes was not revealed. The abdomen is flat and soft, painless.*

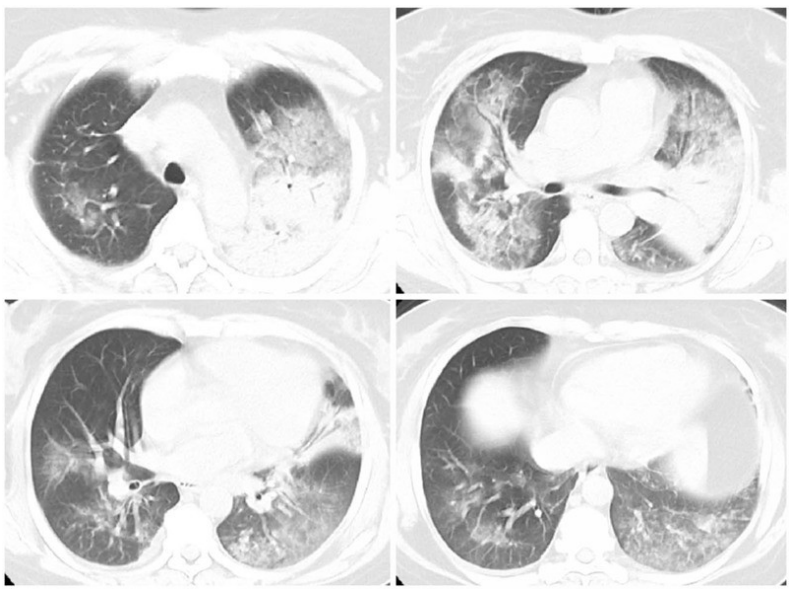
*She had a slight increase in white blood cells (**9.76 x 10^9/L** [4.5-11 x 10^9/L] / 9,760 cells/µL [4500-11,000 cells/µL]) and neutrophils (8.7 x 10^9/L [1.5-8 x 10^9/L] / cells/µL [1,500-8,00 cells/µL]) concentration. Blood gas analysis revealed pO2 59.5 mmHg [75-100 mmHg], pCO2 26 mmHg [35-45 mmHg], $HCO3^-$ **17.8 mmol/L** [23-26 mmol/L] / 17.8 mEq/L [23-26 mEq/L] and PaO2/FiO2 284.8 on oxygen mask (saturation 100%, 10 L/min). In addition, an increase in fibrinogen concentration (**7.4 g/L** [2-4 g/L] / 740 mg/dL [200-400 mg/dL]) and C-reactive protein (11.30 ng/dL [<0.8 ng/dL]) was found.*

*Computed tomography (**Figure 19.2**) revealed multiple patchy consolidation shadows and air bronchgram (i.e., the presence of air in the*



▲ ***Figure 19.1.*** *Computed tomography of the male*

▼ ***Figure 19.2.*** *Computed tomography of the female*



bronchial lumen against the background of consolidation) in both lungs.

*Both patients were diagnosed with severe community-acquired pneumonia and received antibiotic therapy (oral oseltamivir and intravenous moxifloxacin and cefoperazone and sulbactam for injection). They also received intermittent non-invasive artificial ventilation and high-flow oxygenation via nasal cannulae. Bronchoscopic examinations were performed, and sputum and bronchoalveolar lavage samples were sent to the laboratory. Only on 12 November, blood and sputum specimens were found to be positive by PCR and colloidal gold-immunochromatography assay targeting the F1 antigen and by reverse indirect hemagglutination assay (Y. pestis was not able to be isolated by culture). It should be assumed that, since the man worked with soil and the case occurred during an epizootic, he became infected by inhaling plague aerosol accumulated in a rodent burrow, while his wife, caring for him, became infected from sputum. Shortly before, on 8 November, both patients had undergone chest radiographs (**Figure 19.3** and **Figure 19.4**) and the woman was placed on invasive artificial ventilation. After diagnosis, patients were treated separately: the man improved and recovered, while the wife eventually died.*

The *primary pneumonic form* of plague begins suddenly and is characterised by a fulminant course. Georgiy P. Rudnev from the clinical point of view subdivides this form into three stages*: initial, peak* and *terminal.*

The clinical picture at the initial stage is quite varied. Most often the disease starts quickly (as in the described case). Temperature rises sharply, severe headache and recurrent vomiting develop. Chest pain of a cutting character, increased pulse rate, dyspnoea and delirium are possible. Sputum may be vitreous, liquid, odourless or have blood inclusions. There may be no sputum (*dry pneumonic form*).

At the peak stage, a cough is attached, which together with bloody sputum contains a large amount of the pathogen and can cause infection of surrounding people (for example, woman in the case). Signs of toxic damage to the nervous system are aggravated. Depression appears with possible subsequent agitation. Some patients are lethargic, there are disorders of coordination of movements. Tremors appear and articulation becomes difficult. Soporus (stupor) develops, and patients faces take on a characteristic bluish colour and look exhausted. Tachycardia

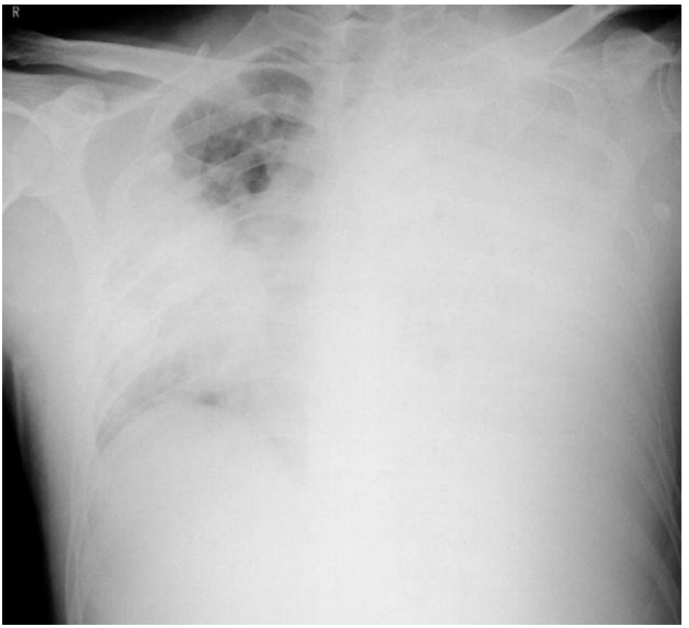


Figure 19.3. *Chest X-ray of the male revealed consolidations on bilateral lungs*

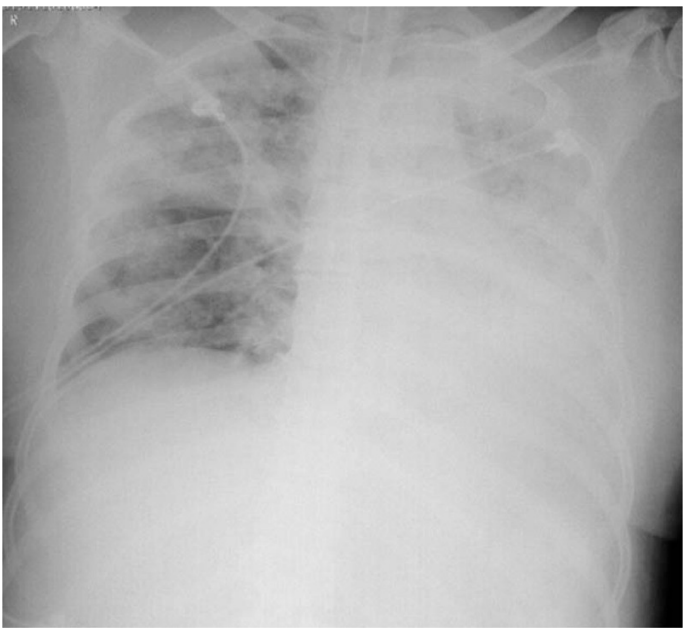


Figure 19.4. *Chest X-ray of the female revealed consolidations on the left lung and patchy shadow on the right lung*

appears, heart tones are muffled. The disease progresses to a generalised form (septicaemia), identical to the primary septicemic form.

In the terminal stage, infectious-toxic shock occurs, which plays a decisive role in the development of the infectious process. Tachycardia and tachypnoea increase, blood pressure decreases and anuria often develops. Nasal and gastric bleeding is possible. Multiple dark-blue haemorrhages (bruises) develop on the skin and mucosa, turning into necrosis.

There is often weakness. Some patients make attempts to get up and run away (plague delirium). Some fall into a coma. Renal failure and DIC develop. The spread of the inflammatory process in the lungs naturally leads to the progression of respiratory failure. Breathing becomes frequent and shallow. Inhalation pain in the chest increases. Most often patients die on the 3-5 day of the disease, which may be preceded by a decrease in body temperature to normal values.

20 PLAGUE MENINGITIS

Case No. 1. *February 1975. The city of Albuquerque, New Mexico, USA. An 11-year-old boy. Hospitalised with a temperature of* ***38.3°C / 101°F****. There is a painful hard mass measuring 10 cm in the right axilla, as well as a laceration on the right forearm and a partially torn nail on the middle finger of the right hand. The history revealed that an inquisitive child had found a dead coyote three days earlier (Y. pestis was found in the animal's corpse). The boy's blood aspirate and lymph node cultures were positive for Y. pestis. On the eighth day of the disease tetracycline therapy was started. After improvement of the condition, the patient was discharged.*

Case No. 2. *July 1975. City of Pecos, New Mexico, USA. A 15-year-old boy. Fever accompanied by general malaise and sweating. On*

examination (without hospitalisation) tetracycline therapy was started for an undiagnosed illness.

The two cases[1] have in common that the first boy was re-hospitalised on the 11th day of illness with lethargy and stiffness of the occipital muscles, and the second was hospitalised on the 9th day of illness with severe temporal headache. Both were found to have bacteria (*Y. pestis*) in the cerebrospinal fluid and were started on antibiotic therapy (chloramphenicol in the former, ampicillin and tetracycline in the latter), which resulted in recovery.

The first case is also interesting due to the Gallium scan[2], which showed[3] not only the possibility of early diagnosis of plague (the "solid mass" accumulated ^{67}Ga), but for the first and probably only time it was possible to visualise the brain in plague meningitis (**Figure 20.1**).

Consider another interesting case that occurred in 1963 in the USA[4].

On 21 March, a 23-year-old male soldier returning from Nha Trang[5], Viet Nam, sought medical attention for fever and a painful lump in his left groin. The examining physician noted a suspicious penile lesion and diagnosed it as lymphogranuloma venereum[6]. Despite antibiotic therapy

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[1] Becker T. M., Poland J. D., et al. *Plague meningitis – a retrospective analysis of cases reported in the United States, 1970–1979*. West J Med. 1987; 147(5):554–557.

[2] A method of nuclear medicine consisting in obtaining an image with a gamma camera (scintillation camera) as a result of intravenous injection of a patient with a preparation containing radioactive ^{67}Ga (in this case ^{67}Ga-citrate), due to its ability to form complexes with bacterial siderophores (*Y. pestis* is yersiniabactin), thus allowing visualisation of bacterial clusters..

[3] Stahly T.L., Shoop J.D. *Plague and the Gallium Scan: Case Report*. Journal of Nuclear Medicine. 1975; 16(11):1031-1032.

[4] Feeley E. J., Kriz J. J. *Plague Meningitis in an American Serviceman*. JAMA. 1965; 191(5):412–413; DOI: 10.1001/jama.1965.03080050058021.

[5] Between 1955 and 1975, there was an armed conflict in the territory of Vietnam, Laos and Cambodia, which went down in history as the Vietnam War, officially fought between the Democratic Republic of Vietnam (supported by the USSR and other communist states) and the Republic of Vietnam (supported by the USA and other anti-communist states). Around the same time (in the 60s), there was a plague epidemic on the territory of Vietnam, in the elimination of which the station in the city of Nha Trang, founded by Alexandre Yersin, played an important role.

[6] Or Durand-Nicolas-Favre disease, a chronic sexually transmitted infectious disease caused by *Chlamydia trachomatis* invasive serotypes L1, L2, L2a, L2b, or L3, characterised by involvement of the femoral, inguinal, iliac and deep pelvic lymph nodes..

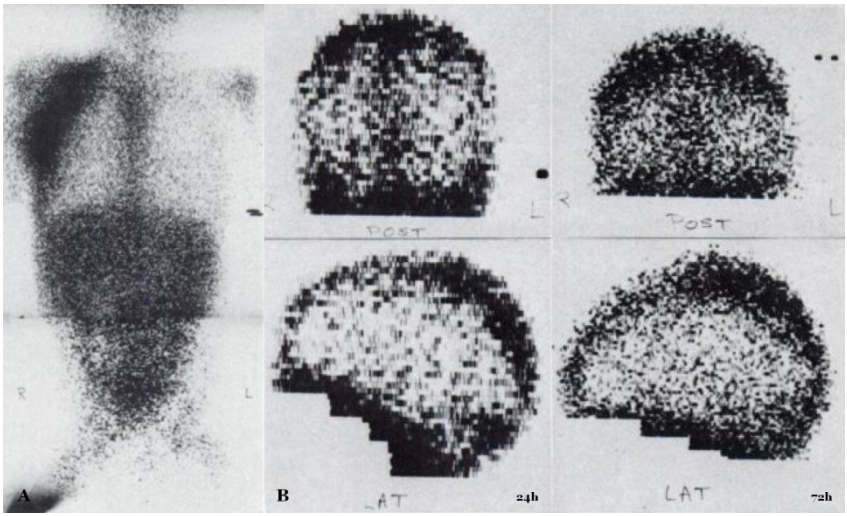


Figure 20.1. *Gallium scan images (by Stahly T. L., 1975; published with permission of the Society of Nuclear Medicine & Molecular Imaging):*
A – whole-body scan; note the abnormal accumulation of ^{67}*Ga in the right axillary nodes;*
B – brain 24 hours (left) and 72 hours (right) after administration of 67Ga-citrate. While the 24-hour result was questionably abnormal, it is the 72-hour imaging that reliably demonstrates increased ^{67}*Ga accumulation in the periphery (in the brain membranes), indicating meningitis*

(oral tetracycline), the groin swelling increased, accompanied by oedema and erythema (i.e. limited reddening of the skin resulting from dermal vascular dilation). This was interpreted as cellulitis and 2.4 million units daily of procaine penicillin G was given intramuscularly with the tetracycline. On 23 March, the patient presented with a frontal headache (without meningeal symptoms), but the groin swelling decreased slightly. The next day, occipital muscle rigidity (i.e., increased tone making movement difficult) developed. Lumbar puncture revealed turbid fluid with a large number of polymorphonuclear cells and a liquor pressure of 330 mm of water column [100-200 mm of water column]. Because the patient had recently returned from Vietnam, the liquor samples were subcutaneously injected into a guinea pig to isolate Y. pestis (stained smear and cultures were negative).

*At the time of transfer to a military field hospital, the patient complained of headache and neck pain, high temperature (**40.1°C / 104.2°F**). There was marked nuchal rigidity, a positive Kernig sign*[1]*, the right fundus showed moderate papillederma without hemorrhage, lymphadenopathy in the left inguinal region combined with erythema and mild induration (i.e. thickening), and a small ulcerating on the underside of the penis near the glans. Repeated examination of the liquor was negative (Gram stain negative and cultures negative smear and culture). The next morning the temperature decreased to **37°C / 98.6°F**, but the condition worsened*

On 2 April, the injected guinea pig was found dead (death on day 8). An post-mortem examination revealed typical signs of plague (buboes in the groin adjacent to the injection site, large friable spleen and haemorrhagic consolidation of the lungs). Smears from the affected organs revealed Gram-negative bacilli with bipolar staining ("safety pin"), and prints on media revealed colonies identified as Y. pestis (culture was sent to the Walter Reed Army Institute of Research for confirmation[2]*. By the time the microorganism was identified, the patient was already asymptomatic, but antibiotic therapy was continued, and he returned to action three weeks later in excellent condition.*

The *meningeal form* may occur during the period of recongestion of the bubonic form and be a relapse (the first two cases), or it may occur at any stage of the infectious process, but is most often a complication of a bacteriemia (or secondary septicemic form) that developed after the bubonic form (the third case). This case is an excellent example of the difficulty of diagnosis, as the bubonic form of plague was diagnosed as

[1] One of the symptoms of early diagnosis of meningitis; named after the Russian physician Woldemar (Vladimir Mikhailovich) Kernig (Владимир Михайлович Керниг; 1840-1917). Methodology: while lying on the back, the patient passively flexes the lower limb in the hip and knee joints at an angle of 90°. Due to the tonic contraction of the tibial flexor muscles, it is impossible for the patient to extend the leg at the knee joint. It should be remembered that in the presence of hemiparesis, the symptom may be a false negative, and in the elderly (in the presence of muscle rigidity) – a false positive.

[2] The organism is a Gram-negative rod (typical short, plump, bipolar) sensitive to *Y. pestis* phage; it reduced nitrate, but did not produce urease; it produced acid without gas in glucose, maltose, mannitol and salicin, but did not ferment glycerol or rhamnose. Cultures are sensitive to chloramphenicol, streptomycin sulfate and aureomycin, but resistant to penicillin and erythromycin. We will talk more about laboratory methods in Act III.

lymphogranuloma venereum due to the lack of laboratory confirmation. An additional complication was the fact that the patient was a soldier serving in Vietnam and should have been vaccinated against plague[1]. However, this was not the case due to frequent transfers[2], and the doctors decided not to specify.

The average age of patients with plague meningitis is 16 years (6 weeks to 64 years), and 68% are male. The characteristic symptoms are fever, stiffness of the occipital muscles and headache. Meningeal symptoms may be present. Rarely, focal neurological abnormalities such as cranial nerve anomalies are present. The reported mortality of patients according to antimicrobial agent is 50% for fluoroquinolones, 19% for aminoglycosides, 14% for sulphonamides[3], 11% for chloramphenicol and 0% for tetracyclines.

21 CLASSIFICATION

So, we have smoothly approached the classification of clinical forms of plague. The first classification was proposed in 1938 by Georgiy P. Rudnev[4], and according to it there are the following forms of the disease:

1. mainly local form:
   - cutaneous
   - bubonic

[1] At that time, vaccines were given with a vaccine containing 1.8-1.2 x 10^9 killed cells (now banned in the US). We will talk more about vaccines in Chapter 26.

[2] Here we should also remember a case from Mongolia, where a vaccinated man died of plague after eating infected marmot.

[3] Not currently in use due to low efficiency.

[4] Руднев Г. П. *Клиника чумы, под ред. д-ра Ф. Р. Чушинского,* Ростовский государственный противочумный институт и кафедра инфекционных болезней Ростовского медицинского института, Ростов-на-Дону: Ростиздат, Тип. им. Коминтерна, 1938, 268 с.

   - cutaneous-bubonic
2. internally disseminated (generalised) form:
   - primary septicemic
   - secondary septicemic
3. externally disseminated form:
   - primary pneumonic
   - secondary pneumonic
   - intestinal

In general, this classification corresponds with what we have considered in Chapters 17-20, with the exception of the intestinal form. And before we talk about it, let us consider the following case.

> *At the beginning of the last century*[1]*, a patient with extensive necrotic skin lesions in the temporal region was brought to the hospital by his relatives. According to them, he had been bitten by a rat during sleep in the temple area a few days earlier. He became ill 2 days later, and rapidly increasing deep tissue necrosis immediately started at the bite site. During the patient's hospital stay, the necrosis spread, and sepsis and death soon ensued. The diagnosis was confirmed bacteriologically: the plague.*

It should be assumed that the cause of the fulminant course of the disease was the primary septicemic form, but at the beginning of the last century clinicians did not fully understand the pathogenesis of plague and probably focused on the "external" manifestations of the disease, which, in this case, was the cutaneous manifestation. However, Georgiy Rudnev himself later expressed doubts about the possibility of independent existence of the cutaneous form. Nevertheless, it is included in modern classifications.

As for the intestinal form, as has been said many times, its existence is also very doubtful, and its inclusion in the classification is probably due to the fact that about half of the patients have gastrointestinal symptoms, which, as has been seen, are not independent. Moreover, even the implementation of the alimentary route of infection (Chapter 16) does not lead to the development of an independent intestinal form, so it is absent in current classifications.

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[1] Dürk H. *Beiträge zur pathologischen Anatomie der Pest.*, Jena, 1904.

The International Statistical Classification of Diseases and Related Health Problems. 11th revision (ICD-11) is currently in use. Let's take a look at it.

ICD-11

Code	Diagnosis	All Index Terms
1B93	Plague	
1B93.0	Bubonic plague	
1B93.1	Cellulocutaneous plague[1]	
1B93.2	Pneumonic plague	Plague pneumonia Plague of lung Pulmonary plague Pneumonia due to *Y. pestis* Primary pneumonic plague
1B93.3	Plague meningitis	
1B93.Y	Other specified plague	Abortive plague Ambulatory plague Asymptomatic plague Minor plague Pestis minor Pharynx plague Tonsil plague - Tonsillar plague Abortive plague Septicaemic plague *without mention of septic shock* - Septic plague - Septicaemic plague NOS - sepsis due to *Y. pestis* Septicaemic plague with septic shock
1B93.Z	Plague, unspecified	

[1] Includes all the types of "cutaneous" forms discussed earlier.

Postcoordination[1].
Has manifestation (use additional code, if desired):
1G40 Sepsis without septic shock
1G41 Sepsis with septic shock

As can be seen, relative to the previous revision (ICD-10), "septicemic plague" (A20.7) has been deleted, being moved to "Other specified plague" (1B93.Y) in the All Index Terms. Otherwise, the changes have been made to the All Index Terms. Of those listed, we have already encountered most of them in the cases reviewed, but "pestis minor", "ambulatory plague" (except for the mention at the end of Chapter 17), and "abortive plague" have not yet been mentioned. It seems time to remedy this.

Russian Empire, Vetlyanskaya stanitsa[2] (now Vetlyanka village, Astrakhan region, Russia), 28 September 1878. Agap Khritonov, a 65-year-old Cossack, suffers from a disease that local physicians cannot identify. The disease is accompanied by headache, general weakness, pain in the side and axillary buboes. After 4 days he dies and the disease spreads further, but with an uncharacteristic moderation in the number of lives taken. It takes several months for doctors to make a correct diagnosis, and then the Imperial government will delay declaring an epidemic (and taking appropriate anti-epidemic measures) for some time to come, as plague is considered a forgotten disease. The introduction of plague into developed countries is accidental and extremely rare[3]. Nevertheless, this plague outbreak would lead not only to a rethinking of the approach to sanitary protection of the state and the beginning of an

[1] Term: The **postcoordination** is a way of adding more information to a classification entity by combining it with other classification entities. For example, you can postcoordinate a diagnosis with a severity, body site, laterality, etc. to add these details.

[2] It is a historical administrative village unit of the Cossack host, that existed in the Russian Empire.

[3] In this connection it is very interesting to read the opinion of a member of the member of the Quarantine Council of Istanbul in 1899: "*the plague virus eludes the best efforts of struggle [against it], it annuls the effects of [our] best efforts and it awaits for the* most *favorable moment, unknown until now to science, for emerging out of its slumber so as to resume its morbid progress*". Do not be surprised by the word "virus' - this term (Lat. "poison") has been known since the 18th century and until the middle of the 20th century denoted any infectious agent.

understanding of natural foci, but also to a crisis of the then dominant of miasma theory[1]. Both domestic and foreign specialists took part in the eradication of the epidemic, and because of its uncharacteristically mild course they called the disease "ambulatory plague" (pestis ambulans), "local plague" (pestis nostras) and "abortive plague" (peste frustre), which were in fact synonyms.

British India, 1896. William John Ritchie Simpson[2], editor of The Indian Medical Gazette and part-time medical officer of health for the city of Calcutta, diagnoses plague in several soldiers of the King's Shropshire Light Infantry temporarily stationed in the city. However, his dreadful diagnosis, which caused nothing but panic, was soon disproved by the military physicians.

In the same year, in the heart of the British Empire, within the walls of the Entomological Society of London (now the Royal Entomological Society), James Cantlie[3] gave a lecture on the experience of plague control, with particular emphasis on the role of rats. At the time it was believed that the rat was not a carrier of the plague, but, like man, was merely an accidental victim, killed by the animal's frequent contact with the ground, which was the natural habitat of the plague bacteria. Consequently, any mass death of people from plague is always accompanied by mass death of rats (remember the words of the Chinese poet Shi Daonan: "*a few days after the death of rats, people fall like collapsing walls*"). However, this was not the case in Calcutta, on the basis of which the lecturer concludes that this form of plague is benign, i.e. "ambulatory plague" or "minor plague" (pestis minor)[4].

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[1] Remind: the theory of "night air", which are vapours from putrefaction processes and other impurities (e.g. vapours from swamps). Penetrating into the air, these vapours are inhaled by humans and cause disease. This theory was opposed to the contagions theory, i.e. small infectious particles responsible for causing epidemics.

[2] 1855–1931; the British (Scottish) physician, specialist in tropical medicine.

[3] 1851–1926; the British (Scottish) physician, surgeon, pioneer of first aid, participant in the eradication of the plague epidemic in the city of Hong Kong (1894). One of the founders of The London School of Tropical Medicine (now The London School of Tropical Medicine) and The Society of Tropical Medicine and Hygiene (now The Royal Society of Tropical Medicine and Hygiene).

[4] In doing so, James Cantlie was inclined to conclude that "*the bacillus of the benign variety attains malignancy by passing through some intermediate host, possibly, but not probably, the rat*".

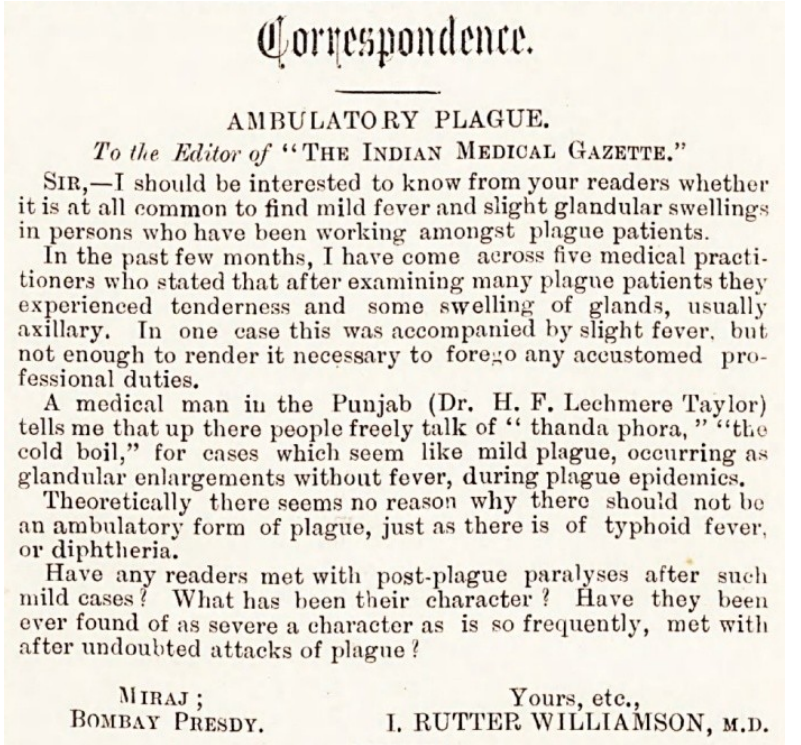
An important problem remained that, in the presence of plague-like symptoms (albeit less pronounced than in what was then called "true plague", plague major), no researcher could confirm the diagnosis bacteriologically, that is, isolate a pure culture of the plague microbe. Therefore, two months later James Lowson[1] strongly criticised James Cantlie's ideas. He argues, based on his own clinical experience, that all stories of pestis minor (buboes and fevers) are nothing more than symptoms of venereal disease in adults and epidemic mumps in children. Nevertheless, he does not deny the existence of "ambulatory plague", which he believes is realised by ingestion of a small amount of toxin but without bacterial contamination, "*as the products of the bacilli may hang about a neighbourhood long enough after all bacilli are dead*"[2].

Speaking of James Lowson's position, we cannot ignore the fact that he had a personal enmity with James Cantlie since the epidemic in the city of Hong Kong (1894), and it is likely that it was the main reason for his rejection of the latter's ideas. As we remember, it was during that outbreak that Kitasato Shibasaburō and Alexandre Yersin discovered *Y. pestis*. In doing so, Alexandre Yersin suggested that the plague microbe could persist in an avirulent form in the ground, regaining its virulence after some time and causing new outbreaks (in fact, this was his explanation for the inter-epizootic periods discussed in detail in Chapter 4). This idea had few supporters, for just five years earlier, together with Émile Roux[3] (of the Pasteur school of science), he had defended the view that the ubiquitous pseudo-diphtheria bacillus (*Corynebacterium pseudodiphtheriticum*) was a weakened form of diphtheria bacillus (*Corynebacterium diphtheriae*), although Robert Koch had argued that they were different species of bacteria. Believing that Alexandre Yersin was wrong again, James Lowson criticised his idea and, as an adherent of the Koch, defended the priority of Kitasato Shibasaburō (as we remember also a representative of this school of science) in the discovery of the causative agent of plague. Although Alexandry Yersin's supporters were then in the minority, the priority was assigned to him (as we

[1] ?-?; the British physician who worked in British Hong Kong.

[2] This view could be due to the existence of so-called VBNC (viable but non-culturable) bacteria, which are not capable of fission, but under certain conditions are capable of changing to a cultivated state (more on this in Chapter 30).

[3] 1853–1933; the French physician, bacteriologist, member of the French Academy of Sciences (1899). He studied diphtheria and other infectious diseases.



Correspondence.

AMBULATORY PLAGUE.

To the Editor of "THE INDIAN MEDICAL GAZETTE."

SIR,—I should be interested to know from your readers whether it is at all common to find mild fever and slight glandular swellings in persons who have been working amongst plague patients.

In the past few months, I have come across five medical practitioners who stated that after examining many plague patients they experienced tenderness and some swelling of glands, usually axillary. In one case this was accompanied by slight fever, but not enough to render it necessary to forego any accustomed professional duties.

A medical man in the Punjab (Dr. H. F. Lechmere Taylor) tells me that up there people freely talk of "thanda phora," "the cold boil," for cases which seem like mild plague, occurring as glandular enlargements without fever, during plague epidemics.

Theoretically there seems no reason why there should not be an ambulatory form of plague, just as there is of typhoid fever, or diphtheria.

Have any readers met with post-plague paralyses after such mild cases? What has been their character? Have they been ever found of as severe a character as is so frequently, met with after undoubted attacks of plague?

MIRAJ;
BOMBAY PRESDY.

Yours, etc.,
I. RUTTER WILLIAMSON, M.D.

Letter by physician John Rutter-Williamson, published in The Indian Medical Gazette

remember, as a result of slander!), and James Cantlie played an important role in this. It may well be that the lecture and debate around pestis minor was simply a convenient excuse to revisit an old feud.

In any case, the possibility of the existence of "pestis minor" ("minor plague"), "ambulatory plague" and "abortive plague" was widely discussed by specialists. As early as October 1903, a letter from John Rutter-Williamson[1] appeared in The Indian Medical Gazette, in which he inquired about the possibility of the existence of "ambulatory plague" in persons working with the sick, saying that this form best explains the presence of thanda phora (the "cold boil"), that is, a slight

[1] ?-?; the British physician.

enlargement of the lymph nodes against a background of moderate fever[1].

Eventually, after examining all available opinions and arguments, which still lacked reliable bacteriological confirmation, the Indian Plague Commission (1907) gave the following clinical definition: "*The patient suffers from headache and sleeplessness, from slight pyrexia, lasting only a few hours, from tenderness and pain over one or more superficial glands [i.e., in the lymph node], usually those in the groin, which are frequently enlarged, and occasionally from nausea and vomiting. The duration may be as brief as only two or three days, but not infrequently the enlargement of the glands is more persistent, and the duration on this account, or because suppuration has taken place in the glands, is protracted to 10, 20 or even 30 days. All cases terminate in recovery*".

Pestis minor and "ambulatory plague" as synonyms of each other continued to exist, being recognised by some specialists (some considered them different forms) and not recognised by others, but in one way or another the diagnosis was made purely clinically, without reliable bacteriological confirmation, except perhaps in one known case.

On 26 February 1904[2], in the city of Manila, Philippines, a 17-year-old boy, an employee of the city's soda factory, suffering from no illness except that for the past two weeks he has had a pale complexion and trouble sleeping, spends the evening outdoors with his friends, as he always does. He played until 11 p.m., after which he went to bed. Around midnight he woke up with severe chest pain and difficulty breathing. The doctor who arrived could do nothing and the young man died 2 hours later. External examination was unremarkable except for a slight enlargement of the inguinal lymph nodes with slight periadenitis of the femoral triangle and chronic skin infection of both legs. A post-mortem examination revealed characteristic changes in the liver, spleen and lungs with foci of inflammation, but the cause of death was pulmonary embolism. Swabs taken from the liver, spleen and lymph nodes showed "bacilli... having a typical appearance, showing bipolar staining and

[1] Interestingly, he cites typhoid and diphtheria as his argument.

[2] Herzog M., Hare C. B. *Latent and ambulatory plague*. JAMA. 1904; XLIII (24):1781–1788; DOI: 10.1001/jama.1904.92500240002k.

rounded end" (as we remind, this is the classic appearance of the plague microbe, see ***Figure 13.8****).*

As we can see, all three terms essentially refer to the mild form of "real plague" (buboes, lung lesions), but was rarely confirmed bacteriologically. Two reasons may explain this. Firstly, and we will discuss this further, not everything that looks like plague is plague. Recall that the painless buboes that are not fused to the skin are characteristic of tularemia, not plague. And in this connection, we cannot ignore the story of Sergei Petrovich Botkin[1], a famous physician, who in 1879 diagnosed plague in the capital's janitor Naum Prokofiev. The dreadful diagnosis provoked panic in the whole of St. Petersburg (Russia) and the residents fled. However, the janitor recovered quickly enough (most likely he had tularemia), which doomed Sergei Botkin to ridicule and accusations of unprofessionalism. And although he remained convinced of the correctness of his diagnosis, who knows, maybe this event was the starting point that led 10 years later to myocardial infarction, from which he died. This is not to say that difficulties in differential diagnosis were exclusively a problem at the time. In Chapters 18 and 20 we have seen contemporary cases of a woman with primary septicemic plague and a male soldier with plague meningitis, both of which took quite a long time to be correctly diagnosed. And both of these cases naturally lead us to the second reason, the difficulty of culture isolation. Of course, in the modern cases cited earlier, the difficulties in culture isolation are due to antibiotic therapy.

However, at the end of the XIX century, the level of bacteriological methods was far from modern methods, because medical science of that time had only just begun to abandon "miasmas" in favour of the existence of "contagions" (then only bacteria, since viruses had not been discovered[2] and fungi were considered plants[1]). What to say, if in text-

[1] Сергей Петрович Боткин, 1832–1889; the Russian physician, therapist, physiologist, health care organiser, author of numerous works on infectious diseases. One of the founders of modern Russian medicine. Chairman of the Society of Russian Physicians (Общество русских врачей; 1878-1889). In Alexei N. Nekrasov's poem «Кому на Руси жить хорошо» (Who Can Be Happy and Free in Russia?), Sergey P. Botkin is dedicated the chapter «Пир на весь мир» (A Feast for the Whole Village) from the second part.

[2] In 1892, the Russian physician Dmitri Iosifovich Ivanovsky (1864-1920), researching tobacco diseases, hypothesised that tobacco mosaic disease was caused by tiny bacteria or a toxin released by them. The point is that the extract of grinded infected tobacco leaves,

books on bacteriology even at the beginning of the XX century we read[2] that the plague microbe is not coloured by Gram! Nevertheless, these forms have survived to the present day and are present in ICD-11 as All Index Terms.

22 DIAGNOSIS

Clinical diagnosis of plague is difficult, especially in the interepidemic period (sporadic cases), because of the similarity of clinical manifestations with a large number of diseases. And if the accuracy of diagnosis without laboratory tests in the bubonic form is difficult, although possible, in the primary pneumonic form it is doubtful, and in the primary septicemic form it is impossible. Recall how, in the cases previously described, plague buboes were mistaken for lymphogranuloma venereum (Chapter 20), primary septicemic form for thrombotic thrombocytopenic purpura (Chapter 18), and primary pneumonic form for lobar pneumonia (Chapter 15) and severe community-acquired pneumonia (Chapter 19). In all cases (with the exception of community-acquired pneumonia), the correct diagnosis was made exclusively bacteriologically, i.e. by isolation and identification of a pure culture of the pathogen.

We said in Chapter 17 that plague can be suspected early in the course of the disease on the basis of epidemiological history alone. In-

when passed through a Chamberland filter (the pores of the filter trap bacteria), retained infectious properties, but did not grow on the cup. Under the microscope, such an extract contained crystal-like bodies. Only in 1939, when the first electron micrographs appeared, this pathogen was identified as tobacco mosaic virus, and the crystal-like bodies were later called "Ivanovsky's crystals". Thus, 1892 is considered to be the beginning of virology.

[1] Until recently, non-vascular plants (e.g. fungi and algae) were considered "lower plants", which is why until today you can still find Departments of lower plants and courses on them in some universities.

[2] See, e.g., Gabrichevsky G. Medical Bacteriology, ed. 4th, posthumous, Moscow: Tipo-lithography of I. N. Kushnerev & Co, 1909, 560 p. (in Russian)

deed, when a flea bites a person in an endemic area, it is obvious to suspect plague. However, it is possible to be infected by other ectoparasites, as well as rodents and mammals. Let's imagine such a situation: a person was bitten by a rat. At the site of the bite there is an ulcer (primary affect), inflammation of regional lymph nodes (lymphadenitis), temperature rises to 40°C / 104°F, there are severe muscle pains and mental agitation. What is it? Is it similar to the case in Chapter 21 when a man was bitten in the head by a rat? Remind: postmortem bacteriological examination showed plague. However, the current case is of sodoku, a disease caused by the Gram-negative bacterium *Spirillum minus* (also known as *Spirillum minor*). To be fair, the incubation period for sodoku averages 10-14 days, although it can range from 3 days to 2 months. A similar incubation period and symptoms are characteristic of felinosis (cat-scratch disease) caused by the Gram-negative bacteria *Bartonella henselae*. As the name suggests, the disease is caused by a scratch, less commonly a bite or close contact with a cat. The difficulty of epidemiological anamnesis in this case is that cats often enough sleep in the bed of the owner and it will be difficult for the patient to determine in the morning whether it is a scratch, a cat bite or a flea bite (from a cat). And in the case of a child it will be difficult to establish where and under what circumstances he got this or that scratch (and who of the parents will pay attention to it). As can be seen from these three examples, it is very difficult to differentiate plague even in the presence of an epidemiological history.

At the same time, when the physician has only *suspicions* of plague, it is *recommended to start antibiotic therapy* without waiting for the results of bacteriological examination, because it can save lives (levofloxacin and moxifloxacin are particularly good drugs in this case). And if Simon Z. Gorelik had lived in the era of antibiotics, he and Abram L. Berlin would most likely be alive (but let us not forget the case of hereditary haemochromatosis from Chapter 14). It seems that this alone obliges us to talk about differential diagnosis in order to understand how difficult it was for doctors without bacteriological studies to diagnose plague and why so many epidemics caused by other microorganisms were attributed to it. After all, Galen[1] argued that if a disease in one place

[1] Γαληνός, 129–216; the Ancient Roman physician and philosopher of Greek origin. After the conquest of the Western part of the Roman Empire, his works were destroyed by

affects many people and many die from it, it is plague. Let us consider with concrete examples that this is far from being true

Cutaneous (Cellulocutaneous) form. Georgiy P. Rudnev writes[1] that the cutaneous form is "*less often seen and usually less well known*". As we remember, it is characterised by the presence of a primary affect. Accordingly, it is differentiated from cutaneous anthrax and tularemia.

Primary affect is possible in some sexually transmitted diseases. In **lymphogranuloma venereum** caused by serovars L1, L2, L2a, L2b, or L3 of *Chlamydia trachomatis* (this is the disease originally diagnosed in the male soldier in Chapter 20), the primary affect is a small, rapidly regressing, painless erosion (i.e., a superficial epithelial defect) that is very often not noticed by the patient. In **chancroid** caused by the Gram-negative bacterium *Haemophilus ducreyi*, the primary affect is a red spot, with a vesicle in the centre, which develops into a pustule and then into a painful purulent ulcer that scars after a few weeks.

In **suppurative lymphadenitis** of staphylococcal and streptococcal genesis, there is no primary affect, but there is local oedema and local inflammation, which is somewhat less than in plague (also the temperature is not so high and the general condition is somewhat milder).

Plague rash is extremely rare, but it should be distinguished from the rash characteristic of **typhus** caused by agents of the typhus group (*Rickettsia prowazekii*, *Rickettsia typhi* and *Rickettsia felis*) and rash characteristic of other **rickettsioses** (**Figure 22.1**), as the vectors of these diseases (ticks and lice) can rarely act as vectors of plague.

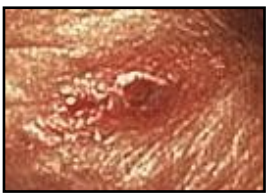
Bubonic form. The bubonic form of plague is differentiated primarily from the bubonic form of tularemia.

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the invaders and thus remained inaccessible to the new Western civilisation (while still relevant in the Eastern part of the Roman Empire and translated into Arabic for the Syrians and Arabs), which only became acquainted with them in the form of translations from Arabic in the 11th century. Nevertheless, his works on anatomy remained relevant until 1543, his theory of the circulation lasted until 1628, and his theory of higher nervous activity exists to the present day. Galen's works were studied in medical universities until the 20th century.

[1] Руднев Г. П. *Клиника чумы, под ред. д-ра Ф. Р. Чушинского,* Ростовский государственный противочумный институт и кафедра инфекционных болезней Ростовского медицинского института, Ростов-на-Дону: Ростиздат, Тип. им. Коминтерна, 1938, 268 с.

Differentiation of cutaneous form

Primary affect		
anthrax	**plague**	**tularemia**
characterised by serous and serous haemorrhagic exudate		
	with pus or pus-like fluid	with pus
a cone-shaped protrusion at the top		
available	not available	not available
new vesicles form after the necrosis		
frequently	non frequently	non frequently
accompanied by peripheral oedema		
significant, can spread to the entire face or arm	insignificant	medium
adjacent tissues		
slightly swollen	dense	dense
regional lymph nodes		
slightly inflamed	may be severely severely inflamed or may not be inflamed	may be severely inflamed or may not be inflamed
		

The buboesis in **suppurative lymphadenitis** of staphylococcal and streptococcal genesis is generally similar to that of tularemia, but, unlike the latter, periadenitis is possible. An important differential diagnostic feature of tularemia and purulent lymphadenitis is the presence of prmary lymphangitis, which does not occur in plague. As Vladimir N.

Differentiation of bubonic form

plague bubo	Tularemia bubo
formed by regional lymph nodes secondary buboes may occur cutaneous fibre involvement	
significant (periadenitis) ↓ bubo is fused to the skin (indistinct boundaries)	none or negligible ↓ the bubo is not fused to the skin (clear boundaries)
serous haemorrhagic impregnation and haemorrhages	
sharply expressed ↓ pathological and anatomical picture of necrosis foci in the form of fields with areas of serous haemorrhagic impregnation	non sharply expressed ↓ pathological picture of clear foci of necrosis («dry»)
acutely painful	painless or mildly painful
	

Lobanov[1] writes, "*in the acute period of the disease, they are visible on external examination as red streaks running from the place of penetration of infectious material towards the regional lymph node*".

[1] Лобанов В. Н. *Патологическая анатомия и патогенез чумы у человека*, М.: Гос. изд. мед. лит. «Медгиз», 1956, 176 с.

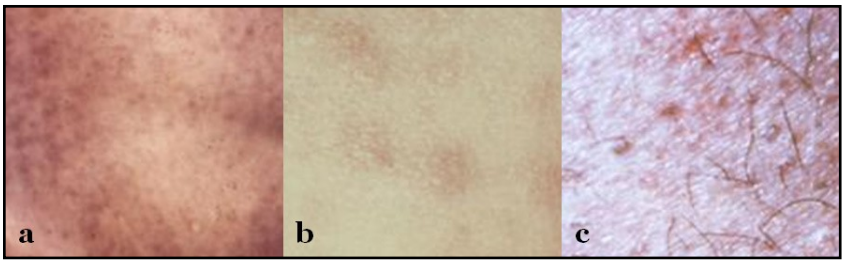


***Figure* 22.1.** *Rash in various rickettsioses: a - typhus (Rickettsia prowazekii), b - Rocky Mountain spotted fever (Rickettsia rickettsia), c - rickettsialpox (Rickettsia akari)*

Some venereal diseases also cause buboes. In **lymphogranuloma venereum**, the inguinal lymph nodes may enlarge, fuse and adhere to the surrounding tissues to form a bubo, the skin over which is usually red (**Figure 22.2**). The buboes may then soften to form a fistula, through which masses of yellow-green pus are discharged (**Figure 22.3**), followed by scarring. It can be seen that this description is very similar to that of the plague buboesis, so it is not surprising that in the case of the male soldier (the case in Chapter 20) it was suspected. The main differential diagnostic features are the duration of the buboes (formed on days 10-14; in plague, on days 2-3) and the lesser severity of the intoxication symptom (occurring after the buboes have "burst"), but when a patient comes in with buboes, it is very difficult to determine on what day they appeared.



***Figure* 22.2.** *Right inguinal bubo in a male with lymphogranuloma venereum. Note how much it looks like the plague bubo (1966)*

In **chancroid**, there may be enlargement of inguinal lymph nodes with slight periadenitis (**Figure 22.4**), which visually resembles a small plague bubo with poorly defined borders (**Figure 17.7**).

In **granuloma inguinale** caused by the Gram-negative bacteria *Klebsiella granulomatis*, small, painless nodules (granulomas) usually form, which then burst and scar. In most cases, granulomas are localised on the penile shaft in male and the labia in female. In rare cases, however, the inguinal lymph nodes may become inflamed with subsequent "bursting" and pus discharge (**Figure 22.5**).



***Figure* 22.3.** *Large fistula on the left inguinal bubo with pus flowing out of it in a male with suppurative lymphadenitis (1968)*

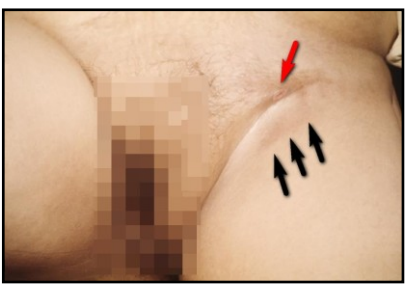


***Figure* 22.4.** *Left inguinal bubo in a female (lower borders marked with black arrows) with chancroid. Note the "breakthrough" site marked with a red arrow (1976)*



***Figure* 22.5.** *"Ruptures" formed at the sites of the right and left inguinal buboes, with pus flowing from them in a male with granuloma inguinale (1979)*

Differentiation of pneumonic form

Anthrax	Plague	Lobar pneumonia
catarrhal symptoms (i.e. cold symptoms)		
present	absent	possible
auscultatory pattern		
moist wheezing, possible pleural friction noise	scanty (does not correlate with the severity of the disease)	moist wheezing, bronchial breathing (coarse, audible on inhalation and exhalation; similar to the sound of pronouncing the letter "kh" with open mouth)
pain syndrome		
insignificant	absent	pronounced
sputum		
mucous with a little bit of blood	liquid or vitreous, may include an admixture of blood	muco-purulent, «rusty», may have a little altered blood
lesions		
scattered large foci ↓ large foci of necrosis on post-mortem examination	scattered small foci ↓ smooth structure (small foci of necrosis) on post-mortem examination	a whole lobe (a substantial part thereof), several or all lobes ↓ granularity on a post-mortem examination
pleural changes		
pleurisy with haemorrhages in the pleural sheets is possible, but without transition from fibrinous to purulent pleurisy	serous pleurisy or serous haemorrhagic pleurisy with a large amount of fluid in the pleural cavity (up to 2 litres)	pleurisy on the side of the affected lung, possibly changing from fibrinous to purulent pleurisy
the infection may spread to the		
trachea and bronchi	trachea and bronchi	bronchi

Pneumonic form. Pneumonic plague is differentiated primarily from anthrax pneumonia and lobar pneumonia.

In **typhus** complicated by pneumonia, lung lesions are extremely similar to those of pneumonic plague, except that plague haemorrhages are larger and more numerous at post-mortem examination. In addition, pleurisy in typhus may be purulent. Changes in the trachea and bronchi are also similar, but fibrinous films on the mucous membranes are rare in typhus. In the case of **influenza** with pneumonia, we should also speak only of a difference in the changes in the trachea and bronchi, expressed by swelling of the mucous membrane and multiple haemorrhages, causing it to have a deep red colour. However, if the pneumonia is fibrinous (lobar) or interstitial (i.e., the inflammatory process occurs in the stroma (interstitial tissue) of the lung), plague should be excluded, as it is not characterised by such a course.

As can be seen from the above, the symptomatic manifestations of plague are similar to those of other diseases, and even the presence of an epidemiological history does not always simplify the clinician's task. In this regard, the World Health Organization has developed[1] the *plague case definition* (latest revision)

Suspected case

clinical presentation suggestive of plague:

- sudden onset of fever, chills, headache, severe malaise, prostration, painful swelling of lymph nodes;

or

- cough with blood-stained sputum, chest pain, difficulty in breathing.

AND

epidemiological context suggesting possible exposure to plague:

- exposure to infected humans or animals;

  ☙ *"exposure" is defined as being in a closed space (e.g. room, vehicle, barrack, jail) with the suspected pneumonic plague patient during the two days prior to the development of symptoms in the suspected pneumonic plague patient until two*

[1] Weekly Epidemiological Record (World Health Organization). 2021; 96(23):238–240.

days after the suspected pneumonic plague patient patient has started appropriate antimicrobial treatment[1].

or

- residence in or travel to a known endemic focus within 10 days prior to onset of the disease.

Probable case

A suspected case;

AND

ONE of the following:

- F1 antigen (i.e. capsule antigen) positive in bubo aspirate, sputum, blood, or post-mortem tissues by rapid diagnostic test based on F1 antigen (F1RDT) or direct fluorescence assay (DFA)[2];
- single anti-F1 serology positive without evidence of previous *Y. pestis* infection or vaccination;
- direct microscopy in a clinical sample, positive for Gram-negative coccobacilli that display bipolar staining with Wayson or Giemsa stain[3].

Confirmed case

A suspected case;

AND

At last ONE of the following:

- isolation of *Y. pestis* from a clinical sample - must have appropriate colony morphology and be identified as *Y. pestis* based by at least two of the following:
  - phage lysis at **20–25°C** / 68-77°F;
  - biochemical profile;
  - F1 antigen detection;

[1] *Interregional meeting on prevention and control of plague*, Antananarivo, Madagascar 1–11 April 2006 (Geneva: World Health Organization; 2006).

[2] We will talk more about laboratory methods in Act III.

[3] Bipolar staining can also be seen with Wright's stain (**Figure 13.8**).

- Seroconversion (i.e. antibodies in the blood) or a 4-fold difference in anti-F1 antibody titer in paired serum samples drawn at least 2 weeks apart;
- *Y. pestis* DNA positive by species-specific PCR on either clinical sample or culture according to standard practice.

23 TREATMENT (RIMS FEVER DISEASE)

Since ancient times, mankind has tried to alleviate the burden of various diseases. We have evidence of the existence of health care systems already in the first civilisations, the most famous of which is probably the ancient Egyptian. Then, over time, medical science developed and improved, and by now we formally have two independently developed systems: The main ("Western") and an additional one, which is still called traditional medicine (non-conventional medicine)[1], because it is based on empirical knowledge accumulated by folk tradition, and is inextricably linked to the philosophical-religious worldview, while the "Western", although it started its way in the walls of monasteries, quickly enough became independent, putting in its basis scientific evidence base. It should be noted that traditional medicine is now most often understood as traditional medical systems developed in Central and South-East Asia (Eastern medicine), probably because of the strong interest of researchers, and also because they are gradually building up an evidence base for their methods, abandoning those whose effectiveness is unconfirmed. The joint use of effective methods of main medicine and traditional medicine is commonly referred to as *integrative medicine*, without which a bright future of world medicine is not possible, which is in line with the strategy of the World Health Organization[2] that

[1] A distinction should be made between the terms "folk medicine" and "traditional medicine". Thus, since the second half of the last century, the former has been understood as an oral medical tradition and the latter as a written one. In some countries, traditional medicine may be termed complementary medicine.

[2] WHO traditional medicine strategy: 2014–2023.

traditional medicine is an important and often underestimated part of the health care.

As we know, bacteria were discovered only at the end of the 18th century, the causative agent of plague at the end of the 19th century, and an effective treatment (antibiotics) appeared only in the middle of the 20th century, marking the so-called era of antibiotics, in which we were lucky to live. However, as we remember, plague has existed since ancient times and people have lived in the territory of plague foci all this time and have developed their own rules of coexistence with this infection. Therefore, it seems right to consider the traditional medicine of the peoples who lived on the territory of ancient plague foci, where the plague appeared, and we will start this small journey into the world of traditional medicine from the land of snow, long closed from prying eyes – Tibet.

Traditional Tibetan medicine (Sowa Rigpa) was formed under the influence of Buddhism, therefore, in addition to the usual diagnostics and treatment, includes Buddhist philosophy, which is expressed in the idea that the cause of all diseases are three poisons – delusion (ignorance), greed (attachment) and aversion (hatred)[3], which, in turn, disturb the balance of the three principles (doshas):

བད་ཀན། (Bad-kan) – a cold ("bile")

མཁྲིས་པ། (mKhris-pa) – a fever ("slime")

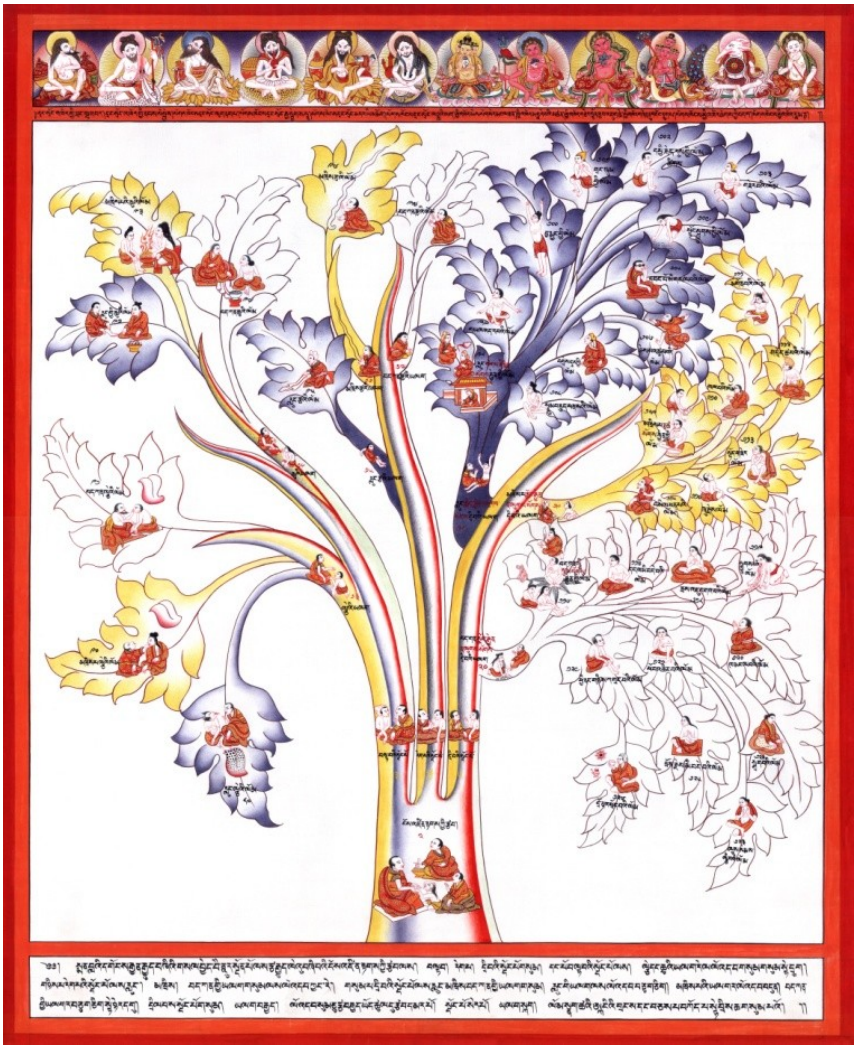
རླུང་། (rLung) – a wind ("energy")

Restoring the balance of doshas (health) is done through treatment. Since medical knowledge is believed to have been imparted by the Bhaiṣajyaguru (Medicine Buddha), the work of the emchi (physician) is based on compassion and a sincere desire to help the patient. Diagnosis

[3] States (defects) inherent from birth in all living beings, which are the root cause of suffering and rebirth of all living things. They are depicted as three animals (pig, bird and snake) biting each other's tails, which symbolises their mutual causality. Thus, we wish to receive only what we like (bird – greed), we do not want to receive what we do not like (snake – disgust), and finally, we believe that we can receive only what we like and not receive what we do not like (pig – delusion). In contrast, in order to get rid of the three poisons, it is necessary to avoid them, and to develop the three good roots of wisdom, generosity, and compassion in ourselves.



Thangka Tree of Medicine, figuratively depicting the structure of Tibetan medicine. Atlas of Tibetan Medicine (No. 2). Courtesy of The Dharmapala Thangka Centre Kathmandu (Nepal). It represents two trunks symbolising health and disease. The left trunk (health) has three branches showing the workings of a healthy human body. The branches show the three doshas (cold, fever and wind), the seven powers of the body (food juice, blood, meat, fat, bones, marrow and seed) and the three impurities (faeces, urine and sweat), the normal functioning of which leads to health and longevity (two flowers above), as well as the ripening of the three fruits (success in karma, wealth and happiness). The right trunk (diseases), which contains at its core the scene of a patient being seen by a doctor, includes nine branches - the cause of diseases, conditions of their development, pathways of penetration, sites of affection, ways of movement in the body, outcome, change over time, and a "summary" in which all diseases are reduced to two types of fever and cold



Tangka Diagnostic Tree, figuratively depicting the main diagnostic methods. Atlas of Tibetan Medicine (No. 3). Courtesy of The Dharmapala Thangka Centre Kathmandu (Nepal). There are three trunks symbolising visual inspection, pulse examination and conversation (history taking). The left trunk (examination) is divided into two branches – tongue examination and urine examination. The branches show the appearance of the tongue and urine in doshas disorder. The middle trunk (examination of the pulse) is divided into three branches, which show the types of pulse in doshas disorders. The right trunk (conversation) is divided into three branches, symbolising the doshas, which show the symptoms that occur when one of the doshas is disturbed

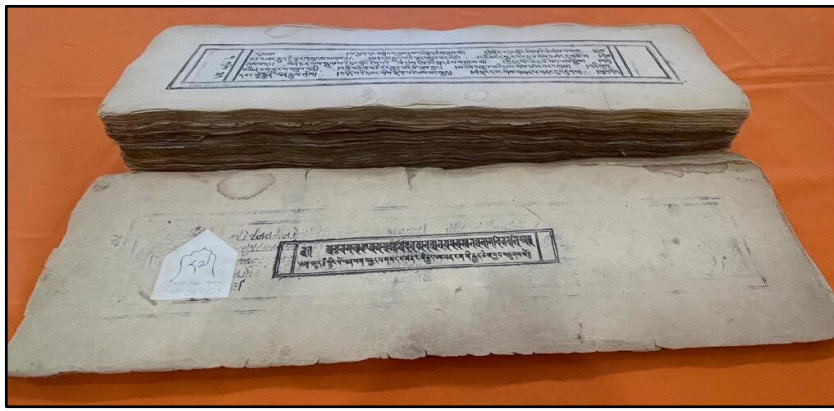
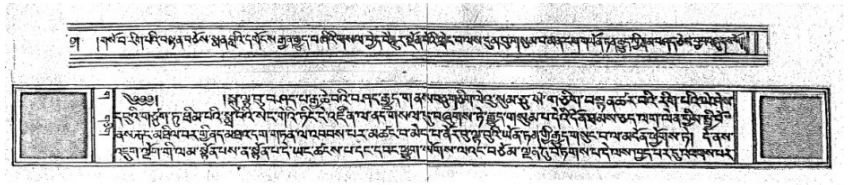


Figure 23.1. *Treatise rGyud-bZhi.*
Courtesy of the Tibetan Medical and Astro.
Institute of H.H. the Dalai Lama (Men-Tsee-Khang)

includes analysis of symptoms, pulse, physical characteristics of urine (colour, odour, appearance of bubbles when shaken), and conversation with the patient (anamnesis), on the basis of which treatment is prescribed, including, on the one hand, lifestyle changes (mental practices and diet), and on the other hand, the administration of herbal and mineral preparations (in the form of powders) and, in severe cases, cauterisation of acupuncture points.

Plague (Tib. བྱི་རིམས – jhi rim) refers to the diseases རིམས (rim), that is, those diseases of fever (the predominant beginning "fever") that are transmitted from person to person. In the rGyud-bZhi (Tib. རྒྱུད་བཞི – "The Four Tantras"; 5th century), the basis of Tibetan medicine (**Figure 23. 1**), the disease is named གཉན་རིམས (nyen rim), indicating the second name གཉན་ཆི་ཐུག་ནག་པོ (nyen chi thuknagpo, lit. "black poison-nyen that came from outside"). In Chapter 23 of the Instructional Tantra of the rGyud-bZhi, it is said that when people commit unwholesome acts due to greed, harm each other, and curse, «*these acts cause a disturbance in celestial beings such as ma mo and mkha'i, who exhale disease*".

In the treatise Vaidurya sngon po (Tib. བཻདཱུརྱ་སྔོན་པོ – «Blue beryl»; 17th



▲ ***Figure* 23.2.** *The title page of the treatise Vaidurya sngon po. Courtesy of the ФГБУН «Институт монголоведения, буддологии и тибетологии Сибирского отделения Российской Академии Наук»*

▼ *Fragment of a thangka illustrating the "rims fever". Atlas of Tibetan Medicine (No.42). Courtesy of The Dharmapala Thangka Centre Kathmandu (Nepal)*



century; **Figure 23.2**)[1], it is additionally reported that the "spirit of diseases" in the form of clouds containing srin (small disease-causing agents, "worms") descends on cities. There they, in the form of "*the odour of disease, dirt and dust*", infect humans either through the nose or through contaminated foodstuffs. Thus we see a description of two

[1] Written in 1687-1688 by the Desi (regent) of Tibet, Sangye Gyatso (1653-1705), it is the first known commentary on the rGyud-bZhi treatise, summarising the experience of various medical schools. The treatise follows the structure of the rGyud-bZhi, but is considerably longer. It elaborates on the meaning of many terms and concepts, contains numerous additions on issues of general and private pathology and pharmacology, and presents the opinions of the authors of various treatises, including those written before the rGyud-bZhi was created. Vaidurya sngon po is the most authoritative medical treatise among the peoples of Transbaikalia who profess Buddhism (known as "Vaidurya Onbo"). Its special feature is the existing illustrative appendix of 77 thangkas (paintings) known as the Atlas of Tibetan Medicine.

transmition – airborne and alimentary. At the same time, contaminated food probably means its improper preparation. Further, from the sick person in the form of "*vapours of disease*" they can be transmitted to another person. Interestingly, srins are described as being very similar to bacteria: in shape they resemble thin rounded copper needles, which are "*undetectable to the simple eye*", spreading "*through the blood instantly from head to toe*".

The symptoms of the disease in the initial stage, according to rGyud-bZhi, are chills, poor concentration, headache, joint pain and confusion. The urine is yellow and cloudy, and the pulse is empty and frequent. The disease spreads through the skin, making its way to the lungs and head. If it spreads through the kidneys, there is urinary retention, kidney and lower back pain. Then, when the disease has spread, the urine becomes mustard oil coloured, the sclerae, tongue, temples and skin become yellow, there is bitterness in the mouth, high body fever, insomnia, loss of appetite. The nails, gums, tongue and lips become whitish-yellow in colour. Pain in the head becomes stabbing, cracks appear on the tongue and lips, plaque, a twisting sensation and soreness on the surface of the liver and gallbladder. A rash may appear on the skin. From the body of the patient goes an unpleasant odour, it is exhausted and without "glow". If the disease has got into the lungs, there is redness of the eyes and red colour of the urine, cough with filthy sputum. As "*there is not enough time for the disorder to mature*", the patient dies within seven or nine days.

The symptom complex described is somewhat similar to the plague. Indeed, the disease most often penetrates through the skin (cutaneous form), travelling to the lungs (pneumonic form) and head, although the latter is understood as psychomotor agitation ("pallor, dizziness, tinnitus") rather than meningeal form. The description of the consequences of the intoxication syndrome (dry lips, "chalky" tongue) and such rare symptoms of the septicemic form as rash and anuria deserve special attention.

However, there is no mention of buboesis, nor of necrosis of the limbs, which occurs in septicemic form. However, it should be noted that the description of pallor of the nails may indirectly refer to pallor of the distal extremities due to thrombosis of small vessels, which is a pre-

cursor to necrosis. In addition, the addition of གཉན་ (nyen, lit. "black poison", i.e. necrotic ulcer) in the name of the disease may be an indirect reference to buboes and the disease nyen is a special form of rims fever diseases. Anyway, already in the treatise Man ngag lhan thabs (Tib. མན་ངག་ལྷན་ཐབས – "Supplement to the Instructional Tantra"; 17th century)[1], about the disease nyen it is specified that from "*meat, bones and vessels outwardly looks [something resembling] a vase*", which in fact is a description of buboes. In addition, we find that the condition of infection of the patient is living in a certain area, season of the year and way of life, which is understood as "careless cooking", which points to the epidemiology of the infection process and, in particular, to the understanding of natural focality, to which Western medicine would come only in the XX century (remind: the founder of the theory of natural focality by Evgeny N. Pavlovsky).

An elaboration of the idea that the disease could be transmitted from person to person is the indication that because the disease is highly contagious, emchi should be careful and make a protective amulet for themselves (**Figure 23.3**). Although the text does not specifically mention it, the general rule was that anyone who contracted rims fever disease was to be isolated.

In Lhan thabs mchan rgyab dang bcas (ལྷན་ཐབས་མཆན་རྒྱབ་དང་བཅས; late 18th century)[2], which is an expanded version of Man ngag lhan thabs, we read that the disease is transmitted by a special kind of srin called par-pa-ta, which, among other things, interferes with the determination of the name of the disease, its clinical forms, and "routes of introduction".

However, preparations have been suggested for the treatment of each form of the disease. Let us consider the preparations described in

[1] Written between 1690 and 1961 by Desi Sangye Gyatso; it is a practical guide to clinic and therapy, supplementing and expanding the Instructional Tantra of the rGyud-bZhi so that it is clear even "*to individuals of low ability*". In modern Tibetology, the treatise is generally regarded as experimental, although it was the most recent work of the author, who systematised the most progressive Tibetan knowledge of that era.

[2] Written in 1789 in Amdo. It is a treatise Man ngag lhan thabs with additions written by emchi Choije Lobsang Wangyel, being a practical guide to the treatment of diseases. After travelling from Amdo to Aginsky datsan (Buryatia), it became the most published and popular among Buryat emchis.

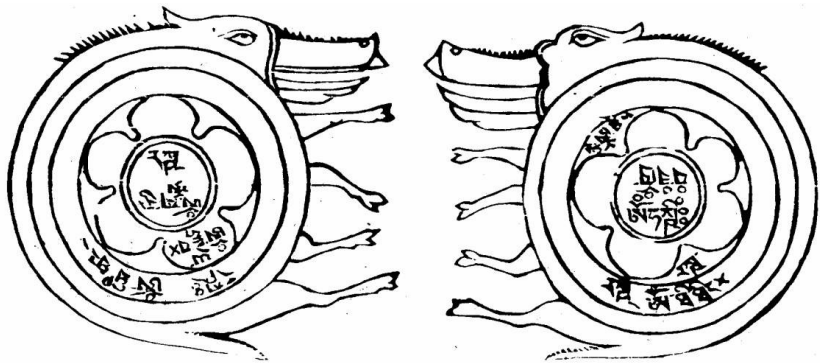


Figure 23.3. *Protective amulet from the Man ngag lhan thabs. Courtesy of the ФГБУН «Институт монголоведения, буддологии и тибетологии Сибирского отделения Российской Академии Наук»*

Chapter 23 of Instructional Tantra of the rGyud-bZhi. In the initial stage, it is suggested to give the patient the preparation མ་ནུ་བཞི་ཐང (ma nu bzhi thang) or the decoction སྨྲེ་ཏྲེས་ལྔ་ཐང (sle tres lnga thang). The former includes[1]:

- ma nu – Inula (*Inula racemosa*),
- kan ta ka ri – *Sambucus sibirica*,
- sle tres – Guduchi (*Tinospora cordifolia*),
- sga-skya – Perfume ginger (*Hedychium spicatum*).

In the second:

- sle tres – Guduchi (*Tinospora cordifolia*),
- a ru ra – Chebulic myrobalan (*Terminalia chebula*),
- bar u ra – Bacheda (*Terminalia bellirica*),
- skyu ru ra – Emblic (*Phyllanthus emblica*),
- rya tig – Felworts (*Swertia chirata*).

In the "*middle stage, when the disease has spread*", it is suggested that the patient be given a decoction including: ཏིག་ཏ (tig ta), གསེར་མེ་ཧོག (dser

[1] Recipes of preparations are given according to: Kosoburov A. A. Men-tsee-khang Recipe Book. Selected Recipes of Tibetan Medicine, Ulan-Ude: The Publishing House of the Buryat Scientific Centre of Siberian Department of the Russian Academy of Sciences, 2005, 96 p. (in Russian).

me tog (Gac, *Momordica cochinchinensis*)), དེ་བ (de ba (Large leaf gentian, *Gentiana macrophylla*)), བར་པ་ཏ (par pa ta (*Hypecoum erectum*)), ལི་ག་དུར (li ga dur (Paashaanbhed, *Bergenia ligulata*)) and ཨ་རུ་ར (a ru ra (Chebulic myrobalan)). If the disease is severely neglected, ག་བུར (ga bur (Camphor tree, *Cinnamomum camphora*)), གི་ཝཾ (gi wam (bezoar)), ཙུ་གང (cu gang (Bamboo manna)), གུར་གུམ (gur kum (Saffron crocus, *Crocus sativus*)), བོང་ང་དཀར་ཟི (bong nga dkar (Atish, *Aconitum heterophyllum*)), ཏིག་ཏ (tig ta), mixed with ཀ་ར (ka ra (sugar)) should be added.

Special attention should be paid to the composition applied "*if the rime has got into the lungs*", and includes: ཙན་དན (tsan dan, (Indian sandalwood, *Santalum album*)), གུར་གུམ (gur kum (Saffron crocus)), ཙུ་གང (cu gang (bamboo manna)), སྐེར་ཤུན (skyer shun (Barberry (genus *Berberis*)), ཤིང་སྨངར (shing mngar (Chinese liquorice, *Glycyrrhiza uralensis*)) и བོང་ང་དཀར་ཟི (bong nga dkar (Atish)), mixed with ཀ་ར (ka ra (sugar).

The properties of the components are given in **Table 23.1**. It should be noted that the component ཏིག་ཏ (tig ta) has not been identified to date, but most likely it is a plant from the genus *Gentiana* or *Swertia* of the Gentianaceae family, and bezoar is a stone-like mass found in the gastrointestinal tract of some animals, in particular, wild goat (*Capra aegagrus*). In ancient times (remind: the rGyud-bZhi treatise dates back to the 5th century) bezoar was attributed unique, one could say, magical properties. Thus, it was believed to be able to save from poisons, which is why it got its name: Pers. پادزهر (pad-zahr, "antidote"). This is probably the reason why it was used in the present composition. Sugar is used as a binding ingredient and also probably to enhance the flavour of the preparation containing bitter plants

The peculiarity of the above drugs, first of all, is their antibiotic activity, as well as anti-inflammatory and protective activity. It should be noted that drugs used at the initial stage of the disease have neuro-, cardio- and hepatoprotective effects, stimulate phagocytic activity, activation of T-killers and differentiation of B-lymphocytes, while drugs used "*when the disease has spread*" have more pronounced nephro- and hepatoprotective, mucolytic and anti-coughing effects, reduce the con-

tent of lipoproteins in the blood (remind: pH6 antigen promotes apolipoprotein B coating of the bacterium to avoid the action of the immune system), which generally corresponds to the pathogenetic features of the course of plague infection.

Table 23.1. *Properties of herbal components of Tibetan medicines*

Plant	Activity	Compounds
Inula (*Inula racemosa*)	anti-inflammatory, *antibiotic*	sesquiterpene lactone
Sambucus sibirica	cardioprotective, neuroprotective	quercetin
Guduchi (*Tinospora cordifolia*)	activation of T-killers, B-cell differentiation (by increasing IL-6 levels); antibiotic, phagocytic (neutrophils and macrophages)	
Perfume ginger (*Hedychium spicatum*)	anti-inflammatory (inhibits the release of TNF-α, IL-1β and IL-6), analgesic, hepatoprotective, *antibiotic*	terpenoids
Camphor tree (*Cinnamomum camphora*)		
Chebulic myrobalan (*Terminalia chebula*)	anti-inflammatory (inhibits the release of TNF-α, IL-1β and IL-6), cardioprotective, hepatoprotective, *antibiotic*	gallic, hebullic and neohebullic acids
Bacheda (*Terminalia bellirica*)		
Emblic (*Phyllanthus emblica*)		
Felworts (*Swertia chirata*)		xanthones and their derivatives, alkaloids, flavonoids, terpenoids
Gac (*Momordica cochinchinensis*)	anti-inflammatory	saponins, saponin glycosides, macrocyclic peptides
Large leaf gentian (*Gentiana macrophylla*)	gastroprotective, hepatoprotective, *antibiotic*	secoiridoids glycosides, xanthones

Table 23.1. *(Continued) Properties of herbal components of Tibetan medicines*

Hypecoum erectum	hepatoprotective, choleretic	Alkaloids
Paashaanbhed (*Bergenia ligulata*)	antipyretic, expectorant, immunomodulatory, *antibiotic*	polyphenols, flavonoids, quinones, sterols, terpenes, lactones, bergenin
Atish (*Aconitum heterophyllum*)	**Poisonous plant!**	
Bamboo manna (Tabasheer), derived from the stem of young bamboo (*Bambusa arundinacea* (Retz.) Willd)	anti-inflammatory, hepatoprotective, nephroprotective	flavonoids, phenolic compounds
Saffron crocus (*Crocus sativus*)	anti-inflammatory, cough suppressant	flavonoids, anthocyanins, saponins, safranal, crocin
Indian sandalwood (*Santalum album*)	anti-inflammatory, *antibiotic*	α- and β-santalol, santalbic acid, fatty acids
Barberry (genus *Berberis*)	hepatoprotective, reduces the content of lipoproteins in the blood (in particular, apolipoprotein B)	alkaloids, flavonoids, vitamin C
Chinese liquorice (*Glycyrrhiza uralensis*)	anti-inflammatory, mucolytic, cough suppressant, *antibiotic*	flavonoids (specifically, liquiritin), saponins

Moreover, the use of Guduchi, which stimulates phagocytic activity at the initial stage of the infectious process, and barberry, which reduces the content of lipoproteins in the blood as the disease progresses, should be noted separately, indicating a sufficiently deep understanding of the pathogenesis. Bamboo, which has a nephroprotective action, cannot be ignored, as it is used only if the disease is highly progressive, and, as we remember, significant kidney damage occurs in the prolonged course of the septicemic form of the disease. Paashaanbhed and Atish, which have expectorant action (remember that atish is poisonous), are also used when the disease progresses, and "*if the rims has got into the lungs*" Paashaanbhed is replaced by Chinese liquorice. On the other hand, it is

impossible not to note a very illogical tendency to reduce the number of plants showing antibiotic activity in the formulations of preparations. Thus, in the preparations used at the first stage, ≥75% of the components exhibit antibiotic activity (3/4 for ma nu bzhi thang and 5/5 for sle tres lnga thang). In the preparation applied "*when the disease has spread*", 71% (5/7) of the components show antibiotic activity, and when the disease has progressed, already 61% (8/13) of the components show antibiotic activity. Finally, in the preparation used "*if the rime has entered the lungs*" only 50% (3/6) of the components show antibiotic activity.

Other preparations are also known, but in order to save precious time, we will limit ourselves to mentioning one more plant called སྦང་རྫི་དོ་དོ། (bang-zi-du-wu) or *Pterocephalus hookeri* (C. B. Clarke) Höeck. The plant is interesting because it was used to treat plague even before the rGyud-bZhi treatise. In addition, like many plants of Tibetan medicine, it has been used in China since the 19th century (under the name 翼首草, yi shou cao) and was even included in the Chinese Pharmacopoeia (1977)[1]. Due to its content of iridoids, triterpenoids and phenylpropanoids, peregolovnik has anti-inflammatory, analgesic, immunomodulatory, neuroprotective activity, and exhibits antibiotic activity.

The traditional Mongolian medicine was influenced by Tibetan medicine when Buddhism entered Mongolia in the 13th century, becoming widespread by the early 17th century. Mongol emchas translated Tibetan treatises into Mongolian and created compilations based on them. The woodcut M-143[2] is an example of such a compilation. Due to the absence of the title page and colophon (the last page), the title of the compilation and the name of the author have not survived (there is only an indirect indication that the text was compiled not by one person, but with the participation of someone else). The first leaf (**Figure 23.4**) of the woodcut begins with the words: "*At this time, when the number of illnesses*

[1] *Committee for the Pharmacopoeia of PR China, Pharmacopoeia of the People's Republic of China*, China Medical Science and Technology Press, Beijing, China, 1977.

[2] A type of book printed by hand from wooden matrices, representing individual sheets assembled in a stack. The woodblock M-143 consists of 11 sheets and was printed on Russian paper, probably in one of the Buryat monasteries (datsan). A notable feature of this woodcut is the system of diacritical marks, which differs from that used in traditional Mongolian woodcuts.

and deaths has increased enormously, because there was no way and possibility of immediate escape from them and it became necessary to find them, if one intends to clarify a little the cause and circumstances of illnesses, then according to the 29th chapter "On Cold Diseases" of the medical sutra "Lhan thabs", ordinary people at the time when five centuries are ending, by virtue of the fact that they desire their own prosperity, go astray from the path of the highest, indulge in ten black sins". The above fragment shows the important role played by the treatise "Man ngag lhan thabs".

In parallel with translations of classical Tibetan treatises, Mongolian emchi began to create their own treatises, written, however, in Tibetan, in which they reflected their unique experience that allowed them to introduce both new methods and new medicinal plants of the Mongolian flora into medical practice. When speaking about traditional Mongolian medicine, it is these treatises that we will be considering.

The Mongols considered plague to be a "nyan" disease, i.e. transmissible from person to person (remember Tib. རིམས, rim). According to legend, in winter, when tarbagan marmots are in hibernation, a polecat sneaks into their burrows, which carries the plague, but does not fall ill with it. The polecat bites the tarbagan marmot, and the plague poison penetrates into the body and sleeps until spring. In spring, when the tarbagan marmot comes out of its burrow, the poison wakes up with the first thunder and starts to work. A person can become infected if he hears the voice of a tarbagan marmot, touches it with his shadow, touches it with a stick or at least inhales its odour. In the treatise Gces bsdus snying nor (Tib. གཅེས་བསྡུས་སྙིང་ནོར།; early 19th century)[1], Chapter 89, we read that "*there is a contagious disease resulting from eating the meat of a diseased tarbagan marmot*". It is manifested by "*pain in the region of the short ribs, [known by the names] phyi bai nad and rtsibs thung lha mo*", i.e. "tarbagan marmot disease" and (literally) "short rib of the goddess". Interestingly, despite the explicit statement that the disease results from "*eating the meat of a diseased tarbagan marmot*", Mongolians continue to eat gophers and marmots and contract the plague from them, with the last known case of infection occurring in August 2023.

[1] Written in Tibetan by the Mongolian emchi Lobsang Choipel as a compilation of the most important information from various medical treatises.



Рис. 23.4. *The woodcut M-143 (Buddhist sutra in Old Mongolian), included in the Museum Fund of the Russian Federation and kept in the Aldan-Maadyr National Museum of the Republic of Tuva (folio 1a). Courtesy of the ГБУ «Национальный музей имени Алдан-Маадыр Республики Тыва»*

In the treatise Ngamatshar dga ston gter mdzod (Tib. ངམཚར་དགའ་སྟོན་གཏེར་མཛོད། ; early 20th century)[1], Chapter 98, we read that infection can occur even as a result of "*listening to the squeak [of the tarbagan marmot], etc. About the signs: there will be [a feeling of] obscuration in the head, shortness of breath, very high fever of the armpit, vomiting brown [masses], weakness, palpitations, above the ribs there are hot swellings of terrible [shape (i.e. bubon), this disease in its course] is close to the strong diseases of gnyan (i.e. nyan)*". All this contributed to the fact that Mongols developed a system of anti-epidemic measures. If a person fell ill in a yurt, it was forbidden to enter and leave it. A cordon was placed outside, and sometimes a black flag was displayed. The entrance was guarded as long as there was smoke coming out of the yurt. When the smoke stopped, it meant the death of all the inhabitants, after which the yurt was set on fire. It happened that the yurt remained unburned. In such a case they did not enter it for almost a year to let the corpse air dissipate, for if it got into the nose, it would certainly lead to infection. And although the Mongols

[1] Written in 1921-1922 in Tibetan by the Mongolian emchi Choizhamts at the Im Khure monastery, it is the last authoritative work on the Mongolian branch of Tibetan medicine, summarising the achievements of Tibetan, Mongolian and Buryat medicine. Apparently, the treatise is addressed to emchi practitioners, as it does not contain chapters on philosophy and theory of medicine, being a reference book on methods of diagnosing diseases with brief descriptions and prescriptions, in which, among other things, one can find preparations sold in pre-revolutionary Russian pharmacies.

smeared their nostrils with aromatic oils[1] for protection, this did not protect them from the corpse air.

As a treatment, the treatise Ngamatshar dga ston gter mdzod recommends using the preparations གོ་རོ་ཙ་ན་བརྒྱད་པ། (go ro tsa na brgyad pa)[2] and གཉན་བཤལ་ཐོག་མདའ། (gnyan bshal thog mda) with the addition of burnt sesame ashes. The first preparation consists of gypsum with clay admixture, Saffron crocus, *Odontites rubra*, *Gentianopsis barbata*, white Indian sandalwood, and clay ochre, Baikal skullcap (*Scutellaria baicalensis*) and *Aconitum heterophyllum*, while the second contains Myrobalan, *Euphorbia fischeriana*, *Euphorbia adenochlora*, mercury, Elecampane (*Inula helenium*) and Castor bean (*Ricinus communis*)[3]. Despite containing plants with therapeutic effects, many of which we are already familiar with, the efficacy of the proposed preparations is questionable due to the poisonous plants (Euphorbia fischeriana, Euphorbia adenochlora, Castor bean) and mercury, which apparently refers to the mineral cinnabar (mercury sulfide).

The author further suggests bloodletting from the snod ka and dpung rca[4] vessels if the disease has fallen into the rmen bu (lymph nodes), and "*when [abscesses (?)] appear to have heads*" (probably the maturation of buboes) one should "*bury the whole body [of the patient] in the ground*" (up to the neck)[5], giving him an infusion of herbs. It is also suggested to drink boiled camel milk with sgog skya (Garlic, *Allium sativum*) and butter.

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[1] The Gces bsdus snying nor (19th century) treatise suggests "drinking a mixture of [plant] sgog [skya s] chang", i.e. garlic chang (tea?), every morning.

[2] The researcher of this treatise Andrei Aleksandrovich Kosoburov (1961-2017) reports that the names of the components in Sanskrit (written in the Tibetan alphabet) are used in the prescription of the drug. And since "go ro tsa na" is "gi wang", the name of the drug in Tibetan would be gi wang brgyad pa.

[3] Recipe of the preparation is given according to: Bazaron E. G., Batorova S. M. *Prescriptions of Tibeto-Mongolian medicine*, Verkhnyaya Ivolga: Publishing House of the Buddhist Institute "Dashi Choinhorlin", 2001, 176 p. (in Russian)

[4] The vessel snod ka refers to the median basilic vein (v. mediana cubiti) or cephalic vein / basilic vein (v. cephalica / basilic a mediana), and the vessel dpung rca (rtsa) refers to the cephalic vein (v. cephalica) or one of its upper branches

[5] The researcher of this treatise and translator into Russian Andrey A. Kosoburov believes that the author is referring to the drawing of vapours of disease (pus?) by the earth when boils ripen (heads appear).

Quite interestingly, the author concludes: "*The fruit (?) of the brug shing or tsag plant is said to be very [good] in preventing the disease in question – [whether this is actually the case (?)], I do not know*". Both plants have not been identified, but the first one is known from the treatise mDzes mtshar mig rgyan (late 18th century)[1] that it cures skin diseases, "*grows in padi and everywhere. The skin of the trunk is blue-green, hard. Just above the rosehip, the brug shing is twined into other trees. Sometimes this plant can be substituted for birch bark*". As we can see, as we move further away from Tibet, the emchi begin to look for substitutes for plants that grow in the region. Thus, among the plants mentioned in the treatise we find dkar po chig thub or Physochlaina (genus *Physochlaina*), which grows on open steppe hillsides and has anti-inflammatory and analgesic properties due to its content of flavonoids, coumarins and iridoids. It should be noted that, according to the reports of physicians who worked in Mongolia in the 1930s, Mongolians died not so much from plague and other infectious diseases as from scurvy that appeared after recovery, which was the result of vitamin deficiency due to the lack of fruits and vegetables in the diet.

The traditional medicine of the Buryats, a Mongolic ethnic group, was formed under the influence of Tibetan and Mongolian medicine when Buddhism spread to Lake Baikal (Russia) in the 16th century, where the flora is very rich in mushrooms and berries, which protects against scurvy. There are also no natural foci of plague, so cases of plague are rare. It is interesting that Buryats called plague itself "бодо" (bodo), genitive case of Buryat "бодын" (bodyn) – cattle, including in this disease also anthrax, which reminds us of the difficulties of differential diagnostics. At the same time, when hunting tarbagan marmots, Buryats killed only healthy-looking animals using rifles. The carcasses of killed tarbagan marmots were carefully examined: incisions were made in the groin area to assess the condition of lymph nodes, and on the legs – to assess the colour of blood. If the inguinal lymph nodes were enlarged or if the blood was dark in colour and thick (stagnant), the tarbagan marmot was left in place.

[1] Written in Tibetan by the Mongolian gegen ("brilliant") Jambaldorje in a monastery in Inner Mongolia (present-day area in China), it is the only illustrated Mongolian treatise containing information on medical theory and medicinal raw materials.

Buryat emchi did not write theoretical treatises (at least, none are known), but in monasteries (datsans) they compiled numerous collections of recipes, called "жоры" (zhoras) and "ман-жоры" (manzhoras), based on similar Mongolian collections. The best-known such compendium is Kunpan dudzi nyingpo (early 20th century)[1], which contains formulas based on the plants of the area, because, as in the case of Mongolian medicine, Buryat emchis also had to find substitutes for some plants. Thus, in the preparation ma nu bzhi thang (rGyud-bZhi), Felworts is replaced by *Gentiana barbata*, and Emblic by the fruit of Siberian crab apple (*Malus baccata*) or Mountain hawthorn (*Crataegus pinnatifida*). Camphor tree is substituted by Hairy rock-cress (*Arabis hirsuta*), Saffron crocus by Scotch marigold (*Calendula officinalis*), *Aconitum heterophyllum* by *Aconitum barbatum* or northern wolf's-bane (*Aconitum excelsum*), various species of Barberry by *Berberis sibirica*, and Gac by Dwarf daylily (*Hemerocallis minor*). Even the Tibetan "king of medicine", Chebulic myrobalan, was replaced by False spiraea (*Sorbaria sorbifolia*).

The traditional medicine of the Kalmyks, another Mongolic ethnic group[2], was also influenced by Tibetan and Mongolian medicine. The Kalmyk emchi also had to find substitutes for some plants, which eventually led to the development of their own medicines, in which wormwood and, in particular, Common wormwood (*Artemisia absinthium*) took an important place. It is used for various diseases, having found its application even in "western" medicine. Another important remedy is "кумыс" (kumis), a fermented milk drink made from mare's milk, which has antibiotic properties. At the same time, due to the small number of emchi and the much larger number of illiterate herbalists, Kalmyk medicine did not offer any of its own methods to combat the plague, called "маалыг" (maal'g) and "тарвһн тахл" (tarvhn takhl), Kalm. "tarbagan marmot epidemic", except for the isolation of sick animals.

[1] Compiled in Tibetan, presumably by Sumati Prajnya (Darkhan-Nanso Lubsan Shirab, d. 1796) and with a number of additions, it was published only on 12 April 1923 in Aginsky datsan, being the largest in volume and the latest Buryat collection containing formulas based on plants growing in this area.

[2] They belong to the Oirat group and are the only Mongolic-speaking people living in Europe and the only representatives of Buddhist culture in the West.

* * *

In the library of the Potala Palace[1], a man sits among many books, talking on various topics with another man dressed in monks' robes of yellow and red colours. The eyes of the interlocutor, framed by the frame of spectacles, are filled with wisdom and participation, and a radiant good-natured smile illuminates his face. A gangs seng ge (Snow Lion) sits majestically next to him, resting its head on its paws as white as the snow in the Himalayas. The gaze is piercing and sad. In spite of everything, they remain here, forever gazing into infinity...

24 TREATMENT

In 951, a plague raged in the Japanese city of Kyoto. In those days, the founder of the Rokuharamitsu-ji temple, the monk Kūya[2], made a statue of Kannon[3], the bodhisattva[4] of mercy, which he carried around the city, serving the sick a special tea made from pickled plums and seaweed brewed on burning bamboo laid out in the shape of a lotus. It

[1] A palace in Lhasa, built in 637 by the King Songtsen Gampo of Tibet (in its present form in the 17th century – on the initiative of H.H. Dalai Lama V). It served as the winter residence of the Dalai Lamas until 1939, when due to the invasion of the Chinese People's Liberation Army and the subsequent annexation of Tibet (called the "Peaceful Liberation of Tibet" by the Chinese government), H.H. Dalai Lama XIV (b. 1935) was forced to flee to India. The Government of Tibet in Exile (Central Tibetan Administration) remains there in the city of Dharamsala (India) to this day.

[2] 空也, 903–972; the itinerant Japanese Buddhist monk of the Heian period, founder of the Kuya school (a branch of the Tendai school), also known as ichi hijiri (holy man of the marketplace) and Amida hijiri. Kūya was known for taking images with him on his travels and added musical rhythm and dance to his prayers, known as odori nembutsu. Presumably the son of Emperor Daigo (885-930).

[3] A revered Japanese Buddhist incarnation of Avalokiteśvara, the "Buddha of compassion"; the statue in question is kept in a temple and displayed for public worship once every twelve years, in the year of the dragon.

[4] The one who, having attained enlightenment (become a Buddha), gave up the transition to nirvana (i.e., eternal life) to help sentient beings attain enlightenment, i.e., to escape the cycle of suffering and rebirths.

is difficult to judge how effective this potion was (although there is evidence of the antibiotic properties of seaweed), but let us dwell a little more on the tradition of public display of statues during epidemics. This action in itself is not new and is present in many cultures. For example, the Emerald Buddha, now preserved in the Thai temple of Wat Phra Kaew, was displayed many times during various calamities such as plague and cholera epidemics until King Mongkut (Rama IV) forbade it, saying: 'Diseases are caused by germs, not by evil spirits or the displeasure of the Buddha'.

What about in the west? Guy de Chauliac[1], who lived in the fourteenth century, reports that bloodletting, purgation (probably a laxative), electuaries (medicines mixed with honey) and syrupy tinctures should be used to treat the plague. Cover the outside of the buboes with a mixture of figs, sliced onions, yeast and oil, which will cause them to open and heal. Another physician, Gentile da Foligno[2], suggests rubbing the buboes with a balm consisting of an extract of an umbrella plant (parsley?), a lily bulb and human excrement, and taking theriac[3] internally.

Time passed, but the only effective way to avoid mass death of people remained only anti-epidemic measures, until in 1890 Emil von Behring and Kitasato Shibasaburō discovered[4] that in the blood of those who had contracted tetanus or diphtheria, substances ("antitoxins") are formed that provide immunity to both the sick and those to whom their blood will be transfused. This is how tetanus and diphtheria serum came into being, and the beginning of serum therapy. And although the very idea of administering immune serum to patients belongs to

[1] 1298–1368; the French physician, "father of surgery", personal physician to popes Clement VI, Innocent VI and Urban V. Author of the famous treatise Chirurgia Magna, which remained the main textbook on surgery until the 17th century.

[2] ?–1348; Italian physician, author of commentaries to the Canon of Medicine by Ibn Sina (Avicenna). He was one of the first European physicians to perform a human postmorton examination (1341). He died of plague.

[3] An infusion known since antiquity (Greek θηριον), used as an antidote. It consisted of a large number of ingredients, including common European adder meat, opium, and castoreum (i.e., an odorous substance secreted by the paraanal glands).

[4] Behring E., Kitasato S. *Über das Zustandekommen der Diphtherie-Immunität und der Tetanus-Immunität bei Thieren*. Dtsch Med Wochenschrift. 1890; 49:1113–1114.

Kitiasato Shibasaburō, only Emil von Behring would be awarded the Nobel Prize in Physiology or Medicine in 1901 ("*for his work on serum therapy, especially its application against diphtheria, by which he has opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and deaths*"). The fact is that Emil von Berin will publish his own article[1], and the Nobel Committee will judge that the idea of serum therapy belongs to him and not to Kitasato Shibasaburō. Of course, the initial success of serum therapy was partial, but with the help of Paul Ehrlich[2], dosages were correctly calculated and purified, highly concentrated sera were produced, which entered clinical practice.

The first plague serum was produced by Alexandre Yersin. As he himself would write[3], a fresh culture of *Y. pestis* is injected into a horse intravenously (to avoid suppuration), "*observing all precautions to avoid embolism. Already 6 hours after the injection, the temperature reaches 40°C [/104°F] and sometimes rises to 41.5°C [/105.8°F]. The horse is despondent, shivering. The fever persists for several days, gradually decreasing, with no increase in lymph nodes. Injections are given in large doses, but at fairly distant intervals, so that the animal recovers completely after each. Indeed, there are often joint swellings, synovitis, which do not fester, but lead to painful lameness. Horses lose a lot of weight during immunisation, so care must be taken not to be too hasty with vaccinations. They react to each one if the dose is strong enough, but the duration of the reaction becomes shorter and shorter*". Three weeks after the first immunisation, serum is isolated from the blood. As can be easily guessed, the choice of animal is due, firstly, to the fact that horses are weakly susceptible animals and rarely get sick in nature, and secondly, that sufficient serum can be obtained from them (a horse contains 40-45 litres of blood).

The first use of serum took place on 26 June 1896 in the city of Canton (present-day Guangzhou, China). The first symptoms of illness in an

[1] Behring E. *Untersuchungen ueber das Zustandekommen der Diphtherie-Immunität bei Thieren.* Dtsch Med Wochenschrift. 1890; 50:1145–1148.

[2] 1854–1915; the Prussian and German physician of Jewish origin, bacteriologist, chemist, "father of immunology". Laureate of the Nobel Prize in Physiology or Medicine (1908). Author of numerous works on infectious diseases, immunology and oncology, one of the founders of modern chemotherapy. During his studies he was considered a rebel, and had poor academic performance.

[3] Yersin A. *Sur la peste bubonique (Séro-thérapie).* Ann Inst Pasteur (Paris). 1897; 1:81–93.

18-year-old Chinese seminarian surnamed Jie appeared early in the morning, and buboesis and vomiting did not take long to appear, foreshadowing the imminent end. Alexandre Yersin injected the patient with 10 ml of serum at 3, 6 and 9 p.m. each. By midnight his condition had already improved and he fell asleep. By 6 a.m. he looked healthy.

After such a grandiose triumph, Alexandre Yersin went to suppress a plague epidemic in Bombay (India), and in 1902 he preferred his academic career to a secluded life in Vietnam, where his favourite pastime was quiet cycling. His labours led to the establishment of a serum production station in Nha Trang, as well as a plantation of Pará rubber tree (*Hevea brasiliensis*), which was used to produce rubber for the Michelin company so that the station could support itself financially. The city of Nha Trang has been mentioned before - it was from there that a serviceman returned with plague meningitis, and the station itself helped save the lives of many Vietnamese from the plague that broke out amidst the 1955-1975 war. Alexandre Yersin died on 1 March 1943. A pagoda was erected near his grave, and altars were erected in local Buddhist temples, before which the locals offer prayers to the scientist, considering him a bodhisattva of wisdom, although he was a Protestant by religion. His spirit is also honoured as the patron of the area (Viet. thành hoàng) by followers of Vietnamese folk religion.

Currently, plague serum is not used in clinical practice due to strong allergic reactions caused by large amounts of foreign protein (horse antibodies)[1], as well as high efficacy only at the initial stages of the infectious process. However, there are developments in the use of monoclonal antibodies to capsule antigen and to LcrV. Experiments[2] conducted on mice have shown efficacy both for prophylaxis (24 hours before infection) and for treatment of bubonic and pneumonic forms of plague.

It would be appropriate to say a few words about Kitasato Shibasaburō. Living in his native Japan, he led the eradication of plague

[1] In the Practical Physician's Manual (1952) we read that serum is administered "*to adults intravenously 50-100 ml subcutaneously or intermuscularly – not more than 300 ml per injection: serum should be administered repeatedly and possibly early - up to 1.5 litres per patient*". Of course, such therapy may shock the modern physician with the possibility of serious post-injection complications (e.g. necrosis).

[2] Andrianaivoarimanana V., Randriantsheno L. N., et al. *Potential human immunotherapeutics for plague*. Immunother Adv. 2021; 1(1):ltab020; DOI: 10.1093/immadv/ltab020.



Tomb of Alexandre Yersin
with a pagoda and offerings (top row) on Remembrance Day (Viet. lễ giỗ),
as well as Linh Sơn Pháp Ấn Temple (middle row), which includes Alexandre Yersin's
office (yellow building), and Long Tuyền Temple (bottom row) with altars and offerings.
Notably, bread and melted cheese are found among the offerings.
The village community of Suoi Sau, Vietnam, 2019
Courtesy of Dr. Elena Vitalievna Gordienko



Koch-Kitasato jinja Shrine in Tokyo, Japan, 2024

outbreaks, developed the system of sanitary supervision and trained a whole host of outstanding students, to whom he often said that the *foundation of medicine lies in the prevention of disease*! He believed that the *mission of the physician was to prevent disease*. This is the theory he formulated as a student and followed for the rest of his life. As a student of Robert Koch, he built a Jinja Shrine in memory of his teacher on the day of his death[1]. When Kitasato Shibasaburō died on 13 June 1931, his students built a Jinja Shrine in his memory, but it was destroyed in a fire during World War II. The two Shrines were merged, and to this day Koch-Kitasato jinja Shrine (Jap. コッホ・北里神社) stands on the grounds of the Kitasato Institute[2]. Both scientists are honoured as kami (patron

[1] Kitasato Shibasaburō placed a strand of hair of Koch, secretly taken from his barber during Koch's visit to Japan, as a shintai ("symbol of the deity"; Jap. 神体) that holds a part of spirit of Robert Koch. It should be clarified here that according to Shinto beliefs, hair contains tama, i.e. the life energy, the spirit of its owner.

[2] It has been in its current location on the Shirokane Campus in Tokyo since 27 May 2015, and was last updated on 26 March 2019. In 1994, a branch of the Koch-Kitasato jinja Shrine was opened on the Kitamoto campus. It is larger in area and has its own gate-torii. Two dates have been set as shrine holidays: 27 May (birthday of Robert Koch) and 13 June (the day of passing of Kitasato Shibasaburō).

deities) of the Institute, and all those associated with it, and bacteriologists.

Linked by the discovery of the plague microbe, so different in nature, did Alexandre Yersin and Kitasato Shibasaburō know that their posthumous veneration would be so similar[1]?

Mankind has known about the properties of moulds and yeast fungi since ancient times and used them in medical practice. For example, there is information about the use of mouldy bread in the treatment of wounds, and the research of Egyptian and Nubian mummies showed a high content of tetracycline, the source of which was probably beer[2]. In the second half of the XIX century, doctors were closely involved in the research of the properties of mould (mycelial) fungi, which were then classified as non-vascular plants. In the 1870s, William Roberts[3] noted that *Penicillum glaucum*, used in the production of some varieties of blue cheese, was not subject to bacterial contamination (i.e. contamination by bacteria)[4], and Alexei Gerasimovich Polotebnov[5] and Vyacheslav Avksent'evich Manassein[6] recommended its use for the treatment of skin diseases.

In parallel, Paul Ehrlich, noticing that some dyes could stain bacteria while others could not, began the search for a synthetic cure for bacterial infections. Focusing on the search for a compound active against the syphilis pathogen (*Treponema pallidum*), by 1907, together with Alfred Bertheim[7], compound 606 (Germ. Ehrlichschen Präparat 606) was synthesised. Tests of the compound were carried out with Hata Sahachirō and were completed by 1909. Although the compound had a number of

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[1] In Japan, until the Meiji Restoration (late 19th century), Shintoism and Buddhism were syncretistic (i.e. combined).

[2] Amelagos G. J. *Take two beers and call me in 1,600 years: ancient Nubians and Egyptians had a way with antibiotics*. Natural History. 2000; 109(4):50–53.

[3] 1830–1899; the English physician, surgeon, nutritionist.

[4] A close relative of this fungus, *Penicillum chrysogenum* (formerly known as *Penicillum notatum*), shows resistance even to the causative agent of anthrax (*Bacillus anthracis*).

[5] Алексей Герасимович Полотебнов, 1838–1907; the Russian physician, founder of Russian dermatology.

[6] Вячеслав Авксентьевич Манассейн, 1841–1901; the Russian physician, editor of the newspaper "Врач" (The physician), where he fought against quackery and witchcraft. He dealt with issues of ethics and deontology.

[7] 1879–1914; the Prussian and Germanic chemist.

contraindications, it went on sale at the end of 1910, under the name Salvarsan. A short time later, they managed to synthesise a safer compound 914, called Neosalvarsan. By the middle of the 20th century, both drugs had already fallen out of use, nevertheless becoming the first synthetic antibiotics.

The era of natural antibiotics began thanks to something that is strictly forbidden in modern laboratories - disorder. In 1929, on one of the forgotten Staphylococcus aureus culture Petri dishes, Alexander Fleming[1] discovered colonies of the fungus *Penicillum notatum* (remind: now *Penicillum chrysogenum*), around which there were clear zones of lysis, i.e. transparent areas where the bacteria did not grow (**Figure 24.1**). He had already seen something similar when he had seeded mucus from his own nose a few years earlier, which led to the discovery of lysozyme. The substance isolated from the fungi, called Penicillin, was considerably more effective than the then existing analogues, but Alexander Fleming was not a chemist and so could not obtain a pure and stable compound, and the article[2] he published for about ten years failed to generate a proper response in the medical community. Only in 1940, when Alexander Fleming himself abandoned his attempts to synthesise the substance, despite the difficulties of wartime, Howard Walter Florey[3] and Ernst Boris Chain[4] (with colleagues) in the walls of Oxford University managed to synthesise a stable substance of the required purity.

On 12 February 1941, Constable Albert Alexander, severely injured in the bombing of Oxford, was given 160 mg of penicillin. Within about 24 hours, his fever was reduced and the patient, who had been considered hopeless, recovered. The synthesised substance was in short supply and, despite extracting its residues from the patient's urine, the penicillin ran out by the fifth day. In the absence of treatment, the patient

[1] 1881–1955; the British physician, bacteriologist. Laureate of the Nobel Prize in Physiology and Medicine (1945). Fellow of the Royal Society of London (1943)..

[2] Fleming A. *On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to their Use in the Isolation of B. influenzæ*. Br J Exp Pathol. 1929; 10(3):226–236.

[3] 1898–1968; the Australian physician. Laureate of the Nobel Prize in Physiology or Medicine (1945). Fellow of the Royal Society of London (1941).

[4] 196–1979; the German biochemist of Jewish origin. Laureate of the Nobel Prize in Physiology or Medicine (1945). Fellow of the Royal Society of London (1949). In 1933, amid growing anti-Semitic sentiment in Nazi Germany, he moved to the UK.

died by 15 March. However, by 1945, thanks to the efforts of American manufacturers, every wounded soldier was able to be treated with penicillin. In the same year, all three were awarded the Nobel Prize in Physiology or Medicine "*for the discovery of penicillin and its curative effect in various infectious diseases*". Penicillin proved to be active against a large number of pathogens, but powerless against *Y. pestis* (the penicillin-binding proteins[1] present in *Y. pestis* are lethal targets for beta-lactam antibiotics)[2].

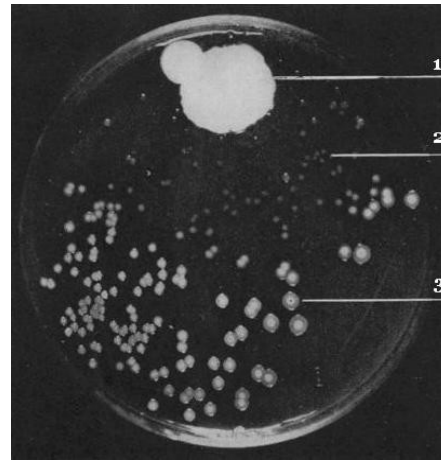


Figure 24.1. *Petri dish with normal (3) and lysed (2) colonies of Staphylococcus aureus, and the germinated fungus Penicillum notatum (1), which led Alexander Fleming to discover the first antibiotic of world (by Fleming A., 1929; published under licence from the World Health Organization)*

It should be realised that by the summer of 1942 the penicillin available in Great Britain and the USA would be sufficient to treat only ten patients. Therefore, in the same year in the USSR, Zinaida Vissarionovna Yermolyeva[3] and Tamara Iosifovna Balezina[1] obtained a

[1] A total of 6 penicillin-binding proteins (PBP) have been found in *Y. pestis*. Interestingly, one of them is conservative, so "*can be used to differentiate this species from other bacteria, including those of the Yersinia genus... Moreover, some minor differences could help to characterize some strains, such as the wild-type isolate P.PB189, which lacked PBP4 [57 kDa]. On the other hand, because of the variability of PBPs under different growth conditions, the contents of PBPs may not be sufficiently reliable to distinguish among individual Yersinia strains*" (see Ferreira R. C., Park J. T., et al. *Interactions of Yersinia pestis penicillin-binding proteins with beta-lactam antibiotics*. Antimicrob Agents Chemother. 1995; 39(8):1853-1855; DOI: 10.1128/AAC.39.8.1853).

[2] It should be noted that the concentration of PBP *Y. pestis* gradually decreases after the transition to 37°C / 98.6°F, indicating a decrease in *Y. pestis* resistance. Moreover, experiments in mice have shown that some new generation beta-lactams are effective and some (e.g. monobactams) are already recommended for human treatment.

[3] Зинаида Виссарионовна Ермольева, 1897/1898–1974; the Russian and Soviet physician, bacteriologist, epidemiologist. Member of the USSR Academy of Medical Sciences. Winner of the Stalin Prize (1943). Author of works on plague and cholera, creator of vac-

more effective analogue of penicillin ("penicillin-crustosin VIEM") from the fungus *Pinicillium crustosum* (Tamara I. Balezina was found in one of the buildings) and set up its mass production, which saved the lives of thousands of Soviet soldiers.

However, the contribution of Tamara I. Balezina is often forgotten, giving all the laurels to her supervisor, but this case is not the only one. In 1943, Albert Schatz[2], working in the laboratory of Selman Abraham Waksman[3], began searching for a compound that would be active against those microorganisms that were resistant to penicillin, especially the causative agent of tuberculosis (*Mycobacterium tuberculosis*). Working alone in an isolated cellar (which is now also forbidden) with the most virulent strain of the time (*M. tuberculosis* H-37), after only three months, on 19 October 1943, he was able to isolate such a compound, calling it streptomycin because it is produced by the bacteria *Streptomyces griseus*. Already in 1947, streptomycin entered clinical practice.

On 12 September 1948, Albert Schatz and Selman A. Waksman were granted a patent for streptomycin (US 2449866) as inventors, but the latter's financial machinations led to a court case that resulted in Albert Schatz being entitled to the royalties due as co-inventor of the patent. The financial issue put the two scientists at odds, which eventually led to the Nobel Prize in Physiology or Medicine in October 1952 "*for his discovery of streptomycin, the first antibiotic effective against tuberculosis*" being awarded only to Selman A. Waksman, and Albert Schatz was forgotten. Only in 2005 The Lancet would write[4] that "*the Nobel committee made a considerable mistake by failing to recognise Schatz's contribution*" (and how can we not remember the life story of Kitasato Shibasaburō?).

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cines. In 1943 with the help of cholera bacteriophage saved the Nazi besieged city of Stalingrad (now Volgograd, Russia).

[1] Тамара Иосифовна Балезина, 1913–2010; the Soviet and Ukrainian physician, bacteriologist, virologist

[2] 1920–2005; the American microbiologist, soil scientist

[3] 1888–1973; the Ukrainian and American biologist and chemist. Laureate of the Nobel Prize in Physiology or Medicine (1952). Member of the US National Academy of Sciences (1942). In 1947 he formulated a definition of the concept of "antibiotic".

[4] *The Nobel cause*, The Lancet Infection Diseases. 2005; 5(11):665; DOI: 10.1016/S1473-3099(05)70245-0.

Streptomycin, invented as a treatment for tuberculosis, was used to treat plague as early as 1948, becoming the first antibiotic active against *Y. pestis*.

In modern clinical practice, plague is divided by severity into *mild*, *moderate* and *severe* plague.

The mild form is observed only in persons living in endemic areas and in vaccinated persons[1]. The severe form begins suddenly and usually has a fulminant course.

Etiotropic therapy includes the use of antibiotics, not only streptomycin, but also representatives of other groups (divided into first- and second-line antibiotics[2]). It should be remembered that the staff of the treatment facility must work in special protective suits, which will be discussed in more detail in Chapter 28.

First-line antibiotics in animals (cats and dogs[3]) are doxycycline and gentamicin, the latter being used mainly in severe cases. Doxycycline can be used to complete treatment, while cephalosporin-type drugs have not shown clinical efficacy. There have been no clinical trials of fluoroquinolones, but experience with their use in enzootic regions favours their efficacy. Doses and duration of use are given in **Table 24.1**.

First-line antibiotics in humans are, for example, streptomycin, gentamicin, doxycycline and others. Doses depending on the form of the disease are given in **Table 24.2** (first-line drugs in **bold**). When choosing a drug (both for humans and animals), the data of antibiotic resistance testing performed on the isolated culture should be taken into account (however, it should be remembered that antibiotic therapy is prescribed for suspected plague **immediately**, i.e. without waiting for identification of the causative agent and its antibiotic resistance testing; treatment should be adjusted after antibiotic resistance data are obtained).

At first glance, **Table 24.1** and **Table 24.2** may seem complicated and unclear because of the large number of symbols. However, after famil-

[1] Remind that vaccinated individuals may be asymptomatic (case in Chapter 16).

[2] Second-line drugs are used when the disease cannot be treated with first-line drugs, as well as when there is a contraindication to the use of first-line drugs (for example, due to the allergy of patient).

[3] Sick farm animals, e.g. camels, cannot be treated (corpses are burnt).

iarising yourself with the "Note" column at the bottom of the table, they all disappear.

Let's consider the method of reading the tables on the example of the antibiotic "streptomycin" (**Table 24.2**). Streptomycin is a first-line treatment and first-generation antibiotic. It's the "Watch" group antibiotic (see "Wa" next to antibiotic name) according to the AWaRe classification of antibiotics (World Health Organization)[1]. It can also be used to treat the pharyngeal form of plague (see "*"). The main route of administration is intramuscular administration, but there are isolated reports of intravenous administration (see "||"; in this method of administration, the antibiotic is included in "Watch" group). In the bubonic form, use 500 mg of antibiotic twice a day for exactly one week (i.e., "D/F/T" from the note). In the case of children (see "*"), a smaller dose is used, namely 15 mg/kg, which can be administered intravenously! Accordingly, for pneumonic and septicemic forms, 520 mg of antibiotic is used three times a day for ten days.

[1] *WHO AWaRe (access, watch, reserve) classification of antibiotics for evaluation and monitoring of use, 2023*. In: *The selection and use of essential medicines 2023: Executive summary of the report of the 24th WHO Expert Committee on the Selection and Use of Essential Medicines*, 24 – 28 April 2023. Geneva: World Health Organization; 2023 (WHO/MHP/HPS/EML/2023.04).

Terms: The **Watch group** includes antibiotic classes that have higher resistance potential and includes most of the highest priority agents among the Critically Important Antimicrobials for Human Medicine and/or antibiotics that are at relatively high risk of selection of bacterial resistance. These medicines should be prioritized as key targets of stewardship programs and monitoring. Selected Watch group antibiotics are recommended as essential first or second choice empiric treatment options for a limited number of specific infectious syndromes and are listed as individual medicines on the WHO Model Lists of Essential Medicines. The **Reserve group** includes antibiotics and antibiotic classes that should be reserved for treatment of confirmed or suspected infections due to multi-drug-resistant organisms. Reserve group antibiotics should be treated as "*last resort*" options. Selected Reserve group antibiotics are listed as individual medicines on the WHO Model Lists of Essential Medicines when they have a favourable risk-benefit profile and proven activity against "Critical Priority" or "High Priority" pathogens identified by the WHO Priority Pathogens List, notably carbapenem resistant Enterobacteriaceae. These antibiotics should be accessible, but their use should be tailored to highly specific patients and settings, when all alternatives have failed or are not suitable. These medicines could be protected and prioritized as key targets of national and international stewardship programs involving monitoring and utilization reporting, to preserve their effectiveness.

Table 24.1. *Antibiotic therapy regimen for pets*

Antibiotic	**Route of administration**	**Treatment regimen** (dose x therapy duration)
Doxycycline	per os	10 mg/kg x 14 days
Gentamicin[1]	IM SQ	2–3 mg/kg x 10 days (3 per day)
Enrofloxacin[1]	IM SQ	5 mg/kg x 10 days
Tetracycline	per os	22 mg/kg x 14 days (3 per day)
Chloramphenicol	per os	50 mg/kg x 14 days (2 per day)
Note: [1] injections may be preferred in the acute stage of the disease, as they avoid contact with oral secretions and also reduce the risk of biting. After clinical improvement, conversion to oral therapy is possible. IM – intramuscular, SQ – subcutaneous, per os – oral		

Table 24.2. *Human antibiotic therapy (monotherapy)*

Antibiotic (generation)	**Route of administration**	**Treatment regimen (D/F/T)**	
		Bubonic form	**Pneumonic and septicemic form**
Aminoglycoside			
Streptomycin[Wa] (I)* *may be used to treat the pharyngeal form; Watch group intravenously only!	IM |IV|	500* / 2 / 7 *children: 15 mg/kg; possibly IV	520 / 3 / 10
Gentamicin (II)* *may be used to treat the pharyngeal form (**not recommended for use in some countries**)	IM IV	160* / 3 / 7 *children: 2,5 mg/kg	160 / 3 / 10

Table 24.2. *(Continued) Human antibiotic therapy (monotherapy)*

Antibiotic (generation)	**Route of administration**	**Treatment regimen (D/F/T)**	
		Bubonic form	**Pneumonic and septicemic form**
Tobramycin[Wa] (II)* *may be used to treat the pharyngeal form (**not recommended for use in some countries**)	IM	100* / 2 / 7 *children: 4,5–7,5 mg/kg every 12 hrs (IV, IM); pharyngeal form: 4,5–7,5 mg/kg every 12 hrs (*children*), 5–7 mg/kg every 12 hrs (*adults*) (IV, IM)	
	IV		200* / 2 / 7 *children: 4,5–7,5 mg/kg every 12 hrs (IV, IM)
Amikacin (III)* *may be used to treat the pharyngeal form (**not recommended for use in some countries**)	IM	500* / 2 / 7 *children and adults with pharyngeal form: 15–20 mg/kg every 12 hrs (IV, IM)	500* / 3 / 10 *children: 15–20 mg/kg every 12 hrs (IV, IM)
Plazomicin[Re] (V)* *may be used to treat the pharyngeal form (**not recommended for use in some countries**)	IV	15* / 1 / 7–10 *mg/kg	15* / 1 / 7–10 *mg/kg
Tetracyclines			
Tetracycline* *may be used to treat the pharyngeal form (children under 8 **only** when other options have been exhausted)	per os (p)	500* / 4 / 10 *⌊children: 10 mg/kg (max 500 mg)⌋	
Doxycycline* *may be used to treat the pharyngeal form; less effective against FI-	per os (p)	100* / 2 / 10 *⌊children (<45 kg): 2,2 mg/kg⌋	100* / 2 / 10–14 *double dose for the first; ⌊children (<45 kg): 2,2 mg/kg⌋

Table 24.2. *(Continued) Human antibiotic therapy (monotherapy)*

Antibiotic (generation)	**Route of administration**	**Treatment regimen (D/F/T)**	
		Bubonic form	**Pneumonic and septicemic form**
Omadacycline[Re] * *may be used to treat the pharyngeal form (**not recommended for use in some countries**)	per os (p)	100* / 1 / 10 *double dose for the first; children first dose 4 mg/kg (max 200 g) then 2 mg/kg (max 100 mg)	
Minocycline[Wa] * *may be used to treat the pharyngeal form (**not recommended for use in some countries**) (children under 8 **only** when other options have been exhausted)	per os (p)	100* / 1 / 10 *double dose for the first	
Eravacycline[Re] (**not recommended for use in some countries**)	IV	1* / 2 / 7 *mg/kg	
Cephalosporins			
Cefotaxime[Wa] (III)	IM	2000 / 3–4 / 7–10	3000 / 3 / 10
	IV		
Ceftriaxone[Wa] (III)	IM	2000 / 1 / 7	2000 / 2 / 7–10
	IV		
Ceftazidime[Wa] (III)	IM	2000 / 2 / 7–10	2000 / 3 / 10
	IV		
Fluoroquinolones			
Ciprofloxacin[Wa] (II)* *may be used to treat the pharyngeal form	IV	400* / 2 / 7–10 *|Children >15 yr are allowed 10 mg/kg (IV) or 15 mg/kg (per os)|	750 / 2 / 10–14

Table 24.2. *(Continued) Human antibiotic therapy (monotherapy)*

Antibiotic (generation)	**Route of administration**	**Treatment regimen (D/F/T)**	
		Bubonic form	**Pneumonic and septicemic form**
[Ciprofloxacin^{Wa} (II)* *may be used to treat the pharyngeal form **]**	per os	0,75* / 1 / 7–10 *|Children >15 yr are allowed 10 mg/kg (IV) or 15 mg/kg (per os)|	
Gemifloxacin^{Wa} (II) (**not recommended for use in some countries**)	per os	320 / 1 / 7–10	320 / 1 / 10–14
Ofloxacin^{Wa} (II)* *may be used to treat the pharyngeal form (**not recommended for use in some countries**)	per os (p)	400* / 2 / 7–10 *|children: 7,5 mg/kg (max 400 mg)|	400* / 2 / 10–14 *|children (<45 kg): 200 mg (>12 yr are allowed 4 mg/kg)|
Pefloxacin^{Wa} (II)	per os (p)	400 / 2 / 7–10	800 / 2 / 10–14
Levofloxacin^{Wa} (III)* *may be used to treat the pharyngeal form and plague meningitis (*the antibiotic of choice until the diagnosis is clear*)	IV per os	750* / 1 / 7–10 *|children (<50 kg): 8 mg/kg; pharyngeal form (neonate >28 days old): 10 mg/kg (IV)|	750* / 1 / 7–10 *|children (<50 kg): 8 mg/kg; neonate (>28 days old): 10 mg/kg (IV)|
Moxifloxacin^{Wa} (IV)* * may be used to treat the pharyngeal form and plague meningitis (*the antibiotic of choice until the diagnosis is clear*)	IV per os	400* / 1 / 7–10 *|neonate (3-23 mo): 6 mg/kg; children (2-5 yr): 5 mg/kg; children (6-11 yr or <45 kg): 4 mg/kg|	
M o n o b a c t a m s			
Aztreonam^{Re}	IM	2000 / 3 / 7–10	

Table 24.2. *(Continued) Human antibiotic therapy (monotherapy)*

Antibiotic (generation)	**Route of administration**	**Treatment regimen (D/F/T)**	
		Bubonic form	**Pneumonic and septicemic form**
Amphenicols			
Chloramphenicol* *may be used to treat the pharyngeal form and plague meningitis (**WHO is not recommended for the bubonic form**) (**not recommended for use in some countries**)	IV	25* / 4 / 7 *mg/kg; may be used to treat in children	25-35* / 3 / 7 *mg/kg; children: 12,5–25 mg/kg every 6 hrs (max 1 g)

Note:
All antibiotics are intended for the treatment of **adults** (including those who are immunocompromised), except where indicated.
First-line antibiotics are indicated in **bold.**
Data based on single messages are labelled with ||.
If it is indicated that the antibiotic can be used for treatment of pharyngeal form and plague meningitis, treatment regimens are used as for the bubonic form (except for separately specified dosages).
D – dose, mg; F – frequency of medication in day (24 hrs);
T – therapy duration.
Wa – Watch grout (AWaRe classification 2023); Re – Reserve group (AWaRe classification 2023)
IM – intramuscular; IV – intravenously;
per os – oral; (p) – parenteral (i.e. bypassing the gastrointestinal tract) administration for the first 2-3 days

It can be seen from **Table 24.2** that doxycycline is less effective in treating plague caused by FI- (i.e. non-capsulate) strains[1], which is important to consider when selecting antibiotic therapy because non-capsulate strains show the same sensitivity to doxycycline during labor-

[1] Similar information is also found for ampicillin and cefoperazone.

atory antibiotic sensitivity testing (more on this in Chapter 35) as strains that synthesise capsular antigen (capsule)[1].

In severe cases, combinations of antibiotics should be used for the first four days and then continued with a single antibiotic. The total treatment time may be increased to 14 days or more. In bubonic plague, the combination of ampicillin/sulbactam is administered intramuscularly (treatment regimen: 2000 + 1000 / 3 / 7-10); in pharyngeal plague, trimethoprim/sulfamethoxazole[2] (5 mg/kg every 8 hours) is administered intravenously or orally to children aged 2 months and older. Possible combinations of antibiotics for the treatment of severe cases of pneumonic and septicemic plague are given in **Table 24.3** (treatment regimens are arranged in cells at the intersection of the antibiotics).

During epidemics, doxycycline (treatment regimen: 0.1 / 2 / 10) and ciprofloxacin[Wa] (treatment regimen: 0.5 / 2 / 10) are used orally. In case of contact (suspected plague) and for prophylactic purposes, preparations from **Table 24.4** are used.

In case of contact of the pathogen with the mucous membranes of the eyes, nose and mouth (so-called *conjunctival infection*), a few drops of antibiotic solutions (**Table 24.4***) are injected into the eyes and nose, and the mouth and throat are rinsed with 70% ethanol.

[1] The reason for this phenomenon, according to Soviet and Russian physicians Andrey Pavlovich Anisimov (b. 1958) and Ivan Alekseevich Dyatlov (b. 1959), may be the absence of pores normally formed by capsular antigen in the macrophage membrane during the second stage of the infection process (see Chapter 13), which is the main pathway for antibiotics to penetrate the macrophage and explains the natural susceptibility of strains synthesising capsular antigen. In contrast, in the case of non-capsulate strains, antibiotics do not have access to the bacteria, which explains the lower efficacy of some antibiotics in the treatment of plague caused by non-capsulate strains.

[2] Or co-trimoxazole.

Table 24.3. *Human antibiotic therapy (combination therapy)*

Antibiotic	**Treatment regimen (route of administration)** the table shows single doses (mg); all antibiotics are administered every 6 hrs for 10 days		
II / **I**	**Ceftriaxone[Wa]**	**Ciprofloxacin[Wa]**	**Rifampicin[Wa]**
Streptomycin[Wa]	500 + 1000 (IV + IM)	500 + 500 (IV* + per os) *possibly IM	500 + 300 (IV* + per os) *possibly IM
Amikacin (**not recommended for use in some countries**)	500 + 1000 (IV + IM)	500 + 500 (IV* + per os) *possibly IM	500 + 300 (IV* + per os) *possibly IM
Gentamicin (**not recommended for use in some countries**)	80 + 1000 (IV + IM)	80 + 500 (IV* + per os) *possibly IM	80 + 300 (IV* + per os) *possibly IM
Rifampicin[Wa]	300 + 1000 (per os + IV)	300 + 500 (per os + per os*) *parenteral administration for the first 2-3 days	
Ceftriaxone[Wa]		1000 + 100 (or 200) (IV* + IV**) *possibly IM **parenteral administration for the first 2-3 days	

Note:
All combinations are for the treatment of **adults** (in CIS countries - children over 15 years old).
Wa – Watch grout (AWaRe classification 2023)
IM – intramuscular; IV – intravenously; per os – oral
In case of combinations of ciprofloxacin with rifampicin, amikacin, gentamicin and ceftriaxone, it is acceptable to replace ciprofloxacin with ofloxacin (200 mg) or pefloxacin (400 mg).

Table 24.4. *Prophylactic antibiotic therapy*

Antibiotic (generation)	**Treatment regimen** Route of administration – per os; duration of administration for prophylaxis – 48 hours after being in the area of possible infection; duration of administration in case of contact (suspected plague) – 7 days	
	Adults (mg / day)	**Children (mg/kg / day)**
Tetracyclines		
Doxycycline	100-200 / 2	2,2* / 2 *if <45 kg; if >45 kg: 100 mg
Tetracycline	500 / 4	10* / 4 *max 500 g
Omadacycline[Re] (**not recommended for use in some countries**)	300 / 1	
Minocycline[Wa] (**not recommended for use in some countries**)	100 / 2	2* / 2 *max 400 mg
Fluoroquinolones		
Ciprofloxacin[Wa] (II)	500–750 / 2	
Pefloxacin[Wa] (II)* *5 days	400 / 2	
Ofloxacin[Wa] (II) (**not recommended for use in some countries**)	200-400 / 2	7,5* / 2 *max 400 mg
Levofloxacin[Wa] (III)	500–750 / 1	8* / 2 *if < 50 kg (max 250 mg); if >50 kg: 500–750 mg
Gemifloxacin[Wa] (IV) (**not recommended for use in some countries**)	0,32 / 1	

Table 24.4. *(Continued) Prophylactic antibiotic therapy*

Antibiotic (generation)	**Treatment regimen**	
	Adults (mg / day)	**Children (mg/kg / day)**
Moxifloxacin[Wa] (IV)	400 / 1	4* / 2 *<45 kg (max 200 mg): 3-23 mo: 6 mg/kg; 2-5 yr: 5 mg/kg; 6-11 yr: 4 mg/kg; >12 yr (<45 kg): 400 mg
Cephalosporins		
Ceftriaxone[Wa] (III)* *IM, 5 days	1000 / 1	
Cefotaxime[Wa] (III)* *IM	1000 / 2	
Ceftazidime[Wa] (III)* *IM	1000 / 2	
Ansamycin		
Rifampicin[Wa]	300 / 2	
Aminoglycoside		
Gentamicin (III)* (**not recommended for use in some countries**) *IM, 5 days	80 / 3	
Amikacin (III)* (**not recommended for use in some countries**) *IM, 5 days	500 / 2	
Combinations		
Co-trimoxazole (trimethoprim/ sulphamethoxazole)	5* / 2 *mg/kg	5* / 2 *>2 mo
Rifampicin[Wa]/ ampicillin	300 + 1 / 2	
Rifampicin[Wa]/ ciprofloxacin[Wa]	300 + 250 / 1	

Table 24.4. *(Continued) Prophylactic antibiotic therapy*

Antibiotic (generation)	**Treatment regimen**	
	Adults (g / day)	**Children (mg/kg / day)**
Rifampicin[Wa]/ofloxacin[Wa] (**not recommended for use in some countries**)	300 + 200 / 1	
Rifampicin[Wa]/pefloxacin[Wa]	300 + 400 / 1	

Note:
First-line antibiotics are indicated in **bold**;
Wa – Watch grout (AWaRe classification 2023);
Re – Reserve group (AWaRe classification 2023)
IM – intramuscularly

Table 24.4*. *Antibiotic therapy for conjunctival infection*

Antibiotic	**Preparation method (eye drops)**	**Concentration, mg/ml**
Gentamicin sulfate	Dissolve the contents of the vial (80 mg) in 20 ml distilled water or dissolve the contents of the ampoule (1 ml = 40 mg) in 10 ml distilled water	4 (or 0.3% solution
Amikacin sulfate	Dissolve the contents of the vial (2 ml = 500 mg) in 125 ml distilled water	4
Ampicillin sodium salt	Dissolve the contents of the vial (500 mg) in 33.2 ml distilled water	15

Note:
Method of application: drop into the eye immediately after the alleged infection, then 3 per day for three days.
Other antibiotics active against *Y. pestis* that are soluble in distilled water or an acceptable (supplied with the medication) solvent may be used.
In the absence of antibiotics, 2% boric acid solution, 20% sodium sulphacil solution (albucid) or 0.5% levomycetin sodium succinate solution are used and injected into the eye.

Of the antibiotics listed in **Table 24.2** – **Table 24.4**, only cephalosporins can be used in *pregnant women* because they are considered relatively safe. However, aztreonam or ciprofloxacin (in the first trimester) have been reported[1] to be safe, **but** the data are from a small sample, so the risk of their use cannot be ruled out.

A number of countries recommend antibiotic therapy for pregnant women, even for prophylactic purposes, despite the risk of side effects to the embryo (**Table 24.5**).

Table 24.5. *Antibiotic therapy for pregnant women*

Antibiotic (generation)	**Route of administration**	**Treatment regimen (D/F)**
Aminoglycoside		
Streptomycin[Wa] (I)	IV IM	1000 / 2
Gentamicin (II) (**not recommended for use in some countries**)	IV IM	5* / 1 *mg/kg
Tobramycin[Wa] (II) (**not recommended for use in some countries**)	IV IM	5–7* / 1 *mg/kg
Amikacin (III) (**not recommended for use in some countries**)	IV IM	15–20* / 1 *mg/kg
Plazomicin[Re] (V) (**not recommended for use in some countries**)	IV	15* / 1 *mg/kg
Fluoroquinolones		
Ciprofloxacin[Wa] (II)	IV	400 / 3
	per os	500 / 3
		750 / 2
Ofloxacin[Wa] (II) (**not recommended for use in some countries**)	per os	400 / 1
Levofloxacin[Wa] (III)	IV per os	750 / 1

[1] Шер С. А., Островская А. В. *Выбор антибактериальных препаратов при беременности*. Педиатрическая фармакология. 2011; 8(1):84–89.

Table 24.5. *(Continued) Antibiotic therapy for pregnant women*

Antibiotic (generation)	Route of administration	Treatment regimen (D/F)
Moxifloxacin[Wa] (IV)	IV per os	400 / 1
Note: D – dose, mg; F – frequency of medication in day (24 hrs) Wa – Watch grout (AWaRe classification 2023); Re – Reserve group (AWaRe classification 2023) IM – intramuscular; IV – intravenous; per os – oral		

New drugs cannot be overlooked. *LpxC inhibitors*, which inhibit the enzyme that catalyses the first stage of lipid A synthesis[1], have shown efficacy against *Y. pestis*, and the drug LPC-069 even showed[2] efficacy in the treatment of bubonic plague in mice at the level of doxycycline. The treatment regimen involves intravenous administration of 200 mg/kg of the drug every 8 hours, which is quite radical (remember: for children weighing less than 45 kg, doxycycline is administered orally at 2.2 mg/kg every 12 hours). Perhaps a molecule with greater efficacy will enter clinical practice. Another class of drugs, *cationic antimicrobial peptides* (remind: they have already been encountered in Chapter 13, being the first barrier that the plague microbe encounters when it enters the human body), which may also be active against *Y. pestis*. At least in vitro studies show[3] their efficacy in combination with antibiotics such as tetracycline and minocycline.

Another promising area of research is the so-called "repurposing of drugs". In particular, a research[4] of the ability of the anticancer drugs

[1] I.e., uridine diphosphate-3-O-(hydroxymyristoyl)-N-acetylglucosamine deacetylase or LxpC.

[2] Lemaître N., Liang X., et al. *Curative treatment of severe Gram-negative bacterial infections by a new class of antibiotics targeting LpxC*. mBio. 2017; 8(4):e00674–17; DOI: 10.1128/mBio.00674-17.

[3] Cote C. K., Blanco I. I., et al. *Combinations of early generation antibiotics and antimicrobial peptides are effective against a broad spectrum of bacterial biothreat agents*. Microb Pathog. 2020; 142:104050; DOI: 10.1016/j.micpath.2020.104050.

[4] Gur D., Chitlaru T., et al. *Screening of an FDA-approved library for novel drugs against Y. pestis*. Antibiotics (Basel). 2021; 10(1):40; DOI: 10.3390/antibiotics10010040.

bleomycin sulfate and streptozocin to inhibit the growth of *Y. pestis* has shown that the standard doses of these drugs, usually used in oncological practice, are several times higher than the minimum suppressive concentration, i.e. the concentration at which the growth of the pathogen is inhibited (more on this in Chapter 35). Moreover, they were also effective against strains resistant to ciprofloxacin and doxycycline, which makes their further investigation very promising. However, it should not be forgotten that results obtained *in vitro* ("on dishes") are not always reproducible *in vivo*: remind that some drugs that inhibit the growth of infraction-free strains are ineffective in treatment (**Table 24.2**). And in this regard, the importance of adequate (correct) antibiotic therapy should be emphasised, as the following case illustrates:

In 1987[1], an 8-year-old American Indian boy was admitted to a small regional hospital with three day hystory of fever and generalised malaise. A day later he developed marked abdominal pain accompanied by an elevated white blood cell count of 29.2 x 10^9/L [4.8-10 x 10^9/L] / ***29,200 cells/μL*** *[4,800-10,000 cells/μL], with 30% polymorphonuclear leukocytes [40-60%] and 57% bands [3-5%]. Appendicitis was initially suspected, but a laparotomy revealed a normal appendix, as well as an easily reducible sigmoid volvulus, marked hepatosplenomegaly (i.e., unnatural enlargement and diffuse liver changes) and numerous enlarged retroperitoneal lymph nodes with Gram-negative bacteria in the biopsy specimen!*

The patient was transferred to a specialised clinic due to deterioration of his condition: severe respiratory distress, disseminated intravascular coagulation and acute renal failure developed. Antibiotic therapy included intravenous administration of chloramphenicol and cefotaxime. At this time, cultures from biopsy specimens were identified as Y. pestis. Fluorescent antibody testing further confirmed the diagnosis of plague.

After two weeks, the patient's condition improved (respiration was normal, blood coagulation system and kidney function normalised), fever was not present for about 10 days, so antibiotic therapy was stopped. However, two days later, the patient again became febrile and

[1] Moreno A. J., Reeves T. A., et al. *Imaging in plague*. Eur J Nucl Med. 1987; 13: 315–317

had right sidedabdominal tenderness. ^{111}In labeled leukocyte scintigraphy (300 μCi) did not reveal any abnormalities within the abdomen. Computed tomography showed a spherical shaped soft tissue density (at the laparotomy site), which was identified by Gallium scan as a conglomerate of necrotised lymph nodes (suppurative buboes). Intravenous antibiotic therapy was resumed and when the fever subsided after five days, the patient was switched to oral tetracycline, after which he recovered completely.

Usually, adequate antibiotic therapy will sterilise abscessed buboes and resorb them, but inadequate antibiotic therapy may result in relapse, which is what happened in the boy, because, as can be seen from **Table 24.2**, chloramphenicol is not recommended for the treatment of bubonic plague and cefotaxime is not effective for children.

Along with etiotropic therapy, ***pathogenetic therapy*** is carried out, which is aimed at detoxification, as well as correction of haemodynamic and metabolic disorders that are observed in the development of toxic shock syndrome. Intensive therapy is based on neutralisation and excretion of toxins, management of hypoxaemia (i.e. reduced oxygen content in the blood), replenishment of electrolyte disorders, correction of haemostasis and maintenance of normal functioning of vital organs. The aim is to maintain the main indicators at the following level (for adult):

Central venous pressure	8–12 mmHg
Mean arterial pressure[1]	≥ 65 mmHg
Diuresis	≥ 0,5 ml kg/hour / ≥ 0,5 cc kg/hour
Saturation (including in case of haemotransfusion)	≥ 70%

It should be remembered that a drop in blood pressure indicates the generalisation of the process, i.e. the beginning of a secondary septicemic form of the disease. Pathogenetic therapy includes normalisation of water-electrolyte balance (infusion support), administration of corticosteroids, cardiac and respiratory support (if necessary). Infusion

[1] Mean arterial pressure (MAP) is calculated by the **formula**:
MAP = (1/3 x SBP) + (2/3 x DBP), where SBP – systolic blood pressure, mmHg; DBP – diastolic blood pressure, mmHg.

therapy is carried out with colloids (maintain the volume of circulating blood) and crystalloids (saline solutions) in a ratio of 1:1. Albumin, hydroxyethyl starch, 4% gelofusine are used as the former, and saline solution (i.e. 0.9% sodium chloride solution), 5-10% glucose solution, Rigner's solution are used as the latter.

Corticosteroids (in particular, hydrocortisone in a dosage of 240-300 mg/day for 5-7 days) can improve haemodynamics. It is possible to use plasmapheresis, haemoplasmafiltration, as well as transfusion of red cell mass.

Vasopressors are used to maintain the cardiovascular system. Norepinephrine is administered at 2-5 mcg/min, bringing the value of mean arterial pressure to target values. Dopamine is administered in 5 ml (4% solution), adding it to 200 ml of saline solution (possibly combined administration of noradrenaline and dopamine).

In case of low cardiac output syndrome, which is characterised by a decrease in cardiac index (CI)[1] to 2 L/min/m^3 or less [2.5-4 L/min/m^2], and systolic blood pressure below 90 mmHg, the inotrope dobutamine should be used at a dose of 250 mg per 500 ml of saline solution. Combination with vasopressors at a calculated dosage of 2.5-40 mg/kg/min is possible.

It is necessary to constantly monitor diuresis, and in case of its disturbance (decrease in urine output) loop diuretics (furosemide, etacrynic acid) are used. The use of osmotic diuretics (mannitol) may aggravate cardiovascular insufficiency.

During intensive care, the patient is provided with a daily caloric intake of 104.6-125-52 kj/kg / 25-30 kcal/kg body weight, and normal blood glucose levels are maintained.

Patients are discharged after complete recovery, but not earlier than 4 weeks in case of bubonic form and not earlier than 6 weeks in case of pneumonic form, as well as three times negative result of bacteriological examination of sputum or blood samples (every day after antibiotic

[1] An indicator of the pumping function of the heart, expressed as the ratio of cardiac minute volume (L/min) to body surface area (m^2), where cardiac minute volume is calculated by the formula: SV x HR (SV – stroke volume, ml; HR – heart rate, min).

withdrawal) or two times examination of bubonic puncture. Medical follow-up after discharge is carried out for three months.

25 RESISTANCE

On 2 August 1996[1]*, a 21-year-old herdsman caught, skinned and ate a sick hare in Qayü village, Shannan Prefecture in Tibet*[2]*. Two days later, he developed a fever with left axillary lymphadenopathy (bubonic plague), followed the next day by headache, chills, chest pain and cough with bloody sputum. On 9 August he died without any treatment. However, he was cared for for some time by friend, a veterinarian (unprotected), who by 11 August had developed symptoms of primary pneumonic plague. He was given unified combination treatment intramuscular injection streptomycin (1000 mg, twice daily), oral trimethoprim/sulfamethoxazole (1000 mg, three times daily); oral tetracycline (500 mg, three times daily). Treatment continued until 19 August, when the patient died. His wife, who had had symptoms of primary pneumonic plague since 16 August, despite similar treatment, died the same day. A village physician who was in close contact with her also became infected - symptoms started on 17 August and he died on 22 August.*

A strain isolated from a veterinarian's wife, Y. pestis strain S19960127 had a mutation in the chromosomal *rpsL* gene that encodes ribosomal S12 protein, which resulted in an amino acid substitution of lysine (Lys) for arginine (Arg) at site 43 (K43R) in the RpsL protein, and made the strain resistant to streptomycin. Of course, all patients received more than just streptomycin, so there is no certainty that death

[1] Dai R., He J., et al. *A novel mechanism of streptomycin resistance in Yersinia pestis: Mutation in the rpsL gene*. PLoS Negl Trop Dis. 2021; 15(4):e0009324; DOI: 10.1371/journal.pntd.0009324.

[2] It is located in the Qinghai-Tibet natural foci.

was due to streptomycin resistance rather than due to severity of the course or lost time (in the pneumonic form, treatment should be started as early as possible, as it may not be effective after 15-20 hours).

In Chapter 7, we discussed that *Y. pestis* may contain conjugative plasmids that allow it to realise the type IV secretion system, i.e. to acquire genes, which may include antibiotic resistance genes. For example, plasmid IP1203 (150 Kb) carries streptomycin resistance genes. On the other hand, antibiotic resistance, as we have seen in the above case, can be the result of gene mutation. The cause of this mutation is currently unknown, but it is definitely not caused by the use of streptomycin, since, for example, in Madagascar (**Table 25.1**; 2013) streptomycin-resistant strains were isolated from patients' sputum before streptomycin was administered. It is also of interest that all patients in Madagascar treated similarly to patients from Tibet survived, whereas in Tibet the lethality was absolute (the strains had different mutations in the *rpsL* gene)[1].

Of course, the problem of antibiotic resistance in the plague microbe is not as acute as in other bacteria, but the existence of antibiotic-resistant strains (**Table 25.1**) is an alarm bell that threatens to complicate both the control and treatment of infection. Artificially created strains (e.g. *Y. pestis* strains KM219, KM244, KM245 and KM258) with resistance to kanamycin, which pose a threat as a possible target of bioterrorism, should not be forgotten. On the other hand, according to Igor V. Domaradsky, he (with colleagues) obtained antibiotic-resistant strain *Y. pestis* strain EV, on the basis of which a vaccine was created, the advantage of which was the possibility of use "*simultaneously with emergency prophylaxis with antibiotics (the conventional vaccine dies in this case)*"[2].

To solve the problem of antibiotic resistance, which has already become relevant for plague, alternative methods of treatment are being developed, among which the use of bacteriophages or *phage therapy* plays an important role. Earlier we have already mentioned lytic pha-

[1] This is in favour of the fact that streptomycin resistance was not the cause of death in the Tibetan patients.

[2] Created as part of the "fifth" problem (bacteriological defence) circa 1970-1973, data not published; cited by: Домарадский И.В. *Перевёртыш (рассказ «неудобного человека»)*. М.: [б. и.], 1995. 176 с.

ges – bacterial viruses with high specificity, whose life cycle most often ends with lysis of the bacterial cell wall (respectively, with its death) and the "emergence" of new bacteriophages.

Table 25.1. *Antibiotic-resistant strains of Y. pestis*

Year	**Location**	**Carrier**	**Mechanism**	**Resistance**	***Y. pestis* strain**
1987	Andapa (Madagascar)	human	*rpsL* gene mutation	streptomycin	12/87
1995	Ambalavao (Madagascar)	human	IP1202 plasmid	multiple[1]	17/95 (IP275)
1995	Ampitana (Madagascar)	human	IP1203 plasmid	streptomycin	16/95
1996	Qayü (Tibet)	human	*rpsL* gene mutation	streptomycin	S19960127
1998	Antananarivo (Madagascar)	rat	pIP2180H plasmid	doxycycline	IP2180H
1999	Kazakhstan	human	?	levomycetin	?
2013	Faratsiho (Madagascar)	human	*rpsL* gene mutation	streptomycin	56/13
2013	Faratsiho (Madagascar)	human	*rpsL* gene mutation	streptomycin	59/13
1960-2007	Mongolia	marmot	?	Multiple[2]	MNG3122

Примечание:

[1] streptomycin, ampicillin, kanamycin, spectinomycin, sulfonamides, tetracycline, minocycline and chloramphenicol (sensitive to sulfamethoxazole/ trimethoprim).

[2] gentamicin, tetracycline, doxycycline, chloramphenicol

The history of bacteriophages began in 1895, when Percy Faraday Frankland[1] conducted experiments[1] on co-culturing *Salmonella typhi* and

[1] 1858–1946; the British chemist. Fellow of the Royal Society of London (1891). Together with his wife Grace Frankland, he isolated the first pure culture of nitrifying (i.e. ammonia-oxidising) bacteria.

Escherichia coli in water and found that when cultured in water from the River Thames, the former died rapidly, for which he could not find an explanation. In 1896 Ernest Hanbury Hankin[2], wishing to research the number of *Vibrio cholerae* in the river Ganges, found that 1 ml of water at the entrance to the city of Agra contained 100,000 bacterial cells, whereas at the exit there were only 90 cells. What's more: "*Unboiled Ganges water kills the cholera microbe in less than 3 hours. Boiled water does not have the same effect. Well water, on the other hand, is a good medium for this microbe, whether boiled or filtered*"[3]. This phenomenon was also not explained at the time. A similar phenomenon was observed two years later by Nikolay Fyodorovich Gamaleya[4], when the anthrax microbe (*Bacillus anthracis*) cultured in distilled water died rapidly, and the liquid acquired the ability to lysis other cultures of the anthrax microbe. He attributed what he saw[5] to the action of bacteriolysin, produced by the decay of the bacteria. Many scientists would describe similar phenomena, and it was not until 1915 that Frederik Twort[6] reported[7] an agent capable of infecting staphylococci, having already identified it as a virus, and two years later Félix d'Hérelle[8], having discovered the virus

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[1] Frankland P. *Ueber das Verhalten des Typhusbacillus und des Bacillus coli communis im Trinkwasser*. Zeitschrift für Hygiene und Infektionskrankheiten 1895; 19(1):393–407; DOI: 10.1007/bf02216789.

[2] 1865–1939; the British physician and bacteriologist. Author of works on cholera and malaria, as well as on the properties of opium and cobra venom. He studied the fauna of India (mainly birds) and Mughal architecture.

[3] Hankin E. H. *L'action bactericide des eaux de la Jumna et du Gange sur le vibrion du cholera*. Annales de l'Institut Pasteur. 1896; 10:511–523.

[4] Николай Фёдорович Гамалея, 1859–1949; Ukrainian and Soviet physician, bacteriologist, epidemiologist. Corresponding Member (1939) and Honorary Member (1940) of the USSR Academy of Sciences, Academician of the USSR Academy of Medical Sciences (1945). Laureate of the Stalin Prize (1943). Author of works on infectious diseases, made a significant contribution to immunology and developed the doctrine of immunity. Scientific director (1930-1938) of the Central Institute of Epidemiology and Microbiology (now the N. F. Gamaleya National Research Center for Epidemiology and Microbiology).

[5] Gamaleya N. F. *Bacteriolysins – ferments destroying bacteria*. Russian Archives of Pathology, Clinical Medicine, and Bacteriology. 1898; 6:607–613 (in Russian)

[6] 1877–1950; the British physician, bacteriologist, author of works on infectious diseases. Fellow of the Royal Society of London (1929)

[7] Twort F. W. *An investigation on the nature of ultra-microscopic viruses*. Lancet. 1915; 186(4814):1241–1243; DOI: 10.1016/S0140-6736(0)20383-3.

[8] 1873–1949; the French and Canadian enthusiast who had no specialised training but became the father of phagology.

of the causative agent of dysentery (*Shigella spp.*)[1], called it a "bacteriophage" (Greek φᾰ́γω – "devouring"; **Figure 25.1**). He also successfully used bacteriophages during the plague and cholera epidemics in Egypt (1921) and proposed their use in the treatment of infectious diseases.

Around the same time, Félix d'Hérelle met Georgiy Grigorievich Eliava[2] and found in his person a colleague, thanks to which in 1923 in the city of Tbilisi (Georgia, USSR) appeared the Institute of bacteriophage (Félix d'Hérelle himself lived in Tbilisi for several years, but against the background of repressions of the 1930s hurriedly left the USSR). The emergence of antibiotics, however, neglected the idea of phage therapy, and its research continued only in the USSR, which played an important role during World War II, when Magdalina Petrovna Pokrovskaya[3] successfully used bacteriophages to treat wounds of soldiers[4].

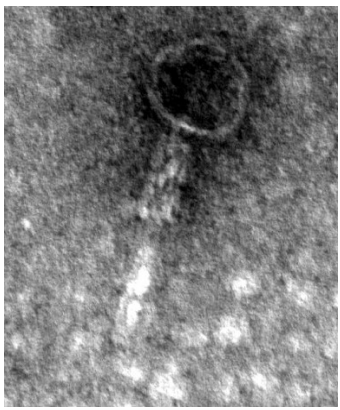


Figure 25.1. *A lytic bacteriophage active against Y. pestis*

However, bacteriophages have not been used in the treatment of plague[5], with the exception of the experience of

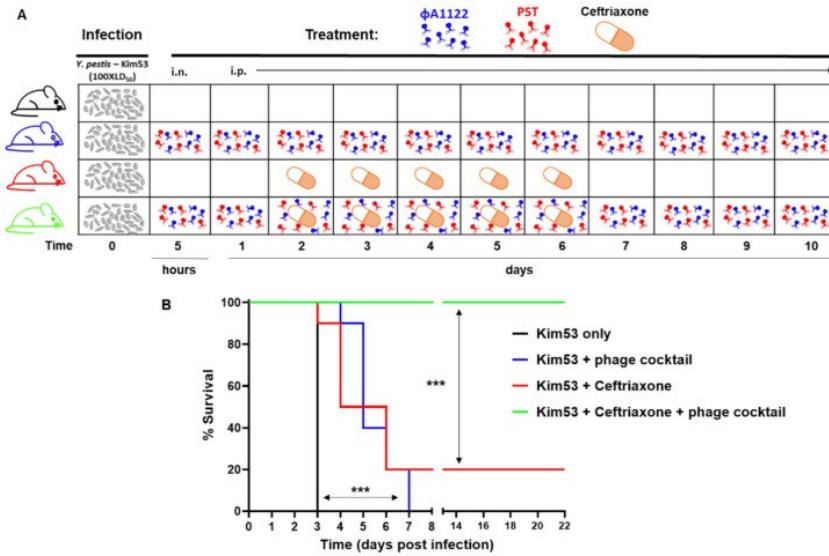
[1] D'Hérelles F. Sur un microbe invisible antagoniste des bacilles dysentériques. Comptes Rendus Academie des Sciences Paris. 1917; 165:373–375.

[2] გიორგი ელიავა, Георгий Георгиевич Элиава, 1892–1937; the Georgian and Soviet physician, bacteriologist, founder of phage therapy in the USSR and founder of the Institute of Bacteriophage (now the George Eliava Institute of Bacteriophage, Microbiology and Virology). As an enemy of the people he was shot 09.07.1937; rehabilitated by the Military Collegium of the Supreme Court of the USSR 22.08.1957 "for lack of corpus delicti".

[3] Магдалина Петровна Покровская, 1901–1980; the Russian and Soviet physician, bacteriologist, epidemiologist. Honoured Scientist of the RSFSR. Deputy Director for scientific work at the Research Anti-Plague Institute of the Caucasus and Transcaucasia (now the Stavropol Research Anti-Plague Institute). Author of the famous book Treatment of Wounds with Bacteriophage (1941), which describes the experience of using bacteriophages in the treatment of wounds (including purulent wounds) during the war. She isolated an anti-plague bacteriophage named in her honour.

[4] Покровская М. П., Каганова Л. С., и др. *Лечение ран бактериофагами*, Москва – Ленинград: Государственное изд-во медицинской литературы «Медгиз», 1941, 60 с.

[5] Currently, lytic bacteriophages are only used for laboratory diagnosis of plague.



Puc. 25.2. *Treatment (A) and survival curves (B) of mice aerogenically infected with Y. pestis strain KIM53. Control mice (black) that did not receive treatment died on day 3. Mice receiving treatment had better survival by day 5 with phagotherapy (blue), however, all died by day 7, whereas 20% of mice on antibiotic therapy (red) survived. Mice receiving the combination treatment survived 100% of the time. Subcutaneous injection of ceftriaxone was used as antibiotic therapy, and intranasal or intraperitoneal injection of a cocktail of phages φA1122 and PST was used as phagotherapy (by Vagima Y., 2022)*

Félix d'Hérelle, which resulted in the recovery of four bubonic plague patients[1]. Experiments[2] carried out on animals have shown that regardless of the form of plague and the method of bacteriophage administration (intranasal, intraperitoneal, intravenous), even in the case of cocktail (i.e. a mixture of different strains of bacteriophages), phagotherapy does not prevent bacteraemia, but only postpones the death of animals,

[1] D'Herelle F. *Essai de traitement de la peste bubonique par le bacteriophage*. Press. Med. 1925; 33:1393–1394.

[2] Filippov A. A., Sergueev K. V., et al. *Bacteriophage therapy of experimental bubonic plague in mice*. Adv Exp Med Biol. 2012; 954:337–348; DOI: 10.1007/978-1-4614-3561-7_41; Vagima Y., Gur D., et al. Phage Therapy Potentiates Second-Line Antibiotic Treatment against Pneumonic Plague. Viruses. 2022; 14(4):688; DOI: 10.3390/v14040688.

while the bacteriophage accumulates in the organs and blood of the animal after 30 minutes.

On the other hand, the combination of phage therapy and antibiotics (at a dose insufficient to cure) ensures survival in pneumonic plague (**Figure 25.2**), which is particularly significant in view of the difficulty of treating this form of the disease. In addition, such combination therapy is possible with second-line drugs (in particular ceftriaxone).

26 PREVENTION

A prophet is not without honor except in his own town, among his relatives and in his own home…

Mark 6:4

Prevention (specific and non-specific) is an effective way to control infectious diseases. As Kitasato Shibasaburō wisely noted: "*In fighting plague, quarantine... however strict and complete, cannot be solely relied on*".

Non-specific prevention includes a set of measures aimed at limiting human contact with the plague microbe (**Figure 26.1**). These include, on the one hand, public education, restrictive measures, and special training of veterinary and medical staff. We have seen from the examples above that people living in natural foci may eat raw marmot organs, and physicians are not always able to recognise plague in both humans and animals (especially when it comes to such a rare phenomenon as primary pneumonic plague in dogs). On the other hand, an important component of non-specific prevention is epidemiological surveillance, which in most countries of the world is limited to timely detection of cases. However, in countries where ancient foci are located (CIS, China and Mongolia), surveillance is traditionally carried out (**Figure 26.2** and **Figure 26.3**), which includes observation of the course of epizootics – identification of the causative agent, as well as counting and timely



Figure 26.1. *Poster in Ea Vi village (Đắk Lắk province) calling for the killing of rats to fight plague (left) and a poster on the Ho Chi Minh City to Dalat road warning of a plague foci (right), Vietnam, 2004*
Courtesy of Dr. Victor Vasilyevich Suntsov, PhD, DSc

regulation of the number of carriers and vectors[1]. Surveillance is carried out by special teams that take environmental samples and capture animals (carriers and vectors). In emergency cases, disinfection (i.e. destruction of the pathogen), disinsection (i.e. destruction of vectors[2]) and deratisation (i.e. destruction of carriers[3]) are carried out. Physical and chemical methods are used for disinfection. The former include incineration, boiling, steam and ultraviolet treatment (etc.), the latter include the use of chlorine-containing (bleaching powder) and oxygen-containing (hydrogen peroxide) preparations, aldehydes (glutaric aldehyde) and alcohols (ethanol), as well as any substances active against

[1] In case of anthropurgic foci, emergency disinfection measures, including killing of carriers and their examination, are carried out (**Figure 26.4**). Interestingly, a large rodent population can sustain a foci for many years. In Vietnam, for example, foci existed until 2005 because cities were infested with rats.

[2] Interestingly, as far back as ancient Egypt, people were advised to cover their bodies with *Moringa oleifera* oil to protect against tick, flea and mosquito bites (Ebers Papyrus, c. 1550 BC).

[3] In this case, rodents.

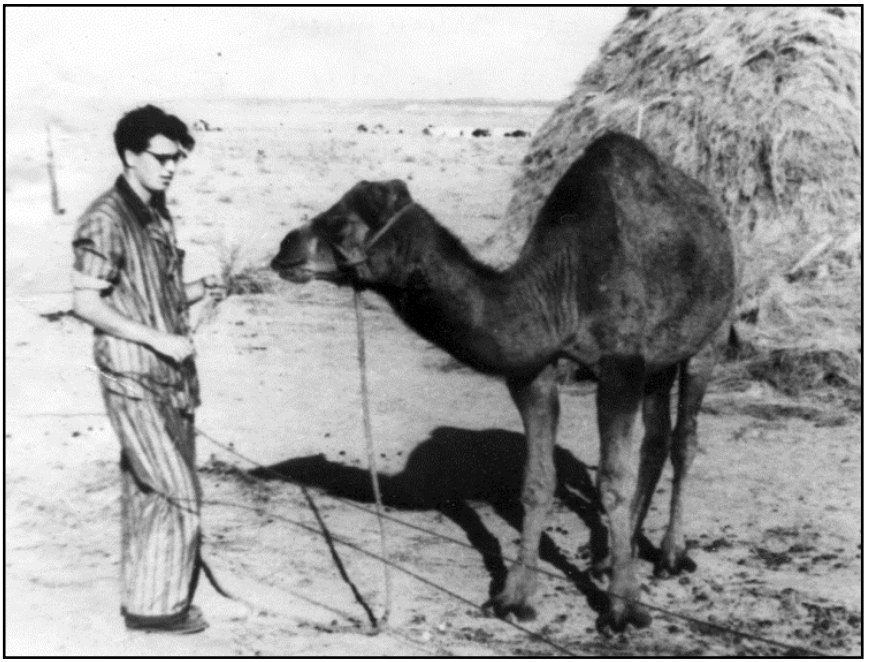


Figure 26.2. *Field work in a plague foci (1954). The photograph, courtesy by Prof. Andrey Pavlovich Anisimov shows his father, Prof. Pavel Ivanovich Anisimov[1], feeding a local camel during one of the "camel expeditions"[2]. As we remember, camels are carriers of plague, but you can get infected only from their meat and milk, not heat-treated. Pay attention to the specialist's clothing (the so-called pyjamas), which will be discussed in more detail in Chapter 28*

[1] Павел Иванович Анисимов, 1929–2019; the Soviet and Russian physician, bacteriologist. Director of the Russian Anti-Plague Institute "Microbe" (1973-1988). One of the founders of Russian genetics of especially dangerous infections, creator (with his collaborators) of diagnostic and prophylactic preparations, in particular, diagnostic plague bacteriophage L-413C and cholera bivalent chemical tablet vaccine (patent RU 2080121 C1, 27.05.1997).

[2] The aim of the expeditions carried out by the staff of the Nebit-Dag branch of the Turkmen Anti-Plague Station (now the Balkanabad branch of the Preventive center on particularly dangerous infections) was to determine the role of camels in plague epidemiology, to study their susceptibility to plague, the mechanism of infection in natural and experimental conditions, to clarify the pathogenesis and clinic.



Figure 26.3. *Research of the population of Himalayan marmot (Marmota himalayana) in the natural plague hotspot of Sichuan Province (Southwestern China)*



Figure 26.4. *A mass research of dead harbour rats in Japan during the third plague pandemic. Such measures made it possible to identify Y. pestis before the first person became ill during an importation into the city of Yokohama in 1902 (by Shibasaburo K., 1906; courtesy of the* 学校法人北里研究所北里柴三郎記念博物館*)*

the pathogen[1]. When carrying out disinsection and deratisation measures, prevention of pollution of nature and preservation of biological diversity are of paramount importance.

The epidemiological potential (EP) of a natural foci (i.e. the risk of human infection) is assessed in terms of the area of the epizootic, its intensity, carrier (and vector) density, and criteria such as the possibility of human contact with the carrier (or vector), hunting of carriers, proximity of human settlements, poaching, cats and dogs living in dwellings. Typically, computer programmes are used for this, but it can be calculated by formula:

$$EP = (S \cdot Y + K(P + M) \cdot V \cdot \sum B_i \ , \% \ ,$$

where S – epizootic area, Y – epizootic intensity, K – fraction of the foci area occupied by settlements of the main carrier, P – density of rodents or their settlements per 1 km², M – flea stock per 1 km², V – virulence of the pathogen strains, B_i – various additional criteria (human contact with fleas, presence of fleas and rodents in the dwelling, presence of cats and dogs in the dwelling, hunting of carriers, poaching, presence of camels, etc.).

It should be understood that the case of infection of even one person is a public health emergency, but depending on the development of the infrastructure of the territory and the intensity of the epidemiological situation in natural foci, the emergency may have different significance (**Table 26.1**).

Chinese poster 见害就除，积极防病，人人与 病害作斗争 ► *(Everyone struggles against diseases and pests), May, 1960 Beginning in the 1950s, the Chinese government mounted the Patriotic Health Campaigns to improve sanitation and public health. In 1958 the campaigns focused on the "the four pests": rats, sparrows, flies, and mosquitoes. Later sparrows were removed from the list, and fleas and lice added. Courtesy of the National Library of Medicine*

[1] It should be remembered that even the extermination of the main carrier cannot lead to the elimination of the natural focus, an example of which is the extermination of the little ground squirrel in the territory of the Caspian North-Western steppe focus in 1960. Already in 1972, the epizootic activity of the centre resumed.



見害就除，积极防病
人人与病害作斗爭！

***Table 26.1**. Criteria for emergency levels*

Criteria	Emergency level			
	Local	**Regional**	**National**	**International**
Area of epizootics (% of total enzootic area[1])	<0,01	0,01–0,1	1–5	>5
Number of population at high risk of infection (% of total population at risk of infection)	<1	1–10	11–40	>40
Value of epidemiological potential (EP,%)	<10	10–30	30–50	>50
Preparedness of health facilities	unsatisfactory (<4)			
Risk of removal (importation)	<10	10–25	25–40	>40

The final evaluation is carried out by the value of the final indicator, which is equal to the sum of all criteria (as a fraction of one) divided by their number (i.e. 5). Thus, it is obtained that the value of the final indicator for the local level is ≤1, for the regional level – from 1.1 to 3, for the national level – from 3.1 to 4, and for the international level – from 4.1 to 5.

"Fireworks at Ryōgoku" ►
ukiyo-e prints 98 from the collection "One Hundred Famous Views of Edo" by Utagawa Hiroshige. Courtesy of the Brooklyn Museum.
The print depicts the Ryōgoku Bridge while enjoying the evening coolness on the Sumida River, a custom accompanied by fireworks and established in 1733 by Shogun Tokugawa Yoshimune as a rite of purification to dispel the evil spirits of plague and famine that swept Japan at the time. Interestingly, fireworks can actually be an effective means of combating plague. For example, Wu Lien-teh used this method during the Manchurian plague epidemic

[1] That is, where there may be an outbreak of disease, such as in a natural foci or in a neighbouring area.


名所江戸百景
両国花火

Specific prevention or immunoprevention is the most effective way to combat infectious diseases. The history of the first plague vaccine begins with Waldemar (Vladimir Aaronovitch) Haffkine[1], who, distinguished from other graduates of the Imperial Novorossiysk University by his brilliant abilities and diligence, but could not find a decent job in the Russian Empire because he did not want to convert from Judaism to Orthodoxy. Thanks to his teacher, Élie Metchnikoff (Ilya Ilyich Mechnikov)[2], in 1889 he found a job at the Pasteur Institute in France, where he worked on the development of vaccines based on strains of bacteria killed by heating. The first development, a cholera vaccine, was tested on himself in 1892 by administering a quadruple dose. Although the medical community was initially very sceptical of the vaccine, the very next year the vaccine saved India from a cholera epidemic. In 1896, a plague epidemic broke out and Waldemar Haffkine was urgently summoned to the Bombay (India). On 10 January 1897 the developed plague vaccine was again tested on himself, also proving to be effective. It was then tested on local prisoners and approved for use. The first plague vaccine[3], called *Haffkine's lymph*, defeated the epidemic[4].

It was prepared as follows: six-day-old stock cultures of *Y. pestis*, grown at 28°C / 82.4°F with frequent shaking, were killed by heating to 65°C / 149°F for one hour. It should be noted, however, that killed (inactivated) vaccines, although stable during storage, induce very short postvaccinal immunity, do not protect against the pneumonic form of plague and are quite reactogenic (i.e. cause side effects). Therefore, al-

[1] Владимир Ааронович Хавкин, 1860–1930, named Mordecai-Wolff at birth (Yiddish מרדכי-וואלף); the Ukrainian and British physician of Jewish origin, immunologist, bacteriologist, prominent figure in the Zionist movement. The Plague Research Laboratory, which he opened in 1899 in Bombay has been named after him since 1925 (Haffkine Institute for training, research and testing).

[2] 1845–1916; the Ukrainian and French physician, bacteriologist, physiologist, pathologist, father of the theory of innate immunity. Honorary member of the St. Petersburg Academy of Sciences (1902). Laureate of the Nobel Prize in Physiology or Medicine (1908).

[3] To be fair, we should point out that even Daniil Samoylovich Samoylovich-Sushchinsky inoculated himself with bubonic pus, which is in fact the first documented vaccination against the plague.

[4] It should be noted that at that time even those who were already sick were vaccinated, which is now considered ineffective.

ready in the 30s of the last century Lev Aleksandrovich Zilber[1] attempted to increase the effectiveness of the vaccine. For this purpose, he heated the microbial suspension in the presence of concentrated sucrose solutions so that the proteins of the bacterial cell did not undergo significant changes due to the sharp denaturation. In this way the AD vaccine (Russ. АД-вакцина) was obtained, but it did not give absolute protection either.

Currently, Commonwealth Serum Laboratories, Ltd (Victoria, Australia) produces a killed whole-cell vaccine[2] containing heat-killed bacteria *Y. pestis* resuspended in physiological solution containing 0.5% phenol to a concentration of 3x109 microorganisms per ml.

At the beginning of the last century, *live vaccines* based on attenuated (i.e. artificially attenuated) strains began to be developed. The first vaccines were derived from the Tjiwidej strain[3] lacking the pCad plasmid and the EV76 strain lacking the *pgm*-region. The first strain isolated by L. Otten[4] in Java, Indonesia, lost virulence after storage on slanted agar with serum for four months. The second strain, isolated by Georges Girard[5] and Jean Robic[6] on the island of Madagascar and named after a girl who died of plague, lost virulence as a result of monthly passages (i.e. crossings) for three years (EV is the initials of the girl and 76 is the

[1] Лев Александрович Зильбер, 1894–1966; the Russian and Soviet physician, bacteriologist, virologist, immunologist. Academician of the USSR Academy of Medical Sciences (1945), laureate of the Stalin Prize and the USSR State Prize in Science and Technology (1967, posthumously). Discoverer of the tick-borne encephalitis virus, one of the creators of the theory of the viral nature of cancer. Co-author of the first Soviet textbook on virology. Participant in the elimination of the plague epidemic in Nagorno-Karabakh (1930). Three times in prison for refusing to participate in the creation of bacteriological weapons.

[2] It is reported that the prototype of this vaccine was the KWC vaccine, first produced in the USA in 1946 as an army vaccine, and its subsequent improvements led to the creation of the United States Pharmacopeia (USP) vaccine, which consists of *Y. pestis* strain 195/P bacteria grown on artificial media, inactivated with formaldehyde and preserved in 0.5% phenol. It was used until 1999 to vaccinate laboratory staff working with *Y. pestis* and persons living in the territory of foci.

[3] The name Tjividej is also encountered; it was with this strain that the Ymt was discovered (discussed in more detail in Chapter 3).

[4] ?-?; the French physician, bacteriologist.

[5] 1888–1985; the French physician, bacteriologist. From 1922 to 1940 he was director of the Institute of Bacteriology of Madagascar (now Institut Pasteur of Antananarivo). From 1941 he was head of the anti-plague service at the Institut Pasteur.

[6] 1893–1968; the French military physician.

number of passages (i.e. crossings) on nutrient dense media required for attenuation). It should be noted that crossing on nutrient media significantly weakens the strain, whereas crossing through an animal may allow it to "recover". This is the effect we saw in the case of the soldier (Chapter 20), when the diagnosis of plague meningitis could only be confirmed using a bioassay animal. However, in the situation of attenuated strains, "comeback" is not possible because they have already lost fragments of the genome.

The EV76 strain was introduced to the USSR in 1936, when Georges Girard transferred it to the Anti-Plague Institute "Microbe". In 1941, Mikhail Mikhailovich Faybich[1] (with colleagues) at the Scientific-Research Institute of Epidemiology and Hygiene of the Red Army (now – Branch Office of 48th Central Scientific Research Institute of the Ministry of Defense of the Russian Federation) on its basis obtained the most immunogenic line NIIEG (Russ. НИИЭГ), which is still used to create vaccines. The undoubted advantage of vaccines based on EV76 and EV line NIIEG strains is the possibility of immunity formation already on the seventh day after vaccination, which lasts up to a year. However, as we remember, in some diseases, in particular haemochromatosis, the use of such a vaccine can be fatal (Chapter 18).

Shortly before that, in 1934, at the Stavropol Research Anti-Plague Institute, Magdalina P. Pokrovskaya developed a vaccine based on her own attenuated strain of AMP ("avirulent mutant pestis"; Russ. АМП), obtained as early as 1929 by exposure of a bacteriophage to a culture of the plague microbe. In contrast to the Tjiwidej and EV76 strains, she obtained her strain purposefully. The fact is that colonies of the plague microbe on Petri dishes (more often said simply: dish) can be in two forms: S-form (i.e. **s**mooth) and R-form (i.e. **r**ough). Strains that form S-form colonies are avirulent, while R-forms are virulent (the reasons for this phenomenon are unknown). Since avirulent S-forms of the plague microbe are usually unstable, Magdalina P. Pokrovskaya set herself the task of obtaining this particular form[2]. By repeatedly exposing the agar culture (i.e. growing on agar, a nutrient dense medium) to the bacterio-

[1] Михаил Михайлович Файбич, 1898–1986; the Belarusian and Soviet military physician, Laureate of the Stalin Prize 3rd degree (1943, 1948).

[2] Tjiwidej and EV76 strains belong to the R-form, and their avirulence is due to loss of part of the genes.

phage, she discovered the appearance of leathery, dense colonies impregnated with a viscous substance on the culture. On resuspension, these colonies grew smooth and transparent like "*dew drops*". This was the first resistant S-form of the plague microbe[1]. Being confident of success, without waiting for the vaccine to be tested on primates, Magdalina P. Pokrovskaya (with colleagues) injected 500 million microbial cells intravenously into herself (there are reports that she was ill with influenza during the experiment). The experiment was successful, and her vaccine saved many lives during the elimination of the plague epidemic in Mongolia (1936-1938).

In 1937, researching the effect of bacteriophages on the R- and S-forms of the plague microbe, Eugenia Ilyinichna Korobkova[2] obtained[3] strain 46-S and a vaccine based on it (the letter S in the name indicates that the strain belongs to the S-form)[4]. The strain[1] obtained two years la-

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[1] Покровская М. П. *Авирулентный мутант B. pestis (культура АМП)*. Вестник микробиологии, эпидемиологии и паразитологии. 1934; 13(1):3–17.

[2] Евгения Ильинична Коробкова, 1893–1970; the Russian and Soviet physician, bacteriologist, laureate of the USSR State Prize (1952). Author of works on plague, cholera and pseudotuberculosis. Thanks to her, the Bacillus Calmette–Guérin (BCG) vaccine was introduced into the USSR medical practice. She developed and introduced the percutaneous method of vaccination against plague.

[3] Коробкова Е. И. *Действие бактериофага на R- и S-варианты чумы и появление авирулентных мутантов*. Вестник микробиологии, эпидемиологии и паразитологии. 1934; XVI(1–2):3–16.

[4] Here is how she herself describes its obtaining: "*one drop of daily broth culture of strain No. 117 was spread with a spatula on the surface of agar; after that one drop of weak phage [i.e. diluted phage, for obtaining which it is necessary to] touch the nacreous plaque of colonies lysed by [phage] with a loop and transfer it to 10-15 cm² of broth] was introduced. After 48 hours, sparsely arranged, shiny, transparent, small colonies appeared on the background of the sterile zone, which, when examined under the microscope, appeared very light-coloured with a smooth convex surface and even edges* [**Figure 26.5**-a] *After another 48 hours, these colonies grew somewhat, but among the healthy colonies untouched by lysis, very pale, flat, dull colonies began to occur, almost melting before the eyes. Sowings of these smooth colonies on broth and agar remained sterile, but broth and agar were thus given lysogenic properties: no strain of plague grew on these media... We sowed one loop of a three-day agar culture of strain No. 46 into such broth. After 24 hours of keeping it at 30°C [or 86°F], the broth became uniformly cloudy. Transferring from it to a cup with agar revealed after 24 hours a pure culture of small, transparent, light-coloured colonies with even edges and smooth convex surface* [**Figure 26.5**-b]. *No deformed or pale melting colonies were visible among these colonies. The suddenly appearing, banal, smooth colonies morphologically bore so little resemblance to plague that at first I found it difficult to associate these colonies with Y. pestis strain No. 46, which grows very characteristically... Further investigation confirmed the plague nature of*

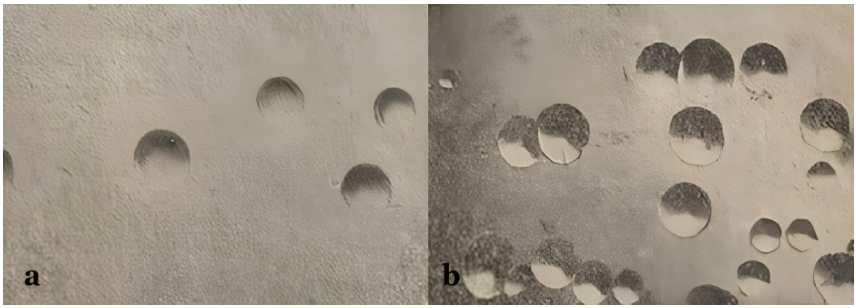


Figure 26.5. *Cultures obtained by Eugenia I. Korobkova:*
a - colonies of S-form of Y. pestis strain 117;
b - colonies of the S-form of Y. pestis strain 46 (by Коробкова Е. И., 1934)

ter by Nikolai Nikolayevich Zhukov-Verezhnikov[2] (with colleagues) was in the S-form (ZhV-S; Russ. ЖВ-S), but was unstable and often reversed (i.e. returned) to the R-form (ZhV-R; Russ. ЖВ-R). Over time, the immunogenicity of the strain began to decline, and because it tended to dissociate (exist in S and R forms), a vaccine based on it was abandoned

Live vaccines in liquid form can only be stored for a couple of months, so it is important to preserve the original strains in order to make vaccines from them when needed. Despite the success of the vaccines of Magdalina P. Pokrovskaya and Eugenia I. Korobkova, the immunogenicity of their strains was lower than that of the R-form strains, and over time it could be lost. In addition, vaccines based on them have a pronounced reactogenicity, which is manifested by the development of general (headache, weakness, fever) and local (swelling, regional lymphadenitis) reactions within the first day. The EV76 and EV line NIIEG strains won against them because they are more stable. They can no longer restore their virulence (reversion is simply impossible with

this variant". It must be said that at the time the nature of bacteriophages was vaguely imagined, so such texts conjure up something magical.

[1] There are reports that this strain may have been a *Y. pseudotuberculosis* strain.

[2] Николай Николаевич Жуков-Вережников, 1908–1981; the Russian and Soviet physician, bacteriologist. Academician (1948) and Vice-President (1949-1953) of the USSR Academy of Medical Sciences. Laureate of the Stalin Prize, 2nd degree (1950). Honoured Scientist of the RSFSR. Director of the Institute of Experimental Biology (1948-1950). Chief forensic medical expert at the Khabarovsk war crimes trials (1949). Associate of Trofim Denisovich Lysenko and participant in the persecution of scientists (rapporteur-accuser at the Pavlovian session).

the loss of a part of the chromosome), and the latter is still used as a production culture to produce a live vaccine[1], the long-term practice of which has clearly demonstrated its immunological efficacy.

Moreover, after Mikhail M. Faibich (with colleagues) developed a method of freeze-drying in a stabilising medium, the shelf life of the drug increased to 3 years! Today, the vaccine (Vaccinum pestosum vivum) is produced in the Russia at the Stavropol Research Anti-Plague Institute (lyophilisate) and the 48th Central Research Institute of the Ministry of Defence of the Russian Federation (tablets), in Kazakhstan at the M. Aikimbayev Kazakh Scientific Center of Quarantine and Zoonotic Diseases (lyophilisate).

At the same time, selective virulence, high reactogenicity[2], limited use for persons with certain hereditary diseases, the need for annual revaccination (but the impossibility of revaccination before the expiry of one year from the date of the previous one), as well as the possibility of loss of immunogenicity and almost complete lack of protection in case of aerosol contamination have led to the search for new methods to create vaccines. The simplest example is *live recombinant vaccines* based on genetically modified strains. There are two approaches to this technology: targeted gene mutation to lose virulence and introduction of avirulence genes into the genome. An example of the first approach[3] is the YopH-mutant strain CO2 (write: CO92Δ*yopH*), which does not synthesise YopH (i.e. is unable to suppress the immune system), and the second approach[4] is the *Y. pestis* strain χ10030(pCD1Ap), which synthesises 6-acyl LPS at 37°C / 98.6°F (i.e. unable to evade the immune system). Candidate vaccines based on both strains provide immunity of mice to bubonic and primary pneumonic forms of plague.

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[1] A system of methods for monitoring activity and harmlessness was developed by Russian and Soviet physician Tamara Ivanovna Anisimova (1929–2022).

[2] It has been shown that deletion of the yp2 gene is an effective strategy to attenuate virulence of *Y. pestis* EV76 while improving immunogenicity (see Guo X., Xin Y., et al. *A novel sORF gene mutant strain of Yersinia pestis vaccine EV76 offers enhanced safety and improved protection against plague*. PLoS Pathog. 2024; 20(3):e1012129; DOI: 10.1371/journal.ppat.1012129).

[3] Bubeck S. S., Dube P. H. *Yersinia pestis CO92 delta yopH is a potent live, attenuated plague vaccine*. Clin Vaccine Immunol. 2007; 14(9):1235–1238; DOI: 10.1128/CVI.00137-07.

[4] Sun W., Roland K. L., et al. *Developing live vaccines against Yersinia pestis*. J Infect Dev Ctries. 2011; 5(9):614-627; DOI: 10.3855/jidc.2030.

Recombinant vector vaccines are created in a similar way when the desired plague microbe gene synthesising an immunogenic factor, such as capsular antigen or LcrV, is inserted into an attenuated bacterium (e.g. *Salmonella typhimuruim*). An example is a polyvalent vaccine candidate based on *Salmonella typhimuruim* strain χ12094, which carries genes synthesising LcrV, pesticin and capsular antigen. The closest relative of the plague microbe, *Yersinia pseudotuberculosis*, can also act as an attenuated bacterium[1].

Another type of vaccine is *subunit vaccines*, which use surface antigens rather than whole bacteria. Such vaccines are promising for people with compromised immune status. They are divided into molecular and chemical. An example of the former is the rF1V vaccine, created[2] at the United States Army Medical Research Institute for Infectious Diseases (USA) and produced since 2009 by DynPort Vaccine Company, LLC. It contains recombinant capsular antigen and LcrV as subunits in an aluminium adjuvant.

The three-component candidate vaccine rF1mutV-PA[3], which contains capsular antigen and LcrV as well as the protective antigen (PA) of Bacillus anthracis, is of great interest, providing immunity against plague and anthrax in mice, rats, and rabbits.

Chemical vaccines are also subunit vaccines, but unlike molecular vaccines, antigenic determinants (chemical complexes) for them are obtained by special processing of cell culture. The first idea about the possibility of using a limited number of antigens to induce the formation of immunity was put forward by Nikolay F. Gamaleya, but only at the beginning of this century in the walls of the Anti-Plague Institute "Microbe", Sarkis Mkrtychevich Dalvadyants[4] (with colleagues) was created

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[1] Derbicse A., Cerdà A., et al. *An encapsulated Yersinia pseudotuberculosis is a highly efficient vaccine against pneumonic plague*. PLoS Negl Trop Dis. 2012; 6(2):e1528; DOI: 10.1371/journal.pntd.0001528.

[2] Heath D. G., Anderson G. W., et al. *Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine*. Vaccine. 1998; 16(11-12):1131-1137; DOI: 10.1016/s0264-410x(98)80110-2.

[3] Tao P., Mahalingam M., et al. *A bivalent Anthrax-Plague vaccine that can protect against two Tier-1 bioterror pathogens, Bacillus anthracis and Yersinia pestis*. Front Immunol. 2017; 8:687; DOI: 10.3389/fimmu.2017.00687.

[4] Саркис Мкртычевич Дальвадянц, 1935–2008; the Soviet and Russian physician of Armenian origin, bacteriologist, isolated the basic somatic antigen of *Y. pestis* and *Y.*

the first plague chemical vaccine candidate, in which the capsular antigen and the basic somatic antigen (i.e., Enterobacterial common antigen; see Chapter 6 for details) are used as chemical complexes.

A promising area of research is *DNA vaccines*, which are constructed from plasmid DNA encoding the pathogen's protective (i.e., immune response-inducing) antigens. When introduced into the human body, with the help of special carriers, DNA penetrates into the human cell and turns it into a kind of factory for the production of protective antigens, which in *Y. pestis* is the capsular antigen or LcrV[1]. To enhance protection, regions encoding other proteins can be added to the plasmid. For example, a DNA vaccine[2] candidate carrying a modified *lcrV* gene and a human tissue plasminogen activator (tPA) signalling sequence protected mice against interanasal infection with lethal doses of *Y. pestis*.

The development of genetic engineering has made it possible to create *plant vaccines* based on transgenic plants in which the genes encoding the synthesis of protective antigens have been transformed (i.e. embedded). Thus, genes encoding the synthesis of capsule antigen and LcrV (in the form of F1-V fusion protein) were transformed[3] into lettuce leaves! However, although the antigen synthesised by lettuce has significant immunogenicity, its amount is small (less than 1% of the total protein contained in the leaves)[4].

There are other types of vaccines, such as *conjugated vaccines*, which bind a low-immunogenic antigen to a high-immunogenic antigen, thus

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pseudotuberculosis. He proved (together with Igor V. Domaradsky) the possibility of using plague live vaccine for immunoprophylaxis of pseudotuberculosis.

[1] It should be noted that the search for protective antigens of bacteria and viruses is an important problem in the creation of vaccines at the present stage, because of the many structures (antigens) of a bacterium or virus, only relatively few will "act" as protective ones.

[2] Wang S., Helman D., et al. *A DNA vaccine producing LcrV antigen in oligomers is effective in protecting mice from lethal mucosal challenge of plague*. Vaccine. 2004; 22(25-26):3348–57; DOI: 10.1016/j.vaccine.2004.02.036.

[3] Rosales-Mendoza S., Soria-Guerra R.E., et al. *Expression of an immunogenic F1-V fusion protein in lettuce as a plant-based vaccine against plague*. Planta. 2010; 232(2):409–16; DOI: 10.1007/s00425-010-1176-z.

[4] Currently, such plants are used to synthesise antigens and then concentrate them, although the idea of growing lettuce, the consumption of which would immunise the population, is very promising.

enhancing the immune response to the former, and *microencapsulated vaccines*, which are biodegradable microspheres (usually lipophilic) containing antigens. An example of the latter is the plague molecular microencapsulated vaccine (PMMV; Russ. ВЧММ), created[1] in 2018 within the walls of the State Research Center for Applied Microbiology (Russia). The vaccine consists of recombinant capsular antigen and LcrV encapsulated in biodegradable microcapsules.

It would be wrong not to mention vaccines based on *bacterial ghosts* (BGs), i.e. cell envelopes devoid of cytoplasmic content but retaining the morphology of all surface structures (**Figure 26.6**), which is a consequence of the ability of some virulent bacteriophages to form transmembrane tunnel structures compounding the inner and outer membranes of the bacterium, through which, due to the action of osmotic pressure, the contents of the bacterium literally "leak" into the external environment. This phenomenon was first described in 1985[2] on bacteriophage φX174. Protein E of this bacteriophage inhibits the activity of the enzyme phosphor-MurNAc-pentapeptide translocase (MraY), which leads to the fusion of the inner and outer membranes in actively growing cells and, as a consequence, the formation of a transmembrane tunnel 40-400 nm in diameter at the equator or poles of the bacterial cell. In the case of the plague microbe, in addition to protein E of bacteriophage φX174, the choline-endolysin systems of bacteriophages λ and L-413C are used, which lead to the formation of mixtures of both retained and completely lost the original form defined by the peptidoglycan framework. It is noteworthy that the combined use of protein E of bacte-

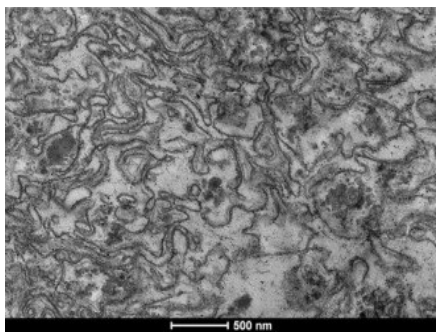


Figure 26.6. *Transmission electron micrographs of bacterial ghost (by Dentovskaya S. V, 2021)*

[1] Дятлов И. А., Анисимов А. П., и др. *Вакцина чумная молекулярная микроинкапсулированная (ВЧММ)*. Бактериология. 2018; 3(1):74–75.

[2] Bläsi U., Henrich B., et al. *Lysis of Escherichia coli by cloned phi X174 gene E depends on its expression*. J Gen Microbiol. 1985; 131(5):1107–1114; DOI: 10.1099/00221287-131-5-1107.

riophage φX174 and the choline-endolysin system of bacteriophage L-413C leads to the formation of structures resembling collapsed sacs. Researches[1] show that bacterial shadows of *Y. pestis* effectively protect guinea pigs when infected with a virulent strain[2], and in combination with capsular antigen and LcrV significantly potentiate the protection of the latter also for mice, which is a prototype for a polygostal three-component vaccine[3].

It should be noted that there are currently no plague vaccines approved by the World Health Organisation (rF1V and HFMM vaccines are candidates for approval). A number of countries have officially authorised the use of live vaccines based on killed *Y. pestis* cells (Australia, New Zealand), attenuated strain EV line NIIEG (CIS countries) and atrain EV-CN (China), rF1V vaccine (USA[4], as an orphan drug) and PMMV vaccine (Russia).

Live vaccines based on attenuated strains are the most effective, as they can provide immunity close to post-infection (i.e. in those who have had the disease), but their use is prohibited in a number of countries (in particular, the USA and the EU). They do not provide long-term protection, but have a limited revaccination period (every 12 months only), because earlier revaccination dates maintain sufficiently intense immunity by not allowing bacteria of the vaccine strain to multiply in sufficient numbers and persist in the human body, increasing immuni-

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[1] Dentovskaya S. V., Vagaiskaya A. S., et al. *Peptidoglycan-Free Bacterial Ghosts Confer Enhanced Protection against Yersinia pestis Infection*. Vaccines (Basel). 2021; 10(1):51; DOI: 10.3390/vaccines10010051; Вагайская А. С. *Бактериальные тени Yersinia pestis*. Дис. … кандидата биологических наук. Оболенск, 2023, 126 с.

[2] Interestingly, hydrolysis of peptidoglycan significantly enhances the protective properties, which is probably due to the possible formation of multiple muramyl dipeptide molecules during hydrolysis, which the innate immune system recognises as pathogen-associated molecular patterns (PAMPs).

[3] Вагайская А. С., Трунякова А. С., и др. *Прототип полигостальной трехкомпонентной вакцины для профилактики чумы*, в сборн. *Материалы VIII Национального конгресса бактериологов (Москва, 27-28 сентября 2023 года)*, Москва: Изд-во «Династия», 2023, 162 с.

[4] The rV10 vaccine-candidate, developed in 2011, representing a truncated LcrV antigen, is currently undergoing US Food and Drug Administration (FDA) pre-Investigational New Drug authorization review for a future phase I trial (see Quenee L. E., Schneewind, O. *Plague vaccines and the molecular basis of immunity against Yersinia pestis*. Hum. Vaccine. 2009; 5:817–823).

ty. For this reason, earlier revaccination is only possible with rF1V and PMMV vaccines.

In come countries, annual vaccine prophylaxis is mandatory for persons who work with the pathogen[1]. In the event of a human disease, the entire population in the area is vaccinated, including children aged two years and older[2]. High-risk populations include:

- livestock keepers with permanent camps in the enzootic area;
- employees of farms located in the enzootic area;
- forage and hay producers;
- hunters;
- family members of livestock breeders, farmers, foragers, hunters who may visit the enzootic area (including children);
- seasonal workers of extractive and processing industries;
- tourists visiting the enzootic area.

Animals such as camels can also be vaccinated (but camel milk can only be consumed after boiling).

Revaccination of the population ceases one year after the last detection of the pathogen, and revaccination of high-risk populations after two years.

Killed whole-cell vaccine (CSL) is most commonly used to vaccinate persons living in foci areas and those visiting enzootic areas. It is administered subcutaneously at the appropriate dosage (**Table 26.2**). Doses are administered at intervals of one to four weeks. It should be remembered that vaccination provides immunity only against the bubonic form of plague.

Live vaccines (Vaccinum pestosum vivum) are given for epidemiological reasons (i.e., when infection is likely). The standard schedule includes revaccination every 12 months. Vaccination is carried out by cutaneous, subcutaneous, intracutaneous and inhalation methods.

[1] In case of contraindications to vaccination, admission to work is carried out by order of the head of the organisation upon written application of the employee.

[2] For persons with reduced immune reactivity (i.e. immunodeficiency), an individualised vaccination schedule should be drawn up to ensure that at least one month, but not more than six months, has elapsed since vaccination at the time of peak epizootic activity in the foci.

Table 26.2. *Vaccination regimens with killed whole-cell vaccine (CSL)*

Age, yr	Volume administered, mL			
	First dose	**Second dose**	**Third dose**	**Booster dose**
6 mo to 2yr	0.1	0.1	0.1	0.1*
3 – 6	0.2	0.2	0.2	0.2*
7 – 11	0.3	0.3	0.3	0.3*
>12	0.5	0.5		0.3**

Note:
*at 6-month intervals after Third dose.
**ar 6-month interval after Second dose. Dose can be reduced to 0.1 mL intradermally for person who had reactions to previous doses of the vaccine.

1.8 ml of saline solution is added to the lyophilisate and dissolved for 3 minutes (the dissolved vaccine is a homogeneous suspension without impurities and flakes). The resulting suspension is taken from the ampoule with a sterile syringe and transferred to a sterile vial containing saline solution, taking into account that the final volume corresponds to the volume specified in the instructions. The diluted vaccine should be used within 2 hours, after which it should be destroyed by boiling for 30 minutes.

Cutaneous method (Eugenia I. Korobkova's method). Vaccination is carried out on the outer surface of the middle third of the upper arm, for which a bifurcated needle is used to lightly scrape the superficial layer of the epidermis in three places (in children in one or two places), having previously treated them with a 70% solution of ethyl alcohol. The distance between the sites is 3-4 cm, the area of the site is 1-1.5 cm^2. The required dose of vaccine is applied to each site with a pipette, after which four horizontal and four vertical incisions 1 cm long are made through each drop of vaccine with a bifurcated needle (the incisions should be shallow, blood protrusion like "dew drops" is allowed). Then the drops are rubbed thoroughly into the skin for a few seconds and allowed to dry for 5 minutes. After 24-48 h, hyperaemia and infiltrate may occur at the vaccine injection site, followed by yellowish crusts along the incisions.

Subcutaneous method. Vaccination is carried out below the angle of the scapula, having previously treated the skin with 70% ethyl alcohol solution. The syringe needle is inserted into the skin at an angle of 45°.

Intracutaneous method. Vaccination is carried out in the area of the outer surface of the upper arm, having previously treated the skin with 70% ethyl alcohol solution. The syringe needle is inserted into the skin at an angle of 10–15°.

Inhalation method. Vaccination is carried out in special rooms with a volume of 50-150 m3 and a height of 2.5-4.5 m with a length to width ratio of no more than 2:1. The room should be well ventilated or equipped with exhaust ventilation. Add 2 ml of 10% lactose solution to the lyophilisate (the temperature of the solution should correspond to the storage temperature of the lyophilisate) and shake until a homogeneous suspension without impurities and flakes is obtained. The obtained suspension is transferred into a sterile vial containing 10% lactose solution, taking into account that the final volume corresponds to the volume specified in the instructions. The obtained microbial suspension in the amount determined by the volume of the room (0.1 ml per 1 m^3 of the room) is poured into the reservoir of a pneumatic atomiser of ejection (vacuum) type. The sprayer is installed vertically, nozzle upwards, in the centre of the room at a height of 80-120 cm from the floor. Spraying is carried out with compressed air at a pressure of 0,12 MPa / 1.21 Bar / 1.2 atm until the suspension poured into the tank is completely used up. Compressed air is supplied to the atomiser until the end of the immunisation session, i.e. 5 min. The number of people immunised in one session is determined on the basis of one person for every 1.4-2 m^3 of room. After the vaccination session, the room should be ventilated for at least 5 minutes.

Age- and route-dependent vaccine doses are given in **Table 26.3** (women who are breastfeeding are vaccinated by the cutaneous method only).

In Russia, the live vaccine is also available in the form of chewable tablets containing 40±10 x 10^9 live bacterial cells (LBC). Persons aged 14 years and older take one tablet, which should be actively sucked or chewed (swallowing is prohibited!). After vaccination it is recommend-

ed not to drink, eat or smoke for half an hour. Revaccination is carried out in a year (in unfavourable epidemic situation in six months).

Camels over 2 years of age are vaccinated with thirty adult doses. Post-vaccination immunity lasts for six months.

Plague molecular microencapsulated vaccine (PMPM) is used for specific prophylaxis of persons (including military personnel) operating in emergency situations, as well as the population living in the territories of natural foci. Persons aged 18 to 55 years are administered 0.5 ml of the preparation subcutaneously. The duration of specific immunity is not less than 90 days (preclinical studies on laboratory animals showed preservation of protective immunity for 180 days after immunisation). It can be used for revaccination after primary vaccination with live vaccine, taking into account the individual integrated indicator of humoral and cellular immunity, which largely contributes to the elimination of adverse reactions of the organism to the introduction of live vaccine.

The **subunit vaccine (rF1V)** is used to prevent infection with the pneumonic form of plague. Individuals aged 18 to 55 years are administered 0.5 ml intramuscularly. Revaccination is carried out on days 28 and 182, then annually thereafter.

The **chemical vaccine** was not approved for use, but the developers recommended its use for revaccination for epidemiological indications not earlier than 3 months after vaccination with live vaccine (recall that revaccination with live vaccine is possible only after 12 months). The drug in the form of 125 mcg is dissolved in 0.5 ml of saline solution and injected subcutaneously. If necessary, revaccination with the chemical vaccine is carried out not only in the third month, but also in the sixth and ninth months.

Table 26.3. *Vaccination regimens with live vaccine (Vaccinum pestosum vivum)*

Age, yr	Doses			
	Intracutaneous	**Subcutaneous**	**Cutaneous**	**Inhalation**
2–6	1/3 of the dose (100 million LBC in 0.1 ml)		1/3 of the dose (1 billion LBC in 0.05 ml, i.e. 1 drop)	
7–9	1/3 of the dose (100 million LBC in 0.1 ml)		2/3 of the dose (2 billion LBC in 0.1 ml, i.e. 2 drop)	
10–13	1/2 of the dose (150 million LBC in 0.1 ml)		1 dose (3 billion LBC in 0.15 ml, i.e. 2 drop)	
14–60	1 dose (300 million LBC in 0.1 ml)	1 dose (300 million LBC in 0.5 ml)	1 dose (3 billion LBC in 0.15 ml, i.e. 3 drop)	1 dose (5 billion LBC in 0.15 ml)
> 60	1/3 of the dose (100 million LBC in 0.1 ml)		1 dose (3 billion LBC in 0.15 ml, i.e. 3 drop)	

ACT III

Altera pars





Fort Alexander I (Plague Fort) in Kronstadt

27 THE FORT

…I have set before you life and death, blessing and cursing: therefore choose life, that both thou and thy seed may live...
Deuteronomy 30:19

A man stands on the deck of a meteor[1] sailing towards the island of Kotlin. A faint breeze ruffles his hair, and an old fort whose walls are covered with a thick layer of soot comes into view. This fort, which bears the name of the Emperor Alexander I of Russia, served for fifteen years as a Special Laboratory where plague and other particularly dangerous infections were researched, and where plague serums and vaccines were produced.

With the outbreak of the third plague pandemic and the resulting threat of the spread of the infection, the government of the Russian Empire decided to establish a centralised operational body in charge of directing and coordinating all anti-plague activities. Such a body was created on 11 January 1897 by the Decree of the last Emperor Nicholas II of Russia, named "Special Commission under the chairmanship of H.H. Duke Alexander Petrovich Oldenburgsky[2] to prevent the introduction of the plague and to combat it, in case of its appearance in the Empire" or abbreviated as KOMOChUM (Russ. КОМОЧУМ).

At one of the first meetings it was decided that it was necessary to produce plague vaccines and serums, which was entrusted to the Imperial Institute of Experimental Medicine, which used horses from the stables of the Duke of Oldenburg. However, the demand for drugs was growing, and the expansion of the area threatened the emergence of an

[1] A motor ship with hydrofoils, due to which hydrodynamic forces keep the vessel above the water surface when travelling, resulting in reduced resistance and, as a consequence, a significant increase in speed. Produced in the USSR since 1961, it became one of the symbols of the city of St. Petersburg (Russia).

[2] Александр Петрович Ольденбургский, Alexander Frederick Constantin of Oldenburg 1844–1932; the Russian statesman of German origin. Great-grandson of the Grand Duke Peter I of Oldenburg and the Emperor Paul I of Russia. Founder and trustee of the Imperial Institute of Experimental Medicine (now the Institute of Experimental Medicine). He took an active part in the creation of the St. Petersburg Pasteur Station.

epidemic in the capital. So through the efforts of the chairman on 26 January of the same year the Institute was handed over the fort "Alexander I", and in the autumn of 1898 the Special Laboratory began work there. At first, the medical staff consisted of only three people: Mikhail Gavrilovich Tartakovsky[1] (head), Nikolai Mikhailovich Berestnev[2] and Danylo K. Zabolotny.

In addition to producing plague vaccine and serum, and training specialists to work with plague and cholera pathogens[3], experiments were conducted here with marmots, monkeys, deer, and even camels. All works were carried out from 9 a.m. to 6 p.m (from noon to 3 p.m. – lunch break). The work was carried out with special precautions. Specialists worked in trousers and dressing gowns, which were soaked in carbolic acid after work. A cap was worn on the head and boots on the feet, which were washed with sulema. Post-mortem examination of the animals was carried out in a special isolated room, and the waste water was disinfected with a steam steriliser (an autoclave). Every visitor had to have a special pass, but he was obliged to leave the fort walls before sunset. In case of the slightest suspicion of the disease, a quarantine was declared. Communication with the outside world was carried out by telephone (to Kronstadt) and telegraph (to St. Petersburg). In addition, a small ferry called "Microbe" ran to Kotlin Island (Kronstadt).

Despite the security measures taken, which were unprecedented for the time, cases of "internal" infection did occur. Thus, on 3 January 1904, Władysław (Vladislav Ivanovich) Turczynowicz-Wyżnikiewicz[4], then the head of the hospital, who conducted experiments on inhalation

[1] Михаил Гаврилович Тартаковский, 1867–1935; the Ukrainian and Soviet veterinarian, bacteriologist, pathologist. Director of the Institute of Comparative Pathology (1923-1932). Author of treatises on plague, rinderpest, pullorum disease, glanders. Repressed.

[2] Николай Михайлович Берестнёв, 1867–1910; the Belarusian physician, bacteriologist. Head of the Special Laboratory (1904-1908), director of the Moscow Bacteriological Institute (1908-1910). Author of treatises on plague, cholera and malaria.

[3] Around the same time, many countries began to open similar courses. For example, by 1910, the Institute of Infectious Diseases (Japan), headed by Kitasato Shibasaburō, had trained 1,293 specialists. Nowadays, professional training in the basics of biological safety and rules of working with pathogens of especially dangerous infections is one of the mandatory conditions for admission to work with them.

[4] Владислав Иванович Турчинович-Выжникевич, 1865–1904; the Polish veterinarian. Author of books on plague and sap. According to legend, an urn (stone vase) with his ashes is kept in the library of the Institute of Experimental Medicine.

and participated in the preparation of plague toxin by rubbing germs, was infected. The disease progressed rapidly (primary pneumonic form), and he died on 7 January 1904. The second victim of plague was Manuil Fyodorovich Schreiber[1], who became infected on 12 February 1907 during the experiments on studying the endotoxic property of plague serum, using pipettes without cotton filters contrary to safety rules (the emulsion with bacteria was swallowed). Two days later, pneumonia developed (primary pneumonic form) and he died on 17 February.

The revolution, which began in 1917, led to the liquidation of KOMOChUM and the Special Laboratory, the equipment of which, thanks to the efforts of Alexei Ilyich Berdnikov[2], was taken to Saratov to the Department of Microbiology of the Imperial Nikolaev University (now Saratov State Medical University). On the basis of this department, on the initiative of Oleksandr Oleksandrovych (Alexander Alexandrovich) Bogomolets[3], the Regional Institute of Microbiology and Epidemiology of the South-East of the RSFSR was opened by the decision of the Board of the People's Commissariat of Health on 15 November 1918. Already in 1919 as a postal (secret) code was given the name "Microbe" (which later became the name), as a tribute to the memory of the Special Laboratory and its ferry. The choice of Saratov is not accidental: it is remote from densely populated cities. Back in 1914, when the Special Laboratory existed, it was said that it was impossible to fight plague from the capital city.

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[1] Мануил Фёдорович Шрейбер, 1866–1907; Ukrainian military physician, bacteriologist, epidemiologist. Author of treatises on plague.

[2] Алексей Ильич Бердников, 1877–?; the Russian physician, bacteriologist. The last head of the Special Laboratory (1916-1917) and the first director of the Institute "Microbe" (1918-1921). Participated in the elimination of the plague epidemic in Manchuria (1910-1911). In 1921 he emigrated to Harbin (China). Fate after 1930 is unknown.

[3] Олександр Олександрович Богомолець, Александр Александрович Богомолец, 1881–1946; the Ukrainian and Soviet physician, bacteriologist, pathologist, founder of Russian pathophysiology, endocrinology and gerontology. President (1930-1946) of the All-Ukrainian Academy of Sciences. Academician (1932) and Vice-President (1942-1945) of the USSR Academy of Sciences. Academician of the USSR Academy of Medical Sciences (1944). Hero of Socialist Labour (1944), laureate of the Stalin Prize (1941). Organiser of the Higher Women's Courses in Saratov (1917). Took part in the creation (the first in the world) of the Institute for Blood Transfusion in Moscow (now the National Medical Research Centre for Hematology). Author of many books in various fields of medicine.

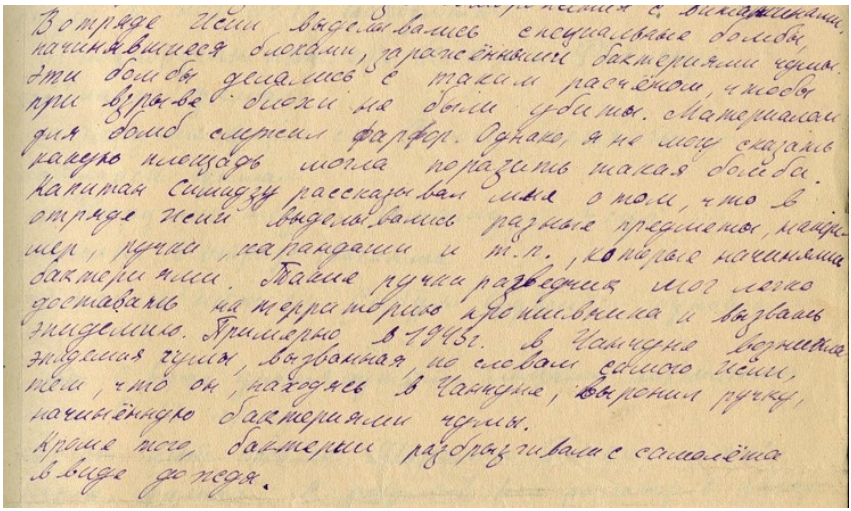
At the same time, institutions were beginning to purposing not to prevent and eliminate epidemics, but to develop *biological (bacteriological) weapons* based on such dangerous microorganisms as *Y. pestis* and *Variola* virus. The first attempts to use them in practice were carried out during the First World War and became inhumane during the Second World War[1]. However, despite the number of innocent human lives lost, development continued, especially in the USA and the USSR during the so-called Cold War[2], which utilised the "results" of Unit 731. The offensive research at Fort Detrick (Maryland, USA) was officially abandoned in 1969, but the development of protective impossible without at least a theoretical understanding (to some extent) of possible methods of attack.

In the USSR, the disparate laboratories were formed into a unified system called "п/я А-1063" (the so-called "Biopreparat") only in 1973, that is, the next year after the adoption of the Convention on the Prohibition of Bacteriological Weapons. The Biopreparat facilities were scattered all over the Soviet Union, and their existence was justified by the need for parity (i.e. equality, conformity) with the Americans. However, as Lev Alexandrovich Fedorov[3] correctly points out, the scale of the system (only one enterprise was capable of producing up to 20 tonnes of bacteria per year!) was so enormous that the question should be "*not about parity, but about the obvious superiority of the USSR over the United States*". "*Unquestioned Soviet leadership in preparing for offensive bacteriological warfare*" became the "*undeclared asymmetric response*". The system lasted until the collapse of the USSR in 1991, and both countries (as well as others) now claim compliance with the Convention. However, research into ways to defend against bacteriological weapons cannot be completely abandoned, since pathogens can become weapons in the hands of terrorists. Nevertheless, the world should endeavour to protect the humanity from particularly dangerous infectious diseases rather than use them in the struggle for geopolitical supremacy.

[1] There is evidence of its use by all participants.

[2] The global confrontation between the USA and the USSR (and their allies) from 1946 to December 1989, when George H.W. Bush (1924-2018), US President, and Mikhail Sergeyevich Gorbachev (1931-2022), General Secretary of the CPSU Central Committee, announced its end at the Malta Summit.

[3] Лев Александрович Фёдоров, 1936–2017; Soviet and Russian military chemist, ecologist, founder and head of the Union "For Chemical Safety".



Fragment (p. 5) of the record of interrogation of Seoei Yamamoto, a prisoner of war, dated 18 July 1949. Courtesy of the Центральный архив Федеральной службы безопасности Российской Федерации (document No. 17): "The unit of Ishii made special bombs filled with fleas infected with Y. pestis. These bombs were made in such a way that the fleas would not be killed by the explosion. The material used for the bombs was porcelain. However, I can't tell you how much area such a bomb could cover. Captain Shimiju told me that the unit of Ishii made various items, such as pens, pencils, etc., which were filled with bacteria. Such pens could be easily transported by a scout into the enemy territory and cause an epidemic. About 1943, there was a plague epidemic in Changchun, which, according to Ishii himself, was caused by dropping a pen filled with plague bacteria while in Changchun. In addition, the bacteria sprayed from the aeroplane in the form of a rain"

Speaking specifically about the *Y. pestis* as a bacteriological weapon agent, it is reported that Fort Detrick quickly abandoned the creation of bacteriological weapons based on *Y. pestis* because the resulting "result" quickly lost virulence. On the contrary, in the "Biopreparat" system, research was conducted in a number of directions: creation of strains carrying genes of some toxin, in particular, myelin toxin (project "Bonfire"; Russ. проект «Костёр»), creation of polyantibiotic-resistant strains (project "Method"; Russ. проект «Метод»), creation of combinations of strains, the spread of which by means of bombs or spraying plants was supposed to confuse doctors because of the contradiction of clinical manifestations. It is difficult to say whether any significant

achievements were made in the USSR (apart from the annual production of 1,500 tonnes of *Y. pestis*), there is information about obtaining antibiotic-resistant strains and strains with increased virulence, as well as techniques for producing an aerosol in which the plague microbe did not lose virulence (actually, what the USA managed to achieve as well).

A typical example is the case of an anthrax (*Bacillus anthracis*) spill in 1979. The Sverdlovsk-19 facility (now the Center for Military-Technical Problems of Bacteriological Defence of the 48th Central Research Institute of the Ministry of Defence of the Russian Federation) was engaged in the production of anthrax spores. At the end of one of the shifts, an specialist removed the protective filter, leaving a note but not making a corresponding entry in the logbook. The next shift began work, and a cloud of spores was blown into the air, carried by the wind to nearby settlements and, in particular, to the city of Sverdlovsk (now Yekaterinburg, Russia). As a result, about 100 people died (officially 64, according to some reports - 500), and outbreaks were reported in 26 settlements towards the city of Chelyabinsk (in the direction of the wind). The official cause of the epidemic was declared to be contaminated meat, even though most of those infected had a pneumonic form of the disease, indicative of aerogenic contamination. The cause was so obvious that as a result the US resumed its bacteriological weapons development programme. Nevertheless, the USSR continued to conceal the truth (the perpetrator did not receive the punishment he deserved), and it was only in 1992, after the collapse of the USSR, that the families of the victims were granted pensions[1].

The military experience formed the basis for the principles of **biological safety** (biosafety). Subsequently, thanks to the work of Arnold Gerhard Wedum[2], published[3] in the early 1950s, and his persistence, these principles were extended to all laboratories and are now applied by all specialists working with *pathogenic biological agents* (PBAs, BAs) or *bio-*

[1] Law of the Russian Federation No. 2667-1 of 4 April 1992.

[2] 1903–1976; American military physician and biosecurity specialist. Director (1944-1969) of the Industrial Health and Safety Division of the U.S. Army Biological Warfare Laboratories, liquidated in 1969. He played a major role in the work of the American Biological Safety Association (ABSA).

[3] Wedum A. G. *Nonautomatic pipetting devices for the microbiologic laboratory*. J Lab Clin Med. 1950; 35(4):648-651; Wedum A.G. *Bacteriological Safety*. Am J Public Health Nations Health. 1953; 43(11):1428–1437; DOI: 10.2105/ajph.43.11.1428.

logical select agents or toxins[1] (BSAT, "select agents"). PBAs are not just microorganisms, but any agents that can cause disease, such as prions or biologically produced poisons. Biosafety principles exist to prevent the spread of infections from laboratories and hospitals, as even the slightest disregard for them can lead to death. We have previously encountered such cases: a researcher who died from a vaccine strain (Chapter 14), Abram L. Berlin who failed to survive an observational study (Chapter 15), Manuil F. Schreiber who used a pipette incorrectly ("pipetting with his mouth"), and many others.[2].

The biosafety conversation should start with the classification of PBAs, as it determines how stringent measures should be applied.

According to the World Health Organization classification[3], all PBAs are classified by risk into four groups:

Risk Group 1: a microorganism that is unlikely to cause human or animal disease (no or low individual and community risk);

Risk Group 2: a PBAs that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available and the risk of spread of infection is limited (moderate individual risk, low community risk);

Risk Group 3: a PBAs that usually causes serious human or animal disease but does not ordinarily spread from one infected individual to another. Effective treatment and preventive measures are available (high individual risk, low community risk);

Risk Group 4: a PBAs that usually causes serious human or animal disease and that can be readily transmitted from one individual to an-

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[1] The term is used in the USA.

[2] To this can be also added the case of intra-laboratory contamination described by Japanese physician Takagi Itsuma (高木 逸磨; 1884-1960). He reports that a physician who contracted pneumonic plague in laboratory *"always worked very diligently and carefully... probably contracted it during frequent animal testing"* (see 高木 逸磨『肺「ペスト」ノ研究室内感染例』,日本傳染病學會雜誌, 1927 年 2 巻 1 号 p. 55-59; DOI: 10.11552/kansenshogakuzasshi1926.2.55).

[3] Laboratory biosafety manual. 3rd ed., World Health Organization, WHO/CDS/CSR/LYO/2004.11, 2004, 181 p.

other, directly or indirectly. Effective treatment and preventive measures are not usually available (high individual and community risk).

According to this classification, *Y. pestis* belongs to Risk Group 3, as effective treatments are available.

It should be realised that each country also has its own classifications. For example, the classification adopted in the CIS countries also divides PBAs by hazard (pathogenicity), but in essence it is an inversion, since **Group I** and **Group II** of pathogenicity include especially dangerous PBAs, **Group III** includes pathogenic PBAs, and **Group IV** includes non-pathogenic and conditionally pathogenic PBAs. According to this classification, *Y. pestis* is the only bacterium classified in Group I of pathogenicity (attenuated strains are classified in Group III).

A different approach is used in the USA. Here, BSAT (i.e. PBAs) are categorised according to the threat they may pose to national security in the hands of terrorists:

Category A: BSAT that can be easily disseminated or transmitted from person to person, result in high mortality rates and have the potential for major public health impact; might cause public panic and social disruption; and require special action for public health preparedness;

Category B: BSAT that are moderately easy to disseminate, result in moderate morbidity rates and low mortality rates and require specific enhancements of diagnostic capacity and enhanced disease surveillance;

Category C: emerging BSAT that could be engineered for mass dissemination in the future because of availability, ease of production and dissemination and potential for high morbidity and mortality rates and major health impact.

As can be easily guessed, *Y. pestis* is in Category A.

Depending on the group (category) of BPAs and the type of work performed with it, laboratories are divided into four **Biosafety levels** (BSLs), where the BSL-1 laboratories have the minimum degree of protection, and the BSL-4 laboratories – the maximum (**Table 27.1**). However, common to all is the separation of the laboratory premises by a special anteroom (sanitary inspection room) where personal clothes and



▲ ***Figure 27.1.*** *The "Biohazard" sign. Such signs are most often placed on the border of zones in CIS countries, as well as on equipment (e.g. BSC)*



▼ ***Figure 27.2.*** *The "Biohazard" sign recommended by the World Health Organization for placement in front of the laboratory entrance. Includes laboratory biosafety level and contact details of the supervisor (responsible person). May include requirements for visitors (e.g. immunisation)*

shoes are changed to work clothes and shoes, and a hygienic shower can be installed. In front of the entrance to the laboratory, as well as on the boundary of the zones a sign "Biohazard" is posted (**Figure 27.1** and **Figure 27.2**).

It should be remembered that different countries may use different designations for similar criteria. For example, in the CIS countries the designation "УББ" (Russ. уровень биологической безопасности)[1] is used, and in Canada – "CL" (Containment level)[2]. Here we will use the international designation adopted by the World Health Organization – "BSL"[3].

As can be seen from Table 27.1, work with *Y. pestis* is carried out in laboratories at a level **no lower** than BSL-3 (**Figure 27.3**). These laboratories are engineering and construction structures with a complex enclosure (barrier) system, whose contribution to

[1] *Санитарные правила и нормы СанПиН 3.3686–21 «Санитарно-эпидемиологические требования по профилактике инфекционных болезней»,* утв. Постановлением Главного государственного санитарного врача Российской Федерации от 28 января 2021 года № 4.

[2] *Canadian Biosafety Standard, Third Edition*. Public Health Agency of Canada (Cat.HP45-7/2022E-PDF), 2022, 166 p.

[3] Laboratory biosafety manual. 3rd ed., World Health Organization, WHO/CDS/CSR/LYO/2004.11, 2004, 181 p.

Table 27.1. *Classification of laboratories by level*

Laboratory level	**Type of work with PBAs**	
	WHO classification	**CIS classification**
Basic laboratories		
BSL-1	*Risk Group 1*: all types of work	*Group IV*: all types of work
BSL-2	*Risk Group 1*: all types of work *Risk Group 2*: all types of work with small volumes and low concentrations	*Group III and Group IV*: all types of work *Group II*: works without accumulation (cultivation or concentration)
Isolated (containment) laboratory		
BSL-3	*Risk Group 2*: all types of work with large volumes and high concentrations *Risk Group 3*: all types of work	*Group II*: all types of work *Group I*: all work with plague and work with viruses, except for the accumulation (cultivation or concentration) of viruses
Maximum isolation laboratory		
BSL-4	*Risk Group 4*: all types of work	*Group I*: all types of work. Work with microorganisms associated with clinical manifestations characteristic of PBAs of Group I and Group II, the taxonomic position of which has not been determined and the degree of danger has not been studied; experimental studies of strains with multiple resistance to antibiotics and chemopreparations; aerobiological studies with PBAs of group I and Group II

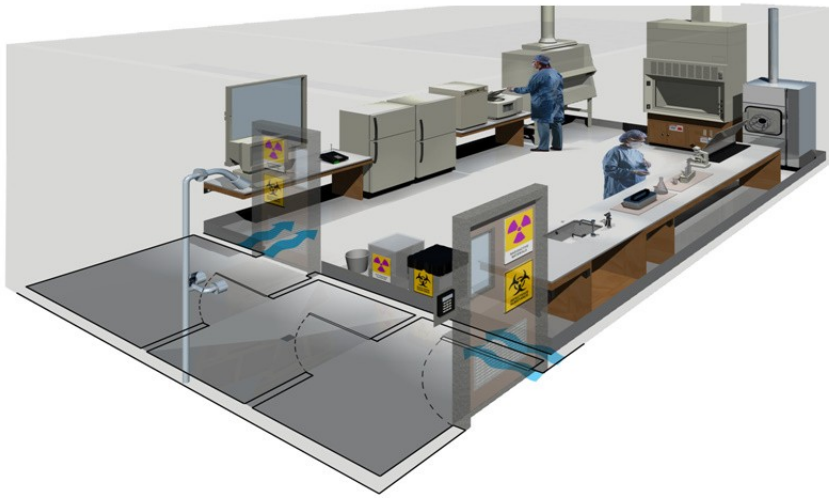


Figure 27.3. *Example of a BSL-3 laboratory.*
You can see the "Biolohazard" signs at the entrance to the laboratory and the boxed room, the code lock for the entrance, the anteroom with shower (note that the "entrance" and "exit" are on the same route, which disrupts flow) and the direction of airflow. The boxed room has a window, which is prohibited in some countries. In this example, there is no pre-box because there is only one laboratory room within the "contagious" area. If a corridor with entrances to the laboratory rooms (boxed rooms) started after the anteroom, there would be a small room (i.e. pre-box) in front of them (between the corridor and the laboratories). The model provided by the CUH2A Laboratory, Princeton, NJ, USA (published under the license from the World Health Organization)

preventing the release of BPA into the external environment is no less than the professionalism of the staff. As a rule, they are located in isolated protected areas. The anteroom of such a laboratory includes two rooms separated by a shower room. In some countries, the laboratory is divided into two zones: the “contagious” zone or “hot” zone and the “clean” zone or “cold” zone. The terms “contagious” zone and “clean” zone are accepted in CIS countries and we will use them further for convenience. In the case of zone division, the wall of the shower room adjacent to the “clean” zone is the boundary of the zones. As a rule, the shower room is arranged in such a way that specialists enter the “contagious” zone bypassing it and exit strictly through it. The passageways of the “contagious” zone are equipped with double doors so that the second door can be opened only after the first door is closed. Specialised

training (knowledge of Biosafety rules is checked by a committee at regular intervals), medical supervision and vaccination (if a vaccine is available for the pathogen and the specialist has no contraindications to vaccination[1]) are mandatory requirements for the staff of such a laboratory. A distinctive feature of work in laboratories of BSL-3 and BSL-4 levels is adherence to the *principle of staff pairing*, according to which work with PBAs is carried out by two specialists, one of them being a supervisor.

In the "clean" zone there are rooms where preparatory work is carried out: washing of clean laboratory utensils, preparation, filling and sterilisation of nutrient media, work with documents. There are also rest rooms here, as continuous work with PBAs is allowed for no more than 4 hours, after which a rest of at least half an hour is allowed[2].

In the "contagious" zone, boxed rooms (i.e. with a small "vestibule" for donning and doffing the hazmat suit) are located where material is received and microbiological and immunological tests are performed. A "Biohazard" sign is also posted at the entrance. In addition, there are rooms for nucleic acid detection, record keeping and material decontamination (autoclave), from which the decontaminated material will go for disposal, and decontaminated instruments and protective clothing will go to the room of the "clean" zone for washing and preparation for work. For these purposes it is most convenient to use a special autoclave, which is installed in the wall of the laboratory in such a way that the loading of material is carried out in the "contagious" zone, and unloading – in the "clean" zone. This is one example of what is known as *flow assurance*, where the laboratory is designed and equipment is placed in such a way that the paths of PBAs, personnel and waste do not overlap. Airlocks are also placed at the boundary of zones to trans-

[1] In such a situation, admission to work is carried out at the personal request of the specialist on the basis of the order of the head of the organisation. At the same time, they are not allowed to work in aerosol laboratories, with material contaminated or suspected of being contaminated with causative agents of Q fever, as well as to work with PBA for which specific treatment methods have not been developed. It should be remembered that some countries do not provide vaccination when working with bacterial pathogens.

[2] In practice, during outbreaks, this rule is broken and most often the laboratory switches to round-the-clock operation. As Igor V. Domaradsky wrote, during a cholera outbreak in Karakalpakstan (now a sovereign republic within Uzbekistan) in the 1960s "*everyone worked almost without sleep, but with great enthusiasm*".

fer equipment and materials when necessary[1]. The airlock design allows for additional decontamination of the air entering from the "contagious" zone before it is opened from the "clean" zone side.

A pressure difference is provided between the "clean" and "contagious" zones: 50 Pa (5 mm water column) / 0.0072 PSI for diagnostic laboratories and 100 Pa (10 mm water column) / 0.015 PSI for research laboratories[2]. The pressure differential is achieved by airtight rooms, outward opening doors and a sophisticated system of supply and exhaust ventilation, where all air entering and leaving the "contagious" zone passes through HEPA filters[3]. As a rule, the incoming air passes through H13 class filters (≥99.95%) and the outgoing air through H14 class filters (≥99.995%). At the same time, in case of work with *Y. pestis* in the laboratories of CIS countries, the pressure difference should be at least 100 Pa and only H14 class filters should be used: one for "inlet" and a cascade of two for "outlet"! The performance of this system is monitored daily by the aerodynamic resistance of the filters (i.e. the pressure difference between before and after the filter).

The liquid waste generated during operation is chemically and thermally disinfected, while the effluent from the hygienic shower is only thermally disinfected. Treatment systems equipped with a backflow prevention device provide collection, disinfection, cooling and discharge of wastewater. Similar systems, consisting of a tank in which wastewater was disinfected after accumulation, were available at the Special Laboratory as early as in the past.

The boxed room is the most protected part of the laboratory (in CIS countries it is also called the maximum protection block or informally "box-in-box"). It is airtight and finished with non-adsorbent, dust-free,

[1] Only keys, stamps and records that have been disinfected (most often by soaking in an anteroom, so all records are made in pencil) may be taken through the anteroom to the "clean" area.

[2] The average air velocity at the zone boundary should be at least 0.4 m/sec / 78.7 ft/min, which is checked every six months.

[3] High efficiency filters used for capturing fine particles. Developed in the 40s of the last century in the USA for capturing radioactive particles at nuclear industry enterprises. In the USSR, filters based on "Petryanov fabric" invented by Igor Vasilievich Petryanov-Sokolov (1907-1996) were used. This fabric consists of chlorinated polyvinyl chloride (perchlorovinyl; $[C_nH_{2n+2-x}Cl_x]$, $n < 2x < 2n$), applied to gauze in a thin layer in the form of polymer fibres.

non-combustible materials that are easy to clean and resistant to disinfectants. The junctions between horizontal and vertical surfaces are rounded and floor waterproofing is applied to the walls to a height of at least one and a half metres. Electricity supply systems are usually fixed to metal structures just below the ceiling. Like all rooms in the "contagious" zone, the box rooms are equipped with ultraviolet lamps, which are switched on after work for half an hour and wiped down at least once a week with a 70% ethanol solution to remove dust.

Biosafety cabinets (BSCs), which are a sealed unit containing a mechanical ventilation system that provides controlled air movement through the unit's internal cavity (plenum) and filters, are housed in boxed rooms. Because BSCs are an effective primary barrier, all works with PBAs is conducted there.

The first analogue of the modern BSC was designed[1] in 1943 by Marinus van den Ende[2] at the National Institute for Medical Research of the Medical Research Council (UK) under the close supervision of the military. Although similar devices had been known since the early 20th century (for example, Robert Koch designed and used a "biocontainment" cabinet for his work), they did not provide a sufficient degree of protection

Currently, BSCs are divided into 3 classes depending on the protection provided to the operator (specialist) and the product (PBA). The Class I BSC provide protection to the specialist but not to the material (the "product"). This is done by sucking air through the front opening with a minimum velocity of 0.36 m/s / 70.87 ft/min (in CIS countries speed from 0.75-1 m/s / 147.6-196.8 ft/min) into the internal space of the cabinet, circulating it over the work surface (table top) and then ejecting it through a filter into the laboratory ventilation system (**Figure 27.4**).

The Class II BSC provides protection for the specialist and the product. This is achieved by sucking air through the front opening into the interior of the cabinet and then entering the pump through a perforation

[1] Van den Ende M. *An apparatus for the safe inoculation of animals with dangerous pathogens*. J Hyg (Lond). 1943; 43(3): 189–195; DOI: 10.1017/s0022172400012808.

[2] 1912—1957; the South African and British physician of Dutch origin, bacteriologist, pathologist. Fellow of the Royal Society of South Africa (1954). Author of books on rickettsiae and "air hygiene".

in the front edge of the work surface. From there the air rises to the upper chamber, where a part of it passes through the filter into the inner space of the cabinet and descends to the working surface in a laminar flow. It is important to realise that part of the flow descends through the glass sash, creating a "curtain" in the front opening, which also protects the operator and the product. Next, through the openings around the perimeter of the working surface, the air flows back into the pump and up into the upper chamber. There, some of the air is recirculated through the filter back into the interior of the cabinet, and some will be discharged through the filter into the room or laboratory ventilation system (**Figure 27.5** and **Figure 27.6**). Depending on the velocity of the incoming flow and air recirculation, Class II BSCs exist in four variants (types A1, A2, B1 and B2). Thus, the cabinet of Type A1 has a minimum air intake velocity of 0.38-0.51 m/s / 74.8-100.39 ft/min (in CIS countries: at least 0.4 m/s / 78.7 ft/min), and cabinets of Type A2, Type B1 and Type B2 have a minimum velocity of 0.51 m/s / 100.4 ft/min. In cabinets Type A1 and Type A2, 70% of the air is recirculated into the interior of the cabinet, whereas in cabinet Type B1 it is 30%. In the cabinet of Type B2 air is not supplied for recirculation: one air stream is sucked through the front opening into the interior of the cabinet and, having passed through the perforation and filter, is discharged into the room or laboratory ventilation system, and the second air stream is sucked from the room into the filter of the upper chamber, from where it descends by laminar flow to the working surface and, having passed through the perforation and filter, is also discharged into the room or laboratory ventilation system.

The Class III BSC was invented in 1943 by Soldier Hubert Kaempf Jr. under the direction of Arnold G. Wedum at Industrial Health and Safety at the U.S. Army Biological Warfare Laboratories, Camp Detrick (now Fort Detrick). This cabinet is an airtight chamber that provides maximum protection, so it is used for work with the most dangerous PBAs. The airflow inside the chamber is supplied from the room (or laboratory ventilation system) through a filter and discharged to the laboratory ventilation system through a system of other filters, while negative pressure (at least 200 Pa or 20 mm of water column relative to the room) is created inside the chamber. The PBA is handled by the operator through special cuffs. The box can be equipped with a water

supply system as well as a tank (or drainage system) for liquid waste (**Figure 27.7**).

BSCs shall be located away from heating devices and strong air flows (supply and exhaust zones), in places where they will not interfere with the flow of PBA and personnel. Their functioning (protective effectiveness) shall be checked annually, as well as after installation (commissioning), changing the filter or moving them more than 10 cm away. Protective effectiveness of BSCs is confirmed only in case of compliance with operational characteristics:

Class I BSC:

- average inflow air velocity should be at least 0.36 m/s / 70.87 ft/min (or 0.75-1 m/s / 147.6-196.8 ft/min in CIS countries) along the entire cross-section of the opening;
- filter protective efficiency corresponds to the control methodology.

Class II BSC:

- average inflow air velocity should be at least 0.38 m/s / 74.8 ft/min (or 0.4 m/s / 78.7 ft/min in CIS countries) at the face of the front opening;
- average downward air velocity 0.25-0.5 m/s / 49.2-98.4 ft/min;
- downward airflow uniformity ±20% of the mean value;
- downward flow direction across the entire cross-section of the cabinet chamber;
- filter protective efficiency corresponds to the control methodology.

Class III BSC:

- average inflow air velocity greater than 0.7 m/s / 137.8 ft/min through the glove port with one glove removed;
- specific air flow rate 0.05 m^3/s per 1 m^3 volume of the working chamber of the cabinet;
- pressure difference in the working chamber of the cabinet in relation to the room is greater than 200 Pa / 0.029 PSI;
- filter protective efficiency corresponds to the control methodology.

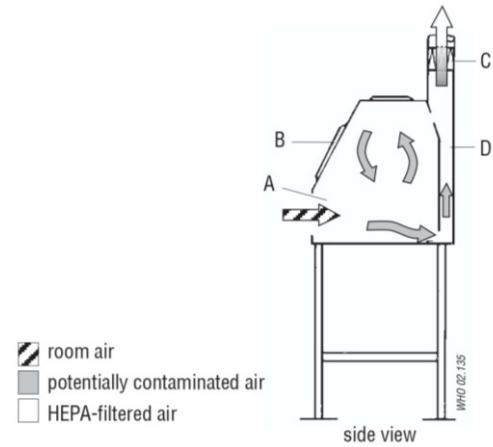


Figure 27.4. *Class I BSC: a room air is sucked in through the front opening (A) at a minimum velocity of 0.36 m/s / 70.87 ft/min (in CIS countries speed from 0.75-1 m/s / 147.6-196.8 ft/min) into the interior of the cabinet, circulates over its working surface (table top) and is discharged through the exhaust duct (D) and filter (C) into the ventilation system. The operator works through the front opening (A), visual control - through the glass (B)*

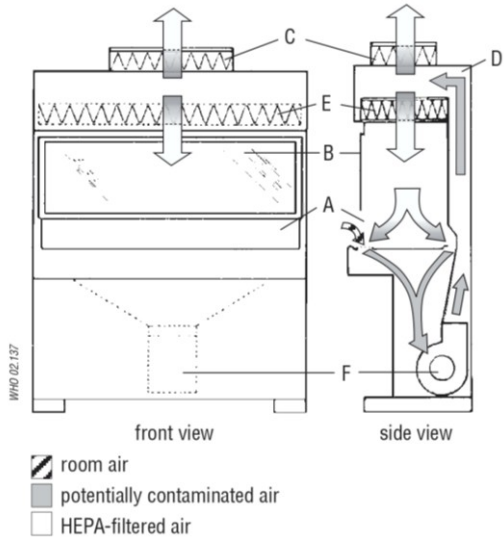


Figure 27.5. *Class II BSC (Type A1): the room air is sucked through the front opening (A) at a minimum velocity of 0.38-0.51 m/s / 74.8-100.39 ft/min (in CIS countries: at least 0.4 m/s / 78.7 ft/min) into the interior of the cabinet, passes through the perforation located on the front edge of the work surface (worktop) into the pump (F), from which it rises into the upper chamber (D). Due to the difference in the size of the filters (C and E), about 70% of the air flows down through the filter (E) in a laminar flow to the work surface (including the glass sash), passing through the perforations located at the edges of the work surface (worktop) to the pump (F), from which it rises to the upper chamber (D). From here, about 70% of the air is recirculated again through the filter (E) and the remaining 30% of the air is discharged into the room through the filter (C) or discharged into the laboratory ventilation system through a special nozzle. The operator works through the front opening (A), visual control through the glass sash (B)*

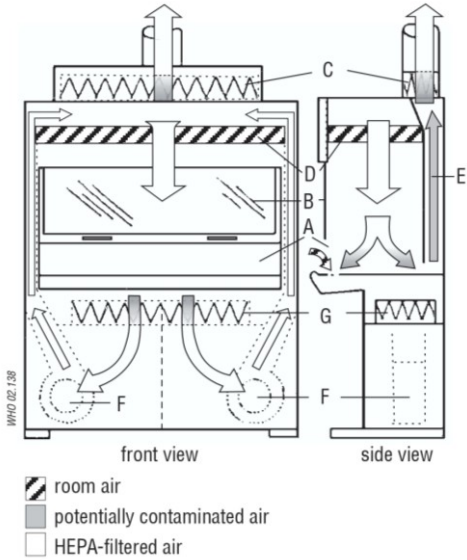
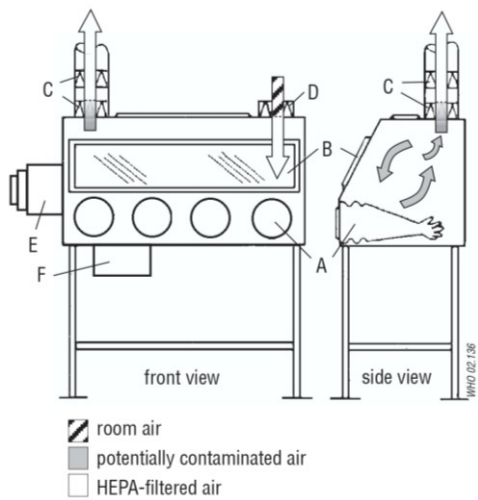


Figure 27.6. *Class II BSC (type B1): the room air is drawn through the front opening (A) at a minimum velocity of 0.51 m/s / 100.4 ft/min into the interior of the cabinet, passes through a perforation located at the front edge of the work surface (table top) into the pumps (F), from which it rises (E) into the upper chamber. Due to the difference in the size of the filters (C and D), about 30% of the air flows laminarly down through the filter (D) onto the work surface (including the glass sash), passing through the perforations located at the edges of the work surface (worktop) into the pumps (F), from which it rises (E) into the upper chamber. From here, about 30% of the air is recirculated through the filter (D) and the remaining 70% of the air is discharged through the filter (C) into the laboratory ventilation system. The operator operates through the front opening (A), visual inspection is through the glass sash (B)*

Figure 27.7. *Class III BSC: the air from the room (or laboratory ventilation system) is supplied through a filter (D) to the internal hermetic space of the cabinet, from where it is discharged to the laboratory ventilation system through filters (C); a negative pressure of about 124.5 Pa (12.45 mm of water column) / 0.018 PSI (in CIS countries: 200 Pa (20 mm of water column) / 0.029 PSI) is created inside the cabinet. The PBA is fed through an airlock (E), operated by means of inbuilt gloves (A). An integrated tank (E) is used for waste material*



Work in the BSC must be carried out by one person (except for special cabinets for pair work). If you are going to work in a new cabinet or a cabinet that has been used by someone else, the first thing you must do is to make sure that there is a certificate of verification of protective effectiveness (it should be posted on the outer surface of the cabinet and indicate that less than one year has passed since the last inspection), and then clean the internal surfaces with a disinfectant solution. It is advisable to wipe non-sterile reusable instruments with 70% ethanol solution[1].

The entire working surface is conditionally divided into areas: "clean", "conditionally dirty" and "dirty", in which the work is performed. For example, Petri dishes with media for plating are placed in the "clean" area, manipulation (culture plating) is performed in the "conditionally dirty" area, and the seeded dishes are stacked in the "dirty" area. There is also a container with disinfectant solution for discharging instruments (handpieces, pipettes, inoculation loops). Another small container with disinfectant solution (for washing hands and wiping seeded cups) is placed in the "clean" area.

It is most convenient to place the "clean" area on the left and the "dirty" area on the right. A tray with low edges is placed in the "conditionally dirty" area, in which a napkin (tightly folded gauze) moistened with disinfectant solution is placed. All work with PBAs is carried out only over this tray. A burner or an alcohol burner (for firing the reusable loop and the neck of the tube) can be placed in the same tray. Despite the fact that the cabinet significantly increases the protection of the specialist and the environment, before each removal of hands from the interior of the cabinet (working chamber), they should be treated with a disinfectant solution. The same should be done with objects (Petri dishes, test tubes) that are removed from the cabinet, for example, to be placed in the thermostat. All reusable instruments should be wiped down with disinfectant solution after finishing work. The napkin lying in the tray ("conditionally dirty" area) is folded in the envelope method (bringing the ends to the centre) and dropped into the container with

[1] This is especially true for automatic pipettes, as dirt accumulated inside the spout (on which the disposable tip is mounted) can significantly affect the quality of work. If you work with crystallising solutions, at least once every few days, run the pipette piston by placing the spout (without the tip) in a cloth heavily moistened with 70% ethanol solution.

the disinfectant solution ("dirty" area). Using a cloth moistened with the disinfectant solution ("clean" area), wipe the work surface (table top), moving from the "clean" area to the "dirty" area (also wipe the tray). Dishes are also wiped, which are placed either in a container for subsequent disinfection (in CIS countries they say "killing") or in a container for transport to the thermostat, observing the principle of staff pairing[1]. Switch on the UV lamp of the cabinet, which is wiped at least once a week with 70% ethanol solution to remove dust. After 30 minutes switch off the lamp and wipe the cabinet surfaces with distilled water.

Working with PBAs requires increased attention and concentration! As long as the dish is open or the material is on the loop, all attention should be paid to it until the dish is closed and the residual material is destroyed. Nevertheless, we are all human beings, so it is possible to make mistakes (including at the stage of packaging of PBAs-suspect material), emergency situations, which in turn lead to *accidents*, i.e. situations leading to a sharp increase in the risk of contamination of personnel, the environment and bystanders. In case of an accident, it is **important** not to conceal it, because in the worst case it can lead to fatalities (as in the case of Sverdlovsk-19), but to strictly follow the procedure depending on the type of accident. The procedure may vary slightly from laboratory to laboratory, but in general it is summarised in the following main steps.

Accident without splashing of PBAs: during rapid roasting of the loop, the cinder fell on the table, a crack was formed on the glassware with PBAs, the loop touched the outer part of the test tube or dish. In this situation the following actions are performed:

Specialist who caused the accident	***Partner (any member of staff who is nearby)***
informs the partner (those nearby) of the accident – "accident" signal	receives an "accident" signal
↓	↓
the place of the accident (where the cinder fell or where the dishes are)	activation of the alarm
	↓
	reports the authorities about the

[1] Dishes are placed in the thermostat with the lids downwards so that the condensate formed does not wet the culture (in the case of work with mycelial fungi, dishes are placed with the lids upwards so that the fungal spores do not fall on the lid).

is covered with a cloth moistened with disinfectant solution
↓
wraps the cinder, utensil or contaminated object in a napkin (if the accident occurred over a tray, the contaminated object, cinder or utensils can be wrapped in the tissue over which the work was carried out, using the envelope method - by bringing the ends of the envelope towards the centre)
↓
immerses in a special tray or bag for autoclaving
↓
treats gloves with disinfectant solution
↓
covers the accident site with a cloth moistened with disinfectant solution for 30 minutes
↓
continues working after reporting the authorities

accident

This type of accident is the most common and, unfortunately, the most concealed. In addition, some experts believe that if PBAs falls on a tray (i.e., a tissue moistened with disinfectant solution) or into a container of a disinfectant solution, it is decontaminated, so it should not be considered to be an accident. Others believe that an aerosol is formed at the time of the fall, so this type of accident should be considered an accident with PBAs splashing.

Another type of accident without splashing of PBAs is when the loop with PBAs touches the hazmat suit. If the glove is touched, a tissue moistened with disinfectant solution is applied to the place of touching and the outer ("upper") glove is removed by turning it out and then immersing it in a container with disinfectant solution (where instru-

ments are dumped). After putting on a new glove, the specialist continues working. If the sleeve of the protective suit is touched, a tissue moistened with disinfectant solution is applied to the place of touching. The specialist reports the accident to his/her partner (who is in the room), who completes the work and, going with the specialist to the pre-box, helps him/her to remove the protective suit, which is placed in a container with a disinfectant solution. With the authorisation of management, they return to work.

Accident with PBAs splashing: breakage of glassware containing PBAs, splashing of bacterial suspension from a pipette or syringe, splashing of biological fluids during autopsy, dripping of biological fluid from the lid of a container during its opening, as well as any events that produce aerosol from PBAs, including work at a vacuum unit during culture drying, work with a centrifuge and transport of PBAs between rooms and to an autoclave room. In case of an accident occurring *inside* the BSC, the following measures must be taken (**important:** *do not panic and perform all movements smoothly (!) so as not to disturb the protective screen - laminar air flow covering the front opening of the BSC*):

Specialist who caused the accident	***Partner (any member of staff who is nearby)***
informs the partner (those nearby) of the accident – "accident" signal	receives an "accident" signal
↓	↓
completes the work (covers the spillage site with a cloth moistened with a disinfectant solution + dumps the pipette (syringe) into a container with a disinfectant solution or wraps broken dishes with a napkin placed on a tray and moistened with a disinfectant solution)	activation of the alarm
↓	↓
treats hands (gloves and suit sleeves) with a disinfectant solution	<u>all staff</u> close the front opening of the cabinet (the method of closing depends on the design) or, if possible, complete the work
↓	↓
removes the outer (top) pair of	

gloves
↓
immerses them in a container with
a disinfectant solution
↓
gently pulls his hands out of the
BSC
↓
closes the front opening of the BSC
(the method of closing depends on
the design)
↓
turns on the UV lamp in the BSC[1]
↓
all stuff go into the pre-box
↓
turn on the room's UV lamp
↓
treat the upper pair of gloves with a disinfectant solution
↓
remove and place in a container with disinfectant solution
↓
carefully remove the suit and place it
into a container with a disinfectant solution
↓
remove the inner (the so-called called bottom gloves) pair of gloves
and place them in a container with a disinfectant solution
↓
treat the hands with 70% ethanol solution
and wash the hands with soap and water
↓
report the authorities about the accident
↓
wait for the arrival of the emergency team and the Biosafety Control
Commission

[1] In practice, it will be morally difficult for the specialist to do this, because the studied culture will die, especially if the patient is expecting its identification, or when it is an experimental culture that took a lot of effort and time to obtain. In this situation, it may be tempting to preserve it or "turn a blind eye" to the accident, but it is absolutely not advisable to do so.

It should be noted that some practitioners treat the sleeves and front of the hazmat suit with a disinfectant solution before removing the suit.

The Commission, once on site, decides on the next course of action. In this case, they may decide to allow the staff to work at other work-places with BSC disinfection after the end of the work.

In the event of an accident *outside* the BSC, the following measures must be taken (**important:** *do not panic*):

Specialist who caused the accident	***Partner (any member of staff who is nearby)***
informs the partner (those nearby) of the accident – "accident" signal	receives an "accident" signal
↓	↓
	activation of the alarm
	↓

all specialists close the front opening of their BSCs and holding their breath, go into the pre-box

↓

turn on the room's UV lamp

↓

treat the upper pair of gloves with a disinfectant solution

↓

remove and place in a container with disinfectant solution

↓

treat open parts of the body with a cotton swab moistened with 70% ethanol solution

↓

treat areas of the suit that may have been exposed to PBAs with a disinfectant solution

↓

carefully remove the suit and place it into a container with a disinfectant solution

↓

remove the inner (called the bottom) pair of gloves and place them in a container with the disinfectant solution

↓

treat the hands with a 70% ethanol solution
and wash the hands with soap and water[1]
↓
report the authorities about the accident
↓
remove work clothes, take a hygienic shower and put on clean clothes
in the anteroom
↓
wait for the arrival of the emergency team and the Biosafety Control
Commission

When the Commission arrives on the scene, they decide on further measures. In this case, they may decide to send the specialists to a medical isolation centre. Further authorisation to work will only be granted after the biological safety knowledge has been verified.

The *accident team* arriving at the accident site carries out disinfection measures in a hazmat suit Type I (discussed below), using a freshly prepared solution of a disinfectant active against the BPA that was handled: for non-spore-forming microorganisms, which includes *Y. pestis*, a 3% chloramine solution is used, and for spore-forming microorganisms a 6% hydrogen peroxide solution with 0.5% surfactant (i.e. for spore-forming microorganisms - 6% hydrogen peroxide solution with 0.5% surfactant).

In the case when the accident occurred in the BSC, two hours after the accident the UV lamp of the BSC is switched off and the front opening may carefully be open. In the working chamber of the cabinet containers with a disinfectant solution and bag(s) are collected for autoclaving. Next to it a container is placed in which the autoclaving bag will be transported for disinfection. If the specialist has not completed the work, the accident site (product spillage) is covered with napkins moistened with a disinfectant solution for 10 minutes. Wipes, fragments of

[1] If the mucous membranes of the eyes, nose and mouth have not been protected, they are rinsed with running water, the nose and throat are rinsed with a 70% ethanol solution, and a 2% solution of boric acid (0.5 g of boric acid per 25 ml of water) is injected into the eyes. When working with bacteria, an antibiotic active against the bacteria is taken, when working with viruses, interferon or an interferon inducer is injected into the eyes and nose, and when working with botulinum toxin, the eyes and mouth are treated with 10 IU/ml of antitoxic serum.

laboratory utensils, as well as instruments and dishes with cultures (i.e. everything inside the cabinet) are collected with forceps (less frequently with tweezers) and placed into a bag for autoclaving. Some specialists believe that visually uncontaminated (not "flooded") instruments can be wiped with a napkin moistened with a disinfectant solution and not sent to autoclave, but it is more correct to proceed from the fact that the aerosol formed during the accident could get on all objects in the working chamber of the cabinet. Further, using a cloth moistened with a disinfectant solution, all surfaces of the internal chamber of the cabinet are treated, including the space under the working surface (table top), which is also treated and placed into a bag for autoclaving. All bags are placed into a container and transported to the autoclave room for disinfection. The UV lamps of BSC and rooms are switched on.

In the event of an accident outside the BSC, two hours after the accident, the UV lamp of the room is switched off and, starting from the threshold (entrance door), spray the disinfectant solution. For this purpose, use a device for disinfection by irrigation method (hydropult and others) or aerosol method. Spraying is carried out in front of the person, spraying the air and all objects, including the ceiling, walls, floor and equipment, slowly moving into the interior of the room. Some specialists believe that irrigation should be carried out from the anteroom, into which the specialists go in their working clothes (possibly contaminated with PBAs). Wipes moistened with a disinfectant solution are applied to the broken dishes fragments, as well as dishes with crops and tools (i.e. everything at the accident site). After two hours, they are placed in an autoclaving bag using a forceps, which is loaded into a container and transported to the autoclave for disinfection. The accident site is additionally wiped with a napkin moistened with the disinfectant solution and the UV lamp of the room is switched on.

Two hours after the accident was cleared, an specialist in a hazmat suit Type IV switches off the UV lamps, washes the BSCs of the remaining disinfectant solution and cleans the room.

An **accident involving damage to the integrity of the hazmat suit** is unlikely in a laboratory environment and is more likely to occur in a hospital environment. In this situation, immediately place a cloth moistened with disinfectant solution on the damaged area, notify your partner (those in the room) and immediately go to the pre-box. There, the

protective suit should be removed according to the schemes described above and the authorities must be reported. The activation of the alarm, as well as the completion of work by other staff, seems inappropriate as there is no risk of infection for them, unlike in previous types of accidents.

It should be noted that damage to the upper pair of gloves (one pair of gloves), most often occurring while working in the BSC, is not considered an accident, as the lower pair of gloves remains intact. In this case, report your partner (those in the room) and replace the damaged pair of gloves with a new pair. It is advisable to keep a supply of gloves right in the room to prevent going to the pre-box. In addition, when working in a suit where the gloves are not an integral part of the sleeves, it is the lower pair of gloves that must be secured with an adhesive tape. If the upper pair of gloves is damaged, they can easily be removed and replaced with new ones.

A special type of accident involving damage to the integrity of a protective suit is damage to the integrity of a pneumatic suit to which air is supplied at low pressure. These suits are most often used to handle large animals, such as monkeys, which can damage them with their claws. If the suit is damaged, the air supplied inside the suit will escape through the damaged area, preventing contaminated air from entering the suit. In this situation, the damage should be repaired immediately using improvised means. A piece of duct tape is an excellent solution, but you can also use a cloth moistened with disinfectant solution, a tourniquet or twist the suit fabric by hand. No matter what you use to repair the damage, the important thing is to repair it. If the suit material allows, it is advisable to put a small piece of duct tape on it in advance to be ready for an accident (and may it never be needed).

A **skin injury accident** is probably the most dangerous type of accident, as there is a high risk of infection. It is also most often combined with another type of accident. For example, a culture flask explodes in the hands of an specialist with a shard cut through the glove, grazing the skin on the palm of the hand. This is, firstly, a skin injury accident and, secondly, as can be easily guessed, a splashing accident. The first measure in such case is to treat the injury site with a disinfectant solution. If you work in a BSC, it is advisable to put your hand into a container with a disinfectant solution (the one in the "clean" area of the

BSC). You can also dip your hand into a glove filled with disinfectant solution. If a foot is injured, it can be put into a container (bucket) of a disinfectant solution that is kept in the room. At the same time report the partner (those in the room) who activates the alarm system. Subsequent actions are similar to those carried out in case of an accident with PBAs splashing. A swab with 70% ethanol solution is applied to the wound site. In the case of work with viruses, the wound is treated with a 5% alcohol iodine solution, and in the case of work with spore-forming bacteria, the wound is flushed with distilled water. Then report the authorities, wait for the arrival of the emergency crew and the Biosafety Control Commission, which decide on further measures depending on the factors such as the type of PBAs, the type of damage and the type of the associated accident.

28 THE SUIT

The first set of the hazmat suit was proposed by Charles de L'Orme[1] in 1619 and was made of Moroccan leather, similar to the military uniform of the time. It consisted of a long coat, hat, gloves and boots soaked in wax and aromatic infusions to ward off disease[2]. The last element of the costume was a characteristic mask, known since the 14th century, with the glasses protecting the eyes and the beak, in which the doctor was supposed to resemble Thoth, the ancient Egyptian god of healing and wisdom. The beak of the mask was filled with cotton wool soaked in infusions, dried flowers and herbs, vinegar, and had two small holes for breathing, thus becoming the prototype of modern respirators and gas masks. The popularity of the mask was so great that it became an integral part of the Venetian Carnival, and the suit was the

[1] ?-?; the French physician, personal physician to King Louis XIII of France.

[2] As already mentioned, at that time it was believed that diseases arose due to the action of stinking night airs, therefore, it was necessary to use essential oils (perfumes) to "repel" them. This was partly logical, as many of the plats from which fragrant infusions were derived had antiseptic properties.



Plague Doctor costume

reason for the appearance of the plague doctor (It. *il Medico della Peste*) in the Commedia dell'arte.

Currently, all work with infectious agents is carried out in the *plague suit* or its equivalent, i.e. *personal protective equipment* (PPE), which we have already seen in the images in Chapter 26. The first prototype of the suit equipped with a means of filtration was designed in 1878 by Victor Vasilievich Pashutin[1]. Depending on the type of work performed, the plague suit is divided into types (**Table 28.1**) and in its maximum configuration (Type I) includes working clothes (trousers and jacket with long sleeves, i.e. the so-called "pyjamas", which we have already seen in **Figure 26.3**, as well as socks in which the trousers of the trousers are tucked!)[2], a cap (small surgical scarf), a large surgical scarf, gloves, pro-

[1] Виктор Васильевич Пашутин, 1845–1901; the Russian physician, pathophysiologist. Author of books on general and experimental pathology, pathological physiology. In 1890-1901 he was Head of the Imperial Military Medical Academy (now the S. M. Kirov Military Medical Academy).

[2] It should be remembered that any presence of jewellery is unacceptable!

tective footwear (boots, waterproof shoe covers)[1], a towel, protective goggles, a respirator and an anti-plague gown. When working with animals, as well as during post-mortem examination, high boots, a rubberised apron and armbands are additionally worn.

Table 28.1. *Equipment of the anti-plague suit*

Element	Type			
	I	II	III	IV
Anti-plague gowns[1]	+	+	+	+
Working clothes	+	+	+	+
Socks	+	+	+	+
Gloves	+	+	+	+
Scarf[2]	+	+	+	+
Safety shoes	+	+	+	
Towel[3]	+	+	+	
Respirator[4]	+	+		
Glasses[4]	+			

Note:
[1] a surgical gown may be used for suit Type IV;
[2] a small surgical scarf or cap is used for suit Type IV, a large surgical scarf or helmet for other types.
[3] is not a part of the suit when working in the hospital environment.
[4] can be replaced by a full-face mask that includes a filtration system (respirator).

The presented sets show that the suit Type I provides protection of the skin of the hands, body surface, face, respiratory and visual organs. The suit Type II provides protection of skin of hands, body surface, face and respiratory organs (without the vision organs). Suit Type III and Type IV provide protection of skin of the hands and body surface (without the face, the respiratory organs and the eyes), and suit Type IV is a light version of suit Type III. Nowadays, instead of the anti-plague suit, its analogues are most often used, selected in accordance with the specified characteristics, which may differ in equipment, but usually differ from the classic anti-plague suit only by the presence of a coverall

[1] Traditional footwear of the anti-plague system in CIS countries are chuviaki (from Adygean. цуакъэ – "shoes"), which are soft leather slippers without heels with a closed heel, from which it is convenient to change into boots in pre-box.

merged with trousers and hood (**Figure 28.1**). In maximally isolated laboratories BSL-4, a pneumatic suit is used.

The presented sets show that the suit Type I provides protection of the skin of the hands, body surface, face, respiratory and visual organs. The suit Type II provides protection of skin of hands, body surface, face and respiratory organs (without the vision organs). Suit Type III and Type IV provide protection of skin of the hands and body surface (without the face, the respiratory organs and the eyes), and suit Type IV is a light version of suit Type III. Nowadays, instead of the anti-plague suit, its analogues are most often used, selected in accordance with the specified characteristics, which may differ in equipment, but usually differ from the classic anti-plague suit only by the presence of a coverall merged with trousers and hood (**Figure 28.1**). In maximally isolated laboratories BSL-4, a pneumatic suit is used.



Figure 28.1. *The anti-plague suit Type I (left) and its analogue (right). Compare them with those worn by the specialists in* ***Figure 26.2*** *and* ***Figure 26.4***



Figure 28.2. *Example of putting on a large scarf*

The type of the suit used depends on the type of activities and work to be carried out. In the case of *Y. pestis*, the suit Type I is used, with the exception of immunisation of animals with killed cultures, during which the suit Type IV, completed with a respirator, is used, and transporting the material to the autoclave requires the suit Type III completed with an apron. During the treatment of bubonic plague patients receiving antibiotic therapy, the use of the suit Type III is permitted.

The order of putting on the suit is not strictly regulated, but it is recommended first to put on the scarf made of cotton fabric, more often calico. As already mentioned, the headscarf can be small (90 x 90 x 125 cm) and large (120 x 120 x 150 cm), and the latter is available in two modifications: 1) a piece of cloth folded diagonally is placed on the head so that the base of the resulting triangle covers the forehead, the corners (ends) are tucked crossed under the chin and tied at the back of the neck (**Figure 28.2**, left); 2) a piece of fabric with a square or rectangular neckline in the middle, with ties (ribbons) sewn to the corners to secure the scarf on the head (**Figure 28.2**, right).

The choice of modification depends on the type of *Personal Respiratory Protection Equipment* (PRPE) being used. If you plan to work in a full-face mask, it is more convenient to use a headscarf without a cut-out (modification 1), because the skirt (i.e. a silicone rubber band around the contour of the mask) will fit tightly on the fabric. For working with goggles and a respirator it is more convenient to use a headscarf with a cut-

out (modification 2), as the goggles and respirator can be put on under the headscarf and the edges of its cut-out will create an additional fixation of the respirator. This is particularly convenient when using the "Petal"[1] (Russ. «Лепесток») type respirators. However, it should be borne in mind that improper fitting can lead to contaminated air being inhaled. For this reason that they are banned in some countries. Nowadays, respirators of protection class FFP3 (maximum protection class) are most often used, although in the 1980s cotton-gauze bandage[2] were still used. As an alternative to the scarf, a helmet (**Figure 28.3**) is used that completely covers the forehead, cheeks, neck and chin. It is also convenient when using a full-face mask.

An anti-plague gowns is made of a water-repellent fabric, such as impregnated cotton fabric. Traditionally, white fabric was used so that contamination could be seen at a glance, but now there are models in different colours. Externally, the anti-plague gowns is similar to the surgical gowns, but with long edges, when the odour goes deep behind each other, and long straps (ties) at the waist and neck. It is also slightly longer than the surgical one (the length reaches the lower third of the shin), but some specialists shorten it slightly to make it comfortable to walk. The sleeves have ties or elasticated cuffs. It is put on in front of a mirror or under



Figure 28.3. *Helmet (ties provide a better fit)*

[1] Filters based on "Petryanov's fabric", have been produced in the USSR since 1957. Simplicity of manufacture, availability of materials and cheapness of production made them extremely popular in the USSR. Despite the dangers of use, respirators of this type are still used by many institutions in the CIS countries.

[2] Currently banned for use. They were made from a 125 cm long and 50 cm wide piece of gauze. A layer of cotton wool about 25 cm long and 17 cm wide (weight of cotton wool 20 g, thickness of the layer 1.5-2 cm) was placed in the middle part of the gauze. The edges of the gauze were wrapped over the cotton wool layer, and the long ends were cut lengthwise, slightly short of the cotton wool layer. Three cotton swabs were additionally placed under the outer edge of the gauze.

the control of a partner, checking that the edges of the scarf do not stick out from under the dressing gown. Ties are tied at the collar and at the waist at the front.

Next, take two pairs of gloves and check them for integrity (tightness). To do this, it is enough to take the glove by the cuff with both hands and throw (fingers) several times over the resulting axis - if the structure is inflated, the glove is intact. The first pair is worn under the sleeves of the dressing gown, and the second pair is worn over the sleeves. For convenience, the gloves are fixed. A towel is placed behind the waistband of the dressing gown (for right-handed people it is more convenient to place it on the left side). When working with animals, as well as during post-mortem examination additionally wear high boots, a rubberised apron and armbands. Boots should be put on last, but if other footwear, such as waterproof booties (**Figure 28.4**), is used, they are to be put on first. Either way, the order of putting on depends on the convenience of the specialist, the main thing is that the suit should provide reliable protection in accordance with the type of the suit.

In contrast to putting on, taking off an anti-plague suit is strictly regulated. It is based on the principle: from the potentially most contaminated to the least contaminated. The elements of the suit are taken off slowly, the removed element is immersed in a tank with a disinfectant solution, hands are washed with a disinfectant solution.

If the work was carried out in a vivarium or a room for post-mortem examination (the sectional room), the apron is treated with a disinfectant solution using top-down movements before taking off. Feet in boots are alternately placed in a basin with a disinfectant solution, also from top to bottom and wiped with a cloth moistened with it. In



Figure 28.4. *Example of putting on waterproof shoe covers*

practice, for convenience, a roller may be used, on which the cloth is wrapped. The other leg is at this moment on a mat moistened with a disinfectant solution.

Then go to the pre-box, where the hands are to be washed in a disinfectant solution for 1-2 minutes, then remove the towel, the armbands and the first pair of gloves. Wash the hands again in a disinfectant solution.

take off your glasses
(unless wearing a full-face mask)

Put into a tank with a disinfectant solution ↓ *wash your hands with a disinfectant solution*

Take off your anti-plague gowns:

– loosen the sleeve straps (this is why it is advisable to wear the first pair of gloves under the gown);
– untie the ties around your neck and waist;
– cross your arms crosswise on your chest and take the dressing gown around your shoulders (i.e. right hand at the left shoulder and left hand at the right shoulder);
– pull off, causing the dressing gown to come off folded inwards;
– roll the dressing gown up ("in a tube")

put into a tank with a disinfectant solution ↓ *wash your hands with a disinfectant solution*

take off your full-face mask
(if the work was done in it)

put into a tank with a disinfectant solution ↓ *wash your hands with a disinfectant solution*

take off your scarf:

– untie the knot (modification 1) or the ties (modification 2);
– gather all ends at the back of the head with the dirty side facing inwards (modifications 1 and 2) or turn inside out (helmet)

put into a tank with a disinfectant solution ↓ *wash your hands with a disinfectant solution*

take of your respirator

put into a tank with a disinfectant solution ↓ *wash your hands with a disinfectant solution*

take off your safety shoes

put into a tank with a disinfectant solution ↓ *wash your hands with a disinfectant solution*

take off the last pair of gloves

The removed gloves are placed into a tank with a disinfectant solution (check for damage if necessary) and hands are treated with 70% ethanol solution and washed with soap and water.

There is a common belief that the respirator is removed before the gown, which is explained by the principle we started with - potentially the respirator is dirtier than the gown and certainly not the scarf. However, as it provides respiratory protection, it is advisable to remove it as late as possible (even after removing protective footwear).

The procedure for donning and doffing an analogue of an anti-plague suit depends on its design, but is generally similar to the principles described above. The only thing that can be added is that in the case of a full coverall, it should be rolled up with the outer side inwards, starting from the hood and gradually going down to the trousers.

Suits are disinfected either by immersion (3% chloramine solution or 6% hydrogen peroxide solution with 0.5% surfactant) or by autoclaving.

29 THE MATERIAL

The only condition under which a case of plague infection can be considered confirmed according to the World Health Organisation is the isolation of *Y. pestis* from the clinical material.

In *patients*, this material includes the contents of the buboes (vesicles, ulcers), pharyngeal secretions, sputum, blood, urine and faeces samples, and, if meningeal form is suspected, cerebrospinal fluid samples. The material is sampled immediately upon admission to the hospital or within two hours of suspicion, but before the start of specific treatment

(antibiotic therapy). Three days after the end of the treatment, daily sampling is carried out until three negative results are obtained (two negative results when examining a punctate from the buboes).

In *contact persons*, only oral and pharyngeal secretions are examined. Material sampling is carried out before prophylactic treatment (antibiotic therapy) and after its completion.

Buboes, pieces of organs (liver, spleen, lungs), blood from the heart, bone fragments containing bone marrow, as well as cerebrospinal fluid, contents of the lungs, bladder, intestines are examined from *cadavers*, which depends on the form of the disease.

Correct sampling affects the quality of the laboratory test and eliminates false negative results[1]. For this reason, when plague is suspected, the material is sampled by medical personnel under the supervision of a specialist of particularly dangerous infections. Let us briefly review the methods of sampling.

Bubo punctate. The buboes and surrounding tissues are treated with 70% ethanol solution, then 5% iodine solution and then ethanol again to remove excess iodine and prevent tissue burns. It is possible to use povidone-iodine (PVP-I) instead of iodine solution. Carefully insert the needle so that its point reaches the centre of the buboesis. If the buboesis is at the vesicle (pustule) stage, the needle is inserted at the edge of vesicles and slowly pushed into the centre. At least 5 ml of the content are withdrawn and the needle is carefully withdrawn. Next, 0.5 ml of sterile nutrient broth (or saline solution) is drawn through the needle into the syringe. This is done because, as a rule, the exudate in the buboesis is located between dense inflamed tissues and it is hardly possible to sample it in sufficient quantity. Most often it fills only the lumen of the needle, so it is "expelled" into a sterile tube using nutrient broth. For convenience, the broth can be drawn into the syringe before the procedure, but then it should either be drawn without a needle or changed to a sterile one. If it is impossible to extract the contents of the bubo, 0.3-0.5 ml of sterile saline solution is injected into the bubo and withdrawn. The last drops of the material (from the needle) are applied to two slides,

[1] It is likely that the difficulty in laboratory diagnosis from the case in Chapter 18 was due to errors made in the collection of the material.

which after drying are fixed (more on this in the next chapter) or unfixed are packed in a sealed container labelled "unfixed smear".

If the bubo has opened (ulcer, carbuncle), then, if necessary, necrotic masses and pus are removed with a sterile napkin, and a napkin moistened with 5% iodine solution (povidone-iodine) is carefully treated with skin around the affected area. A sterile swab is used to gently sample the excretion by wetting it from the centre to the periphery (some specialists recommend samplng the excretion at the centre and at the periphery with two different tissues), after which the swab is placed in a sterile transport medium, e.g. Cary-Blair Medium (see Supplementary Materials for medium prescriptions).

Oral and pharyngeal mucous discharge. It is taken on an empty stomach or 3-4 hours after a meal with a swab. To do this, the tongue is pressed with a sterile wooden stick (spatula) and material is sampled from the posterior surface of the pharynx, tonsils and, if present, areas of inflammation or ulceration of the mucosa. The swab is placed in sterile transport medium. It is important not to touch the mucous membranes of the cheeks, tongue, gums, lips and uvula with the swab when taking the sample.

Phlegm. It is taken into special sterile wide-necked containers (jars) with screw caps.

Blood. The skin over the vein is thoroughly wiped with 70% ethanol solution and iodine solution (povidone-iodine) is applied in a circular motion, starting from the centre. After the iodine dries, a tourniquet is applied and 10-20 ml of blood is taken, of which 5 ml is placed (plating) in 50 ml of broth, and the rest is distributed in tubes: for plating on dense nutrient media (if it is not possible to plating at the bedside of the patient), for biological sampling, for molecular genetic analysis (the tube should additionally contain anticoagulant [1]), for serum production (immunoserological reactions)

Pieces of organs from cadavers (autopsy). Metal parts of instruments (scalpels, scissors, tweezers) are decontaminated - they are wetted with

[1] Typically, a 4% solution of sodium citrate at a ratio of 1:10 to blood volume or a 6% solution of ethylenediaminetetraacetic acid (EDTA) at a ratio of 1:20 to blood volume is used.

95% ethanol solution and set on fire, after which it is necessary to wait for a while for the metal to cool down. Then small fragments are carefully separated and placed in containers with transport medium. To draw *blood from the heart* of an animal, the latter is fixed with tweezers. A red-hot scalpel is used to cauterise the anterior wall of the heart until a white spot appears, after which a syringe needle is used to penetrate the heart cavity and draw the blood. *Urine*, *bile* and *other bodily fluids* are sampled in the same way. On the other hand, if the animal has long since died and the blood is needed only for plating, the apex of the heart is cut off with scissors and touched to the surface of the agar with it (print). To take the *bone marrow*, tweezers and scissors are used to free the middle part of the femur from muscles and tendons. A small sterile napkin moistened with a disinfectant solution is placed under the freed area and the bone is cut with scissors, covering it with a dish lid to avoid splashing of material. In large animals, the bone is cut with a bone saw. The bone section is lifted upwards with tweezers and a loop is used to draw material for culture from the bone-brain canal.

Dried blood and serum samples prepared by impregnating filter paper pre-treated with 0.1% sodium mertiolate solution are also suitable for antibody and antigen testing (see Chapter 33). Dried samples are stored in tubes for 3 weeks at room temperature or up to 3 months at 4°C / 39.2°F. Before examination, 1 ml of buffered saline solution is added to the test tube with the paper and incubated for 40 minutes (the resulting solution corresponds approximately to a serum dilution of 1:10).

In case of rodents, the chest is opened, preserving the diaphragm but removing the lungs. Next, the large vessels and heart are dissected, after which a paper soaked in mertiolate is inserted into the cavity to absorb approximately 0.2 ml of blood.

In a similar way take flushes from the thoracic cavity, for which, after dissection of large vessels and the heart pour into the chest cavity 0.5-1 ml of saline solution with sodium mertiolate at a dilution of 1:4000. Carefully stirred and transferred to a test tube in which the same solution is added, but at a dilution of 1:8000.

In a ward with patients with pneumonic plague, air samples and washings are taken from surfaces.

All material containers are labelled and treated externally with a disinfectant solution. Then they are packed according to the *Triple-Package System*, which implies the presence of three protective layers:

first – a primary container. Watertight and airtight, this is where the sample (material) is stored;

second – a secondary container. Also watertight and leak-proof, contains sufficient absorbent material in case of damage (leakage) of the primary container. Several primary containers (e.g. several samples from one patient) can be placed in one secondary container. One of the referrals is also placed here (the second referral is transferred with the person delivering the container);

third – an outer packaging in which the secondary packaging is placed with sufficient cushioning material to ensure its safety. The minimum dimensions of this packaging shall not be less than 10 x 10 cm.

Packed material is transported to the laboratory in a refrigerator at a temperature not exceeding minus 16°C / 60.8°F, and if this is not possible, it should be delivered within 5-6 hours after the material is taken!



Figure 29.1. *The long-tailed weasel (Neogale frenata) captured in a metal cage in a focus (1975)*



Figure 29.2. *Examination of the gopher captured in a focus under conditions of temporary (field) laboratory for the presence of ectoparasites. The gopher is under the influence of ether. The attentive reader is probably indignant because of the lack of personal protective equipment, but this is how they worked back at the end of the last century. Note that examination is carried out over enamelled dishes from which fleas cannot jump out*

During the epizootological survey of plague foci, laboratory examination of zoological and entomological material and environmental samples is carried out. Carriers (rodents, hares and, rarely, small carnivores) and vectors (fleas and other ectoparasites) captured in the territory of the focus are subject to examination. Live animals are transported in metal cages or crates with mesh covers (**Figure 29.1**). If experimental studies of live carriers are not anticipated, they are killed in a forceps or by other humane means. To avoid loss of ectoparasites, animal carcasses (including those found dead) are transported in calico bags (examined for presence of ectoparasites is carried out at the temporary laboratory base in the foci or in a boxed room for working with infected animals; **Figure 29.2** and **Figure 29.3**). During active epizootics, wild rodents (both alive and dead) are sometimes examined for pharyngeal bacillicarrier by swabbing the pharynx with a swabe). Ectoparasites collected from the foci are delivered in tubes sealed with cotton-gauze plugs, alive or in a preserving solution[1]. Pellets, food residues from nests, rodent burrow soil and water are also sampled and transported using the Triple-Package System.

[1] Sodium chloride solution with gentian violet at a ratio of 1:200000-1:300000.





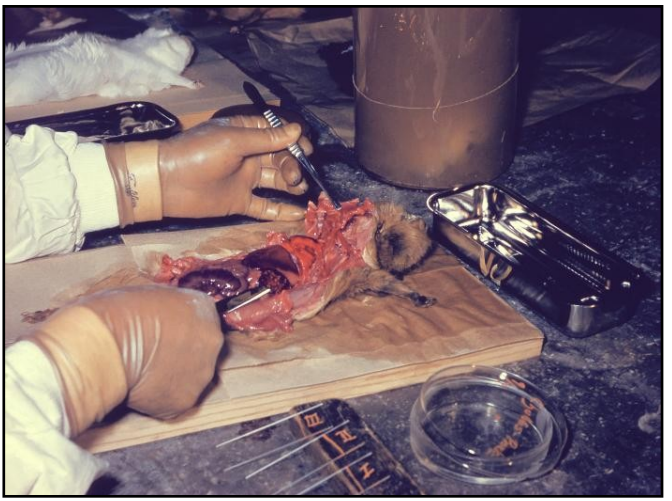




Figure 29.3. *Examination of rats captured in one of the settlements of Tây Ninh province (Vietnam). Note the "street" working conditions - this is how plague specialists worked in South-East Asian countries 20 years ago (now the situation has changed for the better, and Y. pestis has not been isolated in Vietnam since 2005). It is fair to say that with careful work, the risk of aerogenic infection during examination is low, and plague caused by antibiotic-sensitive strains is easily treatable.*
Courtesy of Dr.Victor Vasilyevich Suntsov (left)

If necessary, fragments of animal organs are extracted in a temporary laboratory in the foci (the procedure for post-mortem examination of animals will be discussed below). The liver and spleen are examined, and if pathological anatomical changes of internal organs characteristic of plague are detected, the lungs and lymph nodes are additionally examined (**Figure 29.4** and **Figure 29.5**). From animals found dead, blood from the heart and pericardial bundle of vessels, as well as the bone marrow (from the femur) and very rarely the brain, is also analysed. The transport of samples is carried out according to the Triple-Package System. In case of long transport, preserving liquids (e.g., Broquet's fluid, Cary-Blair medium) are used.

◄ *Capture of the carriers in a focus (courtesy of Ivan Viktorovich Pazizin)*



▲ ***Figure 29.4.*** *Post-mortem examination of the prairie dog (Cynomys sp.), a member of the Chubbs Park, Chaffee County (Colorado, USA) prairie dog colony, during a 1959 complete colonial die off, due to an epizootic plague infection (1959)*

▼ ***Figure 29.5.*** *Post-mortem examination of marmots under field laboratory conditions in the Alai highland natural focus (Kyrgyzstan)*



30 THE CULTURE

Y. pestis is a *facultative anaerobe*[1] and a natural *auxotroph*[2], as it requires amino acids in addition to an energy source, which is a consequence of its sparse habitat outside the host. Most strains of the main subspecies require methionine, phenylalanine and arginine, as well as other amino acids characteristic of strains from different foci (**Table 30.1**). The amino acid requirements of the nonmain subspecies strains are also individualised (**Table 30.2**).

This is caused by mutations in genes. For example, methionine dependence is caused by a mutation in the *metB* gene. The strains of the main subspecies have a single nucleotide deletion (–G) at position 988 of the gene, while the Hissar strains have an insertion (+G) at position 989. In Caucasian strains, mutations in the *argA, apoG, apoF, thiH* and *thiG* genes are responsible for arginine, phenylalanine, tyrosine and thiamine requirements. For this reason, liquid and solid nutrient media, to which various amino acids are added[3], are used for work with cultures of *Y. pestis*, but in practice, media initially rich in various nutrients are more often used.

Sodium sulfite in the amount of 1 ml of 2.5% solution per 100 ml of medium, haemolysed blood in the concentration of 0.05-1.00% are also sometimes added as growth stimulants.

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[1] They grow and develop both in the presence and absence of oxygen due to their ability to switch their energy metabolism from aerobic respiration to fermentation or anaerobic respiration.

[2] They are unable to synthesise organic compounds necessary for growth, which distinguishes them from *Y. pseudotuberculosis*, which is a prototroph. There is evidence that fully prototrophic clones are sometimes found in *Y. pestis* populations (as are auxotrophs, albeit rarely, in *Y. pseudotuberculosis* populations). However, the needs are different. For example, at elevated temperature, *Y. pestis* strains necessarily exhibit isoleucine dependence, whereas *Y. pseudotuberculosis* strains do not. At the same time, the phenomenon of prototrophic *Y. pestis* strains is questioned by a number of specialists who consider it to be a consequence of the erroneous identification of *Y. pseudotuberculosis* strains as *Y. pestis* strains.

[3] For example, in the case of Caucasian strains, thiamine (vitamin B1) is added in the amount of 0.0001 mg per 100 ml of medium.

Table 30.1. *Amino acid requirements (other than methionine, phenylalanine and arginine) of strains of the main subspecies from different natural foci*

Focus	Biovar	Additional requirement
Sarydzhaz (Sari-Dzhas) highland	ANT	leucine, cysteine
Transbaikal steppe		
Upper Naryn highland		cysteine
Alai highland		
Aksai highland		leucine
Central Caucasian highland (Upper Kuban and Kuban-Malka districts)	MED	proline
Tuvinian mountainous (1 – Kargin variant; 2 – Saglian variant)		methionine, cysteine (1) methionine (2)
Pre-Araks lowland		cysteine
Ural-Emba desert		
North-Pre-Aral desert		
Muyun-Kum desert		
Tau-Kum desert		
Pre-Balkhash desert		
Transcaucasian plain-foothill (Bozchel', Kobystan and Mila-Karabakh mezofoci)		cysteine, arginine
Dagestan plain-foothill		
Transcaucasian plain-foothill (Dzheiranchel' mezofocus)		leucine
Pre-Caspian sandy		
Volga-Ural steppe		
Ustyurt desert (northern area)		
Kyzyl-Kum desert		
Pre-Caspian northwestern steppe		leucine, cysteine
Volga-Ural steppe		
Ural-Uil steppe (northern area)		
Pre-Ustyurt desert		
Mangistau desert		

Table 30.2. *Amino acid requirements of nonmain subspecies strains*

Amino acid	cauc.	uleg.	micr.	alt.	tal.	hiss.
arginine	+	−	−	+	±	±
valine	−	−		−	+	−
leucine	±	−	−	+	+	+
methionine	+	−	−	−	+	+
methionine	+	−	?	−	−	−
tyrosine	+*	?	?	?	±	−
threonine	+	±	?	?	+	−
tryptophan	−	−		±	−	−
phenylalanine	+	+	−	+	+	+
cysteine	+*	+	−	+	+	+

Note:
* – only for strains from foci on the territory of Armenia.
± – some strains are auxotrophic (inconsistent data).
? – no data available.

The standard medium recommended by the World Health Organization[1] is sheep blood agar (SBA). Alternatively, brain heart infusion (BHI) agar, nutrient agar and trypticase soy agar can be used, but *Y. pestis* grows more slowly on them. MacConkey agar, Hottinger agar and Martin agar with sodium sulphite and gentian violet, or agars based on casein, fishmeal and Caspian Sea sprat hydrolysates and peptic meat peptone are also used. If it is reliably known that the material is not contaminated with foreign microflora, it is allowed not to add gentian violet. When working in the field, deoxycholate agar is used, since sterilisation is not required and *Y. pestis* can be isolated even at room temperature.

The first attempts to create a *selective agar medium*, *i.e.* specifically designed to isolate a particular bacterium by inhibiting the growth of others, date back to 1917, when Jennie G. Drennan[2] and Oscar Teague[3] sug-

[1] Dennis D. T., Gage K. L., et al. *Plague manual: epidemiology, distribution, surveillance and control*, World Health Organization, WHO/CDS/CSR/EDC/99.2, 1999, 172 p.

[2] ?-?; the American physician, bacteriologist.

[3] ?-?; the American physician, bacteriologist, immunologist.

gested[1] adding gentian violet to the medium. Currently, cefsulodin-irgasan-novobiocin agar (CIN) is used as a selective medium for *Yersinia* spp., but experiments[2] show that due to its strong inhibitory properties, only 5 per cent of the bacteria grow after 36 hours of incubation (i.e. cultivation) at 28°C / 82.4°F. Hektoen enteric agar (HEK) and BIN agar[3], developed on the basis of BHI agar, are also used.

Gentian violet at a concentration of 1:100000-1:800000 is more commonly added to inhibit the growth of foreign microflora, but potassium tellurite at a concentration of 1:300000, sodium deoxychlorate at a concentration of 10 mg/L and fosfomycin at a concentration of 50-100 μg/ml may be used.

To differentiate *Y. pestis* (*i.e.*, in this case, to distinguish it from other bacteria), differential media are used, where different bacteria grow on different colours. This is convenient because bacteria of different species can form similar colonies, and when studying individual isolated colonies on conventional media, there is a possibility that two identical-looking colonies may be mistaken for belonging to the same species of microorganism. The principle of differential media is based on the ability of the microorganism to ferment different substrates, which will be discussed in more detail in the next chapter. An example of such a medium is a coloured differential medium, which allows the differentiation of *Y. pestis* colonies from *Y. pseudotuberculosis* colonies on the basis of the ability of the latter to degrade urea. Thus, in the case of *Y. pestis* the medium acquires a greenish colour, and in the case of *Y. pseudotuberculosis* – a blue colour.

Special attention should be paid to the so-called *chromogenic media*, the composition of which leads to colony staining. The first such medi-

[1] Drennan J. G., Teague O. *A selective medium for the Isolation of B. Pestis from contaminated Plague Lesions and observations on the growth of B. Pestis on Autoclaved Nutrient Agar*. J Med Res. 1917; 36(3):519–532.

[2] Russel P., Nelson M., et al. *Laboratory diagnosis of plague*. Br J Bio Sci. 1997; 5(54): 231–236.

[3] Ber R., Mamroud E., et al. *Development of an improved selective agar medium for isolation of Yersinia pestis*. Appl Environ Microbiol. 2003; 69(10):5787–5792; DOI: 10.1128/AEM.69.10.5787-5792.2003.

um was proposed[1] in 1988 by Lawrence Restaino (and colleagues), and the "R&F® Yersinia pestis Chromogenic Plating Medium" of R&F Products Inc., a company founded by him, contains the chromogenic substrate X-β-D-glucopyranoside, which reacts with β-glucosidase of the plague microbe to form a precipitate that stains the colonies blue due to the pH indicator (**Figure 30.1**). In addition, the medium contains sucrose, L-rhamnose, cellobiose and lactose, which *Y. pestis* does not ferment, but other bacteria of the genus *Yersinia* do, causing their colonies to stain green to yellow colouration of their colonies. The other bacteria either grow in a different colour scheme or are inhibited. Looking ahead, we note that this medium can give false negatives in the case of nonmain subspecies strains.

As liquid nutrient media, Hottinger's broth, Martin's broth, brain heart infusion broth, trypticase-soya broth and lysogeny broth (LB) with addition of gentian violet are used. Alexander Yersin grew a culture of the plague microbe in peptone water.



Figure 30.1. *A culture of Y. pestis grown on R&F® Yersinia pestis Chromogenic Plating Medium (courtesy of the manufacturer)*

For culture isolation ("gold standard"), the material is plated on a liquid (broth), semisolid (0.08-0.7% agar) and solid (1.5-2% agar) nutrient medium depending on the type of material. Liquid and semisolid media are poured into tubes (the latter in the form of a column with a flat or bevelled edge), while solid media are poured into Petri dishes. Plating is carried out in a BSC Class II over a tray containing a cloth moistened with a disinfectant solution. The container into which the material will be plating are signed in advance and all manipulations are carried out in a concentrated manner, without hurrying to avoid accidents. Most often, the material is delivered to the laboratory in a tube (vial), so let's consider the technique of sowing from it.

[1] Frampton E.W., Restaino L., et al. *Evaluation of the β-Glucuronidase Substrate 5-Bromo-4-Chloro-3-Indolyl-β-D-Glucuronide (X-GLUC) in a 24-Hour Direct Plating Method for Escherichia coli*. J Food Prot. 1988; 51(5):402–404; DOI: 10.4315/0362-028X-51.5.402.

The thumb, index and middle fingers of the left hand are used to pick up the tube, with the palm of the hand underneath the tube (the ring finger and little finger may be held underneath). Some specialists prefer to place the palm of the hand on top, but this is inconvenient as it obstructs the view of the test tube contents and creates an emergency situation. The loop or pipette (pipette dispenser) is held in the right hand using the thumb, index and middle fingers. With the ring finger and little finger grasp the stopper of the test tube, pressing it against the palm of the hand, and carefully remove it over the flame of a burner (a spirit lamp). Then quickly burn scorch the neck of the tube in the flame of the burner. Using a sterile loop (disposable or pre-burned[1]) take the material at the tip of the loop and extract it without touching the walls of the test tube (or suck it into the tip of the pipette).

From this point on, the arm should remain stationary (it is best to fix it at the elbow). The loop is placed parallel to the tissue and the pipette is placed so that its tip is as close to the tissue as possible. Next, close the test tube with a cork (left hand) and set it aside[2]. The container in which the material is to be plated is taken, opened, plated, closed and set aside. Then a disposable loop or pipette is immersed in a container with disinfectant solution (which is located in the "dirty" area of the box). In the case of a pipette, the spout is immersed first, the disinfectant solution is drawn into the cavity, and only then is it fully immersed. If the pipette is immersed immediately, it will float in the solution unfilled. In the case of a reusable loop, it is inserted into the burner (to the top of the flame), starting from the loop holder, slowly moving towards the ring of residual material, which is dried, after which it is re-inserted into the burner flame (this time from the ring side) and pierced red hot. If the loop is inserted at once, the charred material, not being decontaminated, may either fly to pieces (accident with PBAs splashing) or fall to the worktop as a char (accident without PBAs splashing).

[1] Pre-firing of the loop is carried out by successively putting the entire length of the loop into the flame (3-4 times) for heating, followed by slow burning to red hot. The integrity of the loop ring is checked. When taking the material, the loop is cooled against the inner wall of the container from which the material is taken (in the case of a Petri dish, this could be the inside of its lid or a clean piece of agar).

[2] Particularly dexterous specialists prefer to grasp the test tube with the thumb, ring finger, and little finger so that the index and middle fingers can grip the stopper to extract it.

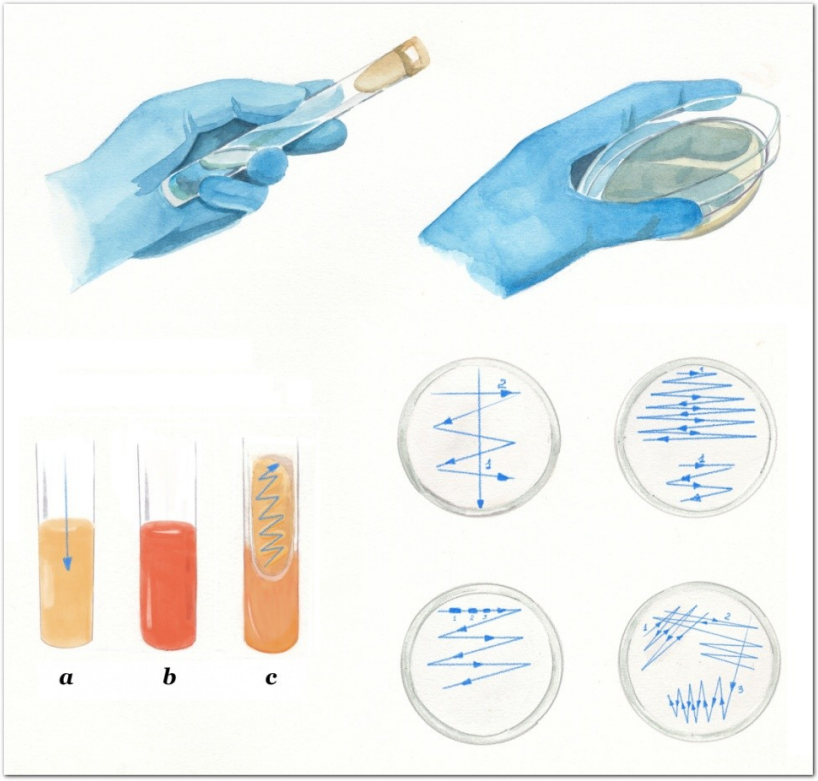


Figure 30.2. *Positioning of the tube and Petri dish in the hand (top row) and culture plating technique (bottom row; loop movements are indicated in blue). Plating is performed in liquid (b) and semi-liquid (a, c) agar, as well as on dishes (in all cases, except for the lower right, the actions are performed without fired of loop; on the upper right dish – spread by "area")*

A test tube or a Petri dish can be used as the container in which the material is plated. In the case of a test tube, it is most convenient to take it together with the test tube with the material. This allows them to be opened at the same time, which significantly reduces the time that the material remains outside the containers on the loop and therefore reduces the risk of accidents. The material is introduced either by puncturing into the thickness of the agar (a needle can be used for this purpose[1]) or on the surface of the bevel part of the agar (in CIS countries:

[1] Some specialists bend the tip of the loop 90° in advance.

"jamb") without touching the glass of the test tube. In the case of liquid medium (broth), the culture is applied to the tube wall at the edge of the broth and gently flushed into the broth in a circular motion. It is most convenient to hold the test tube at an angle of 45° to the surface being worked on (**Figure 30.2**).

In the case of a Petri dish, it is dried (to avoid condensation[1]) and then placed in the palm of the left hand, holding the lid with the thumb and forefinger (**Figure 30.2**). To plating the material, the lid is slightly opened (the lid can be fixed and the bottom of the dish can be lowered) so that the resulting gap is sufficient for free passage of the loop, which should not touch the edges of the dish (accident without PBAs splashing). During plating, the loop slides smoothly over the agar surface, without pressure, without scratching its surface and without touching the sides of the dish (**Figure 30.3**). It is important to spread the material over the agar surface in such a way that so-called isolated colonies - visually round clusters of bacteria originating from a single bacterial cell – are obtained. If the material is introduced with a pipette, it is more convenient to place the dish on a napkin and, having opened it, carefully introduce the material so that there is no splashing (accident with PBAs splashing). Next, the introduced material is spread over the agar surface with a loop or spatula (so-called "bacterial lawn"; or in CIS countries: "continuous lawn")[2].

The bubo punctate is introduced onto the agar surface with a loop or pipette, then spread with a loop (streak plate method) or a spatula (spread plate method). An additional 0.1 ml of material is added to the broth tube and the remaining material is used to infect laboratory animals (see below). If insufficient material is delivered, it should be diluted in 0.3-0.5 ml of broth.

[1] If there is already condensation on the roof, it should be carefully replaced with a dry one. There are different opinions on what to do when condensation has occurred after incubation (i.e. when colonies have grown on a dish). The most appropriate is to place such dish (bottom up) on a napkin, abundantly moistened with disinfectant solution, and next to place a clean dish in the same position. Then carefully swap the bottom of a dish so that the bottom of the clean dish is above the condensate lid, after which this dish (i.e., clean) should be carefully placed in a container with a disinfectant solution.

[2] A number of experts prefer to "roll" the drop across the dish by gently rocking it, but this method requires some skill.

The material from an opened buboes (ulcer, carbuncle) is plated on agar with gentian violet and sodium sulphite or lysed blood.

Mucous secretions of the oral cavity and pharynx are sown on agar with gentian violet using a swab and then placed in a test tube with 2 ml of Hottinger, Martin or LB broth with addition of genzianviolet, thoroughly squeezing against its walls. The resulting suspension is then used to infect animals.

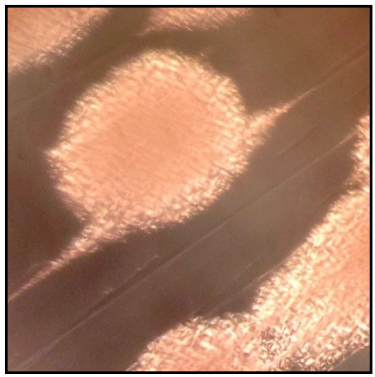


Figure 30.3. *An example of aggressive plating on poorly dried agar, in which an isolated colony "spreads" through a gap in the agar left by the loop*

Sputum, which may contain blood, is introduced onto the agar surface with a loop (2-3 times) or pipette (10 μl), then spread with a loop or a spatula. The residual material on the loop or spatula is transferred to the second and third agar without (!) sterilising. If the sputum is viscous, a lump of it is transferred to the agar surface with sterile tweezers[1] or a large loop, and then spread with a loop or a spatula, transferring the remaining material (without sterilising) to the second agar.

Blood in the form of a clot is carefully transferred to a dish, where sterile needles break its integrity, maximising the release of liquid blood from fibrin. Then 0.1 ml of blood is brought to the agar surface, spread with a spatula, and also brought into a test tube with broth. The blood residue is used to infect animals.

Organ fragments are homogenised with the addition of saline solution. Grinding in a mortar with sterile sand with addition of up to 2 ml of saline solution is possible. It is allowed to combine organs from no more than 10 small animals and 5 large animals for one suspension. In the case of birds, domestic and farm animals and humans, only fragments of one organ may be combined (e.g. left and right lung frag-

[1] Since tweezers, like any metal instruments, cannot be red-hot, its working part (ends) is dipped into a 95% ethanol solution and sterilising in a burner flame (ethanol is kept at a distance to avoid ignition) and then allowed to cool down.

ments). One drop of the resulting suspension (homogenisate) is applied with a loop to the agar surface and streaking. If camel organs are examined, the suspension from each organ is seeded on a separate dish with 100 μg/ml fosfomycin added.

In parallel, it is advisable to plating organs (whole or pieces) on nutrient media during post-mortem examination. In CIS countries, inoculation of bacteria from deceased or euthanized animals is carried out by touching a fresh section of an organ (liver, spleen, lymph node, etc.) to a solid nutrient medium (*plating from the slide organ print*). For this purpose, pre-fired (and cooled) scissors are used to cut off a fragment of an organ, after which tweezers (also pre-fired and cooled) are used to take the cut off fragment and lean it several times with the cut off surface (print) on the agar surface. It is allowed to make prints of several organs on one dish, as well as to plating material from two animals (in this case, the dish is divided into sectors and each sector is signed).

Ectoparasites (except fleas) removed from carriers, as well as collected in their burrows and human dwellings, are washed once with 95% ethanol solution, twice with saline solution and homogenised with saline solution. Grinding in a mortar is possible. One drop of the resulting suspension (homogenisate) is applied with a loop to the agar surface and streaking. Ectoparasites grouped by species, place and object of collection, fatness and phase of development are taken into one pool. Ticks should be taken in a single pool of no more than 30 females and 50 nymphs when hungry, and no more than 3 females, 15 nymphs and 30 larvae when drunk. Before homogenising the females, it is advisable to cut them with scissors (pre-fired and cooled) and, when grinding them in a mortar, to cover it with a dish lid to avoid splashing accidents. Lice are taken in a single pooling of up to 50 specimens.

Fleas are not washed before homogenisation, but are sedated with ether and homogenised with the addition of saline solution. One drop of supernatant is applied with a loop to the agar surface and streaking. Fleas from one animal or one burrow (dwelling) are taken in one pooling, but not more than 20-30 insects of the same species. If they are abundant, it is allowed to take up to 50 fleas in one pooling. Fleas collected from corpses or sick animals (with pathological changes) are examined individually.

Soil of burrows, pellets and food remains in the amount of 50-100 g are homogenised with saline solution to obtain 15-20 ml of suspension, which is gently mixed for 5-10 minutes and allowed to stand for 3-5 minutes. The supernatant is collected, filtered or centrifuged (2-3 minutes at 5000 rpm). The supernatant is taken, centrifuged again (15 minutes at 12000 rpm) and the precipitate is suspended in saline solution. One drop of the suspension is looped onto the agar surface and streaking.

The water is centrifuged (15 minutes at 9000 rpm) and the resulting precipitate is suspended in saline solution. One drop of the obtained suspension is applied with a loop to the agar surface and streaking (it is recommended to plating on BHI agar in case of the presence of viable but non-culturable forms, which will be discussed in more detail below). If the liquid included impurities (e.g. sludge), it should be pre-filtered (filter pore size at least 0.45 nm).

All cultures (Petri dishes upside down) are placed in a thermostat. The optimum temperature for incubation (i.e. cultivation) is 28°C / 82.4°F, because if incubated at the usual 37°C / 98.6°F, colonies form viscous and are more difficult to remove from agar (**Figure 30.4**), which, as we recall, helped Alexandre Yersin get a pure culture[1]. However, in some cases, which will be discussed further on, incubation is also carried out at 37°C / 98.6°F[2]. Remove the plating dishes from the tempera-

[1] Morphological analysis shows that collection strains grown at 28°C / 82.4°F are characterised by the presence of cells homogeneous in morphology (variation in linear dimensions less than 10%) with a roughness of 25 ± 5 nm, which is due to prolonged storage and numerous passivation. When the temperature rises to 30°C / 86°F, the cell volume of such strains increases on average 1.7-fold (roughness increases by 5 nm due to capsule synthesis and increased density of lipopolysaccharide), and when the temperature drops to 4°C / 39.2°F, it decreases 2.1-fold (coccoid-like cells with a roughness of 20 ± 5 nm are formed). Natural strains, on the contrary, are characterised by population heterogeneity at the cellular level (variations of more than 10%), which increases survival. Along with typical cells, more elongated cells are present (roughness 22 ± 5 nm). At the same time, the patterns of changes in cell morphology coincide: when the temperature increases to 30°C / 86°F, the cell volume of such strains increases 1.9-fold on average (roughness increases to 48 ± 11 nm), and when the temperature decreases to 4°C / 39.2°F, it increases 1.9-fold (compacted cells with a roughness of 18 ± 3 nm are formed), which is probably due to the presence of more elongated cells.

[2] It should be remembered that in order to obtain a culture rich in capsular antigen (fraction I), it is not only necessary to incubate the cultures at 37°C / 98.6°F, but also to

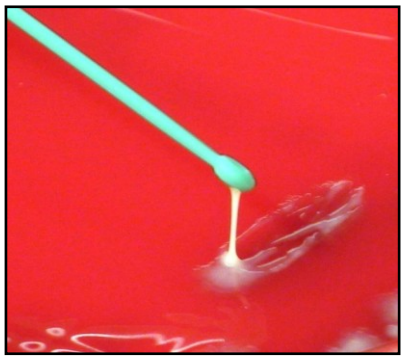


Figure 30.4. *A culture of Y. pestis threads stretch behind the loop*

ture control unit in a position parallel to the table (floor). It is not recommended to turn them upside down because of the risk of condensation water escaping. If there is a lot of condensation water, place the lid in a container with disinfectant solution and carefully replace it with a new one.

The morphology of *Y. pestis* colonies varies slightly depending on the nutrient medium (**Figure 30.5**), but as they incubate, they all go through common stages. If cultures are examined under a microscope, transparent microcolonies, called "*broken glass*" in CIS countries (**Figure 30.6**-a), can be observed after 8-12 hours, and after 18-24 hours they acquire the appearance of flat festooned formations called "*lace shawls*" (**Figure 30.6**-b). In an oblique beam of light, the colonies of the plague microbe are sand-coloured, rough, with irregular edges (**Figure 30.7**). It should be noted that an isolated colony is an already formed biological organism in which cells are divided into dominant and subdominant clusters, so they are able to reproduce their unique architectonics upon resettlement. This "separation of roles" leads to the formation of unique forms visible at low magnification. Note how visually similar colonies of different representatives of *Yersinia spp.* on the dishes are unique in the oblique beam of light, although sometimes this uniqueness is visible only to a specialist with great experience (**Figue 30.6 – Figure 30.8**)! This is why visual methods alone are not relied upon in practice, but more on that later.

By 48 hours, *Y. pestis* forms typical, up to 2 mm in diameter, yellow-brown R-type colonies with a convex darker fine-grained centre and a flat, wavy, festooned edge. It is these colonies that are transferred to dishes for differential diagnosis and into bevel agar tubes to confirm the purity of the culture, its accumulation and storage. As they age, by 72 hours, the centre of the colonies become coarser, opaque, and take on a

use rich nutrient media such as LB agar or Hottinger agar enriched with nutrient components (e.g. medium 199 at a rate of 3 ml of supplement per 100 ml of nutrient medium).

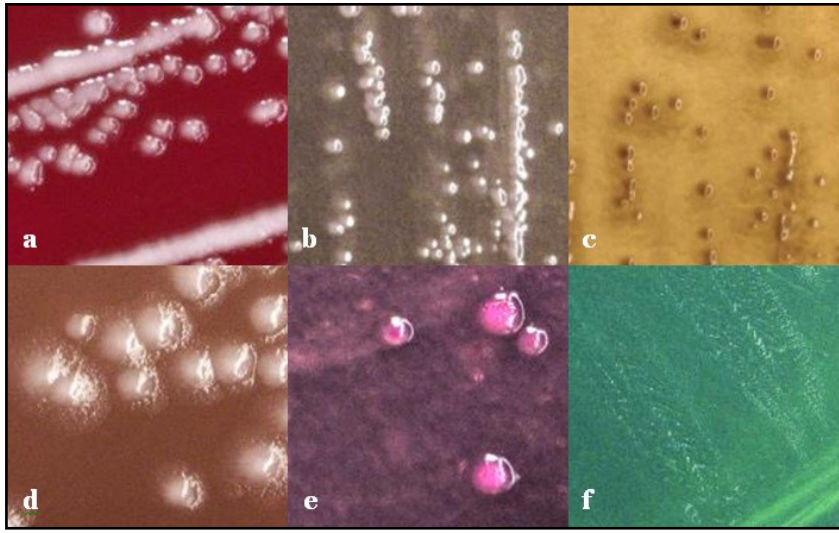


Figure 30.5. *Colonies of Y. pestis colonies grown on SBA (a), McConkey agar (b after 48 hours, c after 72 hours), chocolate agar (d), and selective SIN agar (e) and HEK agar (f)*

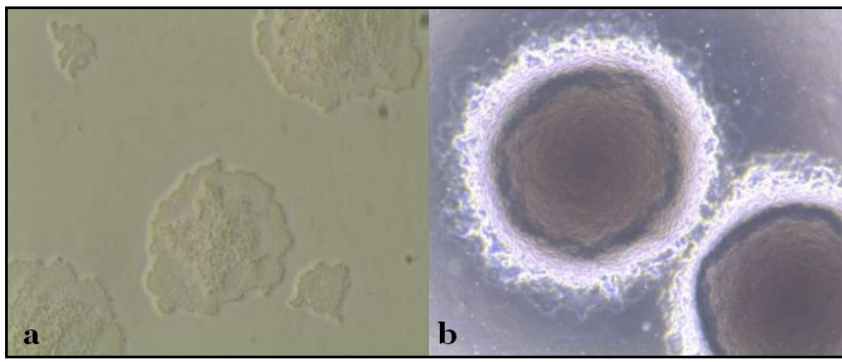


Figure 30.6. *Colonies of Y. pestis at the "broken glass" stage (a; 100x, McConkey agar) and "lace shawls" (b; 20x, McConkey agar).*
Alexandre Yersin wrote that "their edges appear iridescent when viewed"and Kitasato Shibasaburo saw them as bluish in colour

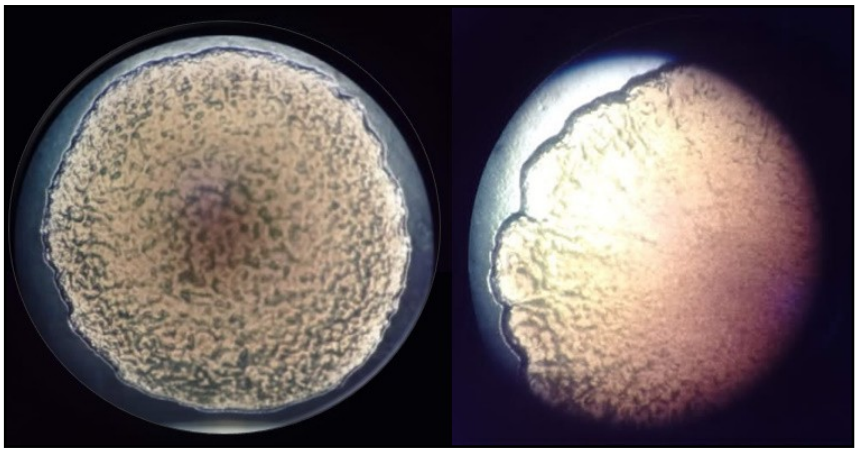


Figure 30.7. *A Y. pestis colony in an oblique beam of light. Alexandre Yersin whrote that "the pulp of the bubo, when inoculated on agar, gives rise to white transparent colonies whose edges seem iridescent when examined under reflected light"*

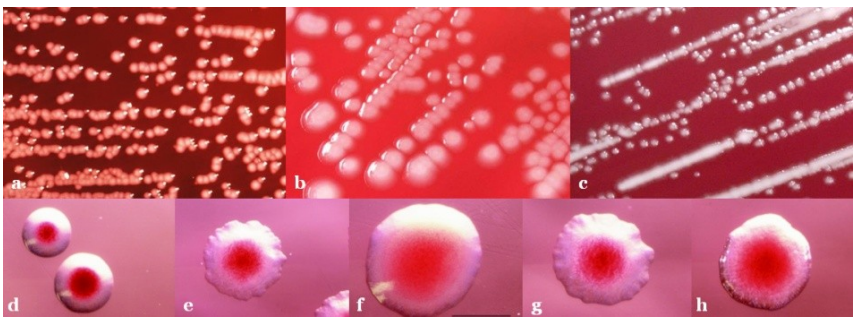


Figure 30.8. *Colonies of human pathogenic Yersinia spp.: Y. enterocolitica (a), Y. pseudotuberculosis (b) and Y. pestis (c) on agar. The bottom row shows isolated colonies of Y. enterocolitica of pathogenic serogroup O:3 (d) and serogroup O:5.27 (e), non-pathogenic serogroup BT1A (f), and former biovariants 3A and 3B – Y. bercovieri (g) and Y. mollaretii (h). Note the characteristic "bull's eye" colony appearance of Y. enterocolitica O:3, as well as the visual similarity between the colonies of Y. enterocolitica O:5,27 and Y. bercovieri (bottom row by Hallanvuo S., 2006; published under licence from The American Society for Microbiology, permission granted through Copyright Clearance Center, Inc.)*

greyish-brown hue (**Figure 30.9**). On bevel agar, *Y. pestis* grows as a succulent greyish-white plaque (**Figure 30.10**).

As a facultative anaerobe, in broth *Y. pestis* grows as a delicate film on the surface or as a loose flaky or powdery precipitate, easily disintegrated by gentle agitation (**Figure 30.11**).

We said earlier that virulent strains of *Y. pestis* exist in the R form, which is particularly well illustrated in **Figure 30.5**. However, it can also exist in the **L-form**[1], which is found in the saline soils of North African foci.

This form is capable of causing latent infection, as well as forming during inadequate antibiotic therapy. In broths, such cultures grow as

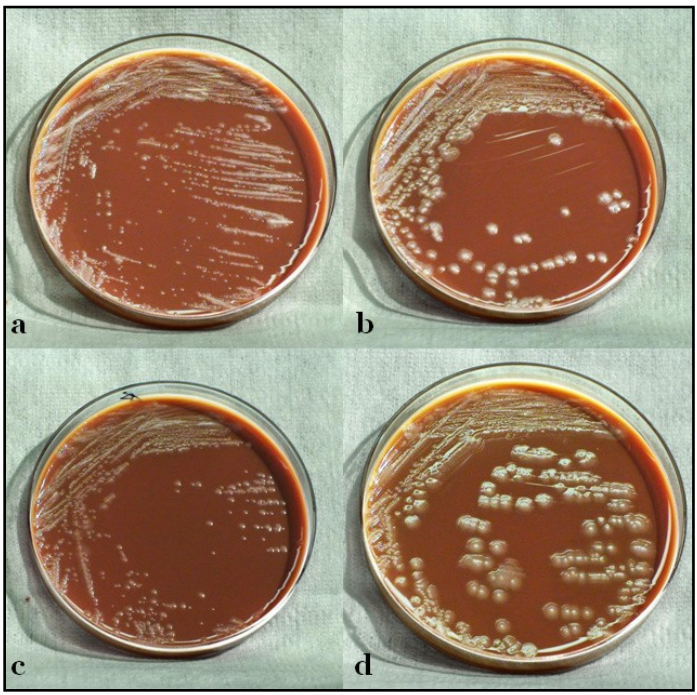


Figure 30.9. *A culture of Y. pestis, which were cultured on chocolate agar medium, for a 48-hour and 72-hour time period, at a temperature of 25°C / 77°F (a, b) at 37°C / 96.8°F (c, d)*

[1] Or L-phase bacteria, or cell wall-deficient bacteria (CWDB). Although these forms of bacteria were first discovered in 1894 by Nikolay Fyodorovich Gamaleya, the letter L refers to the Lister Institute of Preventive Medicine, after whom the German-British microbiologist Emmy Klieneberger-Nobel (1892-1985) named these forms when she discovered them in 1935.



Figure 30.10. *A culture of Y. pestis on bevel agar*

filamentous colonies, while on agar they form small (up to 1 mm) rounded colonies (**Figure 30.12**). Electron microscopy (**Figure 30.13**) shows that the L-forms are rounded and smaller in size (937 x 901 μm) than "normal" *Y. pestis* cells (1340 x 810 μm). In addition, they have a thinner cell wall. Interestingly, *Y. pestis* is not directly exposed to high salt concentrations, but gradually, as if in stages. Such progressive adaptation becomes possible due to the reduction of the surface in contact with salt, which is ensured by the activation of outer membrane proteins, including the "pump" protein TolC, which "pumps" toxic compounds out of the bacterium, as well as the porin OmpF, which regulates osmotic pressure. The sodium–hydrogen antiporter, which provides ion exchange between the cell and the environment (also a virulence factor), also plays an important role in this process.

In addition to L-forms, in response to unfavourable factors, *Y. pestis* can transform into **viable but non-culturable** (VBNC), when the bacterium is in a state of extremely low metabolic activity and unable to divide. The danger of this condition is that the bacterial cell remains alive and under favourable conditions, such as certain manipulations ("reanimation"), can change back to the cultivated form. It is quite possible that long interepizootic periods are caused by the transition of *Y. pestis* into this form. This should be borne in mind when examining environ-



Figure 30.11. *Flake-like growth of Y. pestis in broth by 48 hours of incubation (left) compared to Y. pseudotuberculosis*



Figure 30.12. *Plague microbe culture by 7 weeks of incubation, grown in trypticase-soya broth (a) and SBA (b, right cup). Both media contained 150 g/L NaCl. Note how much larger the colonies of Y. pestis grown on SBA (b, left cup) without the addition of NaCl (by Malek M. A., 2017)*

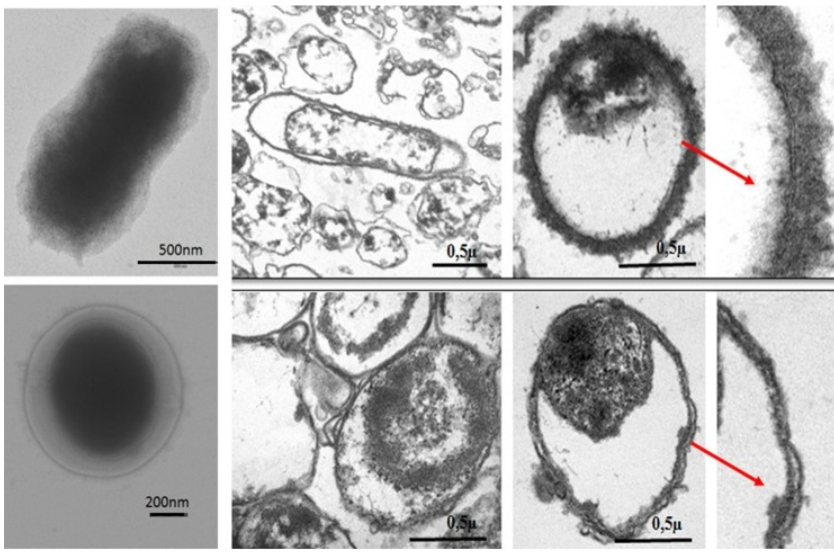


Figure 30.13. *Scanning electron micrograph of Y. pestis cells (top row) and NaCl-exposed Y. pestis cells (bottom row). Red arrows indicate cell walls (by Malek M. A., 2017). It is this arrangement that provides the bipolar staining seen in* ***Figure 13.8***

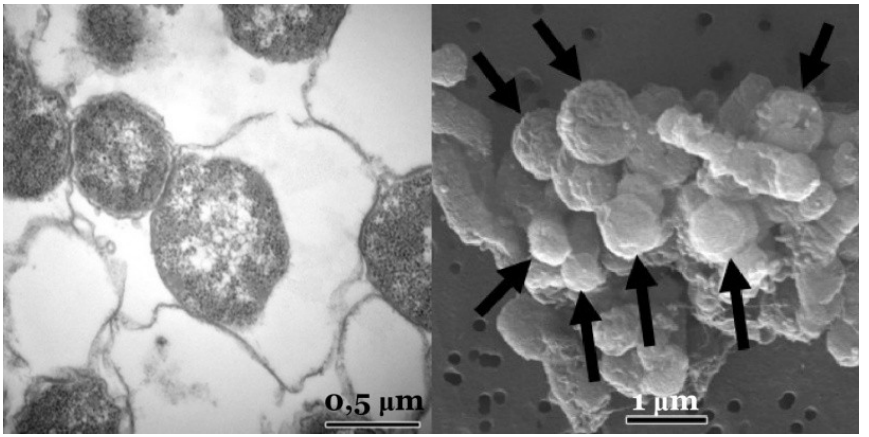


Figure 30.14. *Scanning electron micrograph of a nonculturable Y. pestis (by Pawlowski D. R., 2011). Compare them with the L-form in* ***Figure 30.13***

mental samples (especially water).

It has been experimentally proved[1] that the presence of *Y. pestis* in cold tap water (**4°C** / 39.2°F) is able to convert 10^6 CFU/ml into unculturable form by 28 day[2]. Such cells have morphological changes: the cytoplasm is condensed into a small rounded cytosol, and the periplasmic space is correspondingly much enlarged (**Figure 30.14**). For "resuscitation", the cells were cultured in brain heart infusion broth, but the number of cells grown was small.

* * *

The cultures obtained, as well as samples of clinical material (bubo punctum, blood, sputum, organ sections and impressions) are examined

[1] Pawlowski D. R., Metzger D. J., et al. *Entry of Yersinia pestis into the viable but nonculturable state in a low-temperature tap water microcosm*. PLoS One. 2011; 6(3):e17585; DOI: 10.1371/journal.pone.0017585.

[2] Cell membrane integrity and metabolic activity indicate that the cells are not dead. The former is checked by treating cells with DNAase I nuclease for 24 hours (if membrane integrity is compromised, nuclease destroys genomic DNA), and the latter by incubating cells with a mixture of radioactively labelled amino acids (if metabolic activity is maintained, albeit more slowly, the amino acids will be incorporated into newly translated proteins, which can be determined by the radioactive label).

under a *microscope* for visual detection of the microbe. We have previously seen blood smears (**Figure 7.6** and **Figure 13.8**) and bubo punctum (**Figure 13.6**), but now we will look more closely at the technique used to prepare them.

To prepare smears, degreased slides should be used. For this purpose they are soaked in chromic mixture or 10% solution of sulphuric acid, then boiled for 30 minutes in 5% solution of soda, or 30-40 minutes in 1-3% solution of sodium hydroxide with the addition of soap[1], and in the morning boiled for an hour with the addition of soap. If new glasses are used, the soaking step can be skipped. After boiling the glasses are rinsed repeatedly with water and then treated with alcohol or quick fixative mixture of Nikiforov[2]. In practice, however, degreasing is carried out by thoroughly rubbing the glass with a bar of soap on both sides and polishing with a dry cloth (a towel attached to an anti-plague gowns can be used for this purpose). After degreasing, the glass can only be held by the side edges ("ribs"). The glass is signed on a matt surface with a pencil[3] so that the inscription is not erased during further fixation and placed on a special slide plate, which is placed on a tray with a cloth moistened with disinfectant solution. In practice, a Petri dish with two matches is used instead of a plate. Do not put the glass without matches, because it will be extremely difficult to take such glass without touching its surface. The cup is placed next to a burner, the flame of which ensures the cleanliness of the work.

Using a loop, saline solution is applied to the slide in such a way that the drops (their number corresponds to the number of smears) do not touch each other. Usually two drops are applied. Then a culture is ap-

[1] Previously, it was also soaked overnight in a 5% solution of lysol (i.e. a solution of purified phenols, cresols, xylenols in potassium or sodium soap), but due to the toxicity of lysol it has fallen out of use in some countries.

[2] Proposed by the Russian physician Mikhail Nikiforovich Nikiforov (*Михаил Никифорович Никифоров*; 1858-1915). Preparation method: ethyl alcohol and diethyl ether in equal volumes.

[3] A pencil for glass can be used, which can be made in one of the following ways. Method 1: mix one part of pork fat with six parts of wax, into which, when heated, add Sudan II stain ($C_{18}H_{16}N_2O$) to dark colouring, mix thoroughly and pour into a mould. Method 2: melt seven parts of wax with one part of paraffin, add three parts of castor oil, Sudan III stain ($C_{22}H_{16}N_4O$) until dark colouring is obtained, mix everything thoroughly, boil and pour into a mould.

plied with the fired loop next to the drop, rubbed in a smooth circular motion and introduced into the drop, after which, without firing the loop, it can be introduced into the second drop. This is done in order to obtain a second, less dense smear. The dish is then covered with a lid, leaving a small opening to allow the smears to dry. It is strictly forbidden to dry them over a burner flame or to fix them in it, because in this case microorganisms not only will not die, but there is a risk of their aerosolised spreading. For fixation, the smears are placed in a fixing liquid (96% ethanol solution or, less frequently, quick fixative mixture of Nikiforov) using tweezers, and the tweezers themselves are fired in a torch flame. Smears are fixed for 10-15 minutes (20 minutes for thick smears), but are usually fixed for at least 30 minutes to ensure that the smear is completely decontaminated. After fixation is complete, the smear is removed with tweezers and dried or fired in a torch flame to remove any residual fixative fluid (unless prohibited by the methodology, as in the case of the immunofluorescence method, which will be discussed in more detail later).

To prepare a *sputum* smear, the dish with sputum should be placed as close to the slide as possible, as the material is viscous and may fall off during transfer from the dish, and all manipulations should be carried out over the disinfectant solution. If the sputum is thick, a piece of it is introduced into a drop of saline solution and rubbed in it.

A smear of the contents of a *bubo*, as discussed in the previous chapter, is prepared at the bedside of the patient. If it has not been fixed, it is fixed according to the method described above.

The fixed smear is stained according to the method proposed in 1884 by Hans Christian Joachim Gram[1] (**Gram staining**)[2]:

1) filter paper is placed on the smear with tweezers (it should not hang from the edges), on which gentian violet (1 g in 10 ml of 96% ethanol solution) is poured abundantly[1]);

[1] 1853–1938; the Danish physician, bacteriologist. Author of books on red blood cells. The colouring technique he developed allowed Émile Roux to divide bacteria into Gram-positive and Gram-negative bacteria in 1886.

[2] Gram H. C. *Über die isolierte Färbing der Schizomyceten in Schnitt- und Trockenpräparaten*. Fortschritte der Medizin. 1884; 2:185–189.

2) after two minutes drain the dye, remove the filter paper with tweezers and rinse the smear abundantly with distilled water to remove any dye residues from it;

3) apply Lugol's solution (Lugol's iodine)[2] for two minutes, rinse again with distilled water and pour 96% ethanol solution for half a minute to decolour the smear, then rinse again abundantly with distilled water[3];

4) Pfeiffer's fuchsine[4] (or safranin) is applied for one minute, after which the smear is washed again with distilled water and dried.

Gram-negative bacteria (G-) stain red and Gram-positive bacteria (G+) stain purple. This occurs because in Gram-positive bacteria the cell wall contains an additional lipopolysaccharide (endotoxic) layer that retains the gentian violet and Lugol's solution, preventing it from being washed away[5]. In Gram-negative bacteria, on the other hand, the cell wall is thin, and gentian violet and Lugol's solution are easily washed off and discoloured with alcohol.

In mobile laboratories (in foci) it is advisable to use Gram staining modified by A. I. Sinyov[6]. For this purpose, a solution consisting of 1 g

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[1] Freshly prepared solution should be allowed to stand at room temperature for 24 hours or two hours at 37°C / 98.6°F before use. Normally the solution may have a precipitate, it is not necessary to filter it.

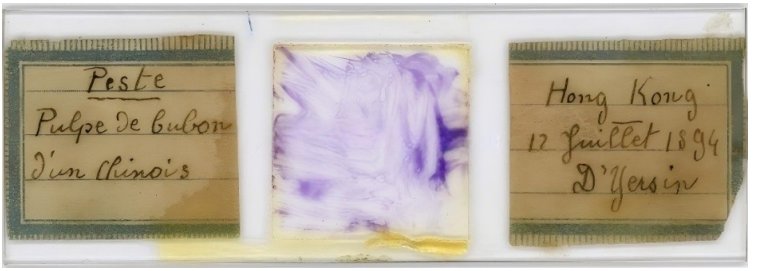
[2] Proposed by the French physician Jean Guillaume Auguste Lugol (1786-1851) as a drug for tuberculosis. Preparation method: dissolve 2 g of potassium iodide in 2 ml of distilled water and after dissolution add 1 g of crystalline iodine (I_2). Shake vigorously until iodine dissolves and add 300 ml of distilled water, then filter.

[3] For beginners, according to Soviet (Russian) physician Nikolai Ivanovich Rozanov (Николай Иванович Розанов; ?-?), it is more appropriate to use at this stage a solution of so-called iodised alcohol (2-4 ml of 10% alcoholic iodine solution per 100 ml of 95% ethanol solution), which is applied for two minutes, after which it is abundantly washed with distilled water.

[4] Proposed by the German physician Richard Friedrich Johannes Pfeiffer (1858-1945) as a modification of the method of German physician Franz Ziehl (1857-1926). Preparation method: to 1 g of thoroughly rubbed dry basic fuchsine add in small portions (each portion is rubbed) 5 g of crystalline carbolic acid or add dropwise 5 ml of 97% carbolic water. Continuing rubbing, add 16-20 drops of glycerin, then 10 ml of ethanol and bring small portions of distilled water to 100 ml. The prepared solution is filtered (it should have a metallic film on its surface) and diluted with distilled water in the ratio 1:10.

[5] Except for *Deinococcus-Thermus spp.*

[6] А. И. Синёв, ?-?; the Soviet (Russian) physician, bacteriologist.



A fixed smear of "the pulp of the bubo of the Chinese man" taken by Alexandre Yersin on 11 July 1894 (21 days after the discovery of the Y. pestis)

of gentian violet (or methyl violet), 5 g of glycerine and 100 ml of 96% ethanol is prepared, with which filter paper strips are impregnated after a day's sedimentation. These strips can be stored for a long time in a dark container with a sealed stopper[1]. 2-3 drops of distilled water are applied to the fixed smear, after which a strip is applied with tweezers and lightly pressed against the glass. After 2 minutes remove and rinse with distilled water. Then continue Gram staining according to the previously indicated scheme from stage 3.

Alternatively, **methylene blue staining** as proposed[2] by Friedrich August Johannes Loeffler[3] is used. Dissolve 3 g of methylene blue in 2 mg of 96% ethanol solution. Add 1 ml of 1% potassium hydroxide solution and 100 ml of distilled water, then mix thoroughly. The prepared paint must mature for several months, so to accelerate the maturation it is poured into a dish half-full, covering it with a loose cotton plug for better aeration. The matured solution is stable and can be kept covered with a glass or rubber stopper for several years. For staining, the solu-

[1] In the absence of filter paper, on the advice of the Soviet (Russian) physician Natalia Nikiforovna Klemparskaya (Наталья Никифоровна Клемпарская; 1914-2003), it is possible to use strips of newsprint soaked in a 10% alcohol solution of gentian violet. Besides, it is expedient to prepare dyeing papers for all solutions: for gentian violet (methyl violet) – to 10 ml of ethanol add 1 g of dye and 0.5 ml of glycerin; for Lugol's solution – to 1 g of crystalline iodine (I_2) add 2.5 g of potassium iodide and 10 ml of distilled water; for fuchsine – to 0.1 g of dye add 0.5 ml of carbolic acid.

[2] Löffler F. *Weitere Untersuchungen über Beizung und Färbung der Geißeln bei den Bakterien*. Centralblatt für Bakteriologie. Mai 1890. Bd. VII.S. 625.

[3] 1852–1915; the Prussian and German physician, bacteriologist, hygienist. One of the founders of medical microbiology, discovered the causative agent of glanders.

tion is applied to the fixed smear so that it covers it completely, kept for a couple of minutes and washed off.

Gram-positive bacteria stain blue and Gram-negative bacteria stain dark blue.

Gram-negative cultures can be easily and quickly identified using a test proposed[1] in 1978 by T. Gregersen[2], based on the ability of the cell wall of Gram-positive bacteria to maintain integrity when exposed to potassium hydroxide. To perform the test, the culture is suspended in a 3% potassium hydroxide solution (similar to smears preparating). From the point of view of biological safety, it is more appropriate to do this in a dish. If, as a result, the suspension has become viscous and mucus threads stretch 1-2 cm behind the loop (i.e., cell wall destruction occurs), the culture tested is Gram-negative. In this case, although the culture is Gram-negative, the test is called positive (such is the paradox).

The World Health Organization's plague case definition also include Wayson and Giemsa staining, which allows the characteristic bipolarity of cells ("English pins") to be seen. Let us consider them.

Romanowsky-Giemsa staining, known as "Giemsa solution for Romanowsky staining" (Germ. Giemsasche Lösung für die Romanowsky Färbung) [3] was developed by Dmitry Leonidovich Romanowsky[4] and Gustav Giemsa[5]. The essence of the method is that the dye[6] is dissolved in distilled water (1-2 drops per 1 ml of water) and poured into a Petri dish, where a slide stand is placed beforehand. Then the smear, previously fixed in methanol, is placed in the Petri dish in

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[1] Gregersen T. *Rapid method for distinction of gram-negative from gram-positive bacteria.* Eur J Appl Microbiol Biotechnol. 1978; 5:123–127.

[2] ?-?; the Danish veterinarian.

[3] To be fair, it should be noted that the staining method was first proposed in 1889 by the Polish physician Czesław Chęciński and became famous thanks to the work of the Russian physician Dmitry Leonidovich Romanowsky. The method was later improved by James Homer Wright and Gustav Giemsa.

[4] Дмитрий Леонидович Романовский, 1861–1921; the Russian physician, therapist, haematologist, bacteriologist. Described the structure of the malaria plasmodium.

[5] 1867–1948; the German chemist, pharmacist, bacteriologist. Author of books on tropical medicine.

[6] Preparation method: a composition consisting of 3.772 g of dimethylthionine chloride (azur B, azur II), 2.165 g of eosin and 1.563 g of methylene blue is dissolved in 750 ml of methanol and 256 ml of glycerol.

such a way that it is in contact with the solution (i.e. with the smear side downwards) and left for up to two hours (but not less than half an hour).

A variation of the method described is the Wright's staining proposed by James Homer Wright[1]. The essence of the method is that a few drops of dye[2] are applied to an unfixed smear placed in a Petri dish and kept for a minute, after which an equal amount of distilled water is added. After 2-3 minutes, the smear is washed with distilled water until a pinkish colour appears.

Wayson's staining, proposed by N. E. Wayson[3], also allows to see bipolar staining of *Y. pestis* cells. The method is reported[4] to be most sensitive in cases of plague meningitis. The essence of the method is that on the smear, previously heated and cooled (remember: this step is not disinfection!), placed in a Petri dish, a few drops of dye[5] are applied and and kept for 10-20 seconds, then poured distilled water and dried.

The following staining methods are used to detect the *capsule*.

Olt's method:

1. a freshly prepared 3% aqueous solution of safranin is applied to the smear and heated over a torch flame;
2. after 3-6 minutes from the onset of vapour, rinse with distilled water and dry.

The microorganisms and the smear background are coloured bright orange, while the capsule is coloured pale yellow. If a 1% aqueous solution of methylene blue (for 2 minutes) is applied to the smear after stain-

[1] 1869–1928; the American physician, pathologist.

[2] Preparation method: 1% solution of methylene blue in 0.5% aqueous solution of sodium bicarbonate is heated to 100°C / 212°F and boiled for one hour, then cooled and filtered. Then 500 ml of 0.1% aqueous eosin solution is added to 100 ml of the filtrate (has a purple colour). The precipitate is filtered, dried and dissolved in methanol (0.1 g of dye per 60 ml of alcohol).

[3] ?-?, the American physician, bacteriologist.

[4] Meyer K. F., Batchelder A. P. *A disease in wild rats caused by Pasteurella muricida*. J Infect Dis. 1926; 39:386–412.

[5] Preparation method: a solution consisting of 0.2 g of basic fuchsin, 0.75 g of methylene blue and 20 ml of 95% ethanol solution is slowly poured into 200 ml of 5% phenol solution, then filtered and stored in an opaque container.

ing with safranin, the microorganisms will turn blue and the capsule will turn pink.

Klett's method:

1. a freshly prepared boiling alcohol solution of methylene blue is applied to the smear, which has just been fired to remove residues of fixing solution (still warm), and heated up;
2. after two minutes, rinse thoroughly with distilled water;
3. apply fuchsine and after 30 seconds wash again with distilled water, then dry.

The microorganisms are coloured dark blue and the capsule is coloured pink.

Ginse's method:

1. a drop of liquid ink is applied to a degreased slide, the culture is introduced into it with a loop and rubbed in a circular motion (without smearing the drop);
2. a smear is made with a ground glass and fixed;
3. Loeffler's methylene blue[1] (for 30 minutes) or Ziehl's fuchsin[2] (for 5 minutes) is applied to the smear, which has just been fired to remove residues of fixing solution (still warm)
4. washed with distilled water and dried.

The microorganisms will stain blue (in the case of methylene blue) or red (in the case of fuchsin) and the capsule will be colourless against a dark background. When staining with this method, a control smear should be made of the ink without bacteria to ensure that there is no contamination. It should be noted that some specialists colour without fired after fixation to avoid damaging the capsules.

Hanging and crushed drop preparations are prepared to study the motility of microorganisms, which requires special care. A Petri dish is placed on a tray with a cloth moistened with disinfectant solution and a slide is placed in it. Apply an agar culture (as described above) or one

[1] Proposed by Friederich Loeffler. Method of preparation: in 100 ml of distilled water with 1 ml of 1% potassium hydroxide solution add 30 ml of filtered saturated alcoholic solution of methylene blue.

[2] Proposed by the the German physician Franz Ziehl (1857-1926). Prepared as Pfeiffer's fuchsine without the last dilution step with distilled water in the ratio of 1:10.

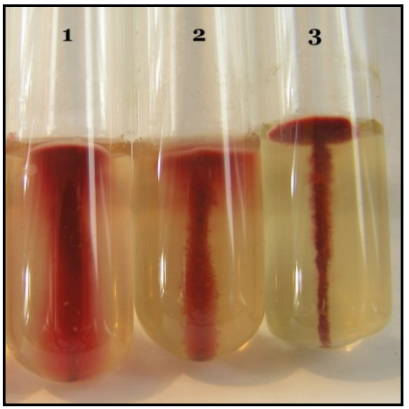


Figure 30.15. *Mobility test (1% solution of 2,3,5-triphenyltetrazolchloride (TTC) was added to the medium, staining the culture): 1, 2 – Y. pseudotuberculosis; 3 - Y. pestis*

drop of broth culture using a loop. It is advisable to use young cultures fresh from the thermostat, since as the culture cools, the mobility of the bacteria will decrease and then stop altogether. After applying the culture to the slide, the cover glass (a glass with a well for a hanging drop) is taken and, having previously applied petroleum jelly to its edges for better fixation, it is placed on the slide in such a way that its corners protrude beyond the edge (i.e. laid rhombically), which will greatly facilitate its removal in future. Motility is studied by microscoping the preparation, whereby bacteria with passive activity will oscillate in one place, whereas bacteria with active motility move around the field of the preparation.

As an alternative (and safer) method, culture in a column of 0.3% semi-liquid Hottinger agar or Martin agar is used. As *Y. pestis* is immobile, growth will only be observed along the course of the loop injection, whereas the motile bacteria diffuse into the nutrient medium in a cloud-like pattern (**Figure 30.15**). To differentiate *Y. pestis* from *Y. pseudotuber-*



Figure 30.16. *Methodology for safe agar pour into the dish (Pour Plate Method)*

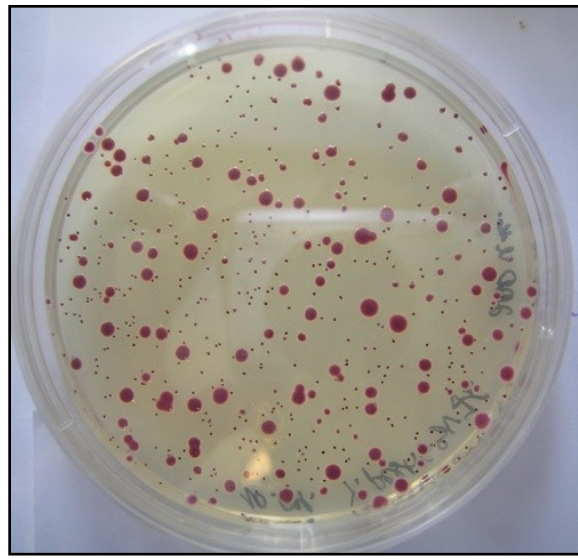


Figure 30.17. *Growth of isolated colonies of a mixture of Y. pseudotuberculosis (large colonies) and Y. pestis (small colonies) in a layer of 0.35% LB agar to which a 1% solution of TTC was added for colony staining (courtesy of Dr. Tatiana Evgenievna Arsenyeva)*

culosis, the culture should be grown at 20-22°C / 68-71.6°F for 48-72 hours, as there may be few motile cells from *Y. pseudotuberculosis* (time is needed for their accumulation and diffusion into the medium). However, atypical strains, which will be discussed in Chapter 38, should be kept in mind.

There is an interesting method by which *Y. pestis* and *Y. pseudotuberculosis*, which very often circulate together in natural foci, can be easily separated. To pre-warmed and cooled to 40-45°C / 104-113°F 0.35% LB-agar, 0.1 ml of the culture (10^6 BC/ml)[1] was added and then quickly poured onto a pre-prepared dish (**Figure 30.16**), which was incubated at 18°C / 64.4°F. About the third day, large and small colonies appear on the dish, corresponding to *Y. pseudotuberculosis* (a culture "spreads" due to motility) and *Y. pestis* (**Figure 30.17**), which can then be "extracted" ("pricked").

Another interesting method, proposed in the middle of the last century, is the addition of malachite green (aniline green) at a concentration of 1:12000 to Hottinger agar, which results in significant growth suppression of *Y. pseudotuberculosis* when 0.1 ml of the tested culture is plat-

[1] I.e., bacterial cell/ml.

ing (10^5 BC/ml). *Y. pestis* colonies grow stained green, and on 5-7 days the medium becomes discoloured.

After examination of the preparations and smears, and their subsequent staining (**Figure 13.8**, **Figure 30.18** and **Figure 30.19**), it can be seen that

Y. pestis is a Gram-negative small (1-3 × 0.5-0.7 μm) immobile bacillus of straight or ovoid, barrel-shaped form, characterised by polymorphism: longer bacilli are found along with the usual coccobacilli and bacilli.
It is coloured bipolarly ("English pin" type).

We said earlier that anti-plague serum are no longer used in clinical practice, but they have found widespread use in laboratory diagnostics. One example of this application is the *immunofluorescence* (IF), in which antibodies are conjugated (i.e. combined) with organic fluorochromes, dyes that cause fluorescence of a sample when exposed to short-wave light (fluorescence microscopy).

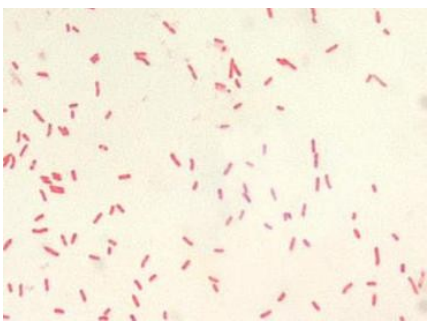


Figure 30.18. *Gram stained culture of Y. pestis*

A distinction is made between the *primary* or *direct* (DIF) and *secondary* (SIF) or *indirect* immunofluorescence method developed by Albert Hewett Coons [1] (DIF with Melvin Kaplan [2], SIF with Thomas Huckle Weller[3]). In the first case, a fluorescent antibody (i.e., antibody, conjugated to a fluorophore) is applied to the sample, resulting in the organi-

[1] 1912–1978; the American physician, immunologist, bacteriologist, pathologist.

[2] Coons A. H., Kaplan M. H. *Localization of antigen in tissue cells; improvements in a method for the detection of antigen by means of fluorescent antibody*. J Exp Med. 1950; 91(1):1–13; DOI: 10.1084/jem.91.1.1.

[3] Weller T. H., Coons A. H. *Fluorescent antibody studies with agents of varicella and herpes zoster propagated in vitro*. Proc Soc Exp Biol Med. 1954; 86(4):789–794; DOI: 10.3181/00379727-86-21235.

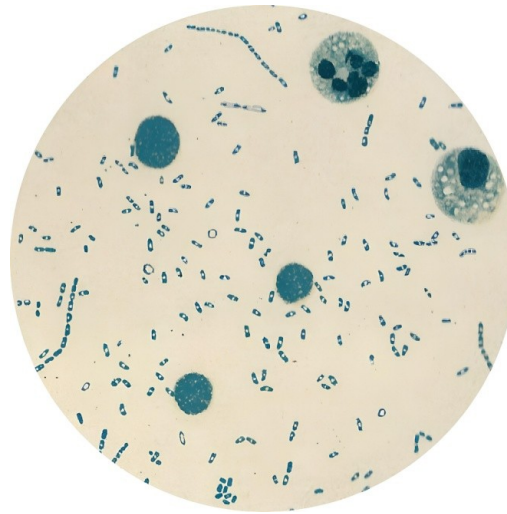


Figure 30.19. *Loeffler stained sputum smear from a patient with primary pneumonic plague (plague epidemic in the city of Bombay in 1897). Bipolarly stained cells of Y. pestis can be seen, some of which are arranged in chains (by Albrecht H., 1898)*

sation of an "antigen-antibody" relationship. This method is considered to be the most sensitive and specific, however, it allows the detection of only one species of microorganism. In the second case, a first antibody is applied to an sample (resulting in the organisation of an "antigen-antibody" relationship), followed by a second antibody that is fluorescent and species-specific against the first antibody (i.e., an "antigen-antibody-(f)antibody" relationship is organised). The advantage of this method is the ability to detect various microorganisms as well as antibodies.

DIF or direct fluorescence assay (DFA), which uses fluorescent antibodies (in CIS countries are referred to as "lum-serum") against the capsular antigen, is used to detect *Y. pestis*. This method is mandatory in the World Health Organization's criteria for plague case detection (probable and confirmed cases) and provides a result within one or two hours. Although simple and easy, this method has two serious limitations: firstly, it does not allow detection of noncapsular (fractionless) strains, and secondly, it requires a high concentration of bacterial cells in the sample (at least 10^5 BC/ml).

For DIF staging, smears from sputum, bubonic contents and blood are prepared, as well as culture smears and smear-prints from animal

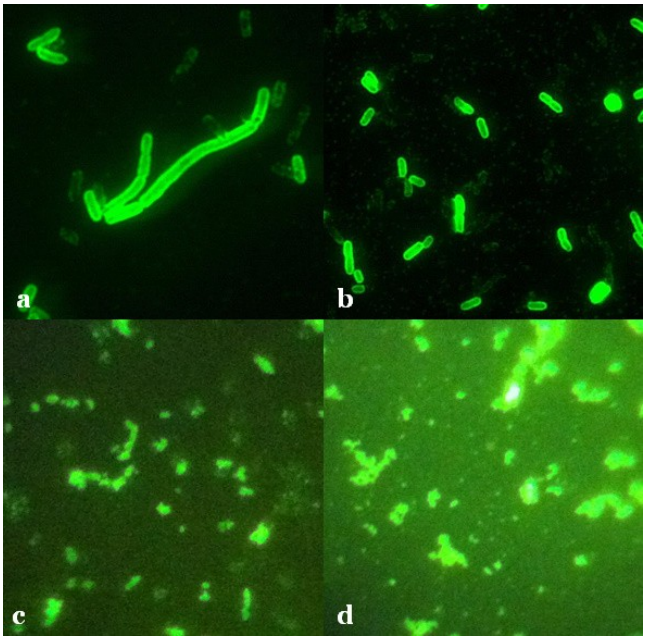


Figure 30.20. *Specific (a and b) and non-specific (c) fluorescent luminescence of Y. pestis bacteria, as well as non-specific luminescence (d) containing the luminescence of foreign inclusions*

organs. The process of preparation differs from that described earlier only in that after fixing the excess alcohol is not fired, but dried out. The smear is then placed in a Petri dish with a lump of damp cotton wool (so-called wet chamber) and a solution of fluorescent antibodies is applied to the surface (some experts recommend staining at 37°C / 98.6°F). After 20-30 minutes, the smear is washed with saline solution and distilled water, and then dried in air (impregnation with filter paper is not allowed).

To quench non-specific luminescence (background) that may arise from impurities in the sample (**Figure 30.6**-d), rhodamine-labelled albumin or normal serum is added to the fluorescent antibody solution instead of saline solution.

Non-luminescent immersion oil is applied to the stained smear (the use of buffered glycerol is allowed) and microscoped using a luminescent microscope. The emerald-green luminescence of cells is considered

specific for *Y. pestis*, when the fluorescent peripheral part of the microbial cell ("rim") is clearly delimited from its central part (**Figure 30.20**).

The results are evaluated according to the so-called "four crosses system":

4+	++++	bright fluorescence of the microbial cell with a clearly recognisable "rim" (positive)
3+	+++	moderate fluorescence of the microbial cell with a clearly recognisable "rim" (positive)
2+	++	weak fluorescence of the microbial cell with a faintly visible "rim" (dubious result)
1+	+	weak fluorescence of the microbial cell without a "rim" (negative)

…A man walks along the rocky shore of the Shimōoda-cho. The small waves roll over one another and play gold in the slanting rays of the evening sun, like the strings of a koto[1]. It is so quiet that one can hear one's own thoughts. This picture of tranquillity is interrupted only by a faintly visible glow - it is the green light of the Amabie[2] yōkai living in the sea trying to chase away the plague, signalling, like the "lum-serum", its arrival…

[1] A traditional Japanese plucked musical instrument consisting of a long zither. It was a solo instrument until the 20th century.

[2] Jap. アマビエ; yōkai in the form of a mermaid with long hair and three legs. According to legend, in May 1846, strange lights began to appear off the coast of the East China Sea in Higo Province (now Kumamoto Prefecture, Japan). When one of the officials arrived to investigate, a yōkai emerged from the water, telling him his name and predicting a good harvest in the next few years. He also specified that if there was an epidemic, his image should be shown to the sick for healing. The story and the image were published in a newspaper, becoming very popular (such one-page newspapers were often hung on trees). In the 20th century, the image was banned because it interfered with sanitation measures (people did not want to follow the orders of sanitary doctors by protecting themselves with images), but in the 21st century, during the COVID-19 pandemic, it was used by the Ministry of Health, Labour and Welfare of Japan as a symbol of pandemic control.



A newspaper with an image of Amabie and an account of his appearance
Courtesy of the 京都大学附属図書館

31 PROPERTIES

Like all bacteria of the Enterobacteriaceae family, the plague microbe is oxidase-negative and catalase-positive, that is, in the former case it does not synthesise cytochrome oxidase or indophenol oxidase, which catalyse the transfer of electrons from hydrogen donors to acceptors, and in the latter case it synthesises catalase, which hydrolyses hydrogen peroxide to form water and oxygen. To investigate *oxidase activity*, a 1% solution of tetramethyl-*p*-phenylenediamine dihydrochloride (50 mg of reagent per 5 ml of distilled water; use within 24 hours!)[1] is used, which is applied to the dish with culture as a drop or impregnated paper. If positive, the colourless solution is stained dark violet[2], but, as may be guessed, in the case of *Y. pestis* there is no staining. To investigate *catalase activity*, a 3% hydrogen peroxide solution is used, which is applied to a freshly prepared (unfixed) culture smear. In case of a positive reaction, the appearance of bubbles can be observed.

Since the set of enzymes catalysing all vital reactions occurring in bacteria is individual for each species (we can say that it is a kind of "taste preferences" of a bacterium), the study of enzymatic activity of each particular strain allows to determine its species affiliation, and for some bacteria, and *Y. pestis* is not an exception here, to differentiate within the species (**Table 31.1** and **Table 31.2**). Of course, the synthesis of enzymes is encoded by genes and we could easily find out about "taste preferences" using modern molecular genetic methods, in particular sequencing, but these methods can only determine the presence of a gene, but not answer the question of whether it works or not. Therefore, such bacteriological studies, which have existed for several centuries and seem at first glance outdated, do not lose their relevance. Let us dwell on them in more detail.

A *fermentation assay for carbohydrates and alcohols* (in CIS countries: "mottled series") is performed to assess the ability of a microorganism

[1] Nadi reactions such as solution α-naphthol with dimethyl paraphenylenediamine can also be used.

[2] It should be borne in mind that commercial test strips often give false negative results, and when applying a drop of reagent to a dish, it is possible that the reagent may touch neighbouring colonies (the solution will wash away some of the cells).

to ferment them to form acid and sometimes gas. Suitable carbohydrates (mono- and disaccharides) and polyatomic alcohols are added to a liquid or semi-liquid medium containing an indicator that changes its colour (and the colour of the medium) under the action of the acid formed. The media used are Phenol Red Broth Base, broth with Andrade's indicator[1], cysteine-trypticase agar, Gissa's medium or special commercial media[2]. Usually, the "mottled series" is divided into short and long series. The former includes glucose, lactose, sucrose, maltose and mannitol, while the latter additionally contains other monosaccharides (arabinose, xylose, rhamnose, etc.) and polyatomic alcohols (glycerol, dulcite, etc.). Plating is carried out according to the previously described methodology, but without fired the loop, because the same material is introduced, and the transfer of carbohydrate is so small that it does not affect the result. The plated media are incubated at least 24 hours at the optimal temperature for the microorganism (in case of *Y. pestis* – at 28°C / 82.4°F), although in practice not all tubes "work", which depends on the medium, indicator and "personal" preferences of the microorganism.

In a positive reaction, when phenol red is used as an indicator, the medium changes colour from pink-red to yellow.

It is recommended to test for *glycerol fermentation* by *Y. pestis* culture on solid medium, because in liquid medium its cleavage is delayed, and this may lead to a false negative result. Therefore, the 24-hour culture is plating on Cole-Belcourt agar (in an "area" or strip) and incubated at 28°C / 82.4°F for up to three days. In case of a positive reaction, the culture grows as red colonies (**Figure 31.1**), quite often staining the agar in the plating area as well (in case of a negative reaction, the cultures are greyish-white).

At the beginning of this book (Chapter 3), we said that strains isolated in foci where rats (bv. orientalis)[3] are the main carriers are unable to

[1] Proposed by the American bacteriologist E. P. Andrade (1890-1906). Preparation method: dissolve 0.5 g of fuchsine in 100 ml of distilled water and add 15 ml of NaOH. When after 24 hours the colour of the solution changes from red to brown, NaOH is added dropwise to a final pH of 7.1-7.2 (the colour of the solution becomes straw-yellow).

[2] Hereinafter, it should be remembered that only legally registered medical devices may be used in medical practice!

[3] The plague caused by these strains was for a time referred to as the "urban" plague.

ferment glycerol[1], and that this was the basis for the first intraspecific classification of *Y. pestis*, proposed by Anna A. Bezsonova. It should be said here that there are strains that dissociate on the basis of glycerol fermentation, that is, they form colonies both capable and incapable of fermenting glycerol (**Figure 31.2**).

However, glycerol fermentation is not the only biochemical property inherent in subspecies of *Y. pestis* that is of some interest for intraspecific differentiation. Thus, strains of non-main subspecies are rhamnose-positive (i.e. fermenting rhamnose; Rha$^+$), which is associated with ecological adaptation of the bacterium to certain host species (Arvicolinae, Pallas's pika)[2]. It should be reminded that this very sign gives a false negative result on chromogenic nutrient medium

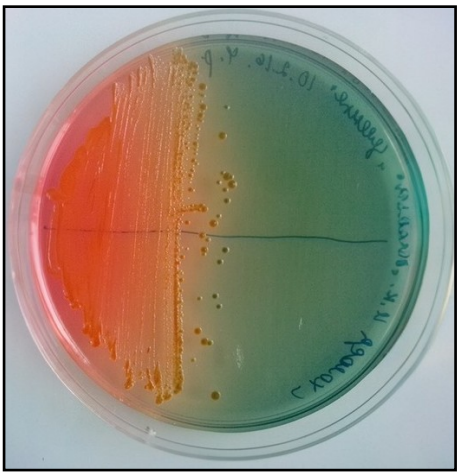


Figure 30.1. *Positive reaction of Y. pestis to glycerol fermentation. Note that single colonies have yellow colouration*

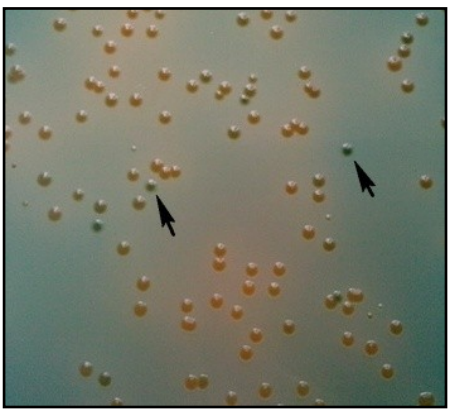


Figure 30.2. *Dissociation of Y. pestis in relation to glycerol fermentation (some glycerol-negative colonies are marked with black arrows)*

[1] The enzyme glycerol-3-phosphate dehydrogenase (GlpD) encoded by the *glpD* gene is responsible for the ability to ferment glycerol. Deletion of this gene in a 0.093 Kb (93 bp) reading frame results in the deletion of 31 amino acids within the N-terminal sequence of the encoded enzyme and, consequently, loss of the trait.

[2] The "vole's" strains, unlike the "classical" Rha$^-$ strains, like *Y. pseudotuberculosis* strains, have an adenine (instead of guanine) at the 671 bp position in the *rhaS* gene.

(attributed to *Y. pseudotuberculosis*)[1]. However, the Ulegei strains circulating in natural foci of Mongolia ferment rhamnose only if the medium contains at least 1% of it, and spontaneous emergence of Rha^- mutants is possible (and vice versa). And since the ability to ferment rhamnose is directly related to the ability to ferment melibiose (Mel^+), this rule applies to it as well.

In addition, rhamnose-positive "vole's" strains isolated in the Altai Mountains and on the Gissar Range from voles (hence the name), formerly called "Mongolian", are unable to ferment arabinose (Ara^-), but with prolonged incubation (up to a month) about half of them recover this property.

It is generally believed that all strains of the plague microbe are unable to ferment lactose, but due to the presence of β-galactosidase, which is usually inactive, some strains are able to ferment lactose at day 50!

The ability to *liquefy gelatin* (proteolytic activity) is investigated using gelatin medium poured into test tubes ("column"). Plating of the material is carried out by puncturing the medium strictly in the centre of tube, not reaching one centimetre to the bottom of the tube, and then incubated for 24-48 hours at room temperature, observing, in case of a positive reaction, liquefaction of the medium at the puncture site. If the cultures are incubated at 35°C / 95°F (gelatin becomes liquid at this temperature), no solidification will occur after cooling.

The *indole* test is used to assess the ability of a bacterium to break down tryptophan. To perform the test, the culture is incubated in broth or agar containing tryptophan. These may be peptone, pancreatic enzymatic hydrolysate of casein, trypticase-soya agar and others. In the case of broth, the culture is incubated for 18-24 hours at 28°C / 82.4°F, after which 2 ml is transferred to a separate tube. Add 5 drops of Kovac's reagent[2] or Ehrlich's reagent[1] (in the latter case 0.5 ml xylene is added beforehand) and shake the tube well.

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[1] The presence of strains of the *Y. pestis* similar to the *Y. pseudotuberculosis* led some specialists in the USSR to propose in 1964 an independent subspecies *semiplenus*, and in 1969 - an independent species *Y. pestoides*, including subspecies *parvocaucasica*, *altaica* and *transbaicalica*!

[2] Proposed by N. Kovacs. Preparation method: dissolve 1 g of p-dimethylaminobenzaldehyde in 95 ml of 95% ethanol solution and slowly under a fume

In case of a positive reaction, a bright red coloured ring is formed at the border of the reagent and the broth literally for a few seconds.

In the case of agar, a filter paper moistened with Ehrlich's reagent or Kovac's reagent is placed on a new Petri dish, onto which the overnight culture is transferred from the agar by a loop.

In case of a positive reaction, after rubbing the culture, a bright pink-red staining appears on the paper within 30 seconds[2].

The *ONPG test* is used to test for lactose fermentation. Lactose fermentation is carried out by permease, which transports lactose inside the bacterial cell, and b-galactosidase, which breaks down lactose into galactose and glucose. O-Nitrophenyl-β-D-galactopyranoside (ONPG) is similar in structure to lactose, so in the presence of β-galactosidase it is cleaved into galactose and O-nitrophenyl (yellow coloured component). A thick bacterial suspension (milk colour) is added to the ONPG-bouillon heated at 37°C / 98.6°F in a water bath to dissolve the salts formed during storage of the solution and incubated at 28°C / 82.4°F for at least one hour (not more than one day). In case of a positive reaction, a yellow staining appears.

The *hydrogen sulphide* test is used to investigate the ability of bacteria to ferment sulphur-containing amino acids (cystine, cysteine and methionine) and some inorganic compounds to sulphur, which interacts with hydrogen to form hydrogen sulphide. This, in turn, is able to react with some iron and lead salts, which is the basis of the test. Iron-containing nutrient media (e.g. Olkenitsky's agar, Kligler agar) and indicator strips with lead acetate are used for staging the test[3].

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cupboard add 20 ml of concentrated hydrochloric acid, stirring constantly. The prepared reagent has a pale yellow colour and can be stored at 2-8°C / 35.6-46.4°F for one year.

[1] Proposed by Paul Ehrlich. Preparation method: dissolve 10 g of p-dimethylaminobenzaldehyde in 150 ml of isobuthyl or isoamyl alcohol and slowly under a fume cupboard add 50 ml of concentrated hydrochloric acid, stirring constantly. The prepared reagent has a pale yellow colour and can be stored at 2-8°C / 35.6-46.4°F for one year.

[2] Indicator media (e.g., McConkey, Endo) should not be used for this method, as their transfer to indicator paper may give uncertain results.

[3] It is prepared by impregnating filter paper strips with a solution containing 30 g of lead acetate and 1 g of sodium hydrogen carbonate dissolved in 100 ml of distilled water.

In case of positive reaction the medium is coloured black and the strip hanging[1] freely over the culture is coloured black-brown. It should be remembered that fermentation of sucrose can inhibit the production of hydrogen sulphide, so Olkenitsky's agar is less sensitive to hydrogen sulphide than Kligner agar

The question about possibility of formation of hydrogen sulphide by *Y. pestis* remained open for a long time, because if incubated in meat-peptone broth containing cysteine in concentration of 0,1-1%, the reaction will be positive. At the same time, it is currently accepted that *Y. pestis* does not form hydrogen sulphide.

The *urease test* is used to test the ability of a bacterium to hydrolyse urea to ammonia and carbon dioxide, i.e. to produce urease. To carry out the test, the culture is sown in Urea Agar Base (Christensen) or Urea broth (Rustigian and Stuart) and incubated at 28°C / 82.4°F, taking into account the results after 2, 4 and 24 hours. In case of positive reaction due to pH shift to alkaline side the medium is coloured red (in case of negative result the medium colour does not change and the observation is continued till the fourth day).

This test is one of the main tests for differentiating *Y. pestis* from *Y. pseudotuberculosis* because *Y. pestis* (unlike *Y. pseudotuberculosis*) does not break down urea[2]. In CIS countries, urease activity is traditionally determined according to the method of Galina Nikolaevna Lenskaya[3]. To 100 ml of saline solution, 2% crystalline urea and 1% alcoholic phenol-

[1] Lead acetate is extremely toxic to bacterial growth, so the test strips should not touch the culture. To perform the test, the strips are placed in a test tube with medium 2.5 cm from the culture, securing the free end of the strip with a cork. This test, although complicated, is about 10 times more sensitive than the culture test.

[2] In some strains, urease is detected by bacterial cell disruption. In addition, it is sometimes detected in freshly isolated strains during the acute epizootic and is lost during attenuation, as well as when cultures are stored without additional selection on nutrient media. The reason for this phenomenon probably lies in the fact that the *ureD* gene of *Y. pestis* contains an insertion of an additional guanine residue in the poly-G-partition, resulting in the formation of a stop codon, which prevents the synthesis of urease. However, sometimes deletion of this extra ("extra") nucleotide can occur, which leads to normal "work" of the *ureD* gene and, as a consequence, to the synthesis of urease.

[3] Галина Николаевна Ленская, 1904–1983; the Russian and Soviet physician, bacteriologist. Laureate of the USSR State Prize, II degree (1952). Author of books on the variability of *Y. pestis*, participant in the elimination of outbreaks of particularly dangerous infections.

phthalein solution are added. The solution is brought to a boil, but not boiled to avoid a decrease in the intensity of the reaction, and then poured into 4 ml tubes. A sufficient amount (approximately 10^9 BC/ml) of a two- or three-day-old culture grown at 28°C / 82.4°F is introduced by looping (applied to the wall at the very edge of the solution and gently "flushed" into the solution in a circular motion). In case of positive reaction, the solution will turn crimson pink due to the alkaline shift in pH.

However, this method is almost never used in practice because of the possibility of spontaneous pH shift and, as a consequence, false positive results (even with careful introduction of *Y. pestis* culture, one can see the solution turning pink for a fraction of a second). Therefore, platings on Urea Agar Base (Christensen) or Urea broth (Rustigian and Stuart), as well as coloured differential medium are used, because after one day of incubation at 28°C / 82.4°F, in the case of *Y. pestise*, the medium changes colour from dark green to red-orange in the case of "column" and blue-green in the case of "bevel". This is due to complete or partial fermentation of the glucose contained in the medium under anaerobic ("column") and aerobic ("bevel") conditions. In the case of *Y. pseudotuberculosis*, the medium will acquire a blue colour due to the breakdown of urea.

The *Voges-Proskauer test* (VP test), proposed[1] in 1898 by Daniel Wilhelm Otto Voges[2] and Bernhard Proskauer[3], is based on the detection of acetoin (acetyl-methylcarbinol), which is an intermediate product of the breakdown of glucose into pyovinic acid. For the test, culture is sown in 5 ml of Clark broth and incubated at 28°C / 82.4°F for at least 48 hours. Transfer 2.5 ml of broth culture into a separate tube, add 0.3 ml of reagent I[4] and 0.1 ml of reagent II[1] in sequence. Shake the tube gently and

[1] Voges O., Proskauer B. *Beitrag zur Ernährungspysiologie und zur Differentialdiagnose der Bakterien der hämorrhagischen Septicämie.* Zeitschrift für Hygiene und Infektionskrankheiten. 1898; 28:20–32; DOI: 10.1007/BF02285362.

[2] 1867–1911; the German physician, bacteriologist.

[3] 1851–1915; the German chemist. Author of books on water hygiene and disinfection.

[4] Preparation method: dissolve 5 g of α-naphthol in 100 ml of ethanol (the solution is almost colourless, remains stable for a year when stored in a dark dish at 2-8°C / 35.6-46.4°F).

leave it for 10-15 minutes, after which the result is taken into account: red (shades of pink) staining - positive result, no staining – negative result.

Nitrification and *denitrification* tests reveal the ability of bacteria to convert nitrogen compounds, namely to *oxidise ammonia* (nitrification) to nitrate, and to produce nitrate reductase, which reduces nitrite to nitrate (and further to nitrogen). To perform the test, 0.5 ml of Griess reagent[2] is added to 1 ml of Hottinger or Martin broth to test for nitrate (the broth should not change colour). In the other two tubes with 1 ml of this broth, the 24-hour agar culture is added to one of them, and 0.1% KNO_3 solution is added to one of them. Both tubes are incubated at 28°C / 82.4°F and after three days 0.5 ml of Griess reagent is added to each tube. In case of positive reaction, colouring from pink to crimson immediately appears, which indicates the presence of nitrite in the medium: in the first test tube (without KNO_3) due to ammonia oxidation (nitrification), and in the second (with KNO_3) - in the process of nitrate reduction (denitrification).

Only some strains of antiqua and orientalis biovars exhibit nitrifying activity. All *Y. pestis* strains exhibit denitrifying activity, with the exception of the medievalis and microtus (xilingolensis and qinghaiensis), Altai, Hissar, Talas and Ulegei strains. This is due to a mutation in the napA gene, in which the TAA triplet is located at position 205 (instead of the GAA triplet), which prevents the synthesis of Nap, the only nitrate reductase of *Y. pestis*[3]. Remind that nitrifying and denitrifying activities, along with the ability to ferment glycerol, are the basis of classification of René Devignant.

The presence of *haemolytic activity* (i.e., the ability to destroy red blood cells) in *Y. pestis* was a subject of discussion for a long time, as

[1] Preparation method: to 40 g of potassium hydroxide add 100 ml of water in a cold bath to avoid overheating (the solution retains stability for a year when stored in glass bottles covered with paraffin or polyethylene at room temperature).

[2] Proposed in 1858 by the German chemist Johann Peter Griess (1829-1888). Preparation: 0.1 g of α-naphthylamine is dissolved in 100 ml of distilled water by boiling for 15 minutes, then cooled. Then 5 ml of glacial acetic acid and 100 ml of 1% sulphanilic acid are added. The mixture is stored in an opaque container.

[3] It should be noted that there are also atypical "vole's" strains, in which the GCC triplet is replaced by the ACC triplet at position 341, which leads to the replacement of hydrophobic alanine with hydrophilic threonine, due to which Nap loses its activity.

many contradictory data had been accumulated. The point in this question was put in 1963 by V. V. Tkachenko[1]. His experiments[2] showed that haemolysis is not caused by all strains, and that it occurs more rapidly at 37°C / 98.6°F than at 28°C / 82.4°F. The haemolysin itself is not similar in its properties to the known haemolysins, which are proteases (α-haemolysin of *Staphylococcus* spp.) or lecithinases (α-toxin of *Clostridium perfringens*). It is thermostable (retains activity even at 4°C / 39.2°F) and physicochemically similar to higher fatty acids (lipids), which can be released when the bacterium is destroyed[3].

To perform the test, 0.1 ml of defibrinated blood is added to a test tube with three-day-old broth culture and incubated for 24 hours at 37°C / 98.6°F. In case of a positive reaction, the medium will become discoloured (**Figure 31.3**). It should be remembered that the most sensitive to haemolysin are the erythrocytes of the dog. Then, in decreasing order, come the erythrocytes of horse, rabbit, pigeon, rat, guinea pig and human, lysis of which occurs not earlier than 72 hours of incubation. The erythrocytes of ram and camel are the most resistant, although the sensitivity of erythrocytes of different animals depends largely on the composition of the nutrient medium.

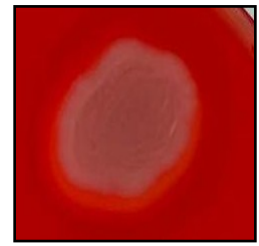


Figure 30.4. *The haemolytic activity test, which in practice is most often performed on blood agar. A good zone of haemolysis (in the form of a brightening of the medium) can be seen*

Contrary to all of the above, post-mortem examination reveal a large number of destroyed erythrocytes inside and outside the vessels, especially in the liver and spleen. How is this possible if the haemolytic activity of *Y. pestis* is low? According to Igor V. Domaradsky, haemolysis in plague "*arises independently of the haemolytic properties of Y. pestis under*

[1] В.В. Ткаченко, ?-?; the Soviet physician, bacteriologist.

[2] Ткаченко В. В. *О химической природе чумного гемолизина*, Дис. … кандидата медицинских наук. Ростов-на-Дону, 1964, 115 с.

[3] This explains the lack of binding of plague haemolysin to water-soluble fractions (e.g., murine toxin) and the retention of haemolytic activity after culture washing. It should be noted that although *Y. pseudotuberculosis* lipids also have haemolytic activity, the bacterium loses this property after washing.

the influence of endogenous haemolytic factors appearing in the sick organism". However, this question remains open up to the present time.

The results of the above tests for *Y. pestis*, a kind of "menu", are shown in **Table 31.1** and **Table 31.2**.

Table 31.1. *Biochemical tests for Y. pestis*

Test	**Result**
Glucose	+
Lactose	+
Glycerol	+ (besides bv. orientalis)
Salicin	±
Urea	– (besides bv. talassica; ± bv. orientalis, Gissar strains)
Rhamnose	+ (besides main subspecies)
Melibiose	+ (besides main subspecies; ± bv. medievalis; ? angola)
Sucrose	–
Mannitol	+
Maltose	+
Arabinose	+ (besides bv. microtus, Altai and Gissar strains)
H_2S	no
Indole	no
Voges-Proskauer	no
Olkenitsky's agar	orange colour
Nitrification	no (besides ± bv. antiqua, orientalis)
Denitrification	протекает (besides bv. medievalis, microtus, Altai, Gissar, Talas and Ulegei strains)
Gelatin	non-liquefying
Milk	uncollapses
Mobility	no
Note: «+»–splitting; «±»–splitting options; «–»–lack of cleavage; ? – no data available	

Table 31.2. *Biochemical tests for Y. pseudotuberculosis u Y. enterocolitica*

Test	Result	
	Y. pseudotuberculosis	***Y. enterocolitica***
Glucose	+	+
Lactose	+	+
Sucrose	–	+ (in biovar 5 only 26-75% of strains)
Rhamnose	+	–
Glycerol	+	+
Sorbitol	–	+ (except for biovar 5)
Mannitol	+	+
Urea	+	+
Indole	no	no (most)
H_2S	no	
Nitrite formation	+	+
Mobility	present at <37°C / 98.6°F	
Voges-Proskauer (22–25°C / 71.6–77°F)	no	no

Alternatively, commercial analysers or indicator papers are used, which may be paper discs impregnated with indicator media, or panels with wells containing dried indicator media. The principle of operation is very simple: the discs are placed in tubes and filled with the culture suspension, and the wells are immediately filled with the culture suspension and incubated, after which the results are recorded (in the case of the analyser, the computer records the result). It is important to realise that the quality of the result depends on the integrity of the manufacturer. On the other hand, sometimes the results of biochemical tests do not match the information presented in the book, as each existing strain is different.

Next, we will talk about additional studies conducted for the definitive identification of *Y. pestis* and differentiation of its subspecies (**Table 31.4**).

Determination of pesticin sensitivity is carried out by the delayed antagonism method. A 24-hour culture of a pesticin-producing strain of *Y.*

pestis (i.e. with pPla plasmid) was plated on the surface of 1.5% Hottinger agar in the centre of the dish using a loop and incubated at 28°C / 82.4°F for 48-72 hours. A filter paper or a swab moistened with chloroform is placed on the lid of the dish (remind: the dish is placed with the lid downwards) to sterilise the culture. After an hour or two, the swabs are removed and the dish is dried with the lid ajar (although the culture is sterilised, it is better not to violate the technique of working with PBAs) until the chloroform smell disappears. Then 4.5 ml of 0.7% agar melted and cooled to 40-47°C / 104-116.6°F and containing 0.5 ml of a four-hour broth culture of the strain under study is applied (poured) onto the agar surface. The cultures are incubated at 28°C / 82.4°F. In case of a positive result, if the strain tested shows sensitivity to the pesticide, there will be a delay in culture growth in the centre of the dish (around the colonies of the producer strain).

Pesticinogenicity (ability to synthesise pesticin) is determined by the reverse method. On the surface of 1.5% Hottinger agar in the centre of the dish, a culture of the strain under study was plated with a loop and incubated at 28°C / 82.4°F for 48-72 hours. A filter paper or swab moistened with chloroform is placed on the lid of the dish to sterilise the culture. After an hour or two, the swabs are removed and the dish is dried, after which 4.5 ml of 0.7% agar, melted and cooled to a temperature of 40-47°C / 104-116.6°F, and containing 0.5 ml of a four-hour broth culture of a pesticin-sensitive strain, is applied (poured) onto the agar surface. The cultures are incubated at 28°C / 82.4°F. In case of a positive result, if the tested strain shows pesticinogenicity, a delay in culture growth will be observed in the centre of the dish (around the colonies of the producer strain).

Determination of plasma coagulase activity. One loop of 24-hour (less often two-day) culture is added to 0.5 ml of blood plasma[1]. As a positive control, a strain of *Y. pestis* strain containing the pPla plasmid (e.g. *Y. pestis* strain EV) is used as a positive control and pure plasma as a negative control. Plates and controls are incubated at 28°C / 82.4°F.

[1] Citrate rabbit plasma is used; purified fibrinogen dissolved in saline solution to a concentration of 10 mg/ml is allowed. It should be noted that a number of specialists recommend the use of whole native human blood plasma, but in some countries it is prohibited by the Law on donation of blood and its components.

The results are recorded every hour, starting with the positive control sample (the negative control sample should remain liquid). As a rule, the blood clot is formed after 1-2 hours, but the observations continue for 24 hours (in case of fibrinogen preparation – for 48 hours), during which time the clot should form and begin to decrease in size due to fibrinolysis. The results are evaluated according to the four crosses system:

4+	++++	formation of a dense clot that fills the entire tube and is difficult to separate from its walls
3+	+++	formation of a rather dense clot, easily separating from the walls of the tube, and a small amount of liquid plasma
2+	++	formation of a well-formed clot and a small amount of liquid plasma
1+	+	formation of a small clot floating in the plasma
	±	formation of a barely visible film on the plasma surface
	–	presence of only liquid plasma in the tube

A positive reaction is generally observed in all strains except Caucasian strains.

Determination of fibrinolytic activity. Saline solution is added to blood plasma to a concentration of 1:8 (less often 1:10[1]) and poured into 0.5 ml test tubes. Then a suspension of the 24-hour culture containing 1 x 10^9 BC/ml is prepared, for which purpose it is introduced into a tube with physiological solution by a loop (applied to the wall at the very edge of the solution and gently "flushed" into the solution by circular movements). To obtain a given concentration, it is more appropriate to introduce one or two loops into a small amount of saline solution and then dilute the resulting concentrated suspension with saline solution. Concentration control is performed by comparing the resulting solution

[1] The dilution is selected by performing the study with a control strain (e.g., *Y. pestis* strain EV) at which the fibrin clot formed will be completely dissolved by 18-20 hours.

with a *McFarland standard*[1] or standart[2] calibrated to The International Reference Preparation for Opacity[3]. So, 0.25 ml of the obtained suspension is put into the tube with plasma, then mixed and 0.1 ml of 0.5% calcium chloride solution is added. As a positive control, a strain of *Y. pestis* strain containing pPla plasmid (e.g. *Y. pestis* strain EV), and as a negative control, pure plasma with calcium chloride solutions (0.25 ml of 0.9% solution and 0.1 ml of 0.5% solution). Plates and controls are incubated at 37°C / 98.6°F.

After 40-60 minutes, clot formation is checked (if it is not formed, the study is repeated), and the results are recorded after 18-20 hours. The results are evaluated according to the four-cross system:

4+	++++	complete dissolution of the fibrin clot
3+	+++	almost complete dissolution of the fibrin clot is observed, with the exception of the film on the surface
2+	++	a small fibrin clot is seen floating in a large amount of plasma
1+	+	a large clot of fibrin in a small amount of plasma
	–	only a fibrin clot is observed (no liquid plasma)

If fibrinolytic activity is to be *quantified*, this study is performed with suspensions of 1 x 10^9, 1 x 10^8, 1 x 10^7, ets. For this purpose, 0.5 ml of the initial dilution (1 x 10^9 BC/ml) is added to a test tube with 4.5 ml of saline solution, thus obtaining the first dilution (10^{-1}), which contains 1 x 10^8 BC/ml[4]. Then 0.5 ml of the solution from the tube with the first

[1] Proposed in 1907 by Joseph McFarland and prepared by mixing a 1% solution of sulphuric acid with a 1.175% solution of barium chloride dihydrate, resulting in the formation of barium sulphate (see McFarland J. *The nephelometer: an instrument for estimating the numbers of bacteria in suspensions used for calculating the opsonic index and for vaccines*. J Am Med Assoc. 1097; 49:1176–1178).

[2] It represents borosilicate glass particles (Pyrex®) suspended in distilled water with a diameter of 0.5-3.5 μm. Standards are available in densities of 5 IU and 10 IU, corresponding to concentrations of 4.75 x 10^6 BC/ml and 0.95 x 10^9 BC/ml for *Y. pestis*, respectively.

[3] *5th International reference preparation of opacity*, WHO International Standard, NIBSC code 76/522, Version 6.0 (2013), 2 p.

[4] It is possible to add 1 ml of the initial suspension in 9 ml of saline solution.

dilution is added to a tube with 4.5 ml of saline solution, i.e. a second dilution (10^{-2}) containing 1 x 10^7 BC/ml is obtained. By continuing the described manipulations, the required dilutions and concentrations are obtained. This process is referred to as *serial dilutions* or, in CIS countries, *titration of the bacterial culture*.

The scheme of the method is given in **Table 31.3**. "Read" it from top to bottom along the lines (up to the double solid line, after which the concentrations are explained). The arrow "→" indicates the transfer of the volume (in this case 0.5 ml) above it to the next tube, the arrow "↑" indicates the removal of the volume (in this case 0.1 ml) from the tube to the dish. The number without the arrow indicates the volume to be added.

Table 31.3. *Titration scheme for quantitative quantification of fibrinolytic activity*

Tube / Reagents	1	2	3	4	5	…	9
Saline solution, ml	4,5	4,5	4,5	4,5	4,5	…	4,5
Culture, ml	$0{,}5\overset{0,5}{\rightarrow}$	$\overset{0,5}{\rightarrow}$	$\overset{0,5}{\rightarrow}$	$\overset{0,5}{\rightarrow}$	$\overset{0,5}{\rightarrow}$	…	↑0,1
Suspension, BC/ml	10^9	10^8	10^7	10^6	10^5	…	10^1
Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	…	10^{-9}

Determination of the pigmentsorption trait, i.e. the ability of the bacterium to sorb acidic dyes and hemin, allows us to determine the presence of an important virulence factor, the *pgm*-region. Conducting a study on hemin agar of Jackson & Burrows. So, 0.1 ml of a suspension of 24-hour culture in saline solution containing 5 x 10^3 BC/ml is pipetted onto the surface of the medium and spread evenly over the agar surface with a spatula. As a negative control, pgm strains (e.g. *Y. pestis* strain EV) are used. Plates are incubated for 5-10 days at 28°C / 82.4°F.

Virulent strains (*pgm*+) tend to grow as small black-brown colonies, while avirulent strains (*pgm*⁻) tend to grow as large colourless colonies.

Pigment sorption can also be detected on Hottinger agar. For this purpose, 0.05 mg/ml of Congo red dye should be added to agar, after which 0.1 ml of suspension of 24-hour culture in saline solution containing 5 x 10^3 BC/ml should be applied, spread evenly over the agar surface

with a spatula and incubated 1-2 days at 28°C / 82.4°F and 2-3 days at 4°C / 39.2°F.

Virulent strains (*pgm+*) grow as red colonies and avirulent strains (*pgm⁻*) grow as colourless colonies[1].

It should be remembered that *Y. pseudotuberculosis* serogroup O1 strains also possess the pigment-sorption trait, and comparison of the nucleotide sequences of the *hms* operon of the two species showed[2] that although the evolutionary process was accompanied by single nucleotide substitutions in all genes of the operon, almost all of them formed only synonymous codons. Thus, substitution of guanine for adenine (G→A) at position 603 in the *hmsH* gene resulted in a change from the AC**G** triplet in *Y. pseudotuberculosis* to the AC**A** triplet in *Y. pestis*, but did not affect the synthesis of an amino acid (threonine). Even the G→A substitution at position 612 (GA**C**$_{612}$/GA**A**$_{612}$) in the same gene only resulted in the substitution of one negatively charged amino acid (isoleucine) for another (valine). Nevertheless, the pigment-sorption sign can be used for differential diagnosis. For this purpose, *Y. pestis* is grown on HmsD agar (hemin-storage phenotype differentiation) and *Y. pseudotuberculosis* on HmsD$_{pstbc}$ agar (at 15°C / 59°F).

Strains of the main subspecies of *Y. pestis* (5) form granular colonies, intensely coloured, with a wavy colourless edge, whereas Altai (1), Hissar (4), Talas (3) and Ulegei (1) strains form loose, less coloured, almost pink colonies. Caucasian strains (2) form the most dense, even-edged and brightly coloured colonies. *Y. pseudotuberculosis* strains (6 and 7) form smooth colonies, slightly less intensely coloured, as well as mixed colonies with both pigmented and non-pigmented segments (**Figure 31.4**).

The *dependence of growth on calcium ions* is investigated on Higuchi-Smith agar. From the culture grown for 18-24 hours at 28°C / 82.4°F,

[1] There are reports that some non-pigmenting strains (notably KIM D27 and UC91309) are able to grow as pale pink colonies (see QueneeL. E., Hermanas T. M., et al. *Hereditary hemochromatosis restores the virulence of plague vaccine strains*. J Infect Dis. 2012; 206(7):1050–1058; DOI: 10.1093/infdis/jis433).

[2] Булгакова Е. Г., Краснов Я. М., и др.*Особенности проявления признака пигментации и структурные различия генов hms-оперона у штаммов Y. pestis и Y. pseudotuberculosis разного происхождения*. Проблемы особо опасных инфекций. 2011; 2:30–35.

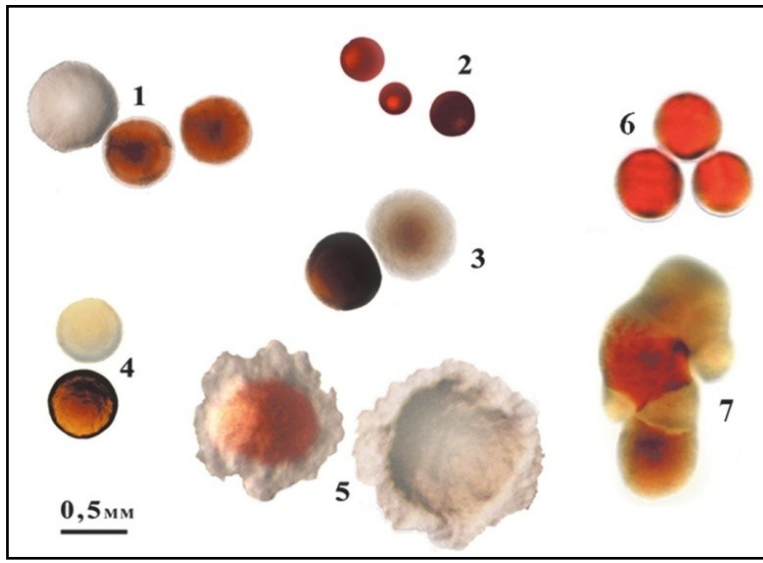


Figure 30.4. *Colonies of Y. pestis and Y. pseudotuberculosis on HmsD agar and HmsD$_{pstbc}$ agar (by Е. Г. Булгакова, 2011):*
1 – Altai strain and Ulegei strain; 2 – Caucasian strain; 3 – Talas strain;
4 – Hissar strain; 5 – strain of the main subspecies (the so-called "sunny side up");
6 – Y. pseudotuberculosis strain 338D (serogroup O1);
7 – Y. pseudotuberculosis strain A17 (serogroup O1)

suspensions in saline solution containing 1 x 10^3, 1 x 10^4 and 5 x 10^5 BC/ml are prepared and plating 0.1 ml on dishes.

The cultures are incubated for 48 hours at 37°C / 98.6°F and colonies (avirulent calcium-independent) are counted. Then incubate for 24 hours at 28°C / 82.4°F and count new colonies (virulent calcium-dependent)[1].

As controls, cultures containing and not containing plasmid pCad are used, plating in the same suspensions of 0.1 ml on Hottinger agar containing 5% blood.

[1] When the cultures were incubated for 71 hours at 37°C / 98.6°F, three types of colonies grew: large (2-2.5 mm), medium (1.5-2 mm) and very small (less than 1 mm). Large and medium colonies are formed by calcium-independent cells.

Table 31.4. *Differential tests for Y. pestis and Y. pseudotuberculosis 01*

Sign	***Y. pestis* subsp. *pestis***	**Caucasian strains**	***Y. pseudotuberculosis o1***
Products pesticin	+	–	–
Pesticin sensitivity	–	+	+
Fibrinolytic activity	+	–	–
Plasma coagulase activity	+	–	–

32 THE STORY OF ONE GRUDGE

Belgium, 1921. The Biological Society is holding a regular meeting in a large hall, which has gathered a large number of scientists and spectators, among whom we can see a man already known to us, closely watching the fierce debate between Jules Jean-Baptiste Vincent Bordet[1] and Richard Bruynoghe[2]. Each of them, together with his group, is trying to prove to his opponent that his understanding of the essence of bacteriophage is the truth. For the former, the bacteriophage is nothing more than a normal bacterial function associated with mutations, whereas for the latter it is a virus! To understand Jules Bordet's point of view, it must be remembered that he was concerned exclusively with immunity and did not even think about bacteriophages. However, after Félix d'Hérelle, being disappointed by the ineffectiveness of vaccines used during the World War I (probably because control was affected by the rush), criticised immunology and Jules Bordet personally, the latter made every effort to prove the invalidity of d'Hérelle's ideas.

[1] 1870–1961; the Belgian physician, bacteriologist, immunologist. Laureate of the Nobel Prize in Medicine or Physiology (1919).

[2] 1881–1957; the Belgian physician, bacteriologist.

But back to the meeting. This time, one of Jules Bordet's opponents is René Appelmans, who has proposed[1] the *liquid method of phage titration* called the "Appelmans protocol", which shows that when the same number of bacteria is introduced into tubes with successive dilutions of bacteriophage, the lysis is observed only in a part of the tubes, which testifies in favour of the existence of the bacteriophage as a particle – a virus.

The *Appelmans protocol* allows to find out the titre of the bacteriophage causing complete lysis of the broth culture. For this purpose, 4.5 ml of broth is poured into ten tubes (one tube is labelled as "control" and the others are numbered from 1 to 9). So, 0.5 ml of bacteriophage is added to the first tube, mixed thoroughly and 0.5 ml is transferred to the second tube, and so continue until the ninth tube, from which 0.5 ml is removed so that all tubes have the same volume. This tube corresponds to the ninth dilution (it can be seen that this process is similar to the serial dilutions (titration) of a bacterial culture). In a separate tube, prepare a bacterial culture suspension (approximately 10^9 BC/ml) and add 0.05 ml (one drop) to all ten tubes. Incubate the cultures in the case of *Y. pestis* at 28°C / 82.4°F, periodically reviewing and recording the transparency in the tubes with dilutions of bacteriophage relative to the control. The tube with the bacteriophage dilution that precedes the dilution in which culture growth is detected (i.e. the last "clean") is the desired titre (in CIS countries it is called the Appelmans titre). The resulting value is expressed as the negative logarithm of 10, where the degree indicates the phage dilution.

A variation of this method is the *method of diagnostic serial dilutions*. The bottom of a Petri dish with 1.5-2% agar is drawn into 8 sectors for convenience (several Petri dishes divided into a smaller number of sectors can be used). In a tube with 4.5 ml of 0.7% agar, previously melted and cooled to 40-47°C / 104-116.6°F, 0.2 ml of culture incubated for 18-20 hours at 28°C / 82.4°F is added, mixed and poured onto a Petri dish[2]. This manipulation is very dangerous, so check the integrity of the tube before opening it. If the agar was melted in a water bath, the tube should be wiped clean. Next, the semi-liquid agar is stirred in the tube,

[1] Appelmans R. *Le dosage du bactériophage*. Compt Rend Soc Biol. 1921; 85:701.

[2] Some specialists instead apply 0.1 ml of culture to the agar surface and rub it thoroughly with a spatula.

making slow circular movements, remove the stopper, while the partner opens the lid of the dish. The tube is brought as close to the agar as possible to avoid splashing, and the semi-liquid agar is carefully poured onto its surface, after which the partner closes the lid of the dish, leaving a small opening for drying. The specialist who poured out the agar carefully reinserts the stopper into the tube, picking up any drops of agar that may have been left on the edge of the tube, after which the closed tube is being autoclaved[1]. Then 0.05 ml (one drop) of whole bacteriophage is added to each sector of solidified and well-dried agar and diluted (10^{-1} to 10^{-7}). The bacteriophage can be applied with a 2 mm diameter loop, burning it each time. Make sure that no liquid remains on the loop, as it may splash during burning. Next, the dishes are allowed to dry and, inverted, incubated for 24 hours at 28°C / 82.4°F. As with the Appelmans titre, the last sector that shows a lysis spot is the diagnostic titre being sought.

A different method was proposed by André Gratia[2], who at the time of the Biological Society meeting was a supporter of Jules Bordet, but later became convinced of the correctness of Richard Bruynoghe's views. His method is to add 0.5 ml dilution of bacteriophage (typically 10^{-1} to 10^{-7}) and 0.05 ml (one drop) of bacterial culture (approximately 10^{9} BC/ml) to the tubes containing 4.5 ml of broth, then pour onto plates with 1.5% Hottinger' agar, dry and incubate at 28°C / 82.4°F. As a result, sterile spots called *plaque-forming units* (PFU, analogous to a bacterial culture's colony-forming units) or, as they say in CIS countries, *negative colonies of bacteriophage* appear on a continuous bacterial lawn (**Figure 32.1**). Their number corresponds to the number of phage particles.

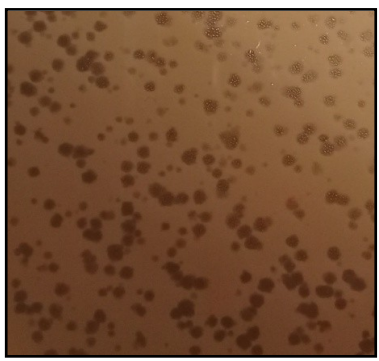


Figure 32.1. *Negative colonies of bacteriophage (second dilution)*

[1] During transportation, tubes should be covered with a cloth moistened with disinfectant solution and autoclaved the same day.

[2] 1893–1950; the Belgian physician, bacteriologist. First researcher of bacteriophages in the USA.

Then, multiplying their number by 10 in the dilution degree, we obtain the number of bacteriophage particles in 1 ml. For example, if 20 negative colonies from the eighth delution are detected, the bacteriophage particles are 2 x 10^9 per 1 мл (20 x 10^8/ml)[1] or 20x10^8 PFU/ml. It is the bacteriophage titration technique that is now most popular!

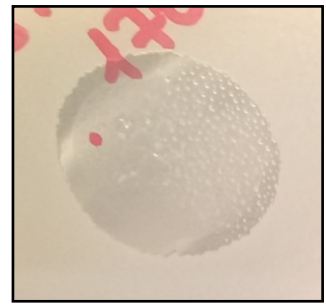


***Figure* 32.2.** *Lysis zone on a dish with a bacterial lawn of Y. pestis strain 1706*

Along with quantitative methods, which allow determining the concentration of bacteriophage required for lysis of the culture, qualitative methods are used to answer the question whether the culture is lysed by a given bacteriophage. Therefore, in diagnostic issues, when it is important to give a result as soon as possible, the determination of culture sensitivity to bacteriophages starts with qualitative methods.

Specifically, 0.1 ml of a bacterial culture (approximately 10^9 BC/ml) grown for 18 hours on agar at 28°C / 82.4°F is added to the tube containing 4.5 ml of 0.7% agar, previously melted and cooled to 40-47°C / 104-116.6°F. Then it has to be stirred evenly, poured onto a plate with 1.5% Hottinger agar and dried. Apply 0.05 ml (one drop) of bacteriophage to the sectors and incubate at 28°C / 82.4°F. Preliminary results (presence of lysis at the site of bacteriophage application) are recorded after 12 hours, and final results are recorded after 24 hours (**Figure 32.2**).

There is also an accelerated method, according to which 0.05 ml (one drop) of the culture and bacteriophage are applied to the first dish with Tumansky's medium (agar), rubbing them on the surface with a spatula. On the second dish with Tumansky's medium plating the test material, and at the very edge of the dish apply one drop (0.05 ml) of bacteriophage, letting it drip to the other edge, forming a "track". The drop of bacteriophage should be applied to the well-dried agar with a Pasteur pipette (touching the surface of the agar) or a loop to avoid splashing.

[1] For accuracy, counting is done on several dishes, calculating the result as an arithmetic mean. If a dish has many negative colonies, it can be divided into sectors, count the number of negative colonies in a sector and multiply by the number of sectors.

Finally, only the test material is plated on the third dish with Tumansky's medium. All dishes are incubated for 24 hours at 28°C / 82.4°F. In case of a positive result, sterile spots are detected on the first dish, a sterile "track" (at the place of bacteriophage application) on the second dish, and bacterial colonies on the third dish (control).

Various lytic bacteriophages active against *Y. pestis* are used for diagnostic purposes. Most often they make connectionto a certain region of the core of LPS[1] (**Table 32.1**, **Figure 32.3**). Pokrovskaya phage and L-413C bacteriophage are used in CIS countries. The first was isolated[2] in 1929 by Magdalena P. Pokrovskaya from tissues infected with plague the gopher of genus *Spermophilus* and belongs to serotype 1 (family *Podoviridae*). The second was isolated[3] in February 1967 from negative colonies formed on hemin agar of Jackson & Burrows as a result of 38 passages of pesticin-positive colonies of *Y. pestis* strain 413 (isolated from Libyan jird (*Meriones libycus*) in the Turkmen SSR in 1964)[4]. It be-

[1] There is indirect evidence for the possibility of binding to pH6 antigen. In particular, it has been shown that pH6 antigen is able to bind 40% of L-413C bacteriophage particles (see Морозова Н. А., Дудина Л. Г. *Участие липополисахарида и pH6 антигена в рецепции бактериофага Л-413С*, в сборн. *Общество. Наука. Инновации (НПК-2023), XXIII Всероссийская (национальная) научно-практическая конференция, приуроченная к 60-летию ВятГУ (12 апреля – 12 июня 2023 г.), Т.2. Технические и естественные науки*, Киров: Вятский государственный университет, 2023, 243 с.).

[2] Покровская М. П. *Чумной бактериофаг в трупах сусликов*. Гигиена и эпидемиология. 1929; 12:31

[3] Ларина В. С., Анисимов П. И., Адамов А.К. *Новый штамм чумного бактериофага для диагностики возбудителя чумы*. Проблемы особо опасных инфекций. 1970; 11:132-136

[4] Here is how the researchers themselves write about it: *"On the 38th passage [of Y. pestis strain 413], 5x6 mm culture lysis spots appeared, irregularly shaped, due to the fusion of several negative colonies. For bacteriophage isolation, agar was cut out from the culture lysis spots and all this was poured into 2 ml of Hottinger's broth (pH=7.2). After 24 hours - chloroform was added, the mixture was shaken thoroughly and left in the refrigerator for 2 hours. The bacterial cells and chloroform were freed from the bacterial cells by centrifugation for 30 min at 1000 rpm. The bacteriophage isolated from these stains was characterised by the formation on sensitive culture of polymorphic negative colonies 0.5 to 2 mm in diameter with a conventionally «transparent» bottom with an irregular edge, a turbid bottom and stains with varying degrees of transparency. Population analysis of the 413/15P+ culture after the 38th passage revealed heterogeneity of the cellular composition of the strain under study. The majority of the cells were sensitive to the isolated phage, while others showed the properties of lysogenic cultures, expressed in the detection of negative colonies of spontaneous phagoproduction on the sensitive culture and resistance to the obtained phage. A phage-sensitive clone was isolated from this culture by selection from single colonies of cultures characterised by the highest phagoproduction, which was taken as an indicator strain. A pure phage line was selected on this strain by 10-fold selection of negative colonies with a «trans-*

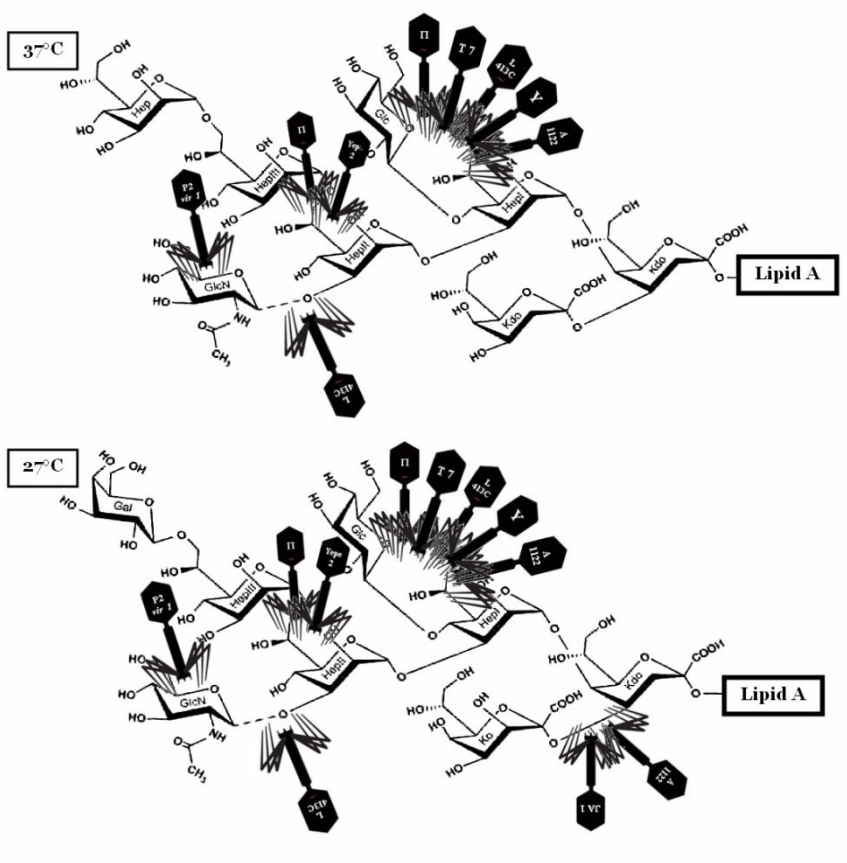


***Figure* 32.3.** *Chemical structures of the Y. pestis LPS core at 27°C / 80.6°F and 37°C / 98.6°F (by Книрель Ю. А., 2012) with marked regions recognised by bacteriophages Pokrovskaya (П), L-413C (L413C), ϕA1122 (A1122), Yepe2, P2 vir1, JA1, T7Yp (T7) and Y. Note that most bacteriophages recognise the HepI/Glc core region. In addition, bacteriophage JA1 recognises the core region only when modified at 27°C / 80.6°F, and bacteriophage ϕA1122 has only one recognition region at this modification*

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parent» bottom. The bacteriophage was brought to a concentration of 3x10^7 phage corpuscles in 1 ml by passivating it 5 times on the indicator strain. Based on the transparency of negative stains, this bacteriophage was named L-413 «C» (clear)... On all studied strains phage L-413C formed negative colonies of the same shape with indistinctly outlined edge, measuring from 1 to 2 mm in diameter. The stains were uniformly «transparent» with isolated microscopic colonies scattered on the surface" (**Figure 32.4**).

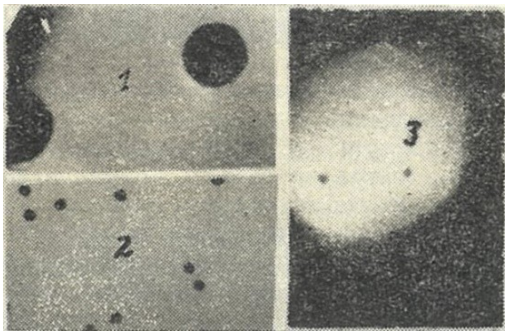


Figure 32.4. *Morphology of negative colonies of bacteriophage L-413C (by Ларина В.С., 1970): 1 - lysis zone, erroneously identified by the authors as "negative colonies of plague virulent phage measuring 8-9 mm («control»)"; 2 - negative colonies of bacteriophage L-413C measuring 1-2 mm; 3 - negative colony of bacteriophage (40x), within which two bacterial colonies are visible*

longs to serotype 2 (family *Myoviridae*) and is highly specific. Pokrovs-kaya phage is closely related to coliphage T7 (active against *Escherichia coli*), is less specific, and depending on dilution lyses 92.5-98.5% of *Y. pestis* cultures and 6.1-19% of *Y. pseudotuberculosis* cultures. Therefore, it is used only when the suspected culture is not lysed by bacteriophage L-413C and in parallel with pseudotuberculosis phage. A positive result in this situation is registered only if the culture is lysed with a diagnostic titre or higher, i.e. it is lysed with a lower concentration of bacteriophage (this is where quantitative methods come into play)[1].

In the USA, bacteriophage φA1122, that was isolated[2] in 1933 from the blood of a patient with bubonic plague and belonging to serotype 1 (family *Podoviridae*), is used. It is interesting, that this bacteriophage, as well as Pokrovskaya phage, is closely related to coliphage T7. It lyses *Y. pestis* cultures at 20°C / 68°F and 37°C / 98.6°F, like *Y. pseudotuberculosis* cultures at temperatures above 28°C / 82.4°F. Moreover, Bacteriophage P, isolated[3] in 1951, and bacteriophage H, isolated[4] in

[1] If lysis occurs at dilutions below the diagnostic titre (i.e. higher concentration of bacteriophage), other diagnostic methods are used.

[2] Advier M. *Etude d'un bactériophage antipesteux*. Bull Soc Pathol Exotiques. 1933; 26:94–99.

[3] Gunnison J. B., Larson A., et al. *Rapid differentiation between Pasteurella pestis and Pasteurella pseudotuberculosis by action of bacteriophage*. J Infect Dis. 1951; 88(3):254–255; DOI: 10.1093/infdis/88.3.254.

[4] Cavanaugh D. C., Quan S.F. *Rapid identification of Pasteurella pestis using specific bacteriophage lyophilized on strips of filter paper; a preliminary report*. Am J Clin Pathol. 1953; 23(6): 619–620.

1953 from sewage at The University of California, Berkeley, which lyses *Y. pestis* cultures at 20°C / 68°F, are also used. Interestingly, both bacteriophages belong to serotype 1 (family *Podoviridae*) and are capable of lysing *Escherichia coli*, as bacteriophage Y, isolated[1] in 1964 in Israel from "turbid plaques" formed on a culture of *Y. pestis* plague microbe at 27°C / 80.6°F.

It is likely that the attentive reader will be interested in bacteriophage H in the sense that earlier (in Chapter 6) we talked about a bacteriophage with the same name, isolated in 1965 by Nikolai N. Novoseltsev. Here we also talk about bacteriophage H, but we mention year 1953 and The University of California, Berkeley. There is no confusion, these are different bacteriophages[2].

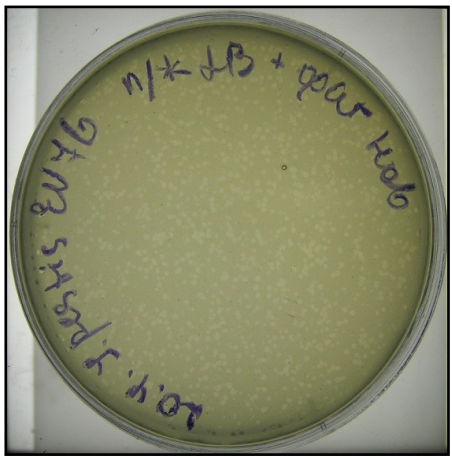


Figure 32.5. *Negative colonies of Novoseltsev's phage (courtesy of Dr. Tatiana E. Arsenyeva)*

In China, the bacteriophage Yep-phi isolated there, belonging to serotype 1 (family *Podoviridae*), is used. This bacteriophage, although closely related to coliphage T7, is not capable of lysing other members of the genus *Yersinia* except for *Y. pestis* (the bacteriophage forms negative colonies only at 26°C / 78.8°F). It is also genetically similar to Berlin, Yepe2[3] and YepMm bacteriophages. However, the lat-

[1] Hertman I. *Bacteriophage common to Pasteurella pestis and Escherichia coli.* J Bacteriol. 1964; 88:1002–1005; DOI: 10.1128/jb.88.4.1002-1005.1964.

[2] Remind that bacteriophages H and H-1 (see Chapter 6) isolated by Nikolai N. Novoseltsev are most likely not moderate but lytic wild-type bacteriophages with unmethylated nucleic acid and, accordingly, phages H-2 and H-3 are not "virulent mutants" but the same bacteriophages, only with methylated nucleic acid. These phages were used for some time as diagnostic phages under the name "Novoseltsev's phage" (**Figure 32.5**). And how can there not be confusion?

[3] Zhao X., Wu W., et al. *The complete genome sequence and proteomics of Yersinia pestis phage Yep-phi.* J Gen Virol. 2011; 92(Pt 1):216–221; DOI: 10.1099/vir.0.026328-0.

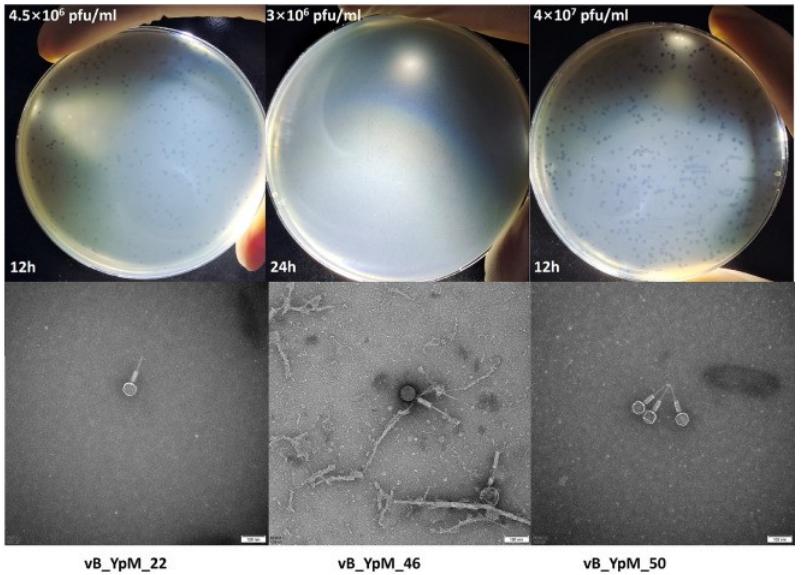


Figure 32.6. *Negative colonies (top row) and transmission electron microscopy (bottom row) of P2-like bacteriophages (by Qi Z., 2011)*

ter, isolated[1] in 2021 from bone marrow of the Himalayan marmot (*Marmota himalayana*)[2], lyses not only *Y. pestis*, but also *Y. pseudotuberculosis* O:1b and *Y. enterocolitica* 1B/O:8, while its genome is 99.99% similar to that of the bacteriophage Yep-phi!

The P2-like bacteriophages vB_YpM_22, vB_YpM_46 and vB_YpM_50, first isolated[3] in 2011 from the blind intestine of the Himalayan marmot (*Marmota himalayana*) in the Qinghai-Tibetan focus, although they have high genetic affinity with bacteriophage L-413C, lysate *Y. pestis* only at 37°C / 98.6°F, so their diagnostic capabilities are limited (**Figure 32.6**).

Bacteriophages P2 *vir* 1, JA1 and T7Yp are also known but not used in diagnosis.

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[1] Liang J., Qin S., et al. *A lytic Yersina pestis bacteriophage obtained from the bone marrow of Marmota himalayana in a Plague-Focus Area in China*. Front Cell Infect Microbiol. 2021; 11:700322; DOI: 10.3389/fcimb.2021.700322.

[2] Isolated during surveillance of the Qinghai-Tibetan focus.

[3] Qi Z., Meng B., et al. *Identification and characterization of P2-like bacteriophages of Yersinia pestis*. Virus Research. 2022. 322:198934; DOI: 10.1016/j.virusres.2022.198934.

Because of the possibility of resistance development that we discussed earlier, the search for new highly specific lytic bacteriophages is ongoing. Thus, new bacteriophages YpEc56, YpEc56D, YpEc57, YpEc58, YpEc1, YpEc2, YpEc11 and YpYeO9 were isolated[1] in 2023 (**Figure 32.7**). They belong to serotype 1 (family *Podoviridae*). The conducted studies showed high specificity of bacteriophages YpEc11 and YpYeO9 only, and the results of full genome sequencing allowed their identification as a new species of the family *Autographiviridae*.

Table 32.1. *Y. pestis LPS core regions recognised by different diagnostic bacteriophages*

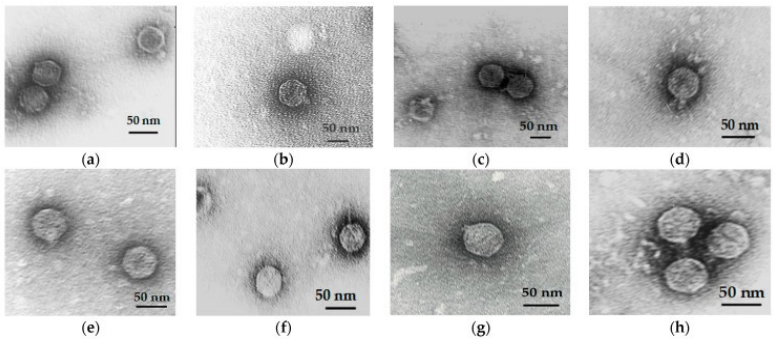
Bacteriophage	**A region of the *Y. pestis* LPS core**
Pokrovskaya	HepIII/HepII – HepI/Glc
L-413C	HepIII/HepII(GlcNAc) – HepI/Glc
φA1122	HepI/Glc – Kdo/Ko
Yepe2	HepIII/HepII
YpsP-PST	
P2 *vir 1*	GlcNAc
JA1	Kdo/Ko
$T7_{Yp}$	HepI/Glc
YpP-R	
Y	

Several years ago, an interesting method for *Y. pestis* detecting was proposed[2] based on the ability of bacteriophages to bind to the bacterium via receptor binding proteins (RBP). In particular, RBPs of bacteriophages L-413C and φA1122, whose *gpH* and *gp17* receptor binding protein genes were binding with the genes of fluorescent proteins mCherry and eGFP, were obtained, which made it possible to obtain fluorescent proteins mCherry-RBP and eGFP-RBP of the named bacteriophages, and to detect *Y. pestis* cells using fluorescence microscopy methods (**Figure 32.8**). This method was simple and fast, but, which is

[1] Suladze T., Jaiani E., et al. *New Bacteriophages with Podoviridal Morphotypes Active against Yersinia pestis: Characterization and Application Potential*. Viruses. 2023; 15(7):1484; DOI: 10.3390/v15071484.

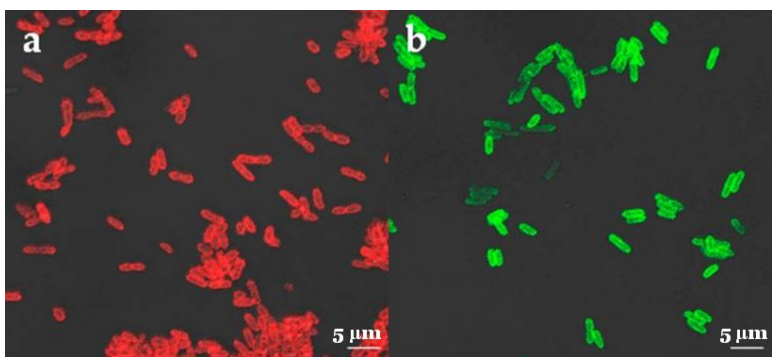
[2] Born F., Braun P., et al. *Specific Detection of Yersinia pestis Based on Receptor Binding Proteins of Phages*. Pathogens. 2020; 9(8):611; DOI: 10.3390/pathogens9080611.

more important, highly specific[1], and the proteins of bacteriophage L-413C showed greater specificity than those of bacteriophage φA1122, since the latter, as well as the bacteriophage itself, can bind to *Y. pseudotuberculosis* at 37°C / 98.6°F. In addition, the presence of a capsule in *Y. pestis* did not significantly affect the ability of mCherry-RBP bacteriophage L-413C to bind to the bacterial cell.



▲ ***Figure 32.7.*** *Bacteriophages YpEc1 (a), YpYeO9 (b), YpEc56D (c), YpEc58 (d), YpEc57 (e), YpEc2 (f), YpEc11 (g) and YpEc56 (h); transmission electron microscopy, 40000x (by Suladze T., 2023)*

▼ ***Figure 32.8.*** *Fluorescence microscopy of mCherry-RBP (a) bacteriophage L-413C and eGFP-RBP (b) bacteriophage ϕA1122 proteins (by Born F., 2020)*



[1] However, it should be noted that resistance to bacteriophage receptor binding proteins acquired by the bacterium jeopardises the specificity and sensitivity of this method, as well as of any method based on diagnostic bacteriophages in general; therefore, negative bacteriophage results should be treated with caution.

33 IMMUNOCHEMISTRY: ANTIGEN – ANTIBODY

In 1944, Archer John Porter Martin[1] and Richard Laurence Millington Synge[2] created a method that allowed a mixture of amino acids to be separated on a thin cellulose carrier. The new method was called *paper chromatography*. A year later, the *lateral flow test* (LFT) or *lateral flow immunochromatographic assay*, or *rapid test*[3] was created on its basis, which allows the presence of the target substance (antigen) in a liquid sample to be detected quickly and easily.

The principle of LFT is the following: when the analysed liquid hits the lower part of the strip, it starts to migrate, combining with the labelled antibodies. Two forms of analysis are distinguished: direct ("sandwich") assay and competitive assay.

In the first case, the sample (analyte) containing antigen (**Ag**) binds to a conjugate – a labelled antibody, usually colloidal gold (**Ab***). As it moves, the formed complex meets the first barrier, where it binds to specific antibodies (**Ab**), forming a "sandwich": **Ab – Ag – Ab***. The excess conjugate (**Ab***) moves on to meet a second barrier where it binds to antibodies (**Ab'**) to that conjugate: **Ab' – Ab***. As a result, if the sample (analyte) contains antigen, the coloured markers (**Ab***) will be retained by both barriers, and only by the second barrier if it does not. Visually, this will be identified as one and two bars (barriers are usually labelled "T" for test and "C" for control). Thus, any result that lacks a control band should be considered invalid. This form of LFT is used for the identification of high molecular weight compounds such as infectious agent antigens and hormones.

In the second case, the test sample (analyte) containing antigen (**Ag**) also binds to the conjugate (**Ab***), but since it has only one binding centre, it cannot bind to the antibodies (**Ab**) of the first barrier, while the excess of the conjugate (**Ab***), reaching the second barrier, binds to the antibodies (**Ab'**) to this conjugate. As a result, if the sample (analyte)

[1] 1910–2002; the British chemist. Laureate of the Nobel Prize in Chemistry (1952).

[2] 1914–1994; the British biochemist. Laureate of the Nobel Prize in Chemistry (1952).

[3] Consden R., Gordon A. H., et al. *Qualitative analysis of proteins: a partition chromatographic method using paper*. Biochem J. 1944; 38(3):224–232; DOI: 10.1042/bj0380224.

contains antigen, only the second barrier (**Ab' – Ab***) will retain coloured markers (**Ab***), and if it does not, both barriers will retain them. Thus, as in the first case, any result that lacks a control bar should be considered invalid, but unlike the first case, a result where two bars are visualised will be considered negative. This form of LFT is used for the identification of low molecular weight compounds such as metabolites of drug compounds found in urine and oral fluid.

In the case of *Y. pestis*, the first form of LFT is used, using the capsular antigen as the specific target (the antigen sought). The ease of use of the system makes it possible to detect quickly (within 20 minutes) and to identify not only *Y. pestis* cells themselves, but also their "traces" (capsular antigen) from environmental objects. It is not for nothing that this test, along with direct fluorescence assay, is mandatory in the World Health Organization's criteria for plague case definition (probable and confirmed cases).

The test itself is a diagnostic panel with a well for sample introduction and "T" and "C" zones for visual evaluation of results. For biosafety purposes, the panel is placed in a petri dish to prevent a possible accident. Both polyclonal and monoclonal antibodies are used as labelled antibodies. The sensitivity of the polyclonal antibody-based on the test system is 10^6 BC/ml or 0.1 μg/ml for capsular antigen. In the case of monoclonal antibodies it is 10^3 BC/ml or 0.004 μg/ml for capsular antigen, however studies show that in case of usage of clinical samples (blood, bronchoalveolar lavage), the sensitivity decreases to 10^4–10^5 BC/ml or 0.01-0.1 μg/ml for capsular antigen (**Figure 33.1**).

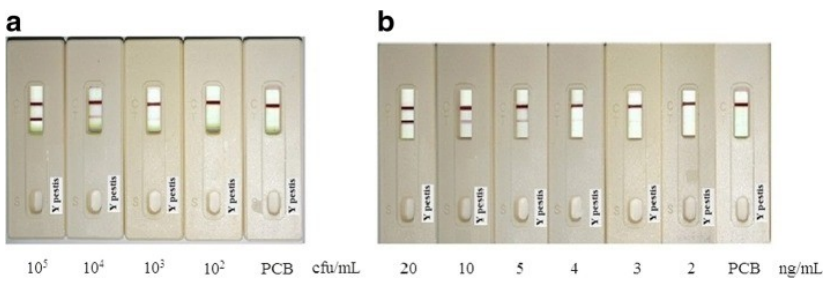


Figure 33.1. *Sensitivity analysis of the panel for Y. pestis (a) and capsule antigen (b). It can be seen that there is a weak band of positive results in the case of 10^3 BC/ml and 4 ng/ml, i.e. 0.004 μg/ml (by Hsu H.-L., 2018)*

Earlier we spoke about the existence of so-called fractionless strains of *Y. pestis*, which, as it is easy to guess, cannot be identified by this method. Therefore, LFT test systems containing monoclonal antibodies to capsule antigen and LcrV are currently being developed[1] (accordingly, the result is counted on three strips). The sensitivity of this test system is 0.001-0.002 μg/ml.

To test the sample[2] for capsule antigen-specific antibodies (IgG), a LFT test is used, which is a 5 mm wide nitrocellulose strip. Instead of antibodies, the test part contains adsorbed purified antigen (capsule antigen or together with LcrV) and the control part contains bovine serum albumin. The sample to be tested is absorbed at the bottom of the strip and rises upwards, interacting sequentially with antigen and albumin. As a result, if the sample contains an antibody, it binds to the antigen, while non-specific antibodies rise above and interact with albumin. Both interactions result in the formation of pink-coloured bands.

It can be seen that all these tests are based on a specific reaction between antigen and antibody, so they are also called *serological reactions*. Independently of each other, in the early 1970s Eva Engvall[3] and Peter Perelmann[4] and Bauke van Weemen[5] and Anton Schuurs[6] developed[7] another method, the *enzyme-linked immunosorbent assay* (ELISA), which detects the "antigen-antibody" complex using a substrate that is cleaved by an enzyme, resulting in staining. And since, fewer complexes are required to achieve staining due to the catalytic properties of enzymes, ELISA is highly sensitive, although more difficult to perform than LFT.

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[1] Hau D., Wade B., et al. *Development of a dual antigen lateral flow immunoassay for detecting Yersinia pestis*. PLoS Negl Trop Dis. 2022; 16(3):e0010287; DOI: 10.1371/journal.pntd.0010287.

[2] Most commonly blood serum diluted with phosphate buffer with 0.5% Twin-80 at a ratio of 1:100.

[3] b. 1940; the Swedish biochemist.

[4] 1919–2005; the Czechoslovakian and Swedish biochemist. Emigrated to Sweden during the German annexation of the Sudetenland in Czechoslovakia (1938-1945).

[5] 1873–1936; the Netherlandish physician.

[6] ?-?; the Netherlandish physician.

[7] Engvall E., Perelmann P. *Enzyme-Linked Immunosorbent Assay, Elisa: III. Quantitation of Specific Antibodies by Enzyme-Labeled Anti-Immunoglobulin in Antigen-Coated Tubes*. J Immunol. 1972; 109(1):129–135; DOI: 10.4049/jimmunol.109.1.129; Van Weemen B. K., Schuurs A. H. W. M. *Immunoassay using antigen-enzyme conjugates*. FEBS Letters. 1971; 15(3):232–236; DOI: 10.1016/0014-5793(71)80319-8.

Nowadays various ELISA methods have been developed , but the most commonly used for plague detection is a variant of *"sandwich" ELISA*. This variant is similar in principle to the direct LFT, which, as we remember, is also called "sandwich" (but this is not to be confused with a direct ELISA). The essence of the "sandwich" ELISA method is that a sample (analyte) containing antigen (**Ag**) is added to a solid carrier (often the bottom of the well on a plate) with immobilised (i.e. attached) antibodies (**Ab**). During incubation, the antigen binds to the antibody and the unreacted reaction components are removed with washing solution. The conjugate, which is an enzyme-labelled specific antibody (**Ab***) capable of binding to the antigen (**Ag**), is then added. The unreacted components are again removed by washing. As a result, if the sample (analyte) contains antigen, it is sandwiched between the immobilised antibodies and the conjugate, forming a "sandwich": **Ab – Ag – At***.

Monoclonal antibodies labelled with horseradish peroxidase (HRP) or other commercially produced peroxidase are used as a conjugate. To visualise the "sandwich", a substrate-indicator solution consisting of hydrogen peroxide and a substance oxidising by interaction with peroxidase and hydrogen peroxide is used, resulting in staining of the solution, which is assessed visually and photometrically. Such substances include, for example, ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), which colours the solution green-blue (wavelength 405-420 nm), and TMB (3,3',5,5'-Tetramethylbenzidine), which colours the solution blue (wavelength 370-650 nm). Sulphuric or orthophosphoric acid are used to stop the reaction.

The sensitivity of the test system in case of *Y. pestis* is 10^6 BC/ml (in the absence of positive results with other microorganisms at concentration of 10^9 BC/ml), and in case of capsular antigen it is 0.004 μg/ml. As in case of LFT, ELISA test systems are being developed for the diagnosis of fractionless strains containing monoclonal antibodies to capsule antigen and LcrV.

The ELISA method is used to examine tissues and organs, as well as blood serum. Sample preparation includes inactivation with sodium mertiolate to a final concentration of 1:10000 (0.01%) with warming for half an hour at 56°C / 132.8°F. Some specialists use formalin for inactivation, adding it to samples to a final concentration of 1% followed by

at least 3 hours exposure or 2% followed by an hour exposure, then warming for half an hour at 72°C / 161.6°F. Next, 100 μl of sample is taken into a 1.5 ml tube and lysing solution prepared with 6M guanidinisothiocyanate or commercially produced solution is added, followed by incubation for 15 minutes at 65°C / 149°F. The sample can then be centrifuged to settle the precipitate (600 rpm for 2-3 minutes) and the supernatant can be collected at the phase boundary.

If pellets are examined, their suspensions are prepared using 1% formalin solution on phosphate-salt buffer with a concentration of 0.1 mol/L, which can be combined into a single sample, which is sedimented, and the supernatant is examined. Excrement of predators is rubbed with 1% formalin solution and examined after 24 hours. Bone remains are examined more often individually (up to 20-25 pieces may be combined), crushed and poured in ten volumes of 1% formalin solution with saline solution.

If the culture on the dish is overgrown with foreign microorganisms, it is kept for 24 hours in the thermostat at 37°C / 98.6°F (to synthesise capsular antigen), then transferred to a dish with 3-4 ml of 2% formalin solution for disinfection. After 24 hours, the fluid is examined. There is another method: a suspension of culture taken from different sectors of the dish is prepared in saline solution, after which formalin is added to a final concentration of 2% and examined after 24 hours.

A cheaper alternative is the *dot enzyme-linked immunosorbent assay* (dot-ELISA) proposed[1] in 1982. The essence of this method is the usage of a nitrocellulose substrate with a dotted antibody (antigen) marked with a coloured marker instead of microplate wells, As a result of the reaction, coloured spots are formed that are easily distinguishable against a white background, thus eliminating the need for a photometer.

The developed[2] test systems with colloidal silver as a marker have a sensitivity in case of *Y. pestis* of 5 x 104 BC/ml and in case of capsular antigen of 0.0048 μg/ml.

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[1] Hawkes R., Niday E. *A dot-immunobinding assay for monoclonal and other antibodies*. Anal Biochem. 1982; 119(1):142–147; DOI: 10.1016/0003-2697(82)90677-7.

[2] Носкова О. А., Загоскина Т. Ю., и др. *Применение ДОТ-иммуноанализа для выявления антигенов чумного микроба в полевом материале*. Проблемы особо опасных инфекций. 2014; 3:69–71

In 1949 Örjan Thomas Ouchterlony[1] published an article[2] where, summarising his own experience of studying diphtheria toxin[3] and the observations of other scientists (in particular, Heinrich Jakob Bechhold[4] as early as 1905[5]), he proposed a method of antigen and antibody identification based on the already known antigen – antibody reaction by measuring diffusion gradients in a gel, called *Ouchterlony double immunodiffusion* or *passive double immunodiffusion*.

In general terms, precipitation (from Latin praecipitatio – "falling down") is a chemical reaction that results in the formation of a precipitate. For example, if a narrow tube is taken and 0.3 ml of immune serum (i.e. serum containing antibodies) is poured carefully along the wall with an equal amount of sample containing antigen, a thin opaque ring of antigen – antibody complex, called *precipitate*, is formed as a result of their interaction. If the sample is an extract of tissue and organ (1-2 g), pre-poured with saline solution (5-10 ml) and boiled for half an hour, this reaction is called *thermoprecipitation* or *Ascoli's thermo precipitation test*, in honour of Alberto Ascoli[6], who proposed it in 1911 for the detection of *Bacillus anthracis*[7].

The difficulty with the Ascoli's thermo precipitation test is that sometimes the ring is difficult to visualise and also, if the sample is added quickly, it does not form at all. Therefore, Örjan Ouchterlony's truly revolutionary idea was to "transfer" this reaction from a test tube to a Petri dish. To perform the reaction, a thin layer of 1.5% agarose gel is poured onto a dish and, after hardening, wells are to be carefully cut into the gel, where the sample shall be tested and the antigen or anti-

[1] 1914–2004; the Swedish physician, bacteriologist. Fellow of the Swedish Academy of Sciences (1968).

[2] Ouchterlony Ö. *Antigen – antibody reactions in gels*. Acta Pathol Microbiol Scand. 1949; 26(4):507–515; DOI: 10.1111/j.1699-0463.1949.tb00751.x.

[3] Ouchterlony Ö. *In vitro method for testing the toxin-producing capacity of Diphtheria bacteria*. Acta Pathol Microbiol Scand. 1949; 26(4):507–515; DOI: 10.1111/j.1699-0463.1948.tb00655.x.

[4] 1866–1937; the German chemist, author of books on colloidal chemistry in medicine. Fellow of the German National Academy of Sciences Leopoldina (1932).

[5] Bechhold H. *Strukturbildung in Gallerten*. Zeitschrift für Physikalische Chemie. 1905; 52U(1):185–199; DOI: 10.1515/zpch-1905-5210.

[6] 1877–1957; the Italian chemist, hygienist.

[7] Ascoli A. *Die Präzipitindiagnose bei Milzbrand*. Centralbl Bakt Parasit Infeckt. 1911; 58:63–70.

body, depending on whatsample is being tested for, are placed. The antigen and antibody placed in the wells diffuse into each other, resulting in a thin band of precipitate between the wells. In multicomponent systems, several precipitation lines appear between the wells with antigens and antibodies: for identical antigens, the precipitation lines merge and for different antigens, they overlap (**Figure 33.2**).

This method is most often used for qualitative analysis, but it can be used semi-quantitatively by using dilutions of the sample being analysed. *Radial immunodiffusion* (RID) or *Mancini method*, developed[1] by Giuliana Mancini[2] in 1964, is also used for antigen quantification. The essence of this method is that a monospecific serum containing an antibody is dispersed in a gel beforehand. Then, after the sample is added to the well, a precipitation ring is formed around it, which diameter is used to estimate the amount of antigen in the sample.

Another simple method of antigen detection is the *agglutination assay*, when antibodies cause adhesion (*agglutinate formation*) and precipitation of corpuscular antigens, i.e. bacteria and particles with antigens adsorbed on them. This reaction is carried out on glass and in test tubes.

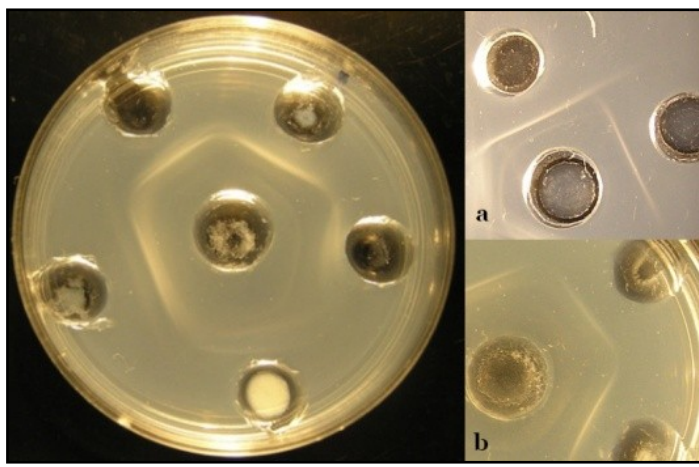


Figure 33.2. *Ouchterlony double immunodiffusion of a culture of Y. pestis culture: general view (left), crossing (a) and merging (b) of the precipitation lines*

[1] Mancini G., Carbonara A. O., et al. *Immunochemical quantitation of antigens by single radial immunodiffusion*. Immunocemistry. 1965; 2(3):235–254; DOI: 10.1016/0019-2791(65)90004-2.

[2] 1934–?; the Italian physician.

In the first variant, three drops of saline solution are applied to a degreased glass placed in a Petri dish and a drop of specific (adsorbed) serum or non-adsorbed serum, previously diluted in the ratio of 1:25-1:5, is added to two of them. Then the loop next to a drop of saline solution is applied incubated at 30°C / 86°F 24-hour culture, rub it with smooth circular movements and put first into a drop of saline solution and then into a nearby drop of serum, stirring in each drop until the formation of a homogeneous suspension. A drop of saline solution is the "culture" control and a drop with serum, where no culture has been added, is the "serum" control. Controls are necessary to exclude spontaneous agglutination of culture and serum. In case of a positive reaction, a flaky precipitate forms in the serum (with culture) drop after 5-10 minutes (**Figure 33.3**). It should be noted that some practitioners perform this reaction directly in a Petri dish (without agar) for convenience.

The described method is considered to be tentative. The expanded method has been proposed by Eugenia I. Korobkova and consists in staging the reaction with whole serum and its dilutions 1:2, 1:4, 1:8 and so on up to 1:128.

It is noteworthy thatthis variant was used to confirm the identity of the strain in the outbreak before the development of molecular genetic methods. For this purpose, blood serum from the first patient was used as serum. If a strain of microorganism isolated from a patient was agglutinated with serum from the first patient, it was possible to say that this patient was infected with the same strain.

In the second variant, proposed[1] by Reuben Leon Khan[2] in 1922 and also considered expanded, 1 ml of saline solution is added to the both identical test tubes. Then 0.1 ml of serum dilution (e.g., 1:50) is added to a separate tube (control) and to the first tube with saline solution (1:100), and then titrated by successive transfers of 1 ml from the tubes with saline solution (i.e., serum dilutions of 1:200, 1:400, 1:800, etc. are obtained). Then, 0.1 ml (two drops) of culture is added to all tubes (except

[1] Khan R. L. *A simple quantitative precipitation reaction for syphilis*. Arch Derm Syphilol. 1922; 5(5):570–578; DOI: 10.1001/archderm.1922.02350300011003.

[2] 1887–1979; Lithuanian and American military physician. He was born in the Lithuanian city of Svira (then in the Russian Empire), but moved with his parents to the USA when he was 12 years old. One of the founders of immunology, author of books on serological reactions.

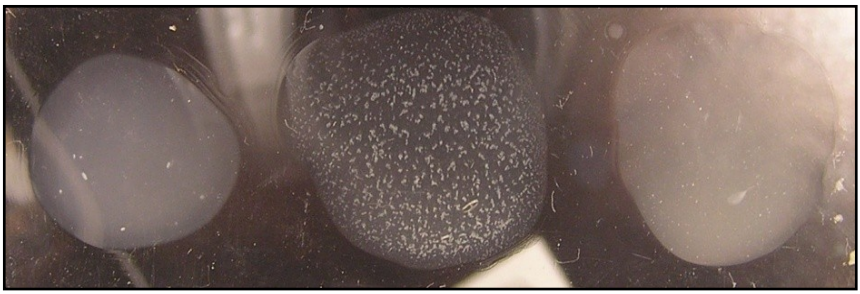


Figure 33.3. *Agglutination assay on the glass: positive reaction (middle) and serum (left) and culture (right) controls*

the control tube), as well as to a separate tube with 1 ml of saline solution (control). The tubes are then gently shaken and incubated at 28°C / 82.4°F. The results are recorded after 2 hours and finally after 24 hours.

The scheme of the method is given in **Table 33.1**. "Read" it, as in **Table 31.3**, from top to bottom, line by line. The arrow "→" indicates the transfer of the volume (in this case 1 ml) stated above it to the next tube, the arrow "↑" indicates the removal of the volume (in this case 1 ml) from the tube to the dish. The number without the arrow indicates the volume to be added. All the following schemes are to be "read" in the same way.

Table 33.1. *Agglutination assay scheme (by R. Kahn)*

Tube / Reagent	1	2	3	4	5	Control	
						"Serum"	"Culture"
Saline solution, ml	1	1	1	1	1	1	1
Serum, ml	$1 \overset{1}{\rightarrow}$	$\overset{1}{\rightarrow}$	$\overset{1}{\rightarrow}$	$\overset{1}{\rightarrow}$	1 ↑	1	
Culture, ml	0,1	0,1	0,1	0,1	0,1		0,1
Incubation at 28°C / 82.4°F							

Already in 1925 Arlyl Noble[1] proposed[2] a modification of this method, used in the laboratory diagnosis of plague and based on the fact that

[1] 1887–1981; American physician, the first female bacteriologist in the USA.

[2] Noble A. *A rapid method for the macroscopic agglutination test*. J Bacteriol. 1927; 14(5):287–300; DOI: 10.1128/jb.14.5.287-300.1927 (The method was presented at the twenty-

if the first stage of agglutination (combination of antigen with antibody) is carried out at high concentrations, the result of the reaction can be obtained faster. Eight tubes are used for staging. In tubes 2 to 8, 0.1 ml of saline solution is added, and in tube 1 - 0.2 ml of whole serum. Then 0.1 ml of serum (dilution 1:2) is transferred from the first tube to the second and further titrated to the eighth tube (1:128), after which 0.1 ml (two drops) of culture is added to each tube and shaken thoroughly for 3-5 minutes. After that, 0.8 ml of saline solution is added to all tubes, thus achieving a final concentration of 1:10, 1:20 and so on. The scheme of the method is given in **Table 33.2**. The results are recorded preliminarily immediately and finally after 15 minutes.

Like many bacteria, pathogenic *Yersinia* spp. are characterised by spontaneous aggregation in liquid media (autoagglutination). In *Y. pseudotuberculosis* and *Y. enterocolitica*, autoagglutination occurs only at 37°C / 38.6°F and is caused by the action of YadA, whereas in case of *Y. pestis*, autoagglutination is provided by an **agglutination factor** isolated[1] in 2010, which is a complex of a protein with a molecular mass of 17.5 kDa and a low molecular weight component. As an autoagglutination control, two tubes with 0.9 ml of saline solution are used, to one of which 0.1 ml (two drops) of culture is added, and to the other 0.1 ml of whole serum is added.

Table 33.2. *Agglutination assay scheme (by A. Noble)*

Tube / Reagent	1	2	3	…	8	Control	
						"Serum"	"Culture"
Saline solution, ml	0,1	0,1	0,1	…	0,1	0,9	0,9
Serum, ml	0,2 $\overset{0,1}{\rightarrow}$	$\overset{0,1}{\rightarrow}$	$\overset{0,1}{\rightarrow}$	…	0,1 ↑	0,1	
Culture, ml	0,1	0,1	0,1	…	0,1		0,1
Shake for 3-5 minutes							
Saline solution, ml	0,8	0,8	0,8	…	0,8		

seventh annual meeting of the Society of American Bacteriologists, Madison, Wisconsin, December 23 to 31, 1925).

[1] Рыкова В. А. *Характеристика фактора аутоагглютинации возбудителя чумы*. Дис. … кандидата биологических наук. Ростов-на-Дону, 2010, 141 с.

The results are evaluated according to the four crosses system:

4+	++++	the solution is clear, there is pronounced precipitate (positive)
3+	+++	the solution is slightly turbid (no complete clarification), precipitate is present (positive)
2+	++	the solution is turbid, precipitate is insignificant (doubtful)
1+	+	the solution is turbid, no precipitate (negative)
"Serum" control		the solution is clear
"Culture" control		the solution is uniformly turbid

In 1946, Anatoly Timofeevich Kravchenko[1] and Mikhail Ignatyevich Sokolov[2] showed[3] that red blood cells on which polysaccharide antigens are adsorbed beforehand acquire the ability to agglutinate in the presence of homologous sera. This was the beginning of the development of *hemagglutination assay*, which are used when isolation of an axenic culture of the pathogen is difficult.

For this purpose, in a Petri dish, a two-day-old culture grown at 37°C / 38.6°F is mixed with 0.05 ml (one drop) of plague antibody monoclonal erythrocyte diagnosticum[4] by loop or as a suspension (10^9 BC/ml) and after 5-7 minutes using a concave mirror the reaction is counted. The results are evaluated according to the four-cross system:

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[1] Анатолий Тимофеевич Кравченко, ?-?; the Soviet (Russian) physician, virologist. First director of the D.I. Ivanovsky Scientific Research Institute of Virology (now the D.I. Ivanovsky Institute of Virology of the N. F. Gamaleya National Research Center of Epidemiology and Microbiology).

[2] Михаил Игнатьевич Соколов, 1908–1975; the Russian and Soviet physician.

[3] Кравченко А. Т., Соколов М. И. *Адсорбция специфических полисахаридов бактерий эритроцитами человека.* Журнал микробиологии. 1946; 12:10–16.

[4] Typically represents a suspension of formalinised ram erythrocytes (red blood cells) sensitized (i.e. loaded) with gamma-globulin plague agglutinating serum. Available in dry form or in 2.5% concentration. The kit also contains "control" erythrocytes, which are a suspension of formalised ram erythrocytes sensitised with horse serum gamma-globulin.

4+	++++	complete clarification of the fluid, agglomeration of all the red blood cells (erythrocyte)
3+	+++	incomplete clarification of the fluid, agglomeration of the bulk of the red blood cells
2+	++	incomplete clarification of the fluid, the bulk of the erythrocytes are not agglomerated
1+	+	single agglomerate particles
	–	absence of fluid clarification and erythrocyte agglomeration

Consider another method called the *co-agglutination* (Co-A) test, based[1] on Samuel Tertius Cowan's[2] discovery in 1939 of the ability of protein A of *Staphylococcus aureus* Cowan 1 cells to adsorb class G antibodies. The mechanism of this process is based on the fact that protein A, due to changes in the *spa* gene, has an affinity for the Fc fragment of immunoglobulins, so if such cells are treated with immune serum, they will be nonspecifically adsorbed by antibodies, which, in turn, will agglutinate with antigens. This method was not used in laboratory diagnosis of plague until 1972, when Nina Vasilievna Bozhko[3] isolated[4] **fraction V** (FV-antigen) from fractionless strains of *Y. pestis*[5]. If this fraction is then immunised in rabbits, IgG-anti-FV-antiserum can be obtained for use as part of both Co-A diagnosticum and in agglutination assay to

[1] Cowan S. T. *Classification of staphylococci by slide agglutination*. J Pat Bacteriol. 1938; 48(1):169–173; DOI: 10.1002/path.1700480117.

[2] 1905–1976; the British physician, bacteriologist.

[3] Нина Васильевна Божко, ?-?; the Soviet physician, bacteriologist.

[4] Божко Н. В. *Получение диагностических препаратов на основе антигенов чумного микроба, выделенных поверхностно-активными веществами*. Автореферат дис. … кандидата биологических наук. Ростов-на-Дону, 1972, 23 с.

[5] Preparation method: to 100 ml of bacterial suspension (10^9 BC/ml) in saline solution add sodium deoxycholate up to a final concentration of 0.005%, then shutelite (i.e. shake at a shaking mode of at least 50-60 vibrations per minute) at room temperature for 3 hours. Then centrifuge for half an hour (10000 rpm) at 4-8°C / 39.2-46.4°F. The supernatant is drained and 100 ml of salinel solution is added to the precipitate and the above steps are repeated four times, adding sodium deoxycholate sequentially to final concentrations of 0.02%, 0.08%, 0.32% and 1.28%. To the obtained supernatant (fifth fraction) 5 volumes of acetone cooled to minus 20°C / minus 4°F are added and left at minus 20°C / minus 4°F for 18-20 hours to form a precipitate. The precipitate is then dissolved in 50 ml distilled water and separated by dialysis for 4-5 days. The resulting fraction V is stored lyophilically dried or bottled at minus 20°C / minus 4°F.

identify fractionless strains of *Y. pestis* (since they do not synthesise capsular antigen) and to differentiate them from strains of *Y. pseudotuberculosis*.

Interestingly, unlike capsule antigen (fraction I), the synthesis of fraction V is independent of the cultivation temperature, and the protein in its composition with the highest immune activity is identified[1] as the *tal* gene-encoded enzyme transaldolase (Tal). A similar gene is responsible for the synthesis of transaldolase in *Y. pseudotuberculosis*, however, unlike *Y. pestis*, its variations occur in different strains of *Y. pseudotuberculosis*, but only in 6-9 nucleotides (about 1%). In case of a maximum variance in 9 nucleotides (*Y. pseudotuberculosis* strains IP31758), the difference in the amino acid sequence with that of *Y. pestis* will be only one amino acid (*Y. pestis* has alanine at position 57, while *Y. pseudotuberculosis* has threonine in this position). The reason why, antibodies to fraction V agglutinate only *Y. pestis* with identical transaldolases, is probably due to the structure of the LPS of *Y. pseudotuberculosis*, which shields antibody access to fraction V.

The *indirect* (IHA) or *passive* (PHA) *hemagglutination assay* is based on the ability of specific adhesion (hemagglutination) of red blood cells, sensitised with a specific antigen or antibody, to a material containing a specific antibody or antigen, respectively. The result of the reaction (assay) is observed in the form of glued erythrocytes falling to the bottom of the plate well.

The sample preparation is similar to ELISA, except that patients' blood usually contains relatively small amounts of capsular antigen, therefore, 150 μl of decontaminated material is heated for half an hour at 75°C / 167°F (instead of 15 minutes at 65°C / 149°F). The sample is then centrifuged for 2-3 minutes at 1000 rpm if a precipitate is formed.

To perform the assay, 50 μl of diluent is added to eight wells of a U or V- bottom shaped 96-well microtiter plate, followed by 50 μl of antigen-containing test material or antibody-containing test serum at a dilution of 1:10 into the first well and titrated to the seventh well. Then, 25

[1] Трухачёв А. Л., Копылов П. Х., и др. *Трансальдолаза – один из наиболее иммунологически активных компонентов препарата «фракция V» Yersinia pestis*, в сб. *IX Всероссийская научно-практическая конференция с международным участием «Молекулярная диагностика – 2017», под ред. В. И. Покровского*. Тамбов: ООО фирма «Юлис», 2017, 544 с.

μl of plague erythrocyte diagnosticum (antibody or antigenic[1]) is added to all eight wells, which is shaken beforehand. The plate is gently shaken to mix the components and incubated for 2 hours at room temperature or half an hour at 37°C / 38.6°F. The method scheme for antigen detection is shown in **Table 33.3** and for antibody detection in **Table 33.4**.

Staging is considered valid if there is no hemagglutination in the control, i.e. small rings or "buttons" are visible at the bottom of the well. In case of positive result, in the first or all wells, the red blood cells are located on the bottom in a uniform layer ("umbrellas"), and in case of negative result, there is no hemagglutination in all wells. A result is considered doubtful, when rings ("buttons") are visualized in a slightly larger diameter in the wells than in control (**Figure 33.4**). In some cases, this may be due to the fact that the test material contains an insignificant amount of specific antibodies, so in order to control specificity, the *indirect (passive) hemagglutination inhibition assays* (IHIA, PHIA) is performed in parallel with IHA (PHA). The essence of this reaction is the specific suppression (inhibition) of hemagglutination by adding specific serum or killed culture in the amount of up to 20 units to the dilutions of the tested material.

For determination of serum (SU) and antigenic (AU) units, 25 μl of diluent liquid is added to eight wells of the plate, then 25 μl of plague agglutinating serum (1:10000) or *Y. pestis* suspension (10^8 BC/ml) is titrated from the first to the seventh wells and 25 μl of plague erythrocyte diagnosticum (antibody or antigenic, respectively) is added to all eight wells. Incubated for 2 hours at room temperature or half an hour at 37°C / 38.6°F. The scheme of the method for determination of SU is given in **Table 33.5**, for determination of AU in **Table 33.6**.

The dilution that causes agglutination of red blood cells sensitised with a specific antigen (antibody) is taken as 1 SU (1 AU) and the dilution in the preceding well is taken as 2 SU (2 AU).

[1] Typically represents a suspension of formalinised ram erythrocytes (red blood cells) sensitized (i.e. loaded) with capsular antigen (Fraction I) of *Y. pestis*. Available in dry form or in 10% concentration. The kit also contains "control" erythrocytes, which are a suspension of formalised ram erythrocytes, but non sensitized.

Table 33.3. *IHA-Ag scheme*

Number of well / **Reagent**	**1**	**2**	**3**	**…**	**7**	**Diagnosticum control**
Diluent (10% normal rabbit serum[1]), µl	50	50	50	…	50	25
Test material, µl	50 $\overset{50}{\rightarrow}$	$\overset{50}{\rightarrow}$	$\overset{50}{\rightarrow}$	…	50 ↑	
Plague antibody monoclonal erythrocyte diagnosticum, µl	25	25	25	…	25	25
Incubation for 2 hours at room temperature or half an hour at 37°C / 38.6°F						

Table 33.4. *IHA-Ab scheme*

Number of well / **Reagent**	**1**	**2**	**3**	**…**	**7**	**Diagnosticum control**
Diluent (10% normal rabbit serum), µl	50	50	50	…	50	25
Test serum, µl	50 $\overset{50}{\rightarrow}$	$\overset{50}{\rightarrow}$	$\overset{50}{\rightarrow}$	…	50 ↑	
Plague antigenic erythrocyte diagnosticum, µl	25	25	25	…	25	25
Incubation for 2 hours at room temperature or half an hour at 37°C / 38.6°F						

[1] Traditionally, 1% normal rabbit serum has been used for hemagglutination assay with bacterial cultures (antigen-containing material) because the effect of antigens causing autoagglutination is eliminated during sample preparation. However, in the case of *Y. pestis*, sample preparation does not completely eliminate the effect of the autoagglutination factor (AF) and therefore either normal rabbit serum at higher concentrations or pre-incubation of samples in 1% rabbit anti-AF serum for one hour is necessary.

Table 33.5. *Scheme for determining SU*

Number of well / Reagent	1	2	3	…	7	Diagnosticum control
Diluent (1% normal rabbit serum), µl	25	25	25	…	25	25
Plague agglutinating serum (1:10000), µl	25 → 25	→ 25	→ 25	…	25 ↑	
Plague antigenic erythrocyte diagnosticum, µl	25	25	25	…	25	25
Final serum dilution	1:20000	1:40000	1:80000	…	1:1280000	
Incubation for 2 hours at room temperature or half an hour at 37°C / 38.6°F						

Table 33.6. *Scheme for determining AU*

Number of well / Reagent	1	2	3	…	7	Diagnosticum control
Diluent (10% normal rabbit serum), µl	25	25	25	…	25	25
Y. pestis suspension (10^8 BC/ml), µl	25 → 25	→ 25	→ 25	…	25 ↑	
Plague antibody monoclonal erythrocyte diagnosticum, µl	25	25	25	…	25	25
Final number of bacterial cells in the well	1250000	625000	312000	…	19531	
Incubation for 2 hours at room temperature or half an hour at 37°C / 38.6°F						

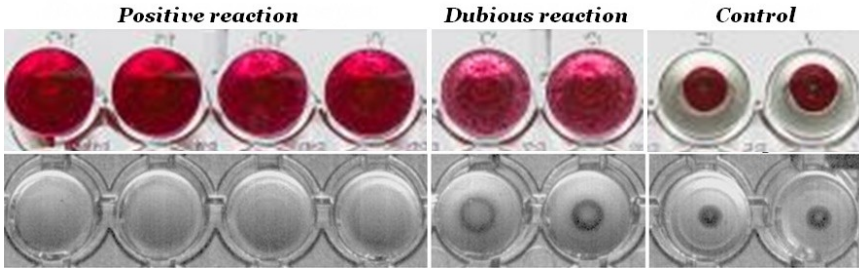


Figure 33.4. *Indirect (passive) hemagglutination assay (top row) and latex agglutination assay (bottom row)*

For IHIA (PHIA), 25 μl of diluent liquid is added into eight wells of the plate, then 25 μl of test material or test serum at a dilution of 1:10 is titrated from the first to the seventh wells and 25 μl of specific immune serum with an activity of 8-16 SU or a suspension of killed culture with an activity of 8-16 AU is added into all eight wells. Next, the plate is incubated for half an hour at 37°C / 38.6°F and 25 μl of plague erythrocyte diagnosticum (antibody or antigenic, respectively) is added to all wells and thenncubated for 2 hours at room temperature or half an hour at 37°C / 38.6°F. The method scheme for antigen detection is shown in **Table 33.7** and for antibody detection in **Table 33.8**.

The results are counted as for IHA (PHA). If the activity of the test material (serum) is absent or significantly reduced (by 4-5 wells) in relation to IHA (PHA), the result of IHA (PHA) is considered specific.

Similarly, *latex agglutination assay* is carried out where polystyrene or polyacrolein latex particles with a diameter of 1.2-1.8 μm are used instead of red blood cells, and phosphate-buffered solution is used as a diluting liquid. The method schemes and recording of results (**Figure 33.4**) are similar for both IHA (PHA) and IHIA (PHIA).

In case of studying the objects of external environment in the diluting liquid add additionally protein sensitizer in dilution 1:100[1] and twin-80 in concentration 1:80000.

[1] Preparation method: to 25 ml of chicken egg white add 75 ml of distilled water and 1.25 g of sodium bicarbonate, then stir for 10-15 minutes, heat for 40-60 minutes at 56°C / 132.8°F and filter (see RU 2198407 C2, 10.02.2003).

Table 33.7. *IHIA-Ag scheme*

Number of well / **Reagent**	**1**	**2**	**3**	**…**	**7**	**Diagnosticum control**
Diluent (10% normal rabbit serum), μl	25	25	25	…	25	25
Test material at a dilution of 1:10, μl	25 →	25 →	25 →	…	25 ↑	
Specific immune serum with an activity of 8–16 SU	25	25	25	…	25	25
Incubation for half an hour at 37°C / 38.6°F						
Plague antibody monoclonal erythrocyte diagnosticum, μl	25	25	25	…	25	25
Incubation for 2 hours at room temperature or half an hour at 37°C / 38.6°F						

Table 33.8. *IHIA-Ab scheme*

Number of well / **Reagent**	**1**	**2**	**3**	**…**	**7**	**Diagnosticum control**
Diluent (1% normal rabbit serum), μl	25	25	25	…	25	25
Test serum at a dilution of 1:10, μl	25 →	25 →	25 →	…	25 ↑	
Suspension of killed culture with an activity of 8–16 AU	25	25	25	…	25	25
Incubation for half an hour at 37°C / 38.6°F						
Plague antigenic erythrocyte diagnosticum, μl	25	25	25	…	25	25
Incubation for 2 hours at room temperature or half an hour at 37°C / 38.6°F						

Let's return to hemagglutination assays. The blood samples tested may contain antibodies and antigens that may influence the results of the assays described earlier. To exclude the effect of antibodies or antigens, the neutralization tests are used: *neutralization antigen assay* (NAg) and *neutralization antibody assay* (NAb), respectively. The essence of these reactions is that the added antibodies with an activity 2 SU (or antigens with an activity 2 AU) neutralise the effect of antigens (antibodies) present in the sample.

To perform the assay, 25 μl of diluent liquid is added to eight wells of the plate, after that 25 μl of test material or test serum at a dilution of 1:10 is titrated from the first to the seventh well and 25 μl of specific immune serum with an activity of 2 SU or killed culture suspension with an activity of 2 AU is added to all eight wells. Next, the plate is incubated for half an hour at 37°C / 38.6°F and 25 μl of plague erythrocyte diagnosticum (antibody or antigenic, respectively) is added to all wells. Incubated for 2 hours at room temperature or half an hour at 37°C / 38.6°F. It can be seen that the technique described is very similar to that for staging IHIA (PHIA). The method scheme for antigen detection is shown in **Table 33.9** and for antibody detection in **Table 33.10**.

If the test material (serum) contains antigens (antibodies), they will neutralise antibodies with an activity of 2 SU (antigens with an activity of 2 AU) and will no longer be able to agglutinate the antigens (antibodies) of the diagnosticum. The result of such a reaction will be negative ("button"). In the reverse situation, when the test material (serum) does not contain antigens (antibodies), the added antibodies with an activity 2 SU (antigens with an activity 2 AU) will agglutinate the antigens (antibodies) of the diagnosticum and the reaction result will be positive ("umbrella"). Thus, for the NAg and NAb assays, a negative result in the well ("button") will be considered positive and, accordingly, a reaction in which agglutination of the control has occurred will be considered valid.

To characterize the epizootic process, it is important to determine the class of immunoglobulins in the blood serum or washes taken according to the method described in Chapter 29, for which serum is treated with an equal volume of 2-mercaptoethanol, which leads to the destruction of disulfide bonds in IgM and, as a consequence, its dissociation into 6-8

Table 33.9. *NAg scheme*

Number of well / Reagent	1	2	3	…	7	Diagnosticum control
Diluent (1% normal rabbit serum), μl	25	25	25	…	25	25
Test serum at a dilution of 1:10, μl	25 $\overset{25}{\rightarrow}$	$\overset{25}{\rightarrow}$	$\overset{25}{\rightarrow}$	…	25 ↑	
Suspension of killed culture with an activity of 2 AU	25	25	25	…	25	25
Incubation for half an hour at 37°C / 38.6°F						
Plague antibody monoclonal erythrocyte diagnosticum, μl	25	25	25	…	25	25
Incubation for 2 hours at room temperature or half an hour at 37°C / 38.6°F						

Table 33.10. *NAb scheme*

Number of well / Reagent	1	2	3	…	7	Diagnosticum control
Diluent (10% normal rabbit serum), μl	25	25	25	…	25	25
Test material at a dilution of 1:10, μl	25 $\overset{25}{\rightarrow}$	$\overset{25}{\rightarrow}$	$\overset{25}{\rightarrow}$	…	25 ↑	
Specific immune serum with an activity of 2 SU	25	25	25	…	25	25
Incubation for half an hour at 37°C / 38.6°F						
Plague antigenic erythrocyte diagnosticum, μl	25	25	25	…	25	25
Incubation for 2 hours at room temperature or half an hour at 37°C / 38.6°F						

monomers that are not of antigenic activity (with IgG being resistant to the substance). Further, hemagglutination assays with serum treated with 2-mercaptoethanol and serum not treated with it (paired sera) allow to determine the total amount of antibodies and to specify their class. This is important primarily because, as we remember, in the first days of the infectious process in the blood IgM and only later IgG are detected, so the detection of IgM in the tested serum indicates a short time elapsed since the day of contact of the animal with *Y. pestis*, and gives grounds for its isolation by bacteriological method[1].

34 STEVEN

Dedicated to Steven, a noble Mouse, who sacrificed himself so that the author would learn the rules of working with laboratory animals

An *animal bioassay*, i.e. infection of a laboratory (bioassay) animal, is an essential part of the laboratory diagnosis of particularly dangerous bacterial infections, without which it is sometimes impossible to isolate the causative agent and make a correct diagnosis. A good example of this case is meningeal plague of the soldier discussed in Chapter 20.

Laboratory animals are kept in *vivarium* (or, in CIS countries, experimental-biological clinics), which are located in separate buildings or in isolated rooms of the laboratory building, have a separate entrance and an autonomous ventilation system. If the vivarium is not located on the ground floor, a separate lift or staircase is provided for it.

As a laboratory, vivarium premises can be divided into two zones, which, for example, in CIS countries are called "clean" and "dirty"[2]

[1] If IgG is detected, isolation of a culture of *Y. pestis* is unlikely.

[2] Note the clear difference in terminology: "contagious" zone in the case of the laboratory, but "dirty" zone in the case of the vivarium!

zones. However, in contrast to the laboratory, this division indicates only the "clean" and "dirty" nature of the work carried out. Thus, in the "clean" zone there are rooms for reception, quarantine and adaptation of clean animals newly arrived from the kennel, storage rooms for clean equipment (cages, drinkers, utensils) and feed, manipulation rooms (e.g. for immunisation), operating room and diagnostic veterinary office, whereas in the "dirty" zone there are isolation rooms for keeping suspicious for infection (or infected) animals, a refrigerated room for storing animal carcasses and other waste, a room for washing and disinfection of equipment, and a room for cleaning and disinfection of animals. In this case, the last rooms are located on the border of the zones to ensure flow. Most often, the flow delimitation is provided by a *two-corridor layout*, where the vivarium rooms are located between two corridors, but a *single-corridor layout* is also possible, where the "clean" and "dirty" zones are located at the opposite ends of the corridor, and its use is regulated by schedule.

Premises where animals are kept are equipped with supply and exhaust ventilation and cages, microclimate control, feeding ration and cleaning schedule are ensured, i.e. conditions ensuring their comfortable keeping are created (**Table 34.1**).

Direct work with animals and samples of zoological and parasitological material is carried out in the *unit for work with infected animals* ("animal laboratory"), which is located in the "dirty" zone of the vivarium or "contagious" zone of the laboratory, separated from other rooms of the zone by a pre-box (the doors of the unit are additionally equipped with a threshold of at least 30 cm).

Table 34.1. *Basic parameters for laboratory animal housing*

Animal species	**Parameter**			
	Temperature, °C / °F	**Relative humidity, %**	**Cell area, cm²**	**Cage height, cm**
Mouse	18–26 / 64.4–78.8	30–70	31–76	13
Rat			110–390	18
Hamster			65–105	16
Guinea pig			390–650	18
Rabbit	16–22 / 60.8–71.6		1500–4000	36

A unit includes rooms for receiving animals from the vivarium and animals delivered from foci in the form of carcasses (in the absence of a vivarium, live animals are also delivered here), rooms for infecting, housing and autopsy of animals, as well as rooms for desinfection of equipment. It is easy to guess that, as in the case of the laboratory, the unit is subdivided into **Animal Biosafety levels** (ABSLs). Work with animals infected (suspicious of being infected) with pathogens of particularly dangerous infections is performed in a unit **not lower than level 3** (ABSL-3), observing the principle of pairing, wearing a type I suit, completed with high boots, rubberised apron and armbands. The rooms of the unit at this level are equipped with open-type BSCs for inoculation, allowing operators to sit opposite each other, and with laminar flow stations for animal care and cage cleaning. Infected animals are kept in rooms equipped with ventilation systems with HEPA filters, more often in the form of racks with cages connected to the general ventilation system of the unit.

White mice (*Mus albus officinarum*) or guinea pigs (*Cavia porcellus*) are most often used for work with *Y. pestis*, the latter being the most sensitive to this infection and therefore more preferable. However, the selective virulence of *Y. pestis* should be borne in mind when choosing an animal. For example, Altai strains are weakly virulent to guinea pigs, whereas they are highly virulent to mice. The same principle is applied to some fractionless strains, which, moreover, while being highly virulent to great gerbils (*Rhombomys opimus*), are weakly virulent to Libyan jirds (*Meriones libycus*) and Tamarisk jirds (*Meriones tamariscinus*).

In this regard the experience[1] of immunisation of a Gerbillinae, which died 252 days after inoculation, is particularly illustrative, with a virulent culture isolated from its lungs and a weakly virulent culture isolated from a perirenal abscess. The reasons for this phenomenon are not fully studied, although some atypicality of the pCad plasmid in Caucasian strains, for example, is among the possibilities. The similarity of some of its genes with the genes of a similar plasmid of *Y. enterocolitica* may indirectly indicate the ability to modify the process of interaction with the animal organism, bringing it closer to that of enteropathogenic *Yersinia* spp.

[1] Хрусцелевский В. П., Хрусцелевская Н. М. *О роли штаммов чумного микроба разной вирулентности в энзоотии чумы*. Проблемы особо опасных инфекций. 1968; 4:186–193.

Animals should enter the unit prepared for the respective manipulation: weighed, with shaved body parts if necessary.

Inoculation is performed in the BSC over a tray with a tissue moistened with disinfectant solution. To reduce the risk of accidents (needle skin damage), the work is carried out by two specialists: one fixes (holds) an animal and the other infects it. A container with disinfectant solution is placed to the right of the infecting person in the BSC, and the necessary equipment (tray with syringe, glass with tweezers, swabs) is placed to the left of the infecting person in the BSC.

Correct fixation of an animal is important during inoculation. An animal is taken out of the cage by hand or with a forceps, depending on the species. For example, a mouse is taken out by the tail, quickly placing it with its paws on the surface for subsequent fixation. It is not recommended to hold the mouse by the tail (with its head down) for a long time, as it may pull up and bite. The rat is usually picked up from the back, with the front paws secured with the hand. A guinea pig is removed in a similar way, but carefully, as it may die because of stress. A hamster is removed with a forceps by pinching its withers - even then it may twist around several times.



Figure 34.1. *Fixation of guinea pig (top) and white mouse (bottom), which poses the risk of puncture*

Special devices are used for the fixation of medium-sized animals, while small animals are more often fixed by hand. *Fixation of a mouse* is carried out by grasping it with one hand by the withers, with a pinching movement when the ears of the animal are pressed with the

thumb and forefinger. The other hand continues to hold the tail. The mouse is stretched slightly during fixation. *Fixation of the guinea pig* is done by grasping it with one hand behind the back (under the chest), with the thumb and forefinger covering the neck and the other fingers fixing the limbs. The other hand holds the back of the body, immobilising the limbs. In practice, these rules are usually slightly deviated from, which creates a risk of accident when inoculation is performed (**Figure 34.1**).

To administer fluid through the probe, a mouse is held by the withers with one hand (as in conventional fixation) and the tail is pressed with the little finger (**Figure 34.2**). A guinea pigis held head up with its back pressed against the palm of the hand.



Figure 34.2. *Mouse fixation for insertion of the transesophageal probe*

Inoculation by skin rubbing (Austrian method). This method is used when the received material contains a lot of foreign microflora (rotting corpse, soil samples). To infect an animal, a part of the body is shaved (1 x 2 cm for mice, 2 x 3 cm for guinea pigs) and moistened with saline solution. It is strictly forbidden to use disinfectant solution, as it may lead to both death of the microorganism and unwanted stress for the animal.

In this type of inoculation, it is extremely important to fix the animal strictly horizontally to eliminate the risk of droplets of material falling (rolling) and splashing. Depending on the consistency of the material, it is applied to the pre-scarified skin with a pipette, tweezers or scalpel and then gently rubbed in smooth movements with the blunt side of the scalpel under the cover of a Petri dish. If the material to be applied is a piece of an organ, either its suspension in saline solution is applied to the skin or several impressions are made. The animal is then placed back to the cage and the used instrumentation is dumped into a container with a disinfectant solution.



Figure 34.3. *Subcutaneous infection of a mouse*

Subcutaneous inoculation is most often carried out in the groin area. For this purpose, the material is drawn into a syringe in advance. To remove trapped air bubbles, the piston is carefully moved back and forth to move them to the area of the needle, after which a small sterile paper bag with cotton wool or gauze is pricked onto the needle with tweezers, into which the air is expelled. It is forbidden to tap the barrel or "bleed" air into the container with the disinfectant solution. Immediately before infection, when the mouse is already fixed by the partner, the paper bag is removed with tweezers and lowered into the container with the disinfectant solution. The same tweezers are used to take a tampon (gauze swab) moistened with saline solution and wipe the injection site, then place it on the partner's hand so that no unnecessary movements are made after infection. Pinch the skin of the animal in the groin area (in the projection of the lymph node) with tweezers and pull away (lift upwards) until a triangular fold is formed (**Figure 34.3**).

The syringe with the material is held horizontally and, without changing position, the needle is inserted into the centre of the fold, after which the syringe plunger is pressed, injecting the contents. Next, a tampon is taken with tweezers, the injection site is covered and the nee-

dle is pulled out of the skin. The animal is placed back to the cage, and the syringe is filled with disinfectant solution and put into a container.

Intraperitoneal inoculation is used to accelerate the infectious process, for which the animal, held with two hands, is moved head downwards so that the abdominal organs are displaced to the diaphragm. Having treated the skin with a tampon moistened with saline solution[1], the skin of the animal is pinched with tweezers on the right lower side of the abdomen, indenting approximately 0.5-0.8 cm from the white line of the abdomen, and slightly pulled away (lifted upwards) until a triangular fold is formed. The syringe with the material is held horizontally and, without changing the position, the needle is inserted into the base of the fold, while feeling the puncture of the skin and peritoneum (the sense of the needle falling into the void). Then the syringe plunger is pressed, injecting the contents, after which a tampon is taken with the tweezers, covering the injection site and the needle is pulled out of the skin. The animal is placed back to the cage, and the syringe is filled with disinfectant solution and put into a container.

These three methods are used in the laboratory diagnosis of plague. Subcutaneous administration is most commonly used, whereas intraperitoneal administration is used to accelerate death, and infection by skin rubbing is used when significant amounts of foreign microflora are suspected. Subcutaneous administration is 0.2 ml for mice and 0.5 ml for guinea pigs, while intraperitoneal administration is 0.3–0.5 ml for mice and 0.5–1 ml



Figure 34.4. *Intravenous infection of a mouse*

[1] Most manuals indicate the need to treat the skin with an ethanol solution, but in practice it is more appropriate to use saline solution, as the animals are clean and the risk of developing negative consequences due to neglect of aseptic rules is minimal, whereas the risk of developing a negative reaction from the animal to the smell of ethanol is very high, which may lead to its anxiety and, as a consequence, accident.

for guinea pigs. If low levels of the pathogen are suspected, the infective dose is increased to 0.5–1 ml for mice and 1.5–2 ml for guinea pigs subcutaneously. As a rule, animals die within 5–7 days. If this does not happen, they are euthanised humanely (most often placed in a chamber with CO_2 or chloroform).

Next, let's consider the inoculation methods used in research practice.

Intravenous inoculation. For this method the tail vein (mouse), the auricular vein (guinea pig), the jugular vein (cat, dog, guinea pig), the femoral vein (cat, dog) or the marginal auricular vein (rabbit) are used. The injection site is carefully treated with a tampon moistened with saline solution to cause the vein to swell, and in the case of the caudal vein, the animal is warmed under a lamp for some time.

Fix the animal with a special device, or, in the case of a mouse, cover it with a crucible leaving outside only the tail , which is slightly stretched (**Figure 34.4**). This method requires a very precise entry into the vein, and it is hard for anyone in the training phase to get it right the first time. If the material is injected past the vein, the tail area will turn white - in this situation, pull the syringe needle slightly towards you and re-enter the vein.

Intradermal inoculation. For this method, the skin is shaved beforehand and the injection site is wiped with a tampon moistened with saline solution. The needle with an outward bevel is inserted at an angle of 10° into the thickness of the superficial layer of the skin for a few millimetres. The material is injected until a bump appears on the skin at the injection site.

Intraocular inoculation (into the conjunctiva of the eye). Firmly fix the animal's head in such a way that the eye into which the material is to be carried out is positioned strictly horizontally. Using tweezers, gently pull back the lower eyelid at the inner corner of the eye and apply a small drop of the material to the conjunctiva of the eye at a distance of not more than one centimetre above the level of the eye. The eyelid is returned to its place and the excess material is blotted off with a tampon and then dipped in a disinfectant solution.

Needle sizes and maximum allowable fluid volumes are given in **Table 34.2**.

Table 34.2. *Needle sizes and maximum allowable fluid volumes depending on the species of laboratory animal*

Animal species	Method of injection		
	intravenously	**intraperitoneally**	**subcutaneously**
mouse	<25 G 0.2–0.5 ml	<23 G 0.5 ml	<23 G 0.5 ml
rat	<23 G 0.5–2.5 ml	<23 G 5 ml	<23 G 5 ml
hamster	<25 G 1 ml	<23 G 3 ml	<23 G 1–2 ml
guinea pig	<23 G 2.5 ml	<21 G 2.5 ml	<23 G 5 ml
rabbit	<23 G до 30 ml	<20 G 30 ml	<20 G 30 ml

Fallen and euthanised animals are subject to post-mortem examination (autopsy), which is performed in the appropriate room of the unit – autopsy (sectional) room.

For dissection prepare a substrate (more often wooden), place there a napkin abundantly moistened with disinfectant solution, cotton wools (3 pieces), needles for fixation of the animal, a container (glass) with 97% ethanol solution, in which scissors and anatomical tweezers are placed (surgical tweezers may tear delicate organ tissues), as well as a burner, a forceps and a container with disinfectant solution. Before starting work, special attention should be paid to the scissors, which should be sharp and have rounded ends, because the quality of work depends on it.

The process of dissection (post-mortem examination) of a laboratory animal (mouse) is shown on the scheme (**Figure 34.5**), supplemented with photographs for clarity, and is reduced to the following stages (the number of the stage coincides with the number on the scheme and photographs):

1. with the help of a forceps, the mouse is placed on a napkin moistened with disinfectant solution on a substrate with the abdomen upwards, after which the limbs are fixed by slightly stretching the spine. Three cotton wools moistened with disinfectant solution are prepared: one, using tweezers, is used to wipe the site of dissection (abdomen of the animal); the second, folded in the form of a boat, is used to cover the opening of the urethra and to place the extracted organs in it; the third is used to clean the instruments (tweezers and scissors) from blood and hair of the animal;
2. the instruments are treated by flambing (dipped in a container with 97% ethanol solution and burnt in a burner flame; to avoid burning of ethanol in the container it is necessary to place a burner at a distance), then in the area of pubic articulation the skin is lifted and an incision is made with scissors. Further, the ends of the scissors in closed form are inserted into the space between the skin and the abdominal wall along the projection of the midline to the sternum and withdrawn, carefully opening and closing them so that the skin is separated from the abdominal wall;
3. treat the scissors with flambing and cut the skin with them according to the type of an envelope (blue line) or apron (red line), after which it is folded back. At this stage, the condition of the lymph nodes is assessed;
4. treat the scissors with flambing and cut the abdominal wall, repeating the operations of steps 2 and 3.
5. assess the condition of internal organs. In the presence of general toxic lesions of the abdominal cavity organs (5*), further evaluation of the organs is not possible;
6. (if necessary) treat the scissors with flambing and cut the chest (by cutting through the cervical vertebrae with scissors, it is possible to take brain samples with a loop along the spinal canal).

Post-mortem examination of guinea pigs is carried out in a similar manner.

In animals killed from plague by percutaneous or subcutaneous inoculation (as well as animals killed from bubonic plague as a result of natural infection), the site of injection of material (at the site of infection) is full of blood, serous impregnation of tissues, suppuration and necrosis are possible. Mucous swelling of the subcutaneous tissue is possible,

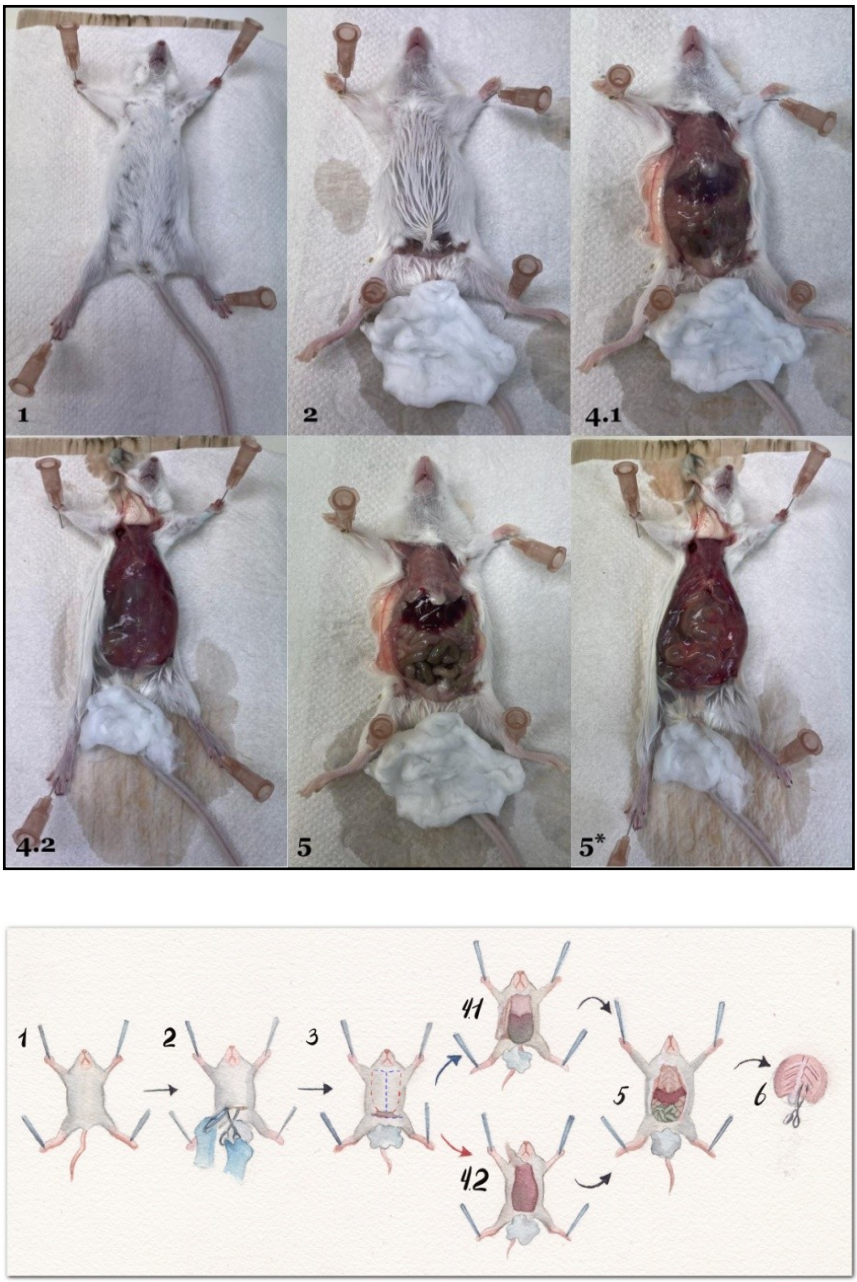


Figure 34.5. *The process of post-mortem examination of a laboratory animal (mouse)*

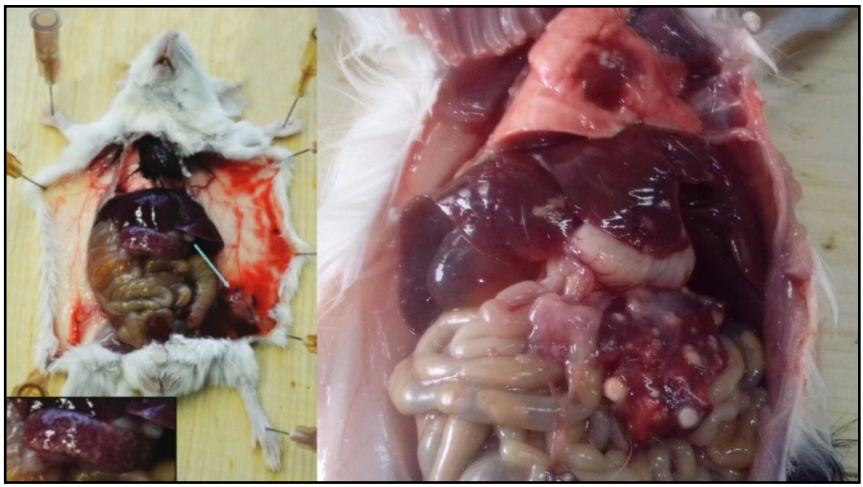


Figure 34.6. *Pathological changes in the internal organs of a mouse (left) and guinea pig (right, infection with a fractionless strain). The arrow marks a lymph node, the spleen in the lower square. Note the barely discernible nodules on the mouse spleen and the huge nodules on the guinea pig spleen (image on the left by Ваеайская А. С., 2021, on the right courtesy of Dr. Tatiana E. Arsenyeva)*

more often localised only at the site of subcutaneous injection. The inguinal lymph node (rarely both) is enlarged, hyperaemic, dense or softened, impregnated with gelatinous serous haemorrhagic fluid. The ipsilateral (i.e., on the same side) axillary lymph node is rarely affected (**Figure 7.12**). In intraperitoneal infection, mucopurulent exudate, enlargement and thickening of mesenteric and para-aortic lymph nodes are found in the abdominal cavity. In intraocular infection, cervical lymph nodes are primarily affected.

In all modes of inoculation, greyish-white nodules are found in the spleen, liver and lungs, which can vary in size from barely visible to large. The number of nodules is also variable (**Figure 34.6**). In addition, the liver may show areas of degeneration of yellowish colour (as it is said in CIS countries: clay liver). The organs are enlarged, with areas of necrosis in some places, and the lungs may have foci of haemorrhage with thickening. The heart may be enlarged and its vessels sharply injected. The same with the vessels of the abdominal cavity (especially the mesentery). It is extremely rare to find a soft exudate in the abdominal cavity.

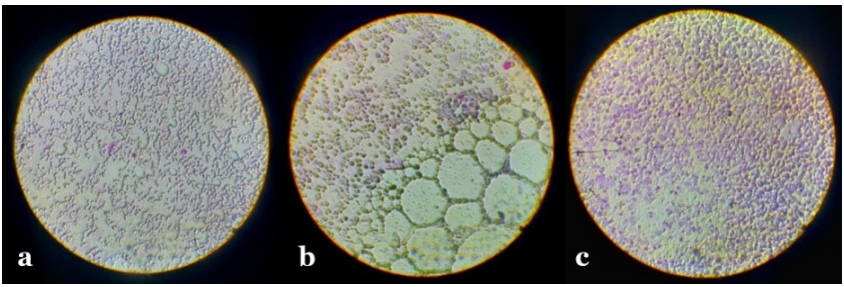


Figure 34.7. *Organs print on glass slide of the liver (a), spleen (b), and lymph node (c). Compare them with the histological sections of these organs given in Chapters 7, 8, 11-14*

Spleen, liver, heart (blood), lungs, brain and bone marrow are used for plating on dense nutrient media (agar). For this purpose, the tweezers and scissors are flambed and cooled, then the organ (or its fragment) is placed on the nutrient medium. In CIS countries, not only plating on dense nutrient media, but also organ prints on glass slide (for the purpose of subsequent staining and microscopy) are performed. A fragment of the organ held with tweezers is cut off with scissors and a few prints (impressions) are made on agar or glass with the fresh cut. Two or three impressions are usually made on agar divided into sectors, followed by dispersal. *One liver print, two lung prints, three spleen prints, four lymph node prints, and blood prints (one strip)* are made on the glass slide, after which the glasses are placed in fixative liquid for half an hour and stained[1] (**Figure 34.7**).

[1] In addition to the staining methods discussed in Chapter 30, a staining method proposed by the Russian and Soviet physician Sergei Nikolayevich Muromtsev (Сергей Николаевич Муромцев; 1898-1960) can be used. Developed as part of the diagnosis of rabies in animals (staining of brain swabs), according to Nikolai I. Rozanov, it is convenient for the examination of organ swabs from bioassay animals in bacterial infections because it allows clear visualisation of bipolarity and capsules. For this purpose, smears are fixed for half an hour in an ethanol solution with formalin at a concentration of 8:2, after which they are stained with pre-prepared dye through filter paper for 30 seconds and washed abundantly with distilled water. The background of the preparation is stained in pink colour, and tissues and microbial cells in shades of blue. To prepare the dye, two solutions are mixed (solution 1: to 20 ml of 96% ethanol solution add 0.15 g of four-water fuchsine (basic fuchsine) and 10 g of crystalline carbolic acid; solution 2: to 200 ml of distilled water add 2.5 g of methylene blue), after which the mixture is filtered through a double paper filter.



Figure 34.8. *Post-mortem examination process in abbreviated form (left to right)*

If direct plating of the material does not result in the growth of *Y. pestis* culture and the bioassay animal has died, a suspension is made from its organs, which is plated on nutrient media and injected into the second animal. Post-mortem examination of the second animal is carried out similarly to the first animal.

After the examination is completed, cotton discs are placed on the animal using tweezers, the limb clips are removed and the corners of the tissue are wrapped in an envelope. At this important moment, it is

not unreasonable to pause for a moment to *mentally thank the animal who died to save human lives*. The tissue-wrapped animal is then placed in a container with tweezers (forceps) for subsequent disinfection and incineration in a crematorium. A cloth soaked in disinfectant solution is used to treat the substrate (both sides) and the surface of the table on which it was placed.

It should be noted that in an outbreak, especially when it is necessary to dissect a sufficient number of animals, an abbreviated method of dissection is used: lay the mouse on its right side, cut the skin and abdominal wall in an arc and extract the spleen for evaluation of its condition and further examination (**Figure 34.8**).

To accelerate the diagnosis, several mice and guinea pigs are infected at the same time, one animal is killed sequentially on the first, second and third days, and post-mortem examination is performed. Platings are made on two dense nutrient media to test one of them with bacteriophage. Special attention is paid to cultures from lymph nodes.

In addition, the diagnosis can be accelerated by decreasing the resistance of animals to *Y. pestis*, for which an emulsion of chicken egg yolk (one yolk diluted in 8 ml of saline solution) administered simultaneously with the test material at the rate of 0.5 ml for a mouse and 1 ml for a guinea pig, as well as hydrocortisone (5 mg per 0.5 ml of saline solution) administered subcutaneously in the thigh area is used.

The diagnosis can also be accelerated by the *antigenuria method*, in which the capsular antigen content in the urine of the animal is determined in vivo (approximately on the second or third day) using the haemagglutination reactions described in the previous chapter. For this purpose, carrots and cabbage are added to the animal's diet, and two sheets of filter paper are placed at the bottom of the cage and changed daily. Urine-stained sections are excised and poured into 4-6 ml of phosphate buffer (0.1 mol/L) with 2% formalin solution. After 24 hours, the contents are thickened and the supernatant is submitted for examination.

35 ANTIBIOTIC SENSITIVITY

Determination of sensitivity to antibiotics of the isolated culture is an important stage of laboratory diagnostics, because it allows to prescribe rational etiotropic therapy and, in case of threatening epidemic situation, to be ready for treatment and prophylaxis. The existence of antibiotic-resistant *Y. pestis* strains, about which much has been said earlier, will make this stage particularly important.

In the early 1960s of the last century Alfred W. Bauer[1], William M. M. Kirby[2], John Charles Sherris[3] and Marvin Turck[4] developed[5] a method for determining the sensitivity (susceptibility) of bacteria to antibiotics based on the ability of antibiotics to diffuse from a paper carrier (disc) into a solid nutrient medium (**Figure 35.1**), inhibiting the growth of the plating bacterial culture. Although similar methods have been known since the discovery of penicillin (**Figure 35.2**), it is this one, called the *disk diffusion test* or *Kirby-Bauer test* (KB test), that is the first standardised by the World Health Organization and the most widely used because of its simplicity and flexibility (the possibility of testing a wide range of antibiotics, in any combination).

The essence of the method is the application of discs with antibiotics on agar, where the test culture was previously plated in a "bacterial lawn". For this purpose, pre-autoclaved and cooled to 48-50°C / 118.4-122°F nutrient agar is poured 25 ml into 100 mm diameter Petri dishes or 20 ml into 90 mm diameter dishes, so that the thickness of the agar is approximately 4 mm, and then left to set at room temperature. Once dried, the dishes are recommended to be used immediately or within a

[1] 1926–1998; the German and American physician, bacteriologist. He was a Nazi soldier during World War II, after which, after graduating from an institute in Germany, he moved to the United States for postgraduate studies.

[2] 1914–1997; the American physician, bacteriologist.

[3] 1921–2021; the British and American physician, bacteriologist.

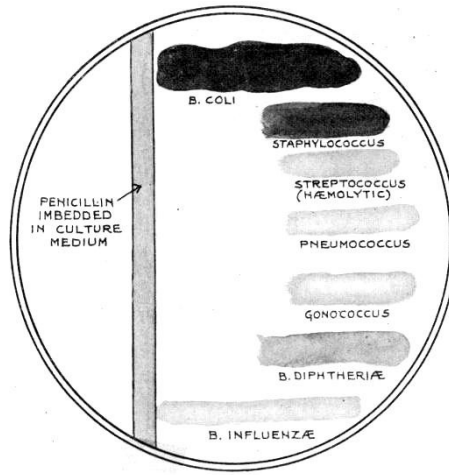
[4] b. 1934; the American physician, infectionist and bacteriologist. Author of books on urinary tract infections and antibiotic resistance in Gram-negative bacteria.

[5] Bauer A. W., Kirby W. M. M., Sherris J. C., Turck M. *Antibiotic susceptibility testing by a standardized single disk method*. Am J Clin Pathol. 1966; 36:493–496.



▲ ***Figure 35.1.*** *Schematic diagram of the process of antibiotic diffusion from the disc into the thickness of the nutrient medium*

▼ ***Figure 35.2.*** *Drawing of the Petri dish in which Alexander Fleming tested the antibiotic properties of the penicillin he discovered (by Fleming A., 1929; published under licence from the World Health Organization)*



day, provided they are stored at 4-8°C / 39.2-46.4°F. Immediately before use, they are dried for 15 minutes at 37°C / 98.6°F to avoid condensation formation.

The medium most commonly used is Mueller–Hinton agar, developed[1] in 1941 by John Howard Mueller[2] and Jane Hinton[3] and accepted

[1] Mueller J. H., Hinton J. *A Protein-Free Medium for Primary Isolation of the Gonococcus and Meningococcus*. Experimental Biology and Medicine. 1941; 48(1): 330–333; DOI: 10.3181/00379727-48-13311.

[2] 1891–1954; the American biochemist, bacteriologist. Fellow of the US National Academy of Sciences (1945). Author of books on bacterial metabolism,

[3] 1919–2003; the American veterinarian (one of the first two African-American women to gain the degree of Doctor of Veterinary Medicine). At the time of medium development, she was a laboratory technician at Harvard University.

as the "gold standard" for this method. Hottinger agar (1.2-1.4 g/litre amine nitrogen, pH 7.0-7.4) and Givental'-Ved'mina's agar (pH 7.2-7.6) are also used. The latter was developed[1] in 1980 by Naum Iosifovich Givental'[2] and Elena Alekseevna Ved'mina[3] (with colleagues). The use of magnesium oxalate (MOX) agar has been proposed to investigate sensitivity to T3SS inhibitors (e.g. imidocarb dipropionate).

Antibiotic discs (commercially) are stored at minus 18°C / minus 0.4°F (those used in the work are usually stored at 4-8°C / 39.2-46.4°F). One hour before work, vials with discs are taken out of the refrigerator to prevent moisture due to abrupt temperature change during opening. A suspension of 24-hour culture of *Y. pestis* in 0.85% isotonic sodium chloride solution (5 x 10^8 BC/ml) of 2 ml is applied to agar and spread evenly with a spatula. After 10-15 minutes, when the agar has dried, use tweezers or an automatic dispenser (**Figure 35.3**) to apply antibiotic discs at least 15-20 mm between the discs and the edges of the cup (usually no more than 6 discs per 100 mm cup), pressing the discs lightly against the agar. The dish with discs as well as the plated dish without discs (culture growth control) are incubated at 28°C / 82.4°F. Preliminary results are recorded after 24 hours and final results after 48 hours, taking into account the characteristic growth of the culture on the control dish.

The results are recorded by measuring the diameter of the inhibition zones (**Figure 35.4**) and comparing them with the boundary values given in **Table 35.1**. To do this, the cups are placed on a dark surface upside down, directing the light beam at an angle of 45°.

The appearance of small colonies in the zone of inhibition may indicate both the presence of foreign microflora (contamination) and heterogeneity of the tested culture (presence of sensitive and insensitive cells in the colony). In this situation, the study is repeated with an axenic culture (purity control) and with colonies grown in the zone of inhibition.

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[1] Гивенталь Н. И., Ведьмина Е. А., и др. *Об унификации методов определения чувствительности микроорганизмов к антибиотикам. Полуколичественная и количественная оценка результатов диффузионного теста.* Антибиотики. 1980; 6:420–424.

[2] Наум Иосифович Гивенталь, 1923–?; the Soviet physician, bacteriologist.

[3] Елена Алексеевна Ведьмина, ?-?; the Soviet physician, bacteriologist.

It should be added that before using a new batch of discs, they should be tested with reference strains [1] (**Table 35.2**) on Mueller–Hinton agar, as discs giving smaller or larger inhibition zone diameters than the reference strains should be discarded (their use may lead to invalid results). A particularly dangerous situation occurs when a defective disc gives a large zone of inhibition, as this runs the risk of assigning a resistant strain to a susceptible strain.

Of all the currently available methods for determining the sensitivity of bacteria to antibiotics in the case of pathogens of particularly dangerous bacterial infections, apart from the disc diffusion method, only the *method of serial dilutions* (MSD) is used, which

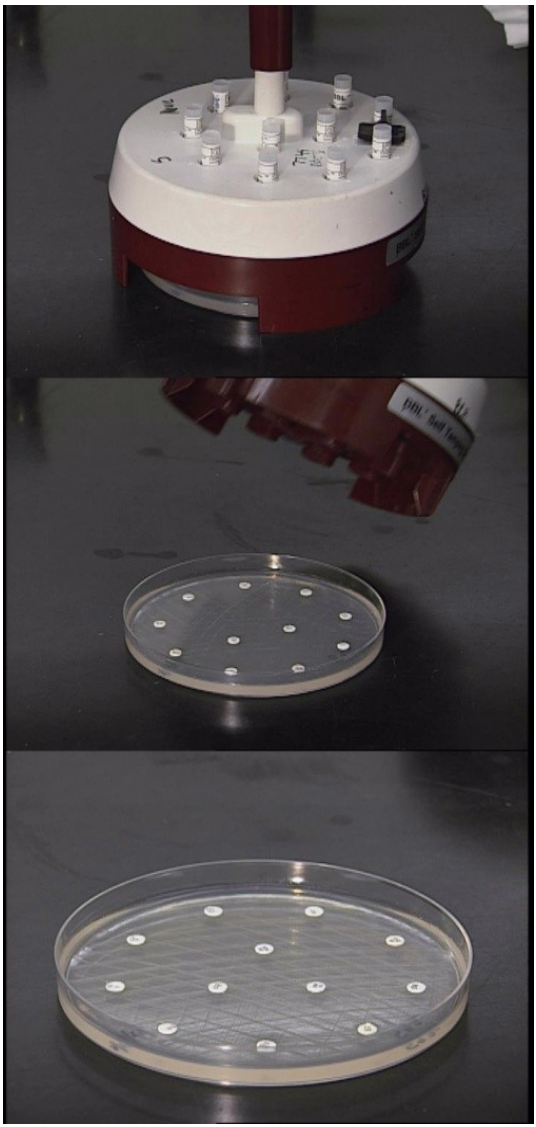


Figure 35.3. *The process of applying antibiotic discs to agar using an automatic dispenser*

[1] Typical strains with well-studied properties and genetic stability are used as reference strains (test strains). For example, in CIS countries, in the case of determining sensitivity to antibiotics, the strain *Y. pestis* strain EV line NIIEG is used, and for internal quality control of antibiotic programme determination – *E. coli* strain ATCC 25922 is used.

allows determining the *minimum inhibitory concentration* (MIC) of the drug by introducing a known amount of it into the nutrient medium.



Figure 35.4. *Dish with zones of inhibition. Note that the zone of inhibition is smaller or absent around some antibiotic discs, indicating partial or complete resistance of the test strain to some antibiotics*

An important step for this method is the preparation of a basic antibiotic solution suitable for the storage and a working solution that will be used immediately for the preparation of nutrient media. Both liquid and solid media can be used, but in practice only solid media are used.

For the main solution use substances with known activity (in an emergency situation it is acceptable to use preparations intended for injection), which are weighed on an analytical scale with an accuracy to the fourth digit and added to the solvent. Calculation of the mass of the preparation is carried out according to the formula:

$$m_{\text{p}} = \frac{C \cdot V}{K_A}, [mg],$$

where C – required preparation concentration, μg/ml; V – volume of solvent, ml; K_A – preparation activity (amount of active ingredient contained in the substance used), μg/ml.

In practice, it is impossible to weigh the exact calculated amount of the substance, so, according to the tradition of analytical chemistry, prepare a sample close to the calculated amount and recalculate the required volume of solvent according to the formula:

$$V_{\text{п}} = \frac{m_{\text{п}} \cdot V_{\text{т}}}{m_{\text{р}}}, [\text{mL}],$$

where $m_п$ – practical weight of preparation, mg; $m_р$ – calculated weight of preparation, mg; $V_т$ – volume of solvent taken to dissolve the calculated weight, ml.

It should be noted that for a number of antibiotics, the solvent will be two substances: solubiliser and diluent (**Table 35.4**). In this case, the preparation is dissolved in a minimum volume of solubiliser and diluted with diluent to the required volume.

Table. 35.4. *Solvents for antibiotic preparations*

<table>
<tr><th rowspan="2">Preparation</th><th colspan="2">Solvent</th></tr>
<tr><th>solubiliser</th><th>diluent</th></tr>
<tr><td>Chloramphenicol</td><td>95% ethanol solution</td><td>distilled water</td></tr>
<tr><td>Rifampicin</td><td>methanol</td><td>distilled water</td></tr>
<tr><td>Sulfonamides</td><td>half a volume of hot distilled water and a minimum amount of NaOH solution (2.5 mol/L)</td><td>distilled water</td></tr>
<tr><td>Trimethoprim</td><td>hydrochloric acid solution (0.05 mol/L) up to 10 % of the final volume</td><td>distilled water</td></tr>
<tr><td>Ampicillin</td><td>phosphate buffer (0.1 mol/L; pH 8)</td><td>phosphate buffer (0.1 mol/L; pH 6)</td></tr>
<tr><td>Nitrofurantoin</td><td colspan="2">phosphate buffer (0,1 mol/L; pH 8)</td></tr>
<tr><td>Norfloxacin</td><td rowspan="3">half a volume of distilled water and add NaOH solution (0.1 mol/L) dropwise until dissolved</td><td rowspan="3">distilled water</td></tr>
<tr><td>Ofloxacin</td></tr>
<tr><td>Nalidixic acid[1]</td></tr>
<tr><td>Other</td><td colspan="2">distilled water</td></tr>
</table>

[1] This drug is not used for the treatment of plague, but *Y. pestis* strains are tested for sensitivity to it, since culture resistance to nalidixic acid may be accompanied by a reduction in the efficacy of fluoroquinolone treatment while remaining sensitive to it on the dish.

For the preparation of working solutions, the required concentrations are obtained from the basic solution by titration, using distilled water as a solvent. At the same time, if preparations are used in prepackaged form (ampoules, vials, tablets), working solutions can be prepared as follows.

In case of *liquid form*, the preparation is dissolved in a known volume of distilled water. For example, if there is a vial containing 5 g of a preparatione, dissolving it in 5 mg of distilled water it will give a solution with a concentration of 100000 μg/ml. Further, by titration it is possible to obtain solutions with concentration of 10000 μg/kg, 1000 μg/kg and so on. In case of dissolution in solubiliser and diluent, the volume is separated. For example, if a capsule of nalidixic acid is available, its concentration (500 mg) is added to 25 ml of distilled water. Then sodium hydroxide solution (0.1 mol/l) is added dropwise until the drug is completely dissolved and 25 ml of distilled water is added, thus obtaining a solution with a concentration of 10000 μg/ml.

In the case of the *solid form*, the preparation is crushed to powder and dissolved in a known volume of distilled water, taking into account a correction factor equal to the ratio of the total mass of the preparation to the mass of the active substance. For example, if a 375 mg tablet contains 250 mg of active ingredient, the correction factor will be 1.5 (375 : 250). Then to prepare a solution with a concentration of 10000 μg/ml, 15 mg of powder obtained from the tablet should be dissolved in 1 ml of distilled water. In the case of combination preparations (e.g. cotrimoxazole, i.e. trimethoprim/sulfamethoxazole), the calculation is based on the main substance (in this case trimethoprim).

Next, Petri dishes are prepared with previously autoclaved and cooled to 48-50°C / 118.4-122°F agar, to which antibiotics are added at double rate concentrations. For this purpose, 20 ml of agar are poured into sterile vials and the required amount of working solutions (**Table 35.5**) are added, starting with the lowest concentration, after which they are thoroughly mixed and poured into a dish.

On the other hand, 2 ml of the antibiotic working solution can be added to 18 ml of agar in pre-prepared serial dilutions, mixed thoroughly and poured into Petri dishes. In this way, the final concentration of

the drug in the agar will be 10 times less than the concentration of the serial dilutions of the working dilution.

Table 35.5. *Concentrations of antibiotic preparations added to agar*

Required preparation concentration in 1 ml agar, μg/ml	**Concentration of preparation in working solution, μg/ml**	**Amount of working solution to be added to 20 ml agar, ml**
0.06	10	0.12
0.125		0.25
0.25		0.5
0.5	100	0.1
1		0.2
2		0.4
4		0.8
8	1000	0.16
16		0.32
32		0.64
64	10000	0.128
128		0.256
256		0.512
Note: For preparation of preparation solutions in concentrations of 1000 μg/ml and 10000 μg/ml, dimexide or 26% ethanol solution is used.		

After drying, the dishes are recommended to be used immediately or within a day, provided they are stored at 4-8°C / 39.2-46.4°F. Immediately before use, they should be dried for 15 minutes at 37°C / 98.6°F to avoid condensation. The medium most commonly used is Mueller–Hinton agar and, less commonly, Hottinger agar.

A suspension from a 24-hour culture of *Y. pestis* in 0.85% isotonic sodium chloride solution (5 x 10^8 BC/ml) is applied dropwise by a light tap of a pipette (approximately 0.005 ml) on the agar surface, starting with

the lowest concentration. Up to 20 strains can be studied in this way (including a control strain for which the MICs are known). Then, after 10-15 minutes, when the agar has dried, the dishes (including a dish without antibiotic to control culture growth) are incubated at 28°C / 82.4°F. Preliminary results are recorded after 36 hours, and final results can be received after 48 hours, taking into account the characteristic growth of the culture on the control dish. The results are recorded by comparing the minimum concentration of the preparation that inhibits growth of the pathogen (i.e. MIC) with the limit values given in **Table 35.1**.

As with the discs, preparations are subject to testing using reference strains on Mueller-Hinton medium.

Based on the results of testing by both methods, the microorganism under investigation is assigned to one of the categories:

sensitive, i.e. the use of the antibiotic in the recommended doses will inhibit the growth and multiplication of the strain;

intermediate, i.e. antibiotic use will inhibit the growth and multiplication of the strain only at the maximum permissible doses, while not excluding the selection of pathogen variants for which even the maximum doses will be ineffective;

resistant, i.e. the use of the antibiotic in the recommended doses will not inhibit the growth and reproduction of the strain, and therefore the use of this antibiotic for treatment is inadmissible.

It is important to remember that clinically orientated sensitivity categories and proven resistance to antibiotics do not always correlate. Therefore, even if there is improvement on the background of the prescribed therapy, after two to three days it is necessary to perform bacteriological control of the effectiveness of etiotropic therapy (culture, bioassay).

Table 35.1. *Allowable diameters of growth zones of inhibition and MICs values for the test strain*

Preparation (antibiotic)	KB test			MSD	
	Concentration in disc, μg	Diameter of growth zones of inhibition, mm		MIC value, μg/ml	
		S	R	S	R
Streptomycin	30	≥18	≤13	≤16	≥64
Kanamycin					
Amikacin		≥17	≤14		
Gentamicin	10			≤4	≥16
Netilmicin	30	≥19			
Tobramycin	10	≥17		≤8	≥32
Tetracycline	30	≥19		≤4	≥16
Doxycycline	10				
Levomycetin	30				
Rifampicin	5	≥15	≤13		≥32
Ampicillin	10	≥19	≤14	≤8	
Ceftriaxone	30	≥25	≤20	≤1	≥4
Cefotaxime					
Ceftibuten					
Ceftazidime					
Cefixime	5				
Cefepime	30				
Aztreonam		≥23	≤17		≥8
Nalidixic acid		≥25	≤20	≤8	≥32
Ciprofloxacin	5		≤19	≤0,1	≥1
Ofloxacin					
Pefloxacin	10	≥22	≤17	≤0,2	≥2
Lomefloxacin				≤0,1	≥1
Levofloxacin	5				
Co-trimoxazole	1.25/23.75	≥21	≤16	≤2/38	≥8/152

Note:
S - sensitive, R - resistant. MIC values and zone diameters between S and R indicate an intermediate category

Table 35.2. *Allowable diameters of growth zones of inhibition for reference strains*

Preparation (antibiotic)	Concentration in disc, μg	Diameter of growth zones of inhibition, mm					
		Mueller–Hinton agar		Hottinger agar		Givental'-Ved'mina's agar	
		E. coli strain ATCC 25922	*Y. pestis* strain EV line NIIEG	*E. coli* strain ATCC 25922	*Y. pestis* strain EV line NIIEG	*E. coli* strain ATCC 25922	*Y. pestis* strain EV line NIIEG
Streptomycin	30	17–25	17–23	17–25	19–25	17–25	19-26
Kanamycin			18–23				
Amikacin		19–26	19–23	19–26	20–26	19–26	20–30
Gentamicin	10		20–27		22–27		25–30
Netilmicin	30	22–30	25–30	22–30	25–30	22–30	30–40
Tobramycin	10	18–26	18–26	18–26		18–26	25–33
Tetracycline	30	18–25	20–27	18–25	20–27	18–25	20–27
Doxycycline	10	18–24		18–24		18–24	
Levomycetin	30	21–27	25–33	21–27	25–33	21–27	25–33
Rifampicin	5	8–10	15–22	8–10	15-20	8–10	15–20
Ampicillin	10	16–22	23–28	16–22	23–28	13–20	25–33
Ceftriaxone	30	29–35	30–40	29–35	30–40	29–35	30–40
Cefotaxime							
Ceftibuten		27–35		27–35	35–45	27–35	
Ceftazidime		25–32	28–35	25–32	30–35	25–32	30–35
Cefixime	5	23–27		23–27		23–27	
Cefepime	30	31–37	31–40	31–37	31–40	31–37	31–37
Aztreonam		28–36	29–37	28–36	30–36	28–36	30–35
Nalidixic acid		22–28	28–35	22–28	30–35	22–28	28–35
Ciprofloxacin	5	30–40	30–35	30–40		30–40	30–35
Ofloxacin		29–33	29–35	29–33	30–35	29–33	29–35
Pefloxacin	10	28–35		28–35	29–35	28–35	30–35
Lomefloxacin		27–33	30–37	27–33	30–37	27–33	30–37
Levofloxacin	5	29–37	30–40	29–37	30–40	29–37	30–40
Co-trimoxazole	1.25/23.75	23–29	23–28	23–29	23–28	23–29	23–28

Table 35.3. *Allowable MIC values for reference strains*

Preparation (antibiotic)	MIC value, μg/ml			
	Mueller–Hinton agar		Hottinger agar	
	E. coli strain ATCC 25922	*Y. pestis* strain EV line NIIEG	*E. coli* strain ATCC 25922	*Y. pestis* strain EV line NIIEG
Streptomycin	1–4	2–8	1–4	1–8
Kanamycin				2–8
Amikacin	0.5–4.0		0.5–4.0	
Gentamicin	0.25–1.0	0.25–2.0	0.25–1.0	0.25–1.0
Netilmicin				
Tobramycin		0.5–2.0		
Tetracycline	0.5–2.0		0.5–2.0	0.5–2.0
Doxycycline		0.25–1.0		0.25–1.0
Levomycetin	2–8	1-4	2–8	1–4
Rifampicin	4–16		4–16	
Ampicillin	2–8	0.5–1.0	2–8	0.5–1.0
Ceftriaxone	0.03–0.12	0.01–0.02	0.03–0.12	0.01–0.02
Cefotaxime				
Ceftibuten	0.12–0.5		0.12–0.5	
Ceftazidime	0.06–0.5	0.02–0.08	0.06–0.5	0.02–0.08
Cefixime	0.25–1.0	0.08–0.16	0.25–1.0	0.08–0.16
Cefepime	0.016–0.12	0.02–0.04	0.016–0.12	0.02–0.04
Aztreonam	0.06–0.25	0.01–0.02	0.06–0.25	0.01–0.02
Nalidixic acid	1–4	0.5–2.0	1–4	0.5–2.0
Ciprofloxacin	0.004–0.016	0.01–0.02	0.004–0.016	0.01–0.02
Ofloxacin	0.015–0.12	0.005–0.01	0.015–0.12	0.005–0.01
Pefloxacin	0.03–0.12	0.02–0.08	0.03–0.12	0.02–0.08
Lomefloxacin		0.01–0.08		0.01–0.08
Levofloxacin	0.008–0.06	0.005–0.01	0.008–0.06	0.005–0.01
Co-trimoxazole	0.5/9.5–2/38	1/19–2/38	0.5/9.5–2/38	1/19–2/38

36 MOLECULAR GENETIC METHODS

The researches[1] of James Dewey Watson[2], Francis Harry Compton Crick[3], Maurice Hugh Frederick Willkins[4] (with colleagues) and Rosalind Elsie Franklin[5] (with colleagues) allowed in 1953 to decipher the structure of DNA, which gave a powerful impetus to the development of molecular biology and the creation of molecular genetic methods of research. Thus, in 1971, Kjell Kleppe[6] (with colleagues) published an article in which he proposed the procedure of replication of a DNA segment using two synthetic primers, calling it *repair replication*. However, its development had not received due attention from the scientific community, so the method, which allows to repeatedly sequentially double the original fragment of the DNA molecule, i.e. *amplify DNA*, was proposed[7] only in 1983 by Kary Banks Mullis[8], called *polymerase chain reaction* (PCR). The essence of PCR is reduced to three steps:

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[1] Watson J. D., Crick F. H. C. *Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid*. Nature. 1953; 171:737–738; DOI: 10.1038/171737a0; Wilkins M. H. F., Stokes A. R, et al. *Molecular Structure of Deoxypentose Nucleic Acids*. Nature. 1953; 171:738–740; DOI: 10.1038/171738a0; Franklin R. E., Gosling R. G. Molecular configuration in sodium thymonucleate. Nature. 1953; 171(4356):740–741; DOI: 10.1038/171740a0.

[2] b. 1928; the American biologist. Laureate of the Nobel Prize in Physiology or Medicine (1962). Head of The Human Genome Project.

[3] 1916–2004; the British and American physicist. During the Second World War he worked on the development of magnetic and acoustic mines. In 1947, he moved, as he later wrote, from the elegance and deep understanding of physics to complex chemical mechanisms whose natural selection had evolved over billions of years, so that he almost had to be born again.

[4] 1916–2004; the Irish-born New Zealand and British physicist. Laureate of the Nobel Prize in Physiology or Medicine (1962).

[5] 1920–1958; the Jewish-born British biophysicist, radiographer.

[6] 1934–1988; the Norwegian chemist and molecular biologist.

[7] US 4683195 A, 28.07.1987; information on the year of method development is given according to the personal website (https://www.karymullis.com/pcr.shtml).

[8] 1944–2019; the American biochemist. Laureate of the Nobel Prize in Chemistry (1993). He had controversial scientific views, in particular, he denied climate change, ozone depletion and the link between HIV and AIDS, explaining his position by conspiracy; he believed in astrology.

1. *Denaturation.* The DNA fragment (DNA matrix)[1] is heated to 95°C / 203°F (half a minute) so that two complementary DNA strands separate as a result of hydrogen bond breakage.
2. *Annealing*. As a result of temperature reduction to about 60°C / 140°F (20-30 seconds), which depends on the primers (short synthetic oligonucleotides 18-30 bases long), the latter binds to complementary fragments on DNA strands. The specificity of PCR depends on the primers, so they are selected to the most unique and, at the same time, conservative section of DNA, so that if mutations occurs, it is not necessary to create new primers and do not get a false negative result.
3. *Extension (elongation)*. The temperature is raised to 70°C / 158°F so that the enzyme DNA-dependent DNA polymerase builds a second strand from deoxynucleoside triphosphates (dNTPs, "building blocks") starting from the 3' end of the primer used as primer "seed".

The temperature mode is provided by a special device – *thermocycler* or *DNA amplifier*. Thus, in theory, as a result of one cycle, two fragments containing the target region are formed from one initial DNA fragment (since the completion is performed by primers), hence, the amount of the reaction product (i.e. new DNA fragments containing the target region, or aplicons) increases exponentially with multiple repetitions. In practice, product yields are usually below 100%, so mathematically the PCR process is expressed as $\mathbf{(1 + E)^n}$, where **E** is the average reaction efficiency, **n** is the number of cycles.

Initially, DNA polymerase had to be added at each cycle after the first step because it was inactivated at high temperature. This made the process complicated, time-consuming and expensive. Therefore, as early as 1988, it was proposed[2] to use a polymerase from the thermophilic bacterium *Thermus aquatiqus*, called *Taq* polymerase (*Taq* or *Taq* pol) and

[1] If the target product is RNA, reverse transcription is carried out before the denaturation step, i.e. the enzyme RNA-dependent DNA polymerase (revertase) is added to RNA and heated at 50°C / 122°F, which leads to the formation of cDNA, which is involved in PCR.

[2] Saiki R. C., Gelfand D. H., et al. *Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase*. Science. 1988; 239(4839):487–491; DOI: 10.1126/science.2448875.

characterised 10 years earlier[1], instead of polymerase from *Escherichia coli*. A significant disadvantage of *Taq* polymerase is a rather high probability of introducing an erroneous nucleotide due to the lack of an error correction mechanism. *Pfu* polymerase, isolated[2] in 1991 from the hyperthermophilic archaea *Pyrococcus furiosus*, has such a mechanism, but has a low speed of operation, so currently a mixture of *Taq* polymerase (for fast operation) and *Pfu* polymerase (for error correction) is used. It is worth noting that at lower temperatures, such as room temperature, a non-specific action of polymerase is possible, leading to primer sticking and dimer formation. Therefore, already in 1994 it was proposed[3] to use antibodies to inactivate polymerase. This modification, called *hot start PCR*, involves "freeing" the polymerase from antibodies in an additional inactivation step (*Initialization*) performed prior to denaturation and involving heating to 96-98°C / 204.8-208.4°F (5 to 15 minutes).

PCR is performed in a boxed PCR laboratory room[4] divided into sequentially arranged working areas: the first area of the laboratory is intended for obtaining (isolation) of nucleic acids from the test material, in the second area the reaction mixture is prepared, and in the third area the nucleic acids are amplified and detected. In CIS countries PCR laboratory is organised differently, but there are also three areas: material sorting and primary processing (area 1), nucleic acid isolation (area 2), amplification (area 3)[5]. Depending on the method, the results are recorded in area 3 or area 4. It is recommended to divide area 3 into two

[1] Chien A., Edgar D. B., et al. *Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus*. J Bacteriol. 1976; 127(3):1550–1557; DOI: 10.1128/jb.127.3.1550-1557.1976.

[2] Lundberg K. S., Shoemaker D. D., et al. *High-fidelity amplification using a thermostable DNA polymerase isolated from Pyrococcus furiosus*. Gene. 1991; 108(1):1–6; DOI: 10.1016/0378-1119(91)90480-Y.

[3] Sharkey D. J., Scalice E. R., et al. *Antibodies as Thermolabile Switches: High Temperature Triggering for the Polymerase Chain Reaction*. Nature Biotechnology. 1994; 12(5):506–509; DOI: 10.1038/nbt0594-506.

[4] After work, the rooms, BSC (PCR cabinet) and tools are treated with a disinfectant solution, which is a 3% solution of hydrogen peroxide or chlorine-containing substances (trichloroisocyanuric acid solution). The use of ethanol solutions for this purpose is not allowed!

[5] If necessary, it is possible to combine area 2 and area 3 in the same room, provided that each stage is operated in a BSC Class II.

separate rooms (sub-area 3a and sub-area 3b). In sub-area 3a, reaction mixtures are prepared and, if necessary, reverse transcription is performed. In sub-area 3b, nucleic acids are amplified.

For detection of amplification results, *gel electrophoresis of nucleic acids* (area 4-1 in CIS countries) is performed, when amplification products[1] are stained with a specific dye, most often ethidium bromide (3,8-diamino-5-ethyl-6-phenyl-6-phenyl-phenanthridium bromide), and placed in an agarose gel (0.7-2% agarose solution in buffer), where, under the action of an external electric field, amplified DNA fragments migrate from a negatively charged cathode to a positively charged anode. Since molecules of different masses migrate at different rates, after a while they will end up in different parts of the gel, which can be seen as streaks due to the dye fluorescing under UV light[2] (**Figure 36.1**). In order to understand the fragment length of the obtained product, a *molecular-weight size marker*, also referred to as *DNA ladder*[3], is additionally introduced into the slit.

For detection in *real-time PCR* (qPCR[4]), area 3 (sub-area 3b), DNA probes with a fluorescent tag (fluorophore) and a quencher complementary to the amplification product are most often used. Since the tag and quencher are sewn to the probe, the signal from the tag is absorbed by the quencher, but during amplification the integrity of the probe is broken and the tag "escapes" into the reaction mixture, where its "meeting"

[1] They should be handled with extreme care so as not to contaminate the workplace!

[2] In a similar way, the presence of plasmids in the tested culture is determined. For this purpose, DNA is extracted from the culture grown on agar for 1-2 days: the culture is emulsified in 100 μl of lytic mixture (50 mM Tris, 3% SDS; bring the pH to 12.6 by adding 2 mol/L NaOH), 10 μl of lysozyme is added and heated for 30 minutes in a water bath at 60°C / 140°F. The culture is then shaken quickly (but gently!) and centrifuged for 30 minutes at 6000/min. Then 100 μl phenol-chloroform mixture (concentration 1:1) is added, shaken quickly (but gently!) and centrifuged for 15 minutes at 6000 rpm. Carefully collect the upper phase without touching the interphase and precipitate. Commercial kits can also be used. Then stained with a specific dye (e.g. ethidium bromide) and applied to a 0.7-0.75% gel for electrophoresis performed on TAE buffer (pH 7.8-7.9). Three bands of plasmid DNA corresponding to pCad (47 Md), pFra (60-100 Md) and pPla (6-8 Md) should be visualised in the electrophoregram (**Figure 36.2**). It is most convenient to compare with the electrophoregram of DNA isolated from a reference strain.

[3] Measured in Dalton (Da, D), more commonly megadaltons (MD, MDa, Md), and base pair (bp).

[4] It is important to note that the acronym "RT-PCR" usually stands for reverse transcription PCR, not real-time PCR.

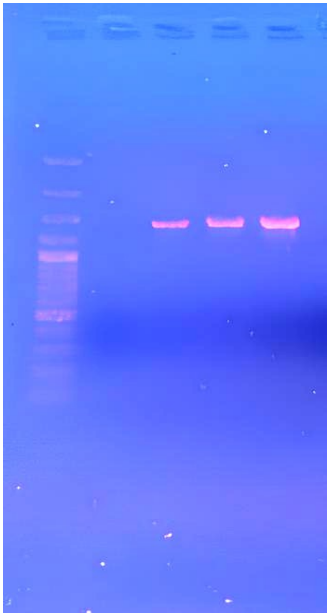


Figure 36.1. *Luminescence of amplification products in the gel (electrophoregram) under UV light. The wells into which the samples were introduced are seen on the top, and the DNA ladder is on the left*

with the quencher is unlikely to occur. This most often occurs during the elongation phase. Detection is performed by excitation of the fluorophore with a laser and registration of the emitted wavelength after each cycle, therefore, the intensity of excitation will increase with each cycle. And since PCR is essentially a polymerisation reaction, where the identified DNA fragment is a kind of reaction monomer, the kinetics of the process (amplification graph) will have a characteristic S-shape: base line, exponential growth and plateau (**Figure 36.3**).

It should be taken into account that by the last cycles the probes may collapse spontaneously and that intense signals from some samples may be picked up by the probes of other samples, which lead to the necessity of introducing a *threshold*, signals below which are not taken into account. The threshold line is set relative to the results of a positive control sample (typically at the 10-20% level). The cycle at which the graph crosses the threshold line is called the *cycle thresholds* (Ct). This is an important characteristic that allows us to judge, albeit roughly, the amount of DNA in the sample. For example, if the Ct of one sample is 6 and that of another is 20, we can safely say that the first sample contains more DNA. The Ct value is also used by test system developers to indicate in the instructions which results should be considered valid and which should be considered invalid.

Y. pestis was first identified by PCR in 1991[1]. Since then, researchers have been developing primers for different targets, and to date the main

[1] Булат С. А., MLFTйлов Н. В., и др. *Геноидентификация видов и сероваров бактерий методом полимеразной цепной реакции с универсальными олигонуклеотидами: реидентификация*

targets are chromosomal regions *3a*[1], *hmsH* and *vlm12/IS216*, as well as the *caf1* gene (plasmid pFra; encodes capsular antigen) and *pla* gene (plasmid pPla; encodes plasminogen activator), which allows the identification of both complete strains and strains lacking one of the species-specific plasmids. The gene *YP_002346399.1* encoding ftsK/SpoIIIE family protein[2] is of interest as a target, since the position 3056 of the gene in the strains of the main subspecies contains adenine, whereas in the strains of the nonmain subspecies it contains guanine, which makes[3] it possible to distinguish the strains of the main subspecies from the strains of the nonmain subspecies on the basis of a difference of only one nucleotide. In addition, test systems based on amplification of fragments of species-specific chromosome regions are being developed for intraspecific differentiation. For example, the deletion in the *AK38_1098* gene (88 bp) is characteristic of the Ulegei strains, while the deletion in the *AK38_1327* gene (90 bp) is characteristic of the Altai strains. The primers designed[4] for these deletions allow identification of these strains by PCR. To differentiate avirulent strains

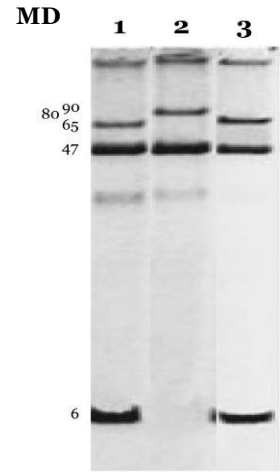


Figure 36.2. *Plasmid profile of typical strains of Y. pestis: 1 - main subspecies; 2 - Caucasian strains; 3 - Altai strains (courtesy of Dr. Tatiana E. Arsenyeva)*

ранее выделенных сероваров Yersinia pseudotuberculosis. Журнал микробиологии, эпидемиологии и иммунобиологии. 1991; 12:2–7.

[1] This region is the prophage genome and is used as a target in many PCR test systems, but to be fair, this region is absent in some strains.

[2] This protein is thought to be associated with DNA segregation (i.e. the distribution of elements of its genetic material into daughter cells).

[3] Трухачёв А. Л., Коновалова Н. В., и др. *Выявление SNP-маркеров и конструирование набора реагентов для внутривидовой дифференциации штаммов Yersinia pestis в ПЦР с помощью аллель-специфических зондов*, в сборн. *Сборник трудов IX Всероссийской научно-практической конференции с международным участием, под ред. В. П. Покровского*, *Т. 1*. Тамбов: ООО фирма «Юлис», 2017, 554 с.

[4] Никифоров К. А., Оглодин Е. Г., и др. *Подвидовая дифференциация штаммов Yersinia pestis методом ПЦР с гибридизационно-флуоресцентным учётом результатов*. Журнал микробиологии, эпидемиологии и иммунобиологии. 2017; 2:22–27; DOI: 10.36233/0372-9311-2017-2-22-27.

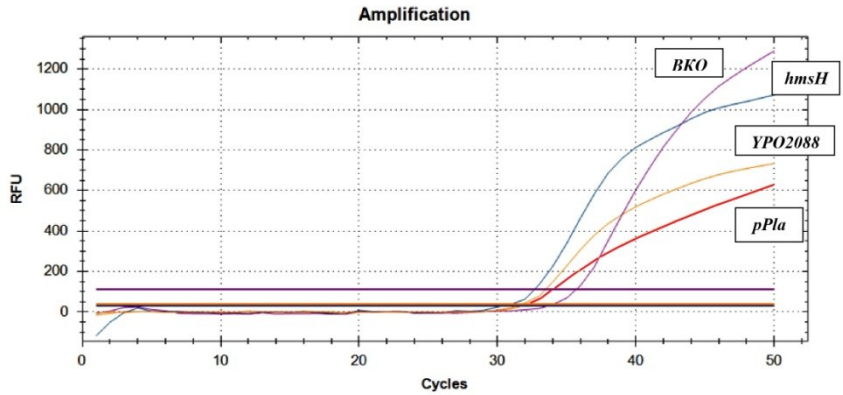


Figure 36.3. *Amplification plot with recorded signals (curves by detection channels) corresponding to a fragment of chromosomal nucleotide sequence hmsH, a fragment of chromosomal gene YPO2088 (encodes methyltransferase) and a specific fragment of nucleotide sequence YPPCP1.09c of species-specific plasmid pPla, as well as to the internal sample control (ISC). Positive controls are omitted for clarity; horizontal lines are the threshold line. The presence of signals on three channels (hmsH, YPO2088, pPla) indicates that the sample contains DNA of a highly virulent, epidemically significant strain of Y. pestis. The presence of only two signals (hmsH and pPla) indicates that the sample contains Y. pestis DNA with reduced epidemic significance, and the presence of only YPO2088 signal - DNA avirulent strain of Y. pestis*

of the main subspecies from virulent ones, the chromosomal *hmsH* genes (*hms* locus of the pigmentation region) and *irp2* (high pathogenicity island of the pigmentation region), as well as the *lcrV* gene of the pCad plasmid are detected (these genes were discussed in detail in Chapter 6).

PCR is a rapid and highly sensitive method, allowing detection of 10^2-10^3 copies/ml of material. It is particularly convenient in situations where bacteriological examination is difficult (isolation of an axenic culture is unlikely or impossible), as well as for rapid diagnostics. At the same time, PCR, like all molecular genetic methods, allows to answer only the question whether or not there is nucleic acid in the sample, not whether the microorganism is alive. And this is very important to realise, because in the case of bacteria, a positive PCR result is not yet the proof of the presence of the pathogen, but PCR can be used to give a preliminary result and also to detect virulence factors.

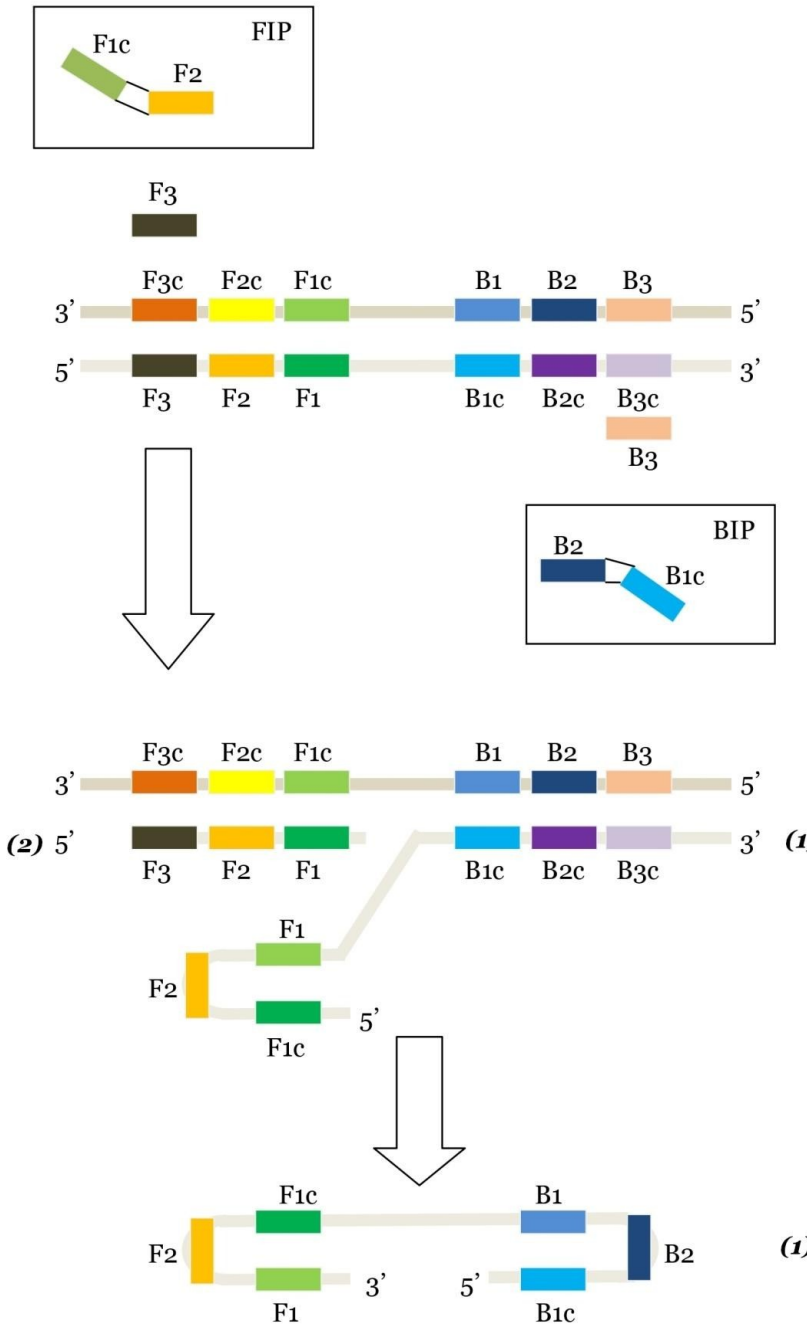
On the other hand, PCR can indirectly confirm the presence of live pathogen cells in a sample using lytic bacteriophages. Knowing the initial concentration of bacteriophages, the presence of live cells of the pathogen in the material can be inferred from the increase in their titre on PCR over time (e.g. after 30, 60 and 90 minutes). In the case of *Y. pestis*, it has been proposed[1] to use bacteriophages L-413C and φ1122, but it should be remembered that, despite the higher specificity of the former, its lytic cycle (i.e. the time from infection of a bacterial cell to the release of phage particles) is 90 minutes, whereas the latter has three lytic cycles during this time. Thus, in the case of bacteriophage L-413C, 115 phage particles will be produced and in the case of φ1122, approximately 10^5. This method is quite simple and allows obtaining results in 4 hours, however, as it has been repeatedly mentioned, it is necessary to remember about phage resistance and specificity of bacteriophages.

Loop-mediated isothermal amplification (LAMP), developed[2] in 2000, is used as a relatively fast (5-30 minutes) alternative to PCR. LAMP is performed at constant temperature (60-65°C / 140-149°F) and the amplification result is detected either visually, by precipitated magnesium pyrophosphate (precipitate), or photometrically (real-time). Four primers (FIP, F3, BIP and B3) recognising a total of six different sequences of target DNA are used (**Figure 36.4**), and a DNA polymerase from the thermophilic bacterium *Bacilus stearothermophilus*, Bst polymerase, is used.

The F2 region of the FIP primer binds to the complementary F2c region of one strand of DNA, causing DNA polymerase to complete the strand (1), displacing the second strand of original DNA. Then primer F3 binds to the complementary F3c region of the original DNA chain, as a result of which DNA polymerase completes the chain (2) again, and the previously completed chain (1) is displaced and the 5'-end "folds" into a loop (F1 – F1c). The BIP primer approaches this chain (1) and binds to the complementary B2c region with its B2 region. DNA polymerase completes the chain (3). Primer B3 binds to the complementary

[1] Sergueev K. V., He Y., et al. *Rapid and sensitive detection of Yersinia pestis using amplification of plague diagnostic bacteriophages monitored by Real-Time PCR*, PLoS One. 2010; 5(6):e11337; DOI: 10.1371/journal.pone.0011337.

[2] Notomi T., Okayama H., et al. *Loop-mediated isothermal amplification of DNA*. Nucleic Acids Research. 2000; 28(15):e63; DOI: 10.1093/nar/28.12.e63.


FIP
F1c
F2
F3
F3c
F2c
F1c
B1
B2
B3
3'
5'
5'
3'
F3
F2
F1
B1c
B2c
B3c
B3
BIP
B2
B1c
(2)
(1)
F1
F2
F1c
5'
3'
B1
B2
(1)

◀ ***Figure 36.4.*** *A summary of LAMP and the primers used in it: FIP, forward internal primer consisting of parts F1c (5') and F2 (3') complementary to F1 region and F2c region; BIP, reverse internal primer consisting of parts B1c (5') and B2 (3') complementary to B1 region and B2c region; F3, forward external primer complementary to F3c region; B3, reverse external primer complementary to B3c region*

B3c site of chain (1), DNA polymerase completes chain (4), which displaces chain (3), resulting in the latter (3) "folding" into two loops at the ends, forming a concatemer (target fragment). The construction of multiple inverted concatemers results in the formation of cauliflower-like structures in space.

A little later, it was proposed[1] to use six primers recognising eight target sites, which, as can be easily guessed, increases specificity. At the same time, the large number of primers determines the main difficulty in creating test systems[2]. LAMP test systems with a sensitivity of 10^4 copies/ml have been developed[3] for *Y. pestis*, with primers for chromosomal *3a* region and *caf1A* gene (pFra plasmid). A method of pathogen detection by bacteriophage titre for LAMP has not been proposed, but theoretically it is also possible.

Rapid detection of *Y. pestis* DNA is not the only challenge that can be met using molecular genetic techniques. Penetrating the DNA structure to trace microevolution is the ultimate feat!

In 1977, Frederick Sanger[4] (with colleagues) proposed[5] a method for determining DNA nucleotide sequences by cutting the chain, called *Sanger sequencing*. The essence of the method is that DNA polymerase,

[1] Nagamine K., Hase T., et al. *Accelerated reaction by loop-mediated isothermal amplification using loop primers*. Mol Cell Probes. 2002; 16(3):223–229; DOI: 10.1006/mcpr.2002.0415.

[2] Most often, computer programmes such as PrimerExplorer are used to select primers (www.primerexplorer.jp).

[3] Singh R., Pal V., et al. *Development of a pair of real-time loop mediated isothermal amplification assays for detection of Yersinia pestis, the causative agent of plague*. Mol Cell Probes. 2020; 54:101670; DOI: 10.1016/j.mcp.2020.101670.

[4] 1918–2013; the British biochemist. Two times Laureate of the Nobel Prize in Chemistry (1958, 1980). Determined the amino acid composition of insulin and other proteins.

[5] Sanger F., Nicklen S., et al. *DNA sequencing with chain-terminating inhibitors*. PNAS. 1977; 74(12):5463–5467; DOI: 10.1073/pnas.74.12.5463.

deoxynucleoside triphosphates (dNTPs, "building blocks") and one of each of the dideoxynucleoside triphosphates (ddNTPs, roughly speaking, one "letter" each) lacking a 3'-hydroxyl group are added, so that DNA polymerase, upon reaching them, breaks the chain ("stop block"), is added to four samples containing fragments of a single DNA strand[1] labelled at one end with a radioactive isotope. Thus, since deoxyribonucleotides are incorporated chaotically, the output in each of the four tubes corresponding to a certain "letter" of DNA will be a set of DNA fragments of different lengths, labelled with a radioactive isotope at one end and a "stop block" at the other. Then electrophoresis is carried out (each row has its own "letter"), area 4-1, on which, with the help of a radioactive marker, there can be seen a ladder on which the sequence of nucleotides in the fragment under study is read "letter by letter".

The method is simple and elegant, so it is still used today, but it is fully automated and performed on special instruments - sequenators. In addition, deoxyribonucleotides labelled with fluorescent tags of different wavelengths are used, which allows the reaction to be carried out in a single tube.

Over time, new sequencing methods[2] have been proposed so by 2001 and 2002 it was allowed the complete genome sequences of *Y. pestis* strain CO92[3] and *Y. pestis* strain KIM[4], respectively, which led to many discoveries, including the discovery of a large number of pseudogenes, independent clusters of genes encoding different fimbriae and adhesins, as well as genes encoding previously unknown surface adhesins, but most interestingly, the ability of the *Y. pestis* genome to frequent intragenomic recombination! The importance of these works is clearly demonstrated by their enormous citation in the scientific literature.

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[1] Since the inception of PCR, fragments have been accrued by aplification.

[2] The principle developed by Frederick Sanger was the basis for technologies that have greatly simplified the sequencing process, among which Oxford Nanopore, Illumina, IonTorrent, DNBSEQ-G50, MGI, and PacBio are currently leading. However, the issue of errors (especially when "reading" large fragments) and interpretation of the obtained data ("assembly" of genomic sequences) is still rather acute.

[3] Parkhill J., Wren B. W., et al. *Genome sequence of Yersinia pestis, the causative agent of plague*. Nature. 2001; 413(6855):523–527; DOI: 10.1038/35097083.

[4] Deng W., Burland V., et al. *Genome Sequence of Yersinia pestis KIM*. J Bacteriol. 2002; 184(16):4601–4611; DOI: 10.1128/JB.184.16.4601-4611.2002.

Sequencing of strains makes it possible to study the evolution of *Y. pestis*, to build the phylogenetic trees discussed at the beginning (Chapter 3), as well as to explain the enzymatic features of some strains and to develop approaches to intraspecific differentiation (that are highly controversial).

In 1998, *multilocus sequence typing* (MLST) was proposed[1] for microbial typing, which is based on analysing the variability of conserved genes, i.e. those genes that accumulate mutations more slowly than others (they are also called *housekeeping genes*). Genes that are used to determine phylogenetic relatedness but do not encode virulence and pathogenicity factors are most often chosen as targets. Most often, the *rha* locus, *aspA*, and *nap* are used as such genes.

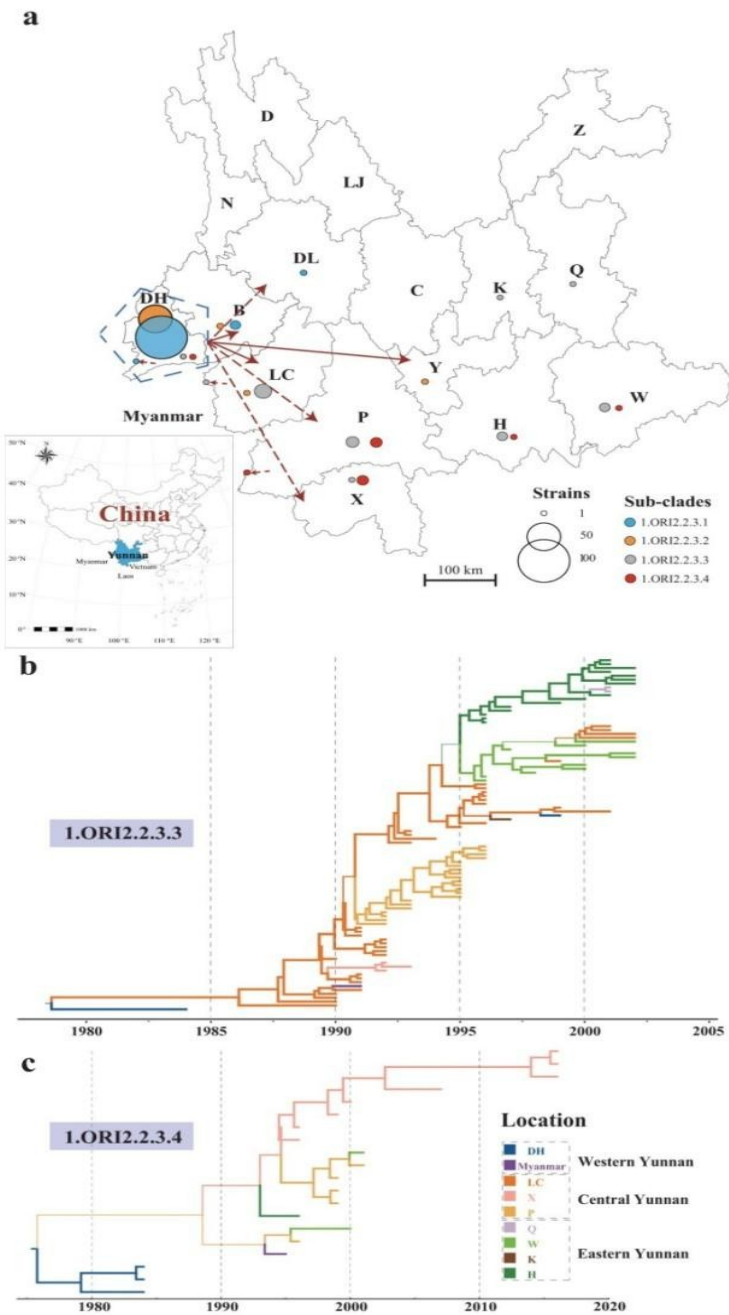
In contrast to MLST, which requires mandatory sequencing of target genes, single nucleotide polymorphism analysis (SNP), which assesses the variability of single nucleotides of a particular genome locus (so-called *single nucleotide polymorphism*), allows for differentiation of the microorganism using simpler methods, such as, for example, real-time PCR. For example, specific SNPs were detected for strains of *Y. pestis* of the main and nonmain subspecies[2], to which the corresponding primers were matched. On the other hand, SNP-types are used by many researchers for intraspecific differentiation and to clarify phylogeny, as mentioned in Chapter 3 (this chapter may have seemed complicated and confusing when you first read it; read it again with your existing knowledge in mind!).

In addition to the variability of genes, the variability of so-called *tandem repeats*, i.e. repetitive stretches of DNA that repeat one after another, is also studied. *Multiple locus VNTR analysis* (MLVA) is based on the study of *variable number tandem repeat* (VNTR), namely errors occurring during VNTR replication process. For this purpose, known loci are am-

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[1] Maiden M. C., Bygraves J. A., et al. *Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms*. PNAS. 1998; 95(6):3140–3145; DOI: 10.1073/pnas.95.6.3140.

[2] Kislichkina A. A., Kadnikova L. A., et al. *Differentiation of Yersinia pseudotuberculosis, Yersinia pestis subsp. pestis and subsp. microti strains and other representatives of Yersinia pseudotuberculosis complex*. Molecular Genetics, Microbiology and Virology. 2017; 32(2):67–74; DOI: 10.3103/S0891416817020070.


a
D
Z
LJ
N
DL
Q
C
K
DH
B
Y
LC
W
Myanmar
P
H
X
China
Yunnan
Strains
1
50
100
Sub-clades
1.ORI2.2.3.1
1.ORI2.2.3.2
1.ORI2.2.3.3
1.ORI2.2.3.4
100 km
b
1.ORI2.2.3.3
1980
1985
1990
1995
2000
2005
c
1.ORI2.2.3.4
Location
DH
Myanmar
LC
X
P
Q
W
K
H
Western Yunnan
Central Yunnan
Eastern Yunnan
1980
1990
2000
2010
2020

plified by PCR and the VNTR variability is compared with a reference. Thus, more than 430 MLVA25 types have been identified to date.

There are also other typing methods, but not all of them are applicable in the case of *Y. pestis*. Thus, the rather popular determination of ribosomal RNA (rRNA) polymorphism, in particular 16S rRNA, is not indicative of *Y. pestis* because of the overlap with *Y. pseudotuberculosis*. Moreover, of the three major rRNA types (23S and 5S in the large subunit, and 16S in the small subunit), only one nucleotide difference was found in the 23S rRNA gene sequence!

All these methods allow us to study the microevolution of *Y. pestis* in order to build the phylogenetic trees we discussed at the very beginning of this book (Chapter 3) or to conduct phylogeographic studies and, in practical epidemiology, to build routes of epidemic transmission (**Figure 36.5**).

37 AFTERWORD

Continuing the discussion on the differentiation of *Y. pestis* from *Y. pseudotuberculosis*, it is impossible not to mention such a method as *matrix–assisted laser desorption/ionisation time–of–flight mass spectrometry*

◄ ***Figure 36.5.*** *Distribution map of Sublinia 2 strains during the epizootic process in the Yunnan Province in China (a) and phylogenetic trees (b, c) of the two major gene variants (SNP types). The blue pentagon denotes the likely region of "onset" of the epizootic, and red arrows indicate direct (solid arrows) and indirect (dashed arrows) routes of spread. Circles denote the number of strains whose groups are indicated by colour. The colours of the phylogenetic tree lines indicate sampling locations (by Qin J., 2023)*

(MALDI-TOF MS). This method, proposed[1] in 1987 by Tanaka Kōichi[2] (and colleagues), consists in applying pulses of laser radiation to a mixture of the substance to be analysed and a matrix - a substance that is selected in such a way as to reduce the destructive properties of the laser radiation on the substance to be analysed and promote its ionization. A so-called plume consisting of various particles (MALDI) is formed above the surface, from which ions are further transported to the sensor via a vacuum tube (TOF) under the action of an electromagnetic current. Since ions have different mass-to-charge ratios (m/z), they are detected by the sensor at different times and with different intensities, which is expressed graphically. Usually bacterial identification is based on protein structures by examining bacterial colonies, but inactivated cells or their protein extracts are used in the case of particularly dangerous bacterial pathogens. Culture of *Y. pestis* is cultured on Hottinger agar for 24-48 hours at 28°C / 82.4°F. The study is most often performed on a matrix of a saturated solution of α-cyano-4-hydroxycinnamic acid (α-HCCA) with 50% acetonitrile and 2.5% trifluoroacetic acid, using 40 laser pulses at 60 Hz (the analysed m/z range is 2000-20000 Da). The mass spectra obtained (**Figure 38.1**) are compared with existing ones in the database.

As can be seen from **Figure 38.1**, differentiation of *Y. pestis* and *Y. pseudotuberculosis* by this method has certain limitations, since most of the peaks (m/z 4830, 5426, 8366, 9658, 10297 and 13268) are common, whereas only the peak m/z 3061 (other information, m/z 3063 or 3065) is specific for *Y. pestis*.

Among the reasons that could lead to different values of mass spectra, it is worth mentioning the natural variability of *Y. pestis*[3], but somehow this peak corresponds to a fragment of plasminogen activator protein. On the other hand, the Caucasian strains have the most intense peak m/z 6474, which is absent in *Y. pestis* of other subspecies but pre-

[1] Tanaka K., Waki H., et al. *Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry*. Rapid Communications in Mass Spectrometry. 1988; 2(8):151–153; DOI: 10.1002/rcm.1290020802.

[2] 田中 耕一, b. 1959; the Japanese engineer, electrician. Laureate of the Nobel Prize in Chemistry (2002).

[3] It should be noted that these values cannot be considered close, if only because the m/z 1755.1, 1755.4 and 1756.3 peaks are considered specific for *Vibrio cholera* O1 classical, O1 El-Tor and O139, respectively.

sent in *Y. pseudotuberculosis*. All this directly indicates the difficulties of laboratory diagnosis, in particular, differentiation from *Y. pseudotuberculosis*, in which the so-called atypical strains of *Y. pestis* play a major role[1].

These are pigmentless (i.e., lost *pgm*-region, avirulent) and plasmidless strains circulating in natural foci, but also rarely isolated in

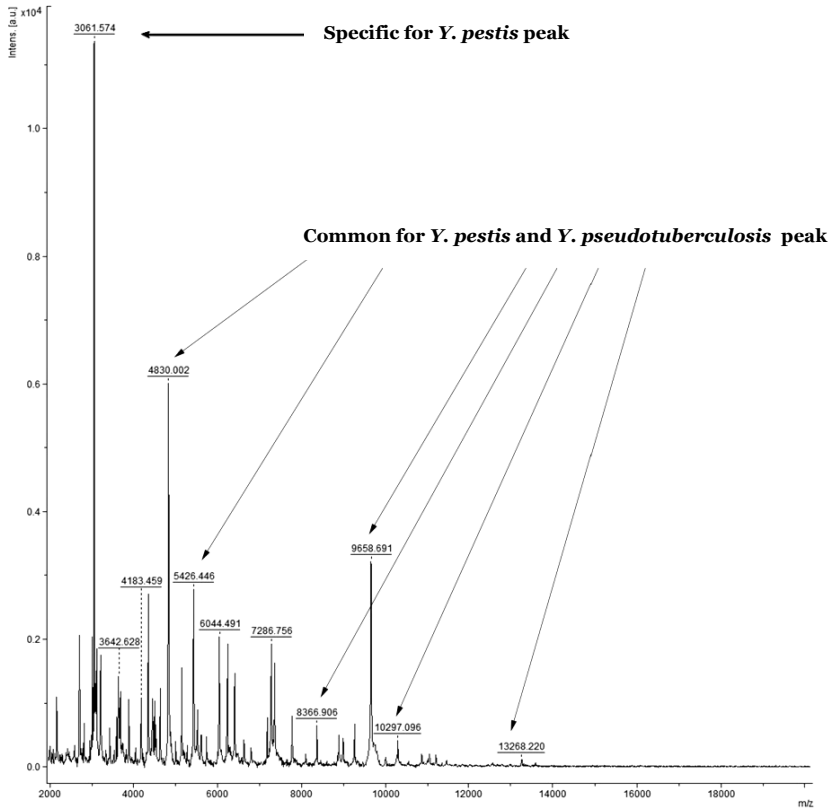


Figure 38.1. *MALDI-TOF Mass spectrum of protein extracts of Y. pestis strain (by Спицын А.Н., 2016)*

[1] Phenotypically, they are so difficult to distinguish that for a time it was even thought that one species was capable of "transform" into the other!

anthropurgical foci, in particular from a rat in the port area of St. Petersburg (Russia), where *Y. pestis* had never been isolated before[1]. Plasmidless strains can be devoid of one, two or even three plasmids[2] (but can have additional, cryptic ones, discussed in Chapter 6). Of all the plasmidless strains, only those that have lost the calcium-dependent plasmid (pCad⁻ strains) are avirulent, whereas the others that have lost pPla and/or pFra may exhibit varying degrees of virulence. As an example, consider the atypical strains isolated in the Southern Pre-Balkhash (Balkhash (Pre-Balkhash) desert focus, Kazakhstan). Thus, Fra⁻ mutants (i.e., lacking the pFra plasmid) are highly virulent to white mice and great gerbils, but avirulent to guinea pigs. P⁻ mutants (i.e. lacking the *pgm*-region) are highly virulent only to small five-toed jerboa. Fra⁻P⁻ mutants (i.e. lacking the pFra plasmid and *pgm*-region) are highly virulent in white mice and jerboas, but avirulent in guinea pigs.

There are also strains with reduced production of capsular antigen (Fra±), which may be due to loss of autonomy of the pFra plasmid as a result of its integration with the chromosome, or damage to the *caf* operon genes (regulatory *cafR*, auxiliary *caf1M* and *caf1A*, or structural *caf1*)[3] while the pFra plasmid remains intact. Usually such strains are isolated at the beginning or more often at the end of epizootics[4]. They are virulent to varying degrees in laboratory animals and can cause a mild or latent course of disease in humans (often atypical) with a prolonged infectious process and a long incubation period[5]. In this regard, it is crucial to remember that the detection of capsular antigen (or antibodies to it) is an important criteria for plague case detection, so the presence of strains lacking it risks false-negative results for patients. In

[1] Initially, they were identified as *Y. pseudotuberculosis*, showed weak virulence to laboratory animals, dissociated in colony form (i.e. formed S- and R-form colonies) and showed signs of bacteriophage damage. As diagnostic methods improved, they were proved to belong to *Y. pestis*, but their origin remained unclear.

[2] Иванова В. С., Лебедева С. А., и др. *Скрининг плазмид у музейных штаммов чумного микроба, выделенных из разных природных очагов*. Молекулярная генетика, микробиология, вирусология. 1990; 3:16–18.

[3] The natural mechanisms of damage in natural strains and the conditions of reversion to Fra+ have not been studied to date.

[4] This is probably due to the fact that such strains have a reduced ability to survive in the flea.

[5] There is also anecdotal evidence of human infection in the Caucasus and China with rhamnose-positive (Rha+) strains.

addition, there are strains in which capsular antigen is detected only in preparations of destroyed cells, i.e. it is synthesised but not secreted (**Figure 38.2**). And such strains have already been isolated from sick people[1].

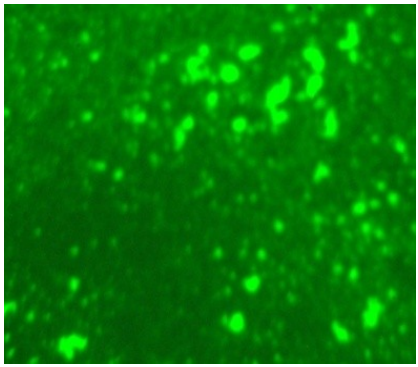


Figure 38.2. *Luminescent luminescence of the capsular antigen of the Fra± strain of Y. pestis. Note that only a small fraction of the cells are luminescent*

On the other hand, such mutants are often isolated in natural foci during the inter-epizootic period because of their high viability. Thus, *Y. pestis* strain 825, isolated in the summer of 1963 in the Bosugen tract (Southern Pribalkhashie, Kazakhstan), was easily transmitted through the chain of the great gerbil – flea – Gerbillinae, and surpassed typical virulent strains in the intensity of block formation in fleas: 61.6% of fleas in the case of atypical and only 29.6% of fleas in the case of typical strains! In other isolated Fra± mutants, this value ranges from 32 to 57%[2]. Although, as we recall, block formation is not the only mechanism of *Y. pestis* transmission, these data are quite revealing.

Since the examination of foci during the inter-epizootic period is primarily aimed at isolation of pure culture, it should be noted that Fra⁻ and Fra± strains quite often form smooth S-shaped colonies (**Figure 38.3**), typical for *Y. pseudotuberculosis*, are worse lysed by diagnostic bacteriophages, and "vole's" strains, classified as Caucasian, are not lysed at all.

We have already mentioned earlier that some strains of *Y. pestis* are able to ferment rhamnose and melibiose, produce urease and have no need for amino acids (prototrophicity) – in short, to be like two drops of water of *Y. pseudotuberculosis*. This similarity is sometimes so pronoun-

[1] Burrows T. W., Bacon G. A. *The effects of lost of different virulence determinants on the virulence and immunogenicity of strains of Pasteurella pestis*. Br J Exp Pathol. 1958; 39(3):278–291.

[2] Ларионов Г. М. *К эпизоотологической роли атипичных штаммов возбудителя чумы, выделяемых в Южном Прибалхашье и некоторых других частях Среднеазиатского пустынного очага.* Автореферат дис. … кандидата медицинских наук, Саратов, 1972, 25 с.

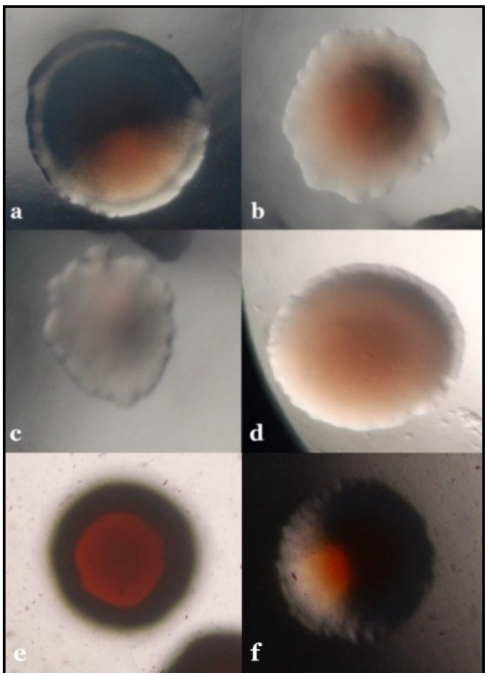


Figure 38.3. *Y. pseudotuberculosis colonies (a – typical, c – atypical) and Y. pestis colonies (b – typical, d, e and f – atypical) in an oblique beam of light (by courtesy of Dr. Tatiana E. Arsenyeva). Note how the atypical colony forms of one microbe resemble the typical colony forms of another, and compare them with* ***Figure 30.6 – Figure 30.8***

ced that before the introduction of molecular genetic methods (sequencing), there were cases of confusion when *Y. pestis* strain were registered as *Y. pseudotuberculosis* strain (and vice versa)[1].

On the other hand, *Y. pestis* and *Y. pseudotuberculosis* quite often circulate together in natural foci and are excreted as a mixture. On dishes, in addition to the typical R- and S-forms of colonies, respectively, they can also form atypical OR, OS, SR and S forms of *Y. pestis* and OR- and R-forms of *Y. pseudotuberculosis* (**Figure 38.3**). These two microbes often cause combined epizootics in carriers and vectors. In this case, pseudotuberculosis in chronic form can cause cross-immunity due to so-

[1] In rare cases, this even led to the appearance of new "averaged" species. For example, "Iolotan" strains isolated in Turkmenistan were at one time attributed to the species "*Y. pseudopestis turkmenica*". However, the advent of molecular genetic methods has often contributed to interesting discoveries. In particular, it was shown that in the *aspA* gene, responsible for the synthesis of AspA (aspartase) protein, in Altai and Hissar strains of *Y. pestis*, the same GTG triplet, which encodes the amino acid valine, is located at positions 1087-1089 as in strains of *Y. pseudotuberculosis*. At the same time, in Caucasian and Ulegei strains, the TCG triplet encoding the amino acid serine is located at this position. It is fair to say that replacing one amino acid with another in the composition of the protein does not affect its activity, namely the catalysis of deamination (i.e. removal of amino groups from the composition of the molecule) of L-aspartate to form fumarate (a component of the tricarboxylic acid cycle).

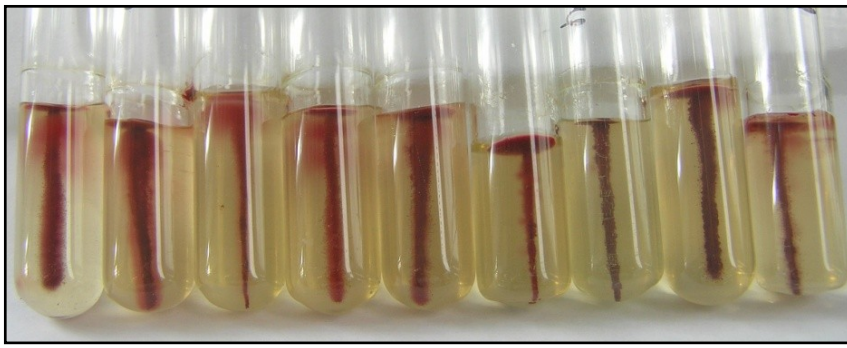


Figure 38.4. *Mobility test of atypical strains of Y. pseudotuberculosis in 0.4% Hottinger agar (1% TTC added to the medium to stain the culture). Note that in the case of some strains there is no motility at all (compare this with the result for Y. pestis in* ***Figure 30.14*** *and, in the light of this information, assess the specificity of the methods given on page* ***377****)*

me common antigens, which will contribute to the emergence of an immune layer of carriers and, as a consequence, reduce the severity of the epizootic process (its "smearing"). It is this close existence that can lead to plasmid exchange and the formation of *Y. pseudotuberculosis* clones with plasmids of *Y. pestis* showing similar properties to *Y. pestis*. For example, in addition to increased virulence, they may be auxotrophic, immobile (**Figure 38.4**), fail to synthesise urease, fail to ferment rhamnose (and melibiose), be lysed by some plague bacteriophages but not by some pseudotuberculosis bacteriophages, and be agglutinated by anti-plague anti-FI sera.

Clones containing the pPla plasmid have no comparative virulence with *Y. pestis*, because the synthesised plasminogen activator cannot switch to the active conformation when interacting with LPS, being shielded by the O-antigen. However, even in the case of serotypes O:8, O:13 and O:14, which have the R-form of LPS (i.e. no O-antigen), activation is weak and occurs only at 37°C / 98.6°F.

In this regard, of course, clones with the pFra plasmid deserve special attention, because, as it has been mentioned many times, the detection of capsular antigen (or antibodies to it) is an important criteria for plague case detection. Cloned *caf* operon genes in the vector plasmid of recombinant clones of *Y. pseudotuberculosis* provide production of a full-

fledged capsular antigen capable of inducing an immune response and, consequently, antibody formation, which is an additional factor reducing the severity of the epizootic process (due to the formation of an immune layer of carriers). In addition, it is quite possible that it was the existence of pFra$^+$ strains of *Y. pseudotuberculosis* that contributed to the formation of false ideas about the "intestinal" form of plague.

Thus, a great similarity between atypical strains of *Y. pestis* and *Y. pseudotuberculosis* can be seen, which to a great extent limits their differentiation, requiring an increase in the efficiency of plague diagnosis. Of course, the increasing introduction of molecular genetic methods of research makes it possible to unambiguously solve the question of belonging of the isolated strain, but this does not diminish the importance of their study, clarification of the mechanisms of interaction and evolution, which is currently still poorly explored. And who knows, maybe you will be the one to penetrate into the most amazing secrets that are still hidden by *Y. pestis*.

No matter what is going on, never give up!.. Develop the heart... Work for peace in your heart and in the world. No matter what is happening, no matter what is going on around you, never give up!

H.H. Dalai Lama XIV

THE FINALE

The Basilica Santa Maria della Salute. The Man in the black overcoat is still standing leaning over the copper disc. Where the source is, thence came salvation. Where there is a beginning, there is an end.

A drop of tear slowly rolled down his cheek and fell on the disc.

Nessun dorma![1]

Nessun dorma!

Let no one sleep! Let them gaze into the boundless expanse of the Universe and see among billions of stars the subtle pattern of the past and future, the interweaving of human destinies and the memory of all events! May the one who has learnt it keep it safely in his heart, carrying it through life! Because one day *e noi dovrem, ahimè, morir, morir*[2], become the sand of time under the silent gaze of the stars…

* * *

My dearest Friend! Here is the end of our journey, and I thank you for being there with me on every page of this book.

THE END



[1] It. "Let no one sleep"; aria of Calaf from the last act of opera Turandot by Giacomo Puccini (1858-1924).

[2] It. "and we will have to, alas, die, die"; too.

Supplementary materials

Brief information on the natural foci of the CIS countries

The natural foci of the suslik's type (01, 02, 03, 14, 15, 17, 37, 38) are located in Russia and Kazakhstan. The plains foci are characterised by periodic activity with long (up to 50 or more years) inter-epizootic periods, whereas in mountain foci epizootics are registered annually or almost annually.

The *Central Caucasian highland focus* (01) was discovered in 1971. Its historical boundaries, probably formed in the Early Holocene period, are determined by the range of the main host, the Caucasian Mountain ground squirrel (*Spermophilus musicus*), and lie on the northern slopes of the central part of the Main Caucasian Range. The area of this focus is 4,600 km^2. The strains of the main subspecies (2.MED0, 2.MED1) with high universal virulence (in the eastern and central parts of the focus, on the right bank of the Baksan River) and weakly virulent or avirulent strains (throughout the entire focus area) were isolated in the focus. The strains isolated from the western part of the focus (Upper-Kuban and Kuban-Malkinsky districts) are characterised by proline auxotrophy (i.e. dependence), while strains isolated from the eastern part of the focus (Baksan-Chegemsky district) are not dependent on proline. In the area between the western and eastern parts (Mal'ko-Baksansky district), both proline-dependent and proline-independent strains are have been found, but in recent years there has been a tendency towards the isolation of the former strains. An additional cryptic plasmid pCKF was isolated by plasmid composition in proline-dependent strains (2.MED1). Epizootics on susliks (*Spermophilus*) are recorded mainly annually from March to October, intensifying in July and August.

The main carrier is the Caucasian Mountain ground squirrel (*Spermophilus musicus*). *Y. pestis* was also isolated from the common vole (*Microtus arvalis*), the Ural field mouse (*Apodemus uralensis*), the house mouse (*Mus musculus*) and *Sorex* sp.

The main vector[1] is *Citellophilus tesquorum*. Also, *Y. pestis* was isolated from *Ctenophthalmus golovi, Frontopsylla semura, Neopsylla setosa, Ctenophthalmus orientalis, Oropsylla ilovaiskii, Rhadinopsylla li.*

The *Terek-Sunzha (Tersko-Sunzhensky) lowland focus* (02) was discovered in 1970. Its historical boundaries, probably formed in the Middle Pleistocene, are determined by the persistent geographical isolation of little ground squirrel (*Spermophilus pygmaeus*) settlements in the Alkhanchurt valley lying between the

[1] Here and hereafter fleas, except in special cases. The division into "main" and "secondary" is conditional, expressing the role of the vector in the focus.

Terek (Tersk) and Sunzha ranges in the foothills of the Greater Caucasus. The area of this focus is 2,480 km². The strains of the main subspecies (2.MED1) with high universal virulence were isolated in the territory of the focus. In 1978, a strain virulent for white mice and weakly virulent for guinea pigs was isolated from a suslik and classified as Caucasian strains (0.PE2). Epizootics were observed in 1970-1971, 2000 and developed against the background of increased abundance and activity of susliks and their fleas in May and July.

The main carrier is the little ground squirrel (*Spermophilus pygmaeus bohemi*). Also, *Y. pestis* was isolated from the Ural field mouse (*Apodemus uralensis*).

The main vectors: *Citellophilus tesquorum*, *Neopsylla setosa*. Also, *Ctenophthalmus golovi* fleas were found infected with plague in the focus.

The *Dagestan plain-foothill focus* (03) was discovered in 1951. Its historical boundaries, formed, according to some data, in the Early Holocene period, are determined by the area of the main carrier, the little ground squirrel (*Spermophilus pygmaeus bohemi*), and fall on the shore of the Caspian Sea, the southern part of the North Dagestan lowland, the coastal lowland and the foothills of the Eastern Caucasus. The area of this focus is 11,150 km². The strains of the main subspecies (2.MED1) with high universal virulence, additionally auxotrophic for cysteine and arginine, were isolated in the focus. In some years, strains auxotrophic for proline were isolated. All manifestations of plague (except for spilt epizootics in 1951-1952) were registered against the background of extremely low numbers of susliks and other rodents and developed in May and June. The only case of human plague was registered in 1951 in the village of Babayurt.

The main carrier is the little ground squirrel (*Spermophilus pygmaeus*). The secondary carriers are considered to be the tamarisk jird (*Meriones tamariscinus*), the house mouse (*Mus musculus*), the social vole (*Microtus socialis*).

The main vectors: *Neopsylla setosa, Citellophilus tesquorum*. Secondary vectors are considered to be *Nosopsyllus laeviceps, Nosopsyllus mokrzeckyi*. Infected *Frontopsylla semura* have been known to be found. One strain isolated from an hard ticks of the genus *Rhipicephalus*.

The *Pre-Caspian northwestern steppe focus* (14) is probably a relic of an ancient focus that occupied vast areas of southern Europe in the Middle Pleistocene. The history of formation of its modern borders is closely connected with fluctuations (i.e. periodic changes) in the level of the Caspian Sea in the Holocene, which is why it is currently located on the right bank of the Volga River and occupies the Sarpinskaya lowland with the Davan gully and the Ergeni upland (*Y. pestis* was first isolated in 1913). From 1933 to 1960 including, extermination of the main carrier was carried out in the territory of the focus, after which the

focus was considered liquidated, but epizootic activity resumed in 1972. In 1987, against the background of desertification of the landscape, reduction of settlements and the number of the main carrier (the little ground squirrel) with a simultaneous increase in the density of *Gerbillinae*, the Pre-Caspian sandy focus (43) was isolated from its composition. The area of this focus is 51152 km^2. The strains of the main subspecies (2.MED1) with high universal virulence were isolated in the focus. Epizootic activity is registered in May and June. Epidemic manifestations were observed from 1878 to 1935 (during this period, 1441 plague patients were identified).

The main carrier is the little ground squirrel (*Spermophilus pygmaeus*). The secondary carriers are considered to be the thick-tailed three-toed jerboa (*Stylodipus telum*), the midday jird (*Meriones meridianus*), the wood mouse (*Apodemus sylvaticus*), the steppe polecat (*Mustela eversmanni*). In years of mass reproduction of mouse-like animals, the latter occupied the role of the main carrier, which was the cause of autumn and winter epidemic complications.

The main vectors: *Neopsylla setosa, Citellophilus tesquorum*. The secondary vectors are considered to be *Frontopsylla setosa, Nosopsyllus laeviceps, Ctenophthalmus pollex* (in the northern and central regions of the focus).

The *Volga-Ural steppe focus* (15) was discovered in 1912. Its historical boundaries, probably formed in the Late Pleistocene, are located in the northern part of the Volga-Ural interfluve, occupying the Caspian Lowland up to the spurs of the Common Syrt. The area of this focus is 85,000 km^2 (20,873 km^2 in Russia, 64,127 km^2 in Kazakhstan). The strains of the main subspecies (2.MED1) with high universal virulence, additionally auxotrophic for leucine and cysteine, were isolated in the focus. It is here that the least atypical strains (only 1.58%) are isolated. The strains are lysed by plague and pseudotuberculosis bacteriophages, are highly sensitive to antibiotics, have no nitrifying and denitrifying activity, are glycerin-positive and sensitive to pesticin. Epizootic activity is registered in April and June. Epidemic manifestations were observed from 1878 to 1933 (during this period, 774 plague patients were identified).

The main carrier is the little ground squirrel (*Spermophilus pygmaeus*). The epizootic process also involves the house mouse (*Mus musculus*), the midday jird (*Meriones meridianus*), the common vole (*Microtus arvalis*), the tamarisk jird (*Meriones tamariscinus*), the Eversmann's hamster (*Allocricetulus eversmanni*), the great jerboa (*Allactaga jaculus*), the small five-toed jerboa (*Allactaga elater*), the lesser white-toothed shrew (*Crocidura suaveolens*), the piebald shrew (*Diplomesodon pulchellum*), the red fox (*Vulpes vulpes*). Infection of domestic animals (cats, dogs) and camels was recorded too.

The main vectors: *Neopsylla setosa, Citellophilus tesquorum*. Natural infection with *Y. pestis* has been established in *Frontopsylla semura, Ctenophthalmus pollex, Ctenophthalmus breviatus, Oropsylla ilovaiskii* has been recorded.

The *Ural-Uil steppe focus*[1] (17) was discovered in 1904 . Its historical boundaries, probably formed in the Late Pleistocene, fall within the area east of the Ural River, bounded on the north and northeast by the Inderskaya Uplands. The area of this focus is 67,400 km². The strains of the main subspecies (2.MED1) with high universal virulence were isolated in the focus. The strains isolated in the southern part of the focus auxotrophic for methionine, threonine and cysteine for growth at 28°C / 82.4°F. The strains circulating in the northern part of the focus require leucine in addition in most cases, while one strain requires leucine, tyrosine and arginine (avirulent for guinea pigs, sensitive to pesticin - similar to strains of the Transcaucasian highland focus). The epizootic state of the focus is characterised by irregularity of epizootic manifestations.

The main carrier is the little ground squirrel (*Spermophilus pygmaeus*). The epizootic process also involves the house mouse (*Mus muscullus*), the russet ground squirrel (*Spermophilus major*), the European hamster (*Cricetus cricetus*), the common vole (*Microtus arvalis*), the social vole (*Microtus socialis*), the steppe lemming (*Lagurus lagurus*), the small five-toed jerboa (*Allactaga elater*), the mid-day jird (*Meriones meridianus*), the tamarisk jird (*Meriones tamariscinus*), the great gerbil (*Rhombomys opimus*), the Eversmann's hamster (*Allocricetulus eversmanni*), the steppe polecat (*Mustela eversmanni*).

The main vectors: *Neopsylla setosa, Citellophilus tesquorum.* Also, *Ctenophthalmus breviatus, Frontopsylla semura, Oropsulla ilovaiskii* are involved in epizootics.

The *Tuvinian mountainous focus* (37) was discovered in 1964. Its borders cover South-Western and Southern Tuva, located along the Chikhachev Ridge in the west, along the perimeter of the Mongun-Taiga mountain knot, along the Tsagan-Shibetu and Tannu-Ola Ranges, stretching along the Russian-Mongolian border for 330 kilometres. At present, the territory of the focus is divided into 11 mesofoci (natural plague foci zone): the *Aspaitin,* the *Mogen-Buren,* the *Kara-Beldyr,* the *Kargin,* the *Tolaylyg,* the *Verkhne-Barlyk,* the *Barlyk,* the *Saglin,* the *Boro-Shay,* the *Chozin* and the *Despen*. Parts of the mesofoci are located in Mongolia. The area of this focus is 10,892 km². The strains of the main subspecies (4.ANT) with high universal virulence, characterised by the presence of an additional cryptic plasmid pTP33 and universal virulence for laboratory animals, were isolated in the focus. According to nutritional requirements, strains are divided into *Kargin variant* (western part), which is characterised by auxotrophy

[1] Previously the Zauralsky steppe focus.

for methionine and cysteine, and *Sagli variant* (eastern part), which is characterised by auxotrophy for methionine only, as well as the ability to form prototrophic mutants at late dates.

The main carrier is the long-tailed ground squirrel (*Spermophilus undulatus*). Secondary carriers: the Daurian pika (*Ochotona dahurica*) and the flat-headed vole (*Alticola strelzowi*). The Pallas's pika (*Ochotona pallasi*) was also involved in the epizootic process between 1967 and 1968, but its role is now of little significance. In 1984, one strain was isolated from the tarbagan marmot (*Marmota sibirica*) in the Barlyk River valley against the background of an intensive spill epizootic on long-tailed ground squirrels. Between 2012 and 2023, 6 cases of tarbagan marmot or their ectopa parasites being found infected with plague were observed. There are also known isolated cases of *Y. pestis* isolated from the narrow-headed vole (*Microtus gregalis*), the steppe polecat (*Mustella eversmanni*) and the Siberian jerboa (*Allactaga saltator*).

The main vector is *Citellophilus tesquorum.* The secondary vectors may be considered to be *Rhadinopsylla li, Oropsylla alaskensis, Frontopsylla elatoides, Neopsylla mana.* Also, *Y. pestis* was isolated from *Frontopsylla hetera, Frontopsylla frontalis, Ctenophyllus hirticrus, Amphalius runatus, Amphipsylla primaris, Ceratophyllus styx avicitelli, Paramonopsyllus scalonae, Paradoxopsyllus scorodumovi, Frontopsylla elata, Frontopsylla wagneri, Amalaraeus penicilliger, Amphipsylla longispina* and *Neopsylla pleskei*; sucking louse *Linognathoides laeviusculus,* as well as larvae and nymphs of gamasid mites and tick *Dermacentor nuttalli*.

The *Transbaikal steppe focus* (38) was discovered in 1911. Its historical boundaries, formed in the Lower Pleistocene, fall on the northern edge of the Central Asian natural plague foci zone. The area of this focus is 18,150 km^2. This focus is a part of a transboundary focus that includes the Khulun-Buir focus (China) and the Khoit-Kherlen focus (Mongolia). The strains of the main subspecies (2.ANT3) with high universal virulence were isolated in the focus. Strains isolated during epizootics among tarbagan marmots (1923-1946) are auxotrophic for methionine, cystine and phenylalanine, whereas strains isolated by 1970 (during epizootics in Daurian ground squirrels) additionally require leucine and cysteine. After 1970, no cultures were isolated (only *Y. pestis* DNA in environmental objects and serological findings). The long inter-epizootic period is attributed to a depression (i.e., decrease) in the number of carriers associated with climatic and anthropogenic factors (extermination activities against marmot and poaching).

The main carrier was, until the 1950s, the tarbagan marmot (*Marmota sibirica*), then the Daurian ground squirrels (*Spermophilus dauricus*). The secondary carriers: the Brandt's vole (*Lasiopodomys brandti*), the narrow-headed vole (*Microtus gregalis*). Sporadic cases of the Siberian jerboa (*Allactaga saltator*), the

steppe polecat (*Mustela eversmanni*) and the mountain weasel (*Mustella altaica*) infections have been recorded.

The main vector is *Citellophilus tesquorum*. The secondary vectors: *Fronopsylla luculenta luculenta, Neopsylla bidentaformis, Neopsylla pleskei, Oropsylla alaskensis (asiatica)*.

The natural foci of the tarbagan's type (31–33, 35, 40) are located in Kyrgyzstan and Kazakhstan.

The *Tian Shan highland focus* includes a group of autonomous foci: the *Sarydzhaz (Sari-Dzhas) highland* (31), the *Upper Naryn highland* (32) and the *Aksai highland* (33). In turn, the Sarydzhaz highland focus (area is 7,400 km^2; 5,000 km^2 in Kyrgyzstan and 2,400 km^2 in Kazakhstan) includes the *Kokpak*, the *Kokzhar* and the *Sarydzhaz mesofoci*; the Upper Naryn highland focus (area is 14,500 km^2) includes the *Ishtyk-Taragai*, the *Uzengeguzh*, the *Akshyirak*, the *Uchkel* and the *Bolgart mesofoci*; the Aksai highland focus (area is 8,500 km^2) includes the *Western*, the *Central*, the *Southern* and the *Eastern Aksai mesofoci*.

The Tian Shan highland focus was discovered in 1914. Its historical boundaries fall within a short strip (up to 100 kilometres) from the Khan-Tengri mountain junction in the east to Lake Chatyrkel in the west. The area of this focus is 30,400 km^2. The strains of the main subspecies with high universal virulence were isolated in the focus: in the territory of the Sarydzhaz highland focus – 0.ANT5 (additionally auxotrophic for leucine and cysteine), in the territory of the Upper Naryn highland focus – 0.ANT3 and 0.ANT5 (additionally auxotrophic for cysteine), in the territory of the Aksai highland focus – 0.ANT3 (additionally auxotrophic for leucine) and 2.MED1.

The main carrier is the gray marmot (*Marmota baibacina*). The secondary carriaers: the narrow-headed vole (*Microtus gregalis*), the grey dwarf hamster (*Cricetulus migratorius*). There have been isolated cases of infestation of the common vole (*Microtus arvalis*), the silver mountain vole (*Alticola argentatus*), the relict ground squirrel (*Spermophilus relictus*), the tolai hare (*Lepus tolai*), the stoat (*Mustela erminea*), the steppe polecat (*Mustela eversmanni*), the European badger (*Meles meles*), the red fox (*Vulpes vulpes*).

The main vectors: *Rhadinopsylla li ventricosa, Oropsylla silantiewi, Citellophilus lebedewi*. Natural infection with *Y. pestis* has been established in *Amalaraeus penicilliger, Amphipsylla asiatica, A. primaris, Chaetopsylla homoea, Neopsylla mana, Neopsylla meridiana, Neopsylla teratura, Rhadinopsylla angusta, Rhadinopsylla dahurica*; and sucking louse *Linognathoides palearcticus*, tick *Ixodes crenulatus*.

The *Alai highland focus* (35) was discovered in 1948. Its historical boundaries initially included only the north-east of the Alai Valley, but as the mountainous regions of Kyrgyzstan were surveyed, three mesofoci were discovered – the

Gulchi (1975), the East Alai and the West Alai (1988). The area of this focus is 3,500 km^2. The strains of the main subspecies (0.ANT3) with high universal virulence were isolated in the territory of the focus. They ferment glycerol and arabinose, but do not ferment rhamnose. Additionally auxotrophic for leucine. They have denitrifying ability, produce pesticin and are insensitive to it. Isolated in June 1968 from Amalaraeus penicilliger fleas collected from the narrow-headed vole (*Microtus gregalis*), *Y. pestis* strain 801 was auxotrophic for leucine and arginine, fermented rhamnose on the third day, did not reduced nitrate and was weakly virulent to guinea pigs. Isolated from the main host in 1961 in the Alai Valley *Y. pestis* strain 296 was prototrophic, while its mutant was highly virulent to laboratory animals.

The main carrier is the long-tailed marmot (*Marmota caudata*). The narrow-headed vole (*Microtus gregalis*), the juniper vole (*Microtus juldaschi*), the silver mountain vole (*Alticola argentatus*), the northern mole vole (*Ellobius talpinus*), the grey dwarf hamster (*Cricetulus migratorius*), the wood mouse (*Apodemus sylvaticus*), the house mouse (*Mus musculus*), the Turkestan rat (*Rattus pyctoris*), the forest dormouse (*Dyromys nitedula*), the Royle's pika (*Ochotona roylei*), the Turkestani red pika (*Ochotona rutila*), the tolai hare (*Lepus tolai*), the steppe polecat (*Mustela eversmanni*), the stoat (*Mustela erminea*), the least weasel (*Mustela nivalis*), the European badger (*Meles meles*) were found infected with the plague microbe in the focus.

The main vectors: *Citellophilus lebedewi, Oropsylla silantiewi, Rhadinopsylla li ventricosa. Pulex irritans* is actively involved in epizootics in the Gulchi mesofocus. Natural infection with *Y. pestis* has been established in *Amalaraeus penicilliger* and *Amphipsylla primaris.*

The *Talass highland focus* (40) was discovered in 1977. Its historical boundaries include the northern slopes of the Talas Ala-Too (Alataw) Range, which contain at least three isolated and probably unconnected mesofoci: the Beshtash, the Manas, and the Suluba-Kair. The area of this focus is 9,900 km^2 (5,500 km^2 in Kyrgyzstan, 4,400 km^2 in Kazakhstan). Currently, the focus is epidemically inactive, but there are prerequisites that determine the possibility of its activation, including the occurrence of highly active vectors of *Pulex irritans*, as well as pronounced migration of fleas from the burrows of the long-tailed marmot (*Marmota caudata*).

In the Beshtash mesofocus (epizootic since 1980), strains classified as Hissar (0.PE4h) were isolated. They are highly virulent for white mice and less virulent for guinea pigs (death occurs only on 9-12 days). The strain isolated in 1985 from the wood mouse (*Apodemus sylvaticus*) in the Sulubkair tract reduced nitrates. *Y. pestis* strains were isolated from the silver mountain vole (*Alticola argentatus*) and the grey dwarf hamster (*Cricetulus migratorius*), and antibodies

to capsule the antigen were detected in the wood mouse (*Apodemus sylvaticus*). One strain was isolated from the lesser white-toothed shrew (*Crocidura suaveolens*) caught in the Tuyuk-Umur tract in May 1988. In addition, the narrow-headed vole (*Microtus gregalis*), the forest dormouse (*Dyromys nitedula*), the northern mole vole (*Ellobius talpinus*) and the long-tailed marmot (*Marmota caudata*) inhabit the area of the mesofocus, but *Y. pestis* has not been isolated from them. The unsuffucient study of the mesofocus does not allow to unambiguously resolve the question about the possible role of individual species as carriers of *Y. pestis*. The possibility of existence of two conjugated plague foci – tarbagan's and vole's – cannot be excluded. The vectors: *Callopsylla caspia* (dominates), *Amphipsylla primaris, Leptopsylla nana, Amphipsylla kuznetzovi, Pectinoctenus nemorosus, Neopsylla pleskei, Frontopsylla protera*.

The strains of the main subspecies (2.MED1) and the strains classified as Talas strain (0.PE4t) were isolated in the Manas mesofocus (epizootics since 1977). Their distinguishing feature is their ability to degrade urea. They synthesise pesticin, but data on sensitivity to their own pesticin are inconsistent. They require cysteine, phenylalanine, threonine, methionine, valine and leucine for growth at 28°C / 82.4°F.

The main carrier is the long-tailed marmot (*Marmota caudata*).

The main vector is *Citellophilus lebedewi.*

The natural foci of the *Gerbillinae*'s type (07, 08–13, 16, 18–30, 42, 45, 46) are located in Russia, Kazakhstan, Turkmenistan, Uzbekistan, Armenia, Azerbaijan and Georgia. Foci No. 07 and 08-13 are grouped as *Transcaucasian natural foci group*; foci No. 16 and 43 are grouped as *Caspian natural foci group*, and foci No. 18-30, 42, 45 and 46 are grouped as *Central Asian desert foci group*.

The *Pre-Araks lowland focus* (07) was discovered in 1967. Its historical boundaries include semi-desert and mid-mountain steppe belts in the left bank of the middle reaches of the Araks River within the Nakhchivan Autonomous Republic (an exclave of Azerbaijan) and adjacent areas of Armenia. The area of this focus is 9,800 km^2. The strains of the main subspecies (2.MED1), additionally auxotrophic for cysteine, and strains classified as Caucasian strain (0.PE2), genetic line IIc, were isolated in the focus. The independence of the Pre-Araks plague focus is relative. This territory is separated from the Iran-Kurdistan focus by the Araks River, which cannot serve as a serious barrier to the penetration of the infectious agent. In 1967, a case of human plague with fatal outcome was reported.

The main carrier is the Vinogradov's jird (*Meriones vinogradovi*). Also, *Y. pestis* was isolated from the Persian jird (*Mieriones persicus*), the Tristram's jird (*Meriones tristrami*), the midday jird (*Meriones meridianus*), the small five-toed

jerboa (*Allactaga elater*), the house mouse (*Mus musculus*), the social vole (*Microtus socialis*).

The main vectors: *Xenopsylla conformis* and *Nosopsyllus iranus.* Natural infection with *Y. pestis* has been established in *Stenoponia tripectinata, Rhadinopsylla cedestis, Coptopsylla lamellifer, Ctenophthalmus secundus, Pulex irritans.* Single strains have been isolated from hard ticks, soft ticks and gamasid mites.

The Transcaucasian plain-foothill focus (includes the *Bozchel* (08), the *Kobystan* (09), the *Milsko-Karabakh* (10), the *Jeyranchol* (11), the *Ganja-Kazakh* (12) and the *Yorsk* (13) *mesofoci*) was discovered in 1914. Its historical boundaries include the territory of the Kura-Aras Lowland and the surrounding foothills of the Greater and Lesser Caucasus. The area of this focus is 26,500 km^2. The strains of the main subspecies (2.MED1) were isolated in the focus. The strains from the Bozchel, the Kobystan and the Milsko-Karabakh mesofoci are additionally auxotrophic for cysteine and arginine, and strains from Jeyranchol mesofocus are additionally auxotrophic for leucine.

The main carrier is *Meriones erythrourus*. The secondary carriers: the Tristram's jird (*Meriones tristrami*), the house mouse (*Mus musculus*).

The main vectors: *Xenopsylla conformis, Nosopsyllus laeviceps*. The presence of *Xenopsylla conformis* imago throughout the year can ensure constant circulation of the pathogen in Gerbillinae populations, whereas the role of *Nosopsyllus laeviceps* fleas increases only in the cold season, when their numbers prevail over *Xenopsylla conformis*. The secondary vactors: *Coptopsylla caucasica, Rhadinopsylla ucrainica, Stenoponia tripectinata*. Several strains were isolated from *Mesopsylla apscheronica, Nosopsyllus consimilis, Nosopsyllus mokrzeckyi, Stenoponia secundus*.

The Volga-Ural sandy focus (16) was discovered in 1922. Its historical boundaries, probably formed in the Late Holocene, making it one of the youngest, are located in the Pre-Caspian lowland between the Ural and Volga rivers. The boundaries coincide with the contours of the zonal sands landscape, including the intrazonal Volga-Akhtubinsk floodplain. The area of this focus is 65,000 km^2 (8,625 km^2 in Russia, 56,375 km^2 in Kazakhstan). The strains of the main subspecies (2.MED1), most often with high universal virulence (weakly virulent not more than 16.6%, avirulent 3.1-10.9%), additionally auxotrophic for leucine, were isolated in the focus. The trains with two additional cryptic plasmids (30 and 70 MDa) were isolated. Single strains ferment rhamnose on 6-10 days, and one strain is able to degrade urea. Most strains are auxotrophic for methionine, threonine, cysteine, phenylalanine and valine. Leucine-independent strains are isolated in the central and northern parts of the focus, and leucine-dependent strains in the southern part of the focus. Epizootics develop in April and May as well as in October and November. Epidemic outbreaks have been known since

1877 and occurred until 1938 (during this period, 2,421 plague patients were identified).

The main carriers: the midday jird (*Meriones meridianus*) and the tamarisk jird (*Meriones tamariscinus*), i.e., a polygostal focus. The secondary carriers: the yellow ground squirrel (*Spermophilus fulvus*), the little ground squirrel (*Spermophilus pigmaeus*), the house mouse (*Mus musculus*). In some periods the brown rat (*Rattus norvegicus*), the northern three-toed jerboa (*Dipus sagitta*), the small five-toed jerboa (*Allactaga elater*), the grey dwarf hamster (*Cricetulus migratorius*), the common vole (*Microtus arvalis*), the steppe lemming (*Lagurus lagurus*), the northern mole vole (*Ellobius talpinus*) and the lesser white-toothed shrew (*Crocidura suaveolens*) was involved in epizootics.

The main vectors: *Xenopsylla conformis, Nosopsyllus laeviceps*. Natural infection with *Y. pestis* has been established in *Coptopsylla lamellifer, Rhadinopsylla cedestis, Rhadinopsylla bivirgis, Ctenophtalmus dolichus, Neopsylla setosa, Citellophilus tesquorum, Fronopsylla semura, Ctenophthalmus pollex, Oropsylla ilovaiskii, Mesopsylla hebes, Amphipsylla rossica*; and ticks *Rhipicephalus schulzei, Hyalomma asiaticum.*

The *Ural-Emba desert focus* (18) was discovered in 1951. Its historical boundaries are located within the North-Eastern Caspian Sea, with a significant part confined to the Caspian lowlands. The area of this focus is 57,700 km^2. The outbreak has been increasing in recent years and has only recently entered the territory of West Kazakhstan oblast together with the great gerbil (*Rhombomys opimus*), which is expanding its range in a northerly direction. Strains of the main subspecies (2.MED1) with high universal virulence were isolated in the focus. The strains are additionally auxotrophic for cysteine. During the acute epizootic phase, the largest number of atypical forms (about 6.5%) is isolated. Infection of humans in the wild was mainly limited to isolated cases. Only in December 1958, 4 people were simultaneously infected as a result of cutting a sick camel.

The main carrier is the great gerbil (*Rhombomys opimus*). Epizootics sporadically involve the little ground squirrel (*Spermophilus pygmaeus*), the midday jird (*Meriones meridianus*) and the tamarisk jird (*Meriones tamariscinus*).

The main vectors: *Xenopsylla skrjabini, Nosopsyllus laeviceps, Coptopsylla lamellifer*. The secondary vectors: *Xenopsylla conformis, Neopsylla setosa, Citellophilus tesquorum*.

The *Pre-Ustyurt desert focus* (19) was discovered in 1957. Its historical boundaries are limited to the Caspian Sea coast in the west, the Chagrai Plateau in the east, River Emba in the north, and the northern and north-western chinks (i.e. steep cliffs) of Ustyurt in the south. The area of this focus is 74,000 km^2. The

strains of the main subspecies (2.MED1) with high universal virulence were isolated in the focus. In the west, mainly in the ancient delta of the Emba the strains are auxotrophic for leucine are isolated.

The main carrier is the great gerbil (*Rhombomys opimus*). The secondary carriers: the midday jird (*Meriones meridianus*), *Meriones erythrourus*, the little ground squirrel (*Spermophilus pygmaeus*).

The main vector is *Xenopsylla skrjabini.* The secondary vactors: *Coptopsylla lamellifer, Nosopsyllus laeviceps.*

The *Ustyurt desert focus* (20) was discovered in 1958. Its historical boundaries include the Ustyurt Plateau. The area of this focus is 158,000 km^2 (88,000 km^2 in Kazakhstan; 70,000 km^2 in Uzbekistan). The strains of the main subspecies (2.MED1) with high universal virulence were isolated in the focus (data on strains isolated from the Kazakhstan part of the focus). In the north (Sam sands, solonchak depressions), strains additionally auxotrophic for leucine are isolated, while in other parts of the focus – mixed strains.

The main carrier is the great gerbil (*Rhombomys opimus*). The secondary carriers: *Meriones erythrourus*, the little ground squirrel (*Spermophilus pygmaeus*).

The main vector is *Xenopsylla skrjabini.* The secondary vectors: *Xenopsylla nuttalli* (south-west areas), *Xenopsylla gerbilli caspica* (south areas), *Nosopsyllus laeviceps, Rhadinopsylla cedestis.*

The *North-Pre-Aral desert focus* (21) was discovered in 1945. Its historical boundaries include the northern coast of the Aral Sea. The area of this focus is 46,500 km^2. The strains of the main subspecies (2.MED1) with high universal virulence with close phylogenetic relationship with strains from Aral-Karakum (Pre-Aral-Kara-Kum) desert focus (24) were isolated in the focus. The strains are additionally auxotrophic for cysteine. The first outbreak of plague in the North-Pre-Aral desert focus was registered in 1945 in the settlement of Avan, located on the Kokaral peninsula (197 patients were identified). Since then, plague patients have been periodically registered in the described territory. The epizootic process runs from April to November (very rarely in the winter months) with peak activity in the spring and summer (April and June) and autumn (September and October) periods.

The main carrier is the great gerbil (*Rhombomys opimus*). Spontaneous carriage has been established in *Meriones erythrourus*, the midday jird (*Meriones meridianus*), the little ground squirrel (*Spermophilus pygmaeus*), the yellow ground squirrel (*Spermophilus fulvus*), the great jerboa (*Allactaga jaculus*), the small five-toed jerboa (*Allactaga elater*), the northern three-toed jerboa (*Dipus sagitta*), the house mouse (*Mus musculus*), the social vole (*Microtus socialis*), the thick-tailed three-toed jerboa (*Scirtopoda telum*), the Eversmann's hamster

(*Allocricetulus eversmanni*), the European hare (*Lepus europaeus*), the steppe polecat (*Mustela eversmanni*), the least weasel (*Mustela nivalis*), the stoat (*Mustela erminea*), the marbled polecat (*Vormela peregusna*), the red fox (*Vulpes vulpes*), the corsac fox (*Vulpes corsak*).

The main vector is *Xenopsylla skrjabini.* The secondary vectors: *Coptopsylla lamellifer, Nosopsyllus laeviceps.*

The *Aryskum-Dariyalyktakyr desert focus*[1] (22) was discovered in 1947. Its historical boundaries are limited to the western edge of the Dariyalyktakyr plain and the Kyzylkiya tract, between the Beleudty River and the dry channel of the Taldyespessai (in the north), the Sarysu River and the floods of the Chu River (in the east), and the Syr Darya River (in the south). The area of this focus is 47,000 km^2. The strains of the main subspecies (2.MED1) with high universal virulence were isolated in the focus.

The main carrier is the great gerbil (*Rhombomys opimus*). The secondary carriers: the midday jird (*Meriones meridianus*), the yellow ground squirrel (*Spermophilus fulvus*). In the clay soils of the northern part of the focus, the leading role is sometimes played by *Meriones erythrourus*, in the sands of Aryskum the midday jird (*Meriones meridianus*) and the yellow ground squirrel (*Spermophilus fulvus*).

The main vector is *Xenopsylla skrjabini, Xenopsylla gerbilli minax* (in the sands of Aryskum). The secondary vectors: *Coptopsylla lamellifer, Nosopsyllus laeviceps.*

The *Mangistau desert focus*[2] (23) was discovered in 1926. Its historical boundaries include the Mangyshlak and Buzachi peninsulas. The area of this focus is 67,000 km^2. The strains of the main subspecies (2.MED1) with high universal virulence were isolated in the focus. Strains auxotrophic for leucine have been isolated in the areas of the Mountainous Mangyshlak and Buzachi Peninsula. The last outbreak of plague was registered in Zhyngyldy settlement in 1999 (3 cases were identified). Since 2002, plague epizootics have been registered continuously.

The main carrier is the great gerbil (*Rhombomys opimus*). The secondary carriers: the midday jird (*Meriones meridianus*), *Meriones erythrourus*, the yellow ground squirrel (*Spermophilus fulvus*).

The main vector is *Xenopsylla skrjabini, Xenopsylla nuttalli.* The secondary vectors: *Coptopsylla lamellifer, Nosopsyllus laeviceps.*

[1] Previosly the Zaaral desert focus.

[2] Previosly the Mangyshlak desert focus.

The Aral-Karakum (Pre-Aral-Kara-Kum) desert (24) was discovered in 1947. Its historical boundaries include the northern desert subzone northeast of the Aral Sea. The area of this focus is 75,000 km². The strains of the main subspecies (2.MED1) with high universal virulence with close phylogenetic relationship with strains from the North-Pre-Aral desert focus (21) were isolated in the focus.

The main carrier is the great gerbil (*Rhombomys opimus*). The secondary carrier: the midday jird (*Meriones meridianus*), *Meriones erythrourus*. Plague carriage has been established in 25 species of wild mammals (maximal number among Central Asian desert foci).

The main vector is *Xenopsylla skrjabini.* The secondary vector: *Coptopsylla lamellifer, Nosopsyllus laeviceps.*

The Karakum (Kara-Kum) desert focus (25) was discovered in 1949. Its historical boundaries are limited to the territory of the Karakum sand desert. The area of this focus is 360,000 km². Strains of the main subspecies (2.MED1) were isolated in the focus. They ferment glycerol but do not ferment lactose, with some strains capable of fermenting rhamnose on days 1-15. Fractionless strains and strains with reduced synthesis of capsular antigen were rarely isolated.

The main carrier is the great gerbil (*Rhombomys opimus*). The secondary carriers: the midday jird (*Meriones meridianus*), *Meriones erythrourus*, the yellow ground squirrel (*Spermophilus fulvus*). Coltures of *Y. pestis* have also been isolated from the tamarisk jird (*Meriones tamariscinus*), the long-clawed ground squirrel (*Spermophilopsis leptodactylus*), the northern three-toed jerboa (*Dipus sagitta*), the comb-toed jerboa (*Paradipus ctenodactylus*), the small five-toed jerboa (*Allactaga elater*), the piebald shrew (*Diplomesodon pulchellum*), the northern mole vole (*Ellobius talpinus*), the tolai hare (*Lepus tolai*), the corsac fox (*Vulpes corsak*), the least weasel (*Mustela nivalis*), the marbled polecat (*Vormela peregusna*), and the dromedary camel (*Camelus dromedarius*), the cat (*Felis catus*).

The main vectors: *Xenopsylla hirtipes, Xenopsylla gerbilli caspica* (in the Sarykamysh depression), *Xenopsylla gerbilli gerbilli* (in the South-Eastern Karakum). the secondary vectors: *Xenopsylla nittalli, Xenopsylla conformis, Coptopsylla lamellifer, Coptopsylla olgae, Nosopsyllus turkmenicus, Oropsylla ilovaiskii, Neopsylla setosa.*

The Kopet-Dag plain-foothill focus (26) was discovered in 1955. Its historical boundaries include the north-western tip of the Turkmen-Khorasan mountain Range. The area of this focus is 15,000 km². The strains of the main subspecies (2.MED1) were isolated in the focus.

The main vector is *Meriones erythrourus*, and in the western and south-western part of the focus the main carrier of the great gerbil (*Rhombomys*

opimus). The secondary carriers: *Meriones afghanus*, the Persian jird (*Meriones persicus*), the social vole (*Meriones socialis*).

The main vectors: *Xenopsylla gerbilli gerbilli, Xenopsylla conformis, Xenopsylla nittalli, Nosopsyllus laeviceps.* Natural infection with *Y. pestis* has been established in *Nosopsyllus turkmenicus altisetus, Coptopsylla lamellifer, Coptopsylla bairamaliensis, Stenoponia vlasovi, Echidnophaga oschanini.*

The Kyzyl-Kum desert focus (27) was discovered in 1924. Its historical boundaries are limited to the Kyzyl-Kum sand desert area. The area of this focus is 385,000 km² (140,000 km² in Kazakhstan, 230,000 km² in Uzbekistan, 15,000 km² in Turkmenistan). The strains of the main subspecies (2.MED1) with high universal virulence were isolated in the focus (the data on the strains isolated from the Kazakh part). In addition, four strains auxotrophic for arginine were isolated, which, despite the presence of all virulence determinants, have low virulence for guinea pigs and white mice. Single strains auxotrophic for lysine and slightly more for leucine have been isolated. Strains whose growth depends only on cysteine have been isolated. Twelve fractionless strains have been isolated, three of which have low virulence for white mice.

The main carrier is the great gerbil (*Rhombomys opimus*). The secondary carriers: the midday jird (*Meriones meridianus*), *Meriones erythrourus*. *Y. pestis* cultures have also been isolated from the tamarisk jird (*Meriones tamariscinus*), the long-clawed ground squirrel (*Spermophilopsis leptodactylus*), the yellow ground squirrel (*Spermophilus fulvus*), the northern three-toed jerboa (*Dipus sagitta*), the small five-toed jerboa (*Allactaga elater*), the Lichtenstein's jerboa (*Eremodipus lichtensteini*), the northern mole vole (*Ellobius talpinus*), the house mouse (*Mus musculus*), the tolai hare (*Lepus tolai*), the marbled polecat (*Vormela peregusna*), and the camel.

The main vectors: *Xenopsylla gerbilli, Xenopsylla hirtipes, Xenopsylla skrjabini.* Natural infection with *Y. pestis* has been established in *Nosopsyllus tersus, Coptopsylla lamellifer, Nosopsyllus turkmenis, Echidnophaga oschanini, Paradoxopsyllus teretifrons, Rhadinopsylla cedestis, Rhadinopsylla social, Coptopsylla olgae, Coptopsylla bairamaliensis, Synosternus longispinus, Stenoponia vlasovi, Stenoponia conspecta.*

The Muyun-Kum desert focus (28) was discovered in 1958. Its historical boundaries are limited by the contours of the Muyunkum sands, located in the northern part of the sandy deserts of the Turan plain in the lower reaches of the Chu-Talas interfluve. The area of this focus is 93,000 km². The strains of the main subspecies (2.MED1) with high universal virulence, most of which do not exhibit nitrifying and dentrifying activity, were isolated in the focus. At the time of isolation, a number of strains showed growth of achromogenic, weakly granular loose colonies. In addition, a highly virulent strain that does not ferment

glycerol and an avirulent strain auxotrophic for arginine, as well as fractionless strains were isolated. Some strains are auxotrophic for arginine, isoleucine and valine, others for cysteine. Summer epizootics are registered only in the years following spring acute and spillover epizootics. There are no officially registered cases of human diseases in Muyun-Kum, but there are known stories of local residents and descriptions in archival documents of Muyun-Kum district about outbreaks similar to this infection. A case of a laboratory technician being infected with a strain of the *Y. pestis* strain of Muyun-Kum during experimental work has been described.

The main carrier is the great gerbil (*Rhombomys opimus*). The secondary carriers: the midday jird (*Meriones meridianus*), *Meriones erythrourus*, the yellow ground squirrel (*Spermophilus fulvus*).

The main vector is *Xenopsylla gerbilli minax*. Also, *Coptopsylla lamellifer, Nosopsyllus laeviseps, Xenopsylla conformis, Echidnophaga oschanini, Cotopsylla trispinus,* mite genus *Ornithodorus, Hyalomma, Haemaphysalis, Rhipicephalus* were found infected with *Y. pestis* in the focus.

The *Taukum (Tau-Kum) desert* (29) was discovered in 1949 (the first epizootic was detected in the autumn of 1964). Its historical boundaries are limited to the contours of the Taukum sand desert. The area of this focus is 30,000 km^2. Strains of the main subspecies (2.MED1) with high universal virulence, some of which do not require threonine but do require cysteine, were isolated in the focus. In recent years, epizootics have been occurring continuously.

The main carrier is the great gerbil (*Rhombomys opimus*). The secondary carriers: the midday jird (*Meriones meridianus*), *Meriones erythrourus* (in the Chu-Ili Range).

The main vectors: *Xenopsylla skrjabini, Xenopsylla hirtipes, Xenopsylla gerbilli minax*. The secondary vectors: *Xenopsylla conformis, Paradoxopsyllus teretifrons, Coptopsylla lamellifer* (in autumn), *Ctenophtalmus dolichus* (in autumn).

The *Balkhash (Pre-Balkhash) desert focus* (30) was discovered in 1948. Its historical boundaries, formed during alpine orogenesis in the Tertiary period, include the Balkhash-Alakol Basin. The area of this focus is 70,000 km^2 (there is a tendency for further expansion of the focus in the eastern direction). The strains of the main subspecies (2.MED1), auxotrophic for cysteine, were isolated in the focus. Some of the isolated strains are also auxotrophic for tryptophan, while others are auxotrophic for threonine. Fractionless strains and strains with reduced synthesis of capsule antigen were isolated. In 1993, an S-form strain was isolated from fleas of the great gerbil (*Rhombomys opimus*). In 1998, glycerol-

negative strains dissociated by glycerol fermentation (i.e., having both glycerol-positive and glycerol-negative cells[1]) were isolated in Ili Basin.

The main carrier is the great gerbil (*Rhombomys opimus*). The secondary carrier is the midday jird (*Meriones meridianus*). In the Prikaratal part (since 1990) and in the Iliya Basin, the red-cheeked ground squirrel (*Spermophilus erythrogenys*) is a secondary carrier. *Y. pestis* cultures have also been isolated from the tamarisk jird (*Meriones tamariscinus*), the long-clawed ground squirrel (*Spermophilopsis leptodactylus*), the northern three-toed jerboa (*Dipus sagitta*), the Lichtenstein's jerboa (*Eremodipus lichtensteini*), the piebald shrew (*Diplomesodon pulchellum*), the tolai hare (*Lepus tolai*), the corsac fox (*Vulpes corsak*), the least weasel (*Mustela nivalis*).

The main vectors: *Xenopsylla skrjabini, Xenopsylla hirtipes, Xenopsylla gerbilli minax.* The secondary vectors: *Xenopsylla conformis, Paradoxopsyllus teretifrons, Paradoxopsyllus repandus* (in the Iliya Basin).

The *Betpakdala (Betpak-Dala) desert focus* (42) was discovered in 1959. Its historical boundaries include the Betpak-Dala Desert. The area of this focus is 60,000 km^2 (there is a tendency for further expansion of the focus in a northerly direction). The strains of the main subspecies (2.MED1) with high universal virulence were isolated in the focus.

The main carrier is *Meriones erythrourus*. The secondary carrier is the red-cheeked ground squirrel (*Spermophilus erythrogenys*). In desalinated areas the main carrier may be the great gerbil (*Rhombomys opimus*) and the midday jird (*Meriones meridianus*); and the yellow ground squirrel (*Spermophilus fulvus*) in the Chulak-Espe tract.

The main vectors: *Xenopsylla conformis, Xenopsylla gerbilli minax, Xenopsylla skrjabini.*

The *Pre-Caspian sandy focus* (43) was separated in 1987 from the Pre-Caspian northwestern steppe focus (14) due to desertification of the landscape, reduction of settlements and the number of the main carrier (the little ground squirrel) with a simultaneous increase in the density of *Gerbillinae*. Its borders include the eastern part of the North-Western Caspian Sea, starting from the lower reaches of the Volga River in the north-east to the lower reaches of the Terek River in the south, bounding the coastal line of the Caspian Sea in the east. The area of this focus is 63,276 km^2. The strains of the main subspecies (2.MED1) with high universal virulence, additionally auxotrophic for leucine, of which about 7% are atypical (P$^-$ and pCad$^-$), were isolated in the focus. Seasonal manifestations of plague in carrier populations are confined to April and June and October and

[1] This phenomenon is illustrated in **Figure 31.3**.

November. Major epidemic outbreaks occurred from 1923 to 1936. Human cases of plague were recorded in 1947, 1948, 1979 (during this period, 128 plague patients were identified).

The main carriers: the midday jird (*Meriones meridianus*), the tamarisk jird (*Meriones tamariscinus*), the little ground squirrel (*Spermophilus pigmaeus*). Until 1954, the last animal was the only main carrier. The secondary carriers: the grey dwarf hamster (*Cricetulus migratorius*), the social vole (*Microtus socialis*), the common vole (*Microtus arvalis*), the house mouse (*Mus musculus*), the striped field mouse (*Apodemus agrarius*), the great jerboa (*Allactaga jaculus*), the small five-toed jerboa (*Allactaga elater*), the thick-tailed three-toed jerboa (*Scirtopoda telum*), the muskrat (*Ondatra zibethica*), the lesser white-toothed shrew (*Crocidura suaveolens*). In 1936, 1938, 1946 and 1988, *Y. pestis* was isolated from a brown rat (*Rattus norvegicus*).

The main vectors: *Citellophilus tesquorum, Neopsylla setosa, Nosopsyllus laeviseps, Xenopsylla conformis.* Also, *Nosopsyllus mokrzeckyi, Frontopsylla semura, Mesopsylla hebes, Nosopsyllus consimilis, Amphipsylla rossica* are periodically included in the epizootic process.

The *Alakol'sky (Pre-Alakol) lowland focus* (45) was discovered in 2000. Its historical boundaries include part of the Balkhash-Alakol lowlands. The area of this focus is 2,850 km^2. Strains of the main subspecies (2.MED1) were isolated in the focus.

The main carrier is the great gerbil (*Rhombomys opimus*). The secondary carriers: the midday jird (*Meriones meridianus*), *Meriones erythrourus*, the red-cheeked ground squirrel (*Spermophilus erythrogenys*).

The main vectors: *Xenopsylla gerbilli minax, Xenopsylla skrjabini.*

The *Ili intermountain focus* (46) was discovered in 2000. Its historical boundaries include the Ili district. Separated from the Balkhash (Pre-Balkhash) desert focus area (30) due to the lack of association of the populations of the main carrier in the Ili basin with the settlements of this species in the Southern Pre-Balkhash region and differences in the cyclicity of plague epizootics. The area of this focus is 23,900 km^2. The strains of the main subspecies (2.MED1) with high universal virulence were isolated in the focus. In addition, three strains belonging to the 0.ANT lineage (branches 0.ANT3 and 0.ANT5) and one strain belonging to the 1.IN1 branch were isolated in the focus.

The main carrier is the great gerbil (*Rhombomys opimus*). The secondary carrier is *Meriones erythrourus*.

The main vectors: *Xenopsylla gerbilli, Xenopsylla hirtipes.* The secondary vector is *Xenopsylla conformis* (parasitised by *Meriones erythrourus*).

The natural foci of the vole's type (04–06, 34, 39) are located in Russia, Tajikistan, Georgia and Armenia.

The *Transcaucasian highland focus*, comprising the *Gyumri*[1] (04), the *Prisevan* (05) and the *Zangezur-Karabakh* (06) *mesofoci*, was discovered in 1958. Its historical boundaries include the central, north-western and south-eastern parts of the Transcaucasian Highlands. The area of this focus is 26,430 km^2. The strains classified as Caucasian strains (0.PE2), which ferment rhamnose on 1-2 days, are highly virulent for white mice and common voles, but weakly virulent for guinea pigs, little ground squirrels and midday jirds, were isolated in the focus. They are characterised by rapid growth rates on nutrient media (by the end of the first day). Some strains are auxotrophic for leucine and cysteine. The strains from the Gyumri mesofocus belong to genetic lineage III, from the Prisevan mesofocus – to genetic lineage IIa, from the Zangezur-Karabakh mesofocus – to genetic lineage IIb. Epizootics occur in all seasons of the year except winter, more often in late spring and autumn. Single cases of human plague were registered in 1958, 1969 and 1975.

The main carrier is the common vole (*Microtus arvalis*). The epizootic process also included the social vole (*Microtus socialis*), *Microtus nivalis*, the European water vole (*Arvicola terrestris*), the grey dwarf hamster (*Cricetulus migratorius*), the Ciscaucasian hamster (*Mesocricetus raddei*), the wood mouse (*Apodemus sylvaticus*), the house mouse (*Mus musculus*), the forest dormouse (*Dyromys nitedula*), the Asia Minor ground squirrel (*Spermophilus xanthoprymnus*), the Eurasian water shrew (*Neomys fodiens*), the least weasel (*Mustela nivalis*).

The main vector is *Callopsylla caspia*. Spontaneous infection with *Y. pestis* has also been noted in *Amphipsylla rossica, Ctenopthalmus teres, Frontopsylla elata caucasica, Nosopsyllus consimilis, Ctenopthalmus wladimiri*.

The *Gissar highland focus* (34) was discovered in 1970. Its historical boundaries include the northern macroslope of the Hissar (Gissar) Range. The area of this focus is 400 km^2. The strains classified as Hissar strain (0.PE4h) were isolated in the focus, most of which are auxotrophic for arginine and have weak urease activity, some of which are sensitive to pesticin. A significant proportion of strains (17-75%) are sensitive to pesticin (including their own). When working with these cultures, media supplemented with cysteine, methionine, phenylalanine, leucine should be used.

The main carrier is *Microtus carruthersi*. The secondary carriers: the long-tailed marmot (*Marmota caudata*), the silver mountain vole (*Alticola argentatus*), the grey dwarf hamster (*Cricetulus migratorius*), the wood mouse (*Apodemus sylvaticus*). From 1970 to 1991, the forest dormouse (*Dyromys nitedula*), the least

[1] Previosly the Leninkan mountainous mesofocus.

weasel (*Mustela nivalis*) and the stoat (*Mustela erminea*) also served as occasional carriers. Antibodies to capsule antigen were detected in a wheatear (*Oenanthe* sp.).

The main vectors: *Callopsylla caspia, Neopsylla pleskei, Amphipsylla phaiomydis*. Also, *Y. pestis* isolation from *Frontopsylla elata* и *Laelaptidae* gamasid mites.

The East-Caucasian highland focus (39) was discovered in 1977. Its historical boundaries include the northern slopes of the Eastern Caucasus mountain Ranges. The area of this focus is 23,420 km^2. The strains classified as Caucasian strains (0.PE2), genetic line I, which are similar to strains isolated from the Transcaucasian highland focus, were isolated in the territory of the focus. They show selective virulence. In particular, a much larger number of strains are weakly virulent for white mice. The so-called "Dagestan highland" strains grow on media supplemented with arginine, phenylalanine, leucine, thiamine and sodium sulphate. They are heterogeneous in relation to calcium: out of 32 strains studied, calcium-dependent cells prevailed in 27 strains, whereas calcium-independent cells prevailed in 5 strains (from 56 to 100%). Plague manifestations in *Arvicolinae* settlements are localised, short-lived, of low intensity and confined to July and September.

The main carrier is the common vole (*Microtus arvalis*). Spontaneous carriage has been established in the Caucasian snow vole (*Chionomys gud*), the Major's pine vole (*Microtus majori*), the Daghestan pine vole (*Microtus daghestanicus*), the European water vole (*Arvicola terrestris*), the grey dwarf hamster (*Cricetulus migratorius*), the wood mouse (*Apodemus sylvaticus*), the house mouse (*Mus musculus*).

The main vector is *Callopsylla caspia*. Also, *Y. pestis* has been noted in *Megabortis turbidus, Amalaraeus dissimilis, Frontopsylla elata caucasica*; and *Ctenopthalmus intermedius* and *Ctenopthalmus golovi* at the Kokmadag section.

The natural foci of the "combined" type (36 and 44) are located in Russia and Kazakhstan.

The Gorno-Altaisk high-mountain focus (36) of combined pika's and suslik's type which is a part of the transboundary Sailugem natural plague focus was discovered in 1961. Its historical territory includes the southern part of the Altai Republic. The area of this focus is 11,597 km^2. In the territory of the focus, epizootics were associated with Pallas's pika settlements until 2012. The strains classified as Altai strains (0.PE4a) were isolated, with atypical strains occasionally occurring among them. The strains of 0.PE4a-1-1 cluster were isolated in the territory of the Kurai mesofocus in 2009-2019, the strains of 0.PE4a-1-2 cluster were isolated in the territory of the Tarkhatinsky mesofocus in 2012-2020, and strains of 0.PE4a-2-2-2 cluster were isolated in the territory of the Ulandryk

mesofocus in 1965-2010. In 2012, a strain of the main subspecies (4.ANT) was isolated from the corpse of the long-tailed ground squirrel (*Spermophilus undulatus*) in the Big Sary-Gobo tract, presumably imported from the KhuKh-Serkh-Munkh-Khairakhan focus (Mongolia). The most likely mechanism of introduction was the transfer of infected fleas by birds, possibly by the isabelline wheatear (*Oenanthe isabellina*). Subsequently, *Y. pestis* of the main subspecies was spread to grey marmot (*Marmota baibacina*) settlements almost throughout the whole territory of the focus.

Currently, the focus is polygostal, the main role in epizootics is played by the gray marmot (*Marmona baibacina*) and the Pallas's pika (*Ochotona pricei*). The Daurian pika (*Ochotona daurica*), the flat-headed vole (*Alticola strelzovi*), the long-tailed ground squirrel (*Spermophilus undulatus*), the alpine pika (*Ochotona alpina*), the winter white dwarf hamster (*Phodopus sungorus*), the Siberian jerboa (*Allactaga saltator*), the steppe polecat (*Mustela eversmanni*), the tolai hare (*Lepus tolai*) are also included in the epizootic process.

The main vectors: *Oropsylla silantiewi, Amphalius runatus, Ctenophyllus hirticrus, Frontopsylla hetera, Paradoxopsyllus scorodumovi, Rhadinopsylla dahurica, Amphipsylla primaris*. Natural infection with *Y. pestis* has been established in *Rhadinopsylla li transbaikalica, Paramonopsyllus scalonae, Paradoxopsyllus dashidorzhii, Paradoxopsyllus kalabukhovi, Citellophilus tesquorum altaicus, Frontopsylla frontalis, Rhadinopsylla altaica, Oropsylla alaskensis, Chaetopsylla homoea, Callopsylla caspia, Wagnerina tuvensis, Frontopsylla elata, Ceratophyllus styx, Amphipsylla kuznetzovi, Neopsylla mana, Paraneopsylla ioffi, Wangeria longicauda, Amphipsylla longispina.*

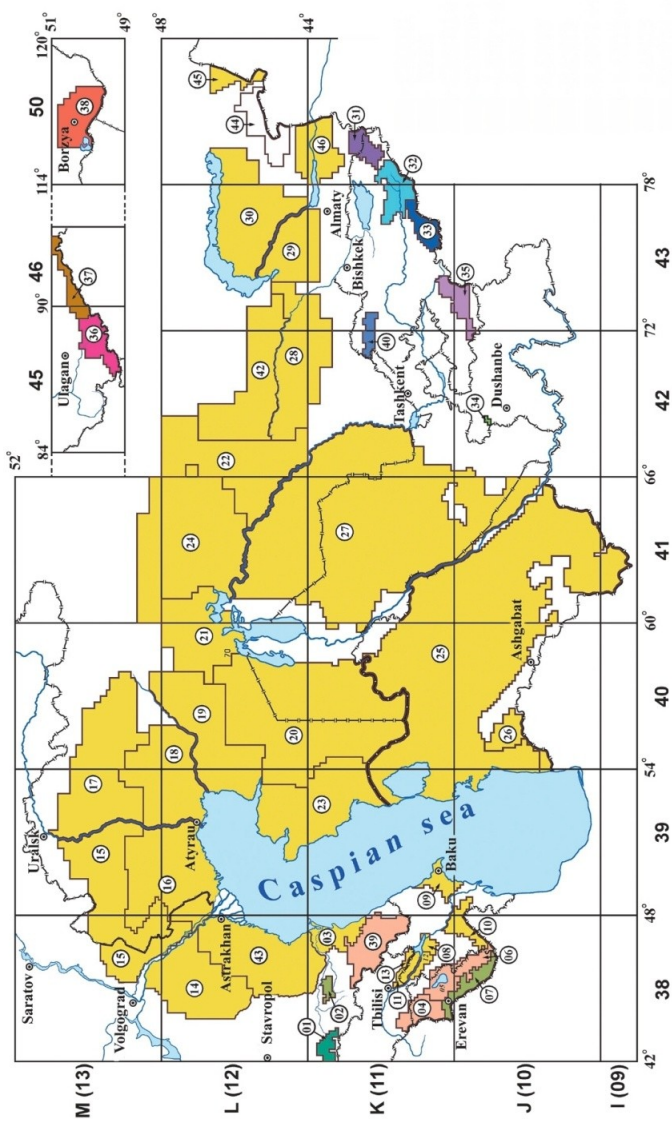
The *Dzungar mountainous focus* (44) was discovered at the end of the last century. It has a "combined" type (vote's and suslik's and marmot's). Its historical boundaries include the Dzungarian Alatau mountains, located within the boundaries of Almaty Region. The area of this focus is about 15,500 km^2. To date, no cultures of *Y. pestis* have been isolated in the territory of the focus, and the territory has been recognised as plague enzootic on the basis of positive results of serological and molecular-genetic methods. No epidemic manifestations have been registered in this territory.

The main carriers: the Tien Shan red-backed vole (*Myodes centralis*), the long-tailed ground squirrel (*Spermophilus undulatus*) and apparently the gray marmot (*Marmota baibacina*).

The main vectors: *Ctenophthalmus assimilis, Citellophilus tesquorum, Frontopsylla elata, Oropsylla silantiewi.*

It should be noted that from the Chinese side, the Dzungar mountainous focus is adjacent to the Boro-Khorin focus, with the main carriers the long-tailed

ground squirrel (*Spermophilus undulatus*) and grey marmot (*Marmota baibacina*), the main vectors *Oropsylla silantiewi* and *Citellophilus tesquorum* and circulation of *Y. pestis* of the main subspecies (0.ANT).



Overview map of natural plague foci of the CIS countries (by Г. А. Ерошенко, 2022)

Brief information on the natural foci of Mongolia

The natural foci of the tarbagan's type

The *Tavyn-Bodo-Ul (Tavan-Bogdo-Uil) focus* (MN01) was discovered in 2010. Its historical boundaries include Bayan-Ölgii aimag (Tsengel Somon) adjacent to the Tavyn-Boпdo-Ul mountain Range. The strains of the main subspecies (4.ANT), distinguished by the presence of an additional cryptic plasmid pTR33, as well as strains classified as Ulegei strains (0.PE5/2-2) were isolated in the focus. The main carrier is the gray marmot (*Marmota baibacina*). The main vector is *Oropsylla silantiewi*.

The *Kharkhira-Turgen focus* (MN03) was discovered in 1946. Its historical boundaries include the Ubsunur (Uvs) aimag (Bukhmuren, Sagil, Turgen, Taryalan, and Khovd somons). The strains of the main subspecies (3.ANT2; not isolated since 1969) were isolated in the focus. The main carrier is undetermined. *Y. pestis* was isolated from the tarbagan marmot (*Marmota sibirica*), the long-tailed ground squirrel (*Urocitellus undulatus*), the Pallas's pika (*Ochotona pallasi*) and the Daurian pika (*Ochotona dauurica*). Vectors are *Rhadinopsylla dahurica*, *Oropsylla silantiewi, Ctenophyllus hirticrus.*

The *Tsengel-Khairakhan focus* (MN04) was discovered in 1989. Its historical boundaries include the western part of Bayan-Ulgii aimag (Altai, Sagsai somons) on the Tsengel-Khairakhan ridge along the left bank of the Sagsai River. The strains of the main subspecies (3.ANT) and the strains classified as Ulegei (0.PE5/2-2) were isolated in the focus. The main carrier is the gray marmot (*Marmota baibacina*). The main vector is *Oropsylla silantiewi*.

The *Ezerleg-Buyant-Ul (Ezerleg-Buyant-Uil) focus* (MN06) was discovered in 1989. Its historical boundaries include part of Bayan-Ulgii aimag (Sagsai Somon) west and southwest of Lake Tolbo-Nur (Tolbo, Buyant, Altai Somons). The strains of the main subspecies (3.ANT) and the strains classified as Ulegei (0.PE5/2-1) were isolated in the focus. The main carrier is the gray marmot (*Marmota baibacina*). The main vector is *Oropsylla silantiewi*.

The *KhuKh-Serkh-Munkh-Khairakhan focus* (MN07) was discovered in 1982. Its historical boundaries include Bayan-Ulgii and Kobdos-Skoym aimags. The outbreak includes 8 mesofoci, which are sometimes recognised as independent foci. The strains of the main subspecies (3.ANT), including those differing by the presence of an additional cryptic plasmid pTR33 (4.ANT), as well as the strains classified as Ulegi (0.PE5/2-1), were isolated in the focus. The main carrier is the gray marmot (*Marmota baibacina*). In the south-west of the focus the The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Ulan-Sunduy (Ikh-Ulan-Davan, Buratin) focus* (MN08) was discovered in the mid-1980s. Its historical boundaries include the Hovd aimag. It is believed that epizootic events in the Bugat-nuur Lake area are territorially related to this focus and not to the KhuKh-Serkh-Munkh-Khairakhan focus (MN07). The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Sutay (Sutai-Darvin) focus* (MN09) was discovered in 1952. Its historical boundaries include the Sutai mountain junction (Darvi and Tsetseg somons of Hovd aimag, Darvi and Tonkhil somons of Gobi-Altai aimag). The strains of the main subspecies (3.ANT) were isolated in the focus. One strain classified as Altai (0.PE4a-2-1) was isolated from an unspecified flea species. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Khasagd focus* (MN10) was discovered in 1948. A number of researchers distinguish this focus as a part of the unified focus of the Mongolian Altai. Its historical boundaries include the Kha-sagd-Bogd-uul mountain Range. The strains of the main subspecies (3.ANT) were isolated in the focus. Main carriers: the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Taishir focus* (MN11) was discovered in the middle of the last century. A number of researchers distinguish this focus as a part of the unified focus of the Mongolian Altai. Its historical boundaries include the Taishiri mountain Range. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Burkhai-Buday focus* (MN12) was discovered in 1972. A number of researchers distinguish this focus as a part of the unified focus of the Mongolian Altai. Its historical boundaries are limited from the Azargyn Nuruu Range in the west almost to the Gichgeny Nuruu Range in the east. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). In 1973, the extinction of a secondary carrier, the Pallas's pika (*Ochotona pallasi*), from unknown causes was noted. The main vector is *Oropsylla silantiewi*.

The *Azh-Bogdin focus* (MN13) was discovered in the middle of the last century. A number of researchers distinguish this focus as a part of the unified focus of the Mongolian Altai. Its historical boundaries include the Azh-Bogd-uul mountain system. The strains of the main subspecies (3.ANT) and the strains classified as Ulegei (0.PE5/1) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Gichgen focus* (MN14) was discovered in 1954. A number of researchers distinguish this focus as a part of the unified focus of the Mongolian Altai. Its historical boundaries include the Gichgenii Nuruu Range. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Khan-Khukhey focus* (MN17) was discovered in 1944. Its historical boundaries include the eastern part of the Han-Huhei Range. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Songin-Tudev focus* (MN18) was discovered in 1954. Its historical boundaries include the northern part of Zavkhan aimag (Songino, Bayankhairkhan, Tudevtei somons). The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Yaru-Bogdyn focus* (MN19) was discovered in 1893. Its historical boundaries include the western macro-slope of the Khangai mountain system. The strains of the main subspecies (3.ANT) and the strains classified as Altai (0.PE4a-2-1) were isolated in the focus. The strains of the main subspecies (4.ANT), characterised by the presence of an additional cryptic plasmid pTR33, were also isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The secondary carrier is the long-tailed ground squirrel (*Urocitellus undulatus*). The main vector is *Oropsylla silantiewi*.

The *Buyant-Gol (Buyant-Otgon) focus* (MN20) was discovered in 1936. Its historical boundaries include the Buyant-Gol River basin and adjacent mountain ranges in the southeastern part of Zawhang aimag. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Tes-Shara Gol (Thesijn-Sharagol) focus* (MN21) was discovered in 1965. Its historical boundaries include the Sangilen mountain spurs and other ranges in the southwestern part of the Khubsugul aimag (Tsetserleg, Tsagaan-Uul, Arbulag somons). The strains of the main subspecies (3.ANT) and the strains classified as Altai (0.PE4a-2-1) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Bulnay (Telmen-Sogot, Sogot-Bulnay) focus* (MN22) was discovered in 1919. Its historical boundaries include Taimen and Bulnai somon of Zavkhan aimag. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Terkhin focus* (MN23) was discovered in 1880. Its historical boundaries include the southern slopes of the Tarbagatai Range. The strains of the main subspecies (3.ANT) were isolated in the focus. In 1966, a strain resistant to streptomycin was isolated. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Khanuin-Gol focus* (MN24) was discovered in 1894. Its historical boundaries include the north of Arkhangai aimag. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Chuluut-Tamir focus* (MN25) was discovered in the middle of the last century. Its historical boundaries include the south-west of Arkhangai aimag. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Shara-Usyn focus* (MN26) was discovered in 1942. Its historical boundaries include the northern regions of South Hangai. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Khuremal focus* (MN27) was discovered in 1928. Its historical boundaries include the west of Bayankhongor aimag. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Zag-Baidragin focus* (MN28) was discovered in 1953. Its historical boundaries include south-west Khangai (Bayan-Khongor aimag). The strains of the main subspecies (3.ANT) and strains classified as Altai (0.PE4a-2-1) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Dalan focus* (MN29) was discovered in 1949. Its historical boundaries include the spurs of the Dalan Range in Bayan-Khongor aimag. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Tuin Gol (Tuingol) focus* (MN30) was discovered in 1980. Its historical boundaries include the Tuin Gol and Ulziit Gol river basins. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The secondary carrier is the long-tailed ground squirrel (*Urocitellus undulatus*). The main vector is *Oropsylla silantiewi*.

The *Taats-Ongii focus* (MN31) was discovered in the middle of the last century. Its historical boundaries include the north-west of the Ubur-Khangai ai-mak

(most of it is located in the upper part of the Orkhon River valley). Some researchers recognise the territory of Khairkhandulan Somon as an independent Shar-Murui focus. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Bayan-Under focus* (MN33) was discovered in 1899. Its historical boundaries include the northeast of Ubur-Khangai, southwest of Central and southeast of Arkhangai aimags. The strains of the main subspecies (3.ANT) and the strains classified as Altai (0.PE4a-2-1) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The secondary carrier is the Brandt's vole (*Lasiopodomys brandtii*). The main vector is *Oropsylla silantiewi*. The secondary vector is *Neopsylla pleskei*.

The *Baga-Gaderyn-Ul (Baga-Gazryn-Chuluut) focus* (MN34) was discovered in the middle of the last century. Its historical boundaries include the northern part of North Gobi aimag and the south of Central aimag. The strains of the main subspecies (3.ANT) and the strains classified as Altai (0.PE4a-2-1) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The secondary carrier is the Brandt's vole (*Lasiopodomys brandtii*). The main vector is *Oropsylla silantiewi*. The secondary vector is *Neopsylla pleskei*.

The *Serven Dzamar-Ul focus* (MN35) was discovered in 1938. Its historical boundaries include the north-west of Central Aimak. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Nalgar-Ul focus* (MN36) was discovered in 1894. Its historical boundaries include the west of Central aimag. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Bogd-Khan-Ul focus* (MN37) was discovered in 1947. Its historical boundaries include the spurs of the Bogdo-Khan-Ul mountain Range of the central part of Central aimag. The strains of the main subspecies (3.ANT) were isolated in the focus. Between 2002 and 2010, the strains classified as Altai (0.PE4a-2-1), were isolated from Brandt's voles (*Lasiopodomys brandtii*). The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Baga-Khentii focus* (MN38) was discovered in the middle of the last century. Its historical boundaries include the mountains north of Ulaanbaatar. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Mungun-Mort focus* (MN39) was discovered in 1952. Its historical boundaries include north-eastwards from Ulaanbaatar in Mungunmorit Somon. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Khurkhin (Kherlen) focus* (MN40) was discovered in 1896. Its historical boundaries include the south-east of Khentei. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Zun-Khentey (Idermeg-Batnorov) focus* (MN41) was discovered in 1929. Its historical boundaries include the north-east of Khentei between the Onon and Kerulen rivers. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Otsol-Janhar focus* (MN42) was discovered in 1888. Its historical boundaries include the junction of the Khenti, East Gobi and North Gobi aimags. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Khoit-Kherlen focus* (MN43) was discovered in 1886. It is part of a transboundary centre that includes the Khulun-Buir focus (China) and the Transbaikal steppe focus (Russia). The strains of the main subspecies (2.ANT3) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Darigan focus* (MN45) was discovered in 1899 году. Its historical boundaries include the southern part of Subhatora aimag. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is undetermined. And *Y. pestis* was isolated from the tarbagan marmot (*Marmota sibirica*), the Daurian ground squirrel (*Spermophilus dauricus*), the Brandt's vole (*Lasiopodomys brandtii*) and the Daurian pika (*Ochotona dauurica*). Vectors are *Rhadinopsylla dahurica, Oropsylla silantiewi, Ctenophyllus hirticrus, Neopsylla pleskei.*

Natural foci of the *Gerbillinae*'s type

The *Southern Gobi (Khan khongor) focus* (MN16) was discovered in 1978. Its historical boundaries include the desert areas north of the Gurvan-Saikhan Range. The strains of the main subspecies (2.MED) were isolated in the focus. The main carrier is the great gerbil (*Rhombomys opimus*). The main vector is *Xenopsylla scrjabini*.

The *Tamsag-Bulag focus* (MN44) was discovered in 1947. Its historical boundaries include Eastern Aimag. The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the Mongolian gerbil (*Meriones unguiculatus*). Secondary carriers: the Daurian ground squirrels (*Spermophilus dauricus*), the Brandt's vole (*Lasiopodomys brandtii*). Main vectors are *Xenopsylla scrjabini, Nosopsyllus laeviceps, Neopsylla pleskei.*

The *Dzamyn-Ud focus* (MN46) was discovered in 1953. Its historical boundaries include the East Gobi aimag (Erdene and Urgen Somons). This focus is part of a transboundary focus that includes the Erlian focus (China). The strains of the main subspecies (3.ANT) were isolated in the focus. The main carrier is the Mongolian gerbil (*Meriones unguiculatus*). Main vectors are *Xenopsylla scrjabini, Nosopsyllus laeviceps, Neopsylla pleskei.*

Natural foci of the vole's type

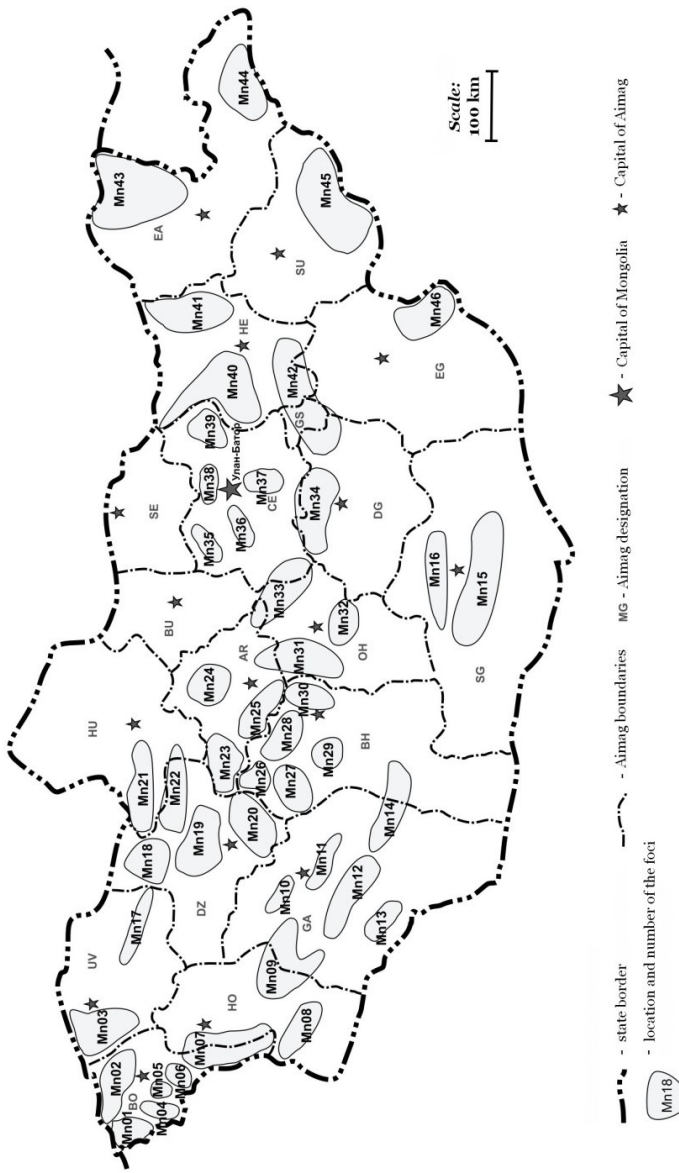
The *Sailugem focus* (MN02) was discovered in 1953. Its historical boundaries include Bayan-Ulgii aimag (Ulankhus, Tsagannur, Nogannur somons) on the southern macro-slope of the Sailugem Range. The strains classified as Altai (0.PE4a-2-1) and Ulegei (0.PE5/2-2) were isolated in the focus. The Ulegei strains are characterised by slow growth on nutrient media. The main carrier is the Pallas's pika (*Ochotona pallasi*). The secondary carrier is the gray marmot (*Marmota baibacina*). The main vector is *Paradoxopsyllus scorodumovi.*

The *Bukhen-Ul focus* (MN05) was discovered in 1968. Its historical boundaries include the central part of Bayan-Ulgii aimag (Sagsai Somon) along the right bank of the Sagsai River. The strains classified as Ulegei (0.PE5/2-2) were isolated in the focus. The 0.PE5/2-1 subbranch is formed by the strains isolated in 1974-1986, and the 0.PE5/2-2 subbranch by the strains isolated in 1972-1990. The main carrier is the Pallas's pika (*Ochotona pallasi*). The main vector is *Paradoxopsyllus dashidorzhii.*

The *Gurvan-Saikhan focus* (MN15) was discovered in 1971. Its historical boundaries include the Gichgenii Nuruu Range. The strains of the main subspecies (3.ANT) and the strains classified as Ulegei (0.PE5/1) were isolated in the focus. The main carrier is the Pallas's pika (*Ochotona pallasi*). The secondary carrier is the Mongolian gerbil (*Meriones unguiculatus*). The main vector is *Paradoxopsyllus dashidorzhii*.

The *Khankhegsh focus* (MN32) was discovered in the middle of the last century. Its historical boundaries include the foothills of the Khanhegsheen Range in Southeast Khangai. The strains classified as Altai (0.PE4a-2-1) and Ulegei (0.PE5/1) were isolated in the focus. The strains of the main subspecies (3.ANT) are rarely isolated. The main carrier is the Brandt's vole (*Lasiopodomys brandtii*).

The secondary carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Neopsylla pleskei*. The secondary vector is *Oropsylla silantiewi*.



Overview map of natural plague foci of Mongolia
(by Вержуцкий Д. Б., 2019)

Brief information on the natural foci of China and Tibet

The natural foci of the suslik's type

The *Manchurian (Central Manchurian, Songhua-Liaohe Song Liao Plains) focus* (2) was discovered in 1898. Its historical boundaries include Heilujiang, Jilin, Liaoning provinces, the northern part of Hebei province and partly the eastern part of the Inner Mongolia Autonomous Region. The area of this focus is 120,000 km^2. The strains of the main subspecies (2.ANT3) were isolated in the focus. The main carrier is the Daurian ground squirrels (*Spermophilus dauricus*). The secondary carriers are the Daurian pika (*Ochotona dauurica*), the northern three-toed jerboa (*Dipus sagitta*), the false zokor (*Myospalax aspalax*), the Chinese striped hamster (*Cricetulus barabensis*), the Campbell's dwarf hamster (*Phodopus campbelli*), the Brandt's vole (*Lasiopodomys brandti*), the striped field mouse (*Apodemus agrarius*), the house mouse (*Mus musculus*), the brown rat (*Rattus norvegicus*), азиатский the European badger (*Meles leucurus*), the steppe polecat (*Mustella eversmanii*), the least weasel (*Mustella nivalis*). The main vector is *Citellophilus tesquorum sungaris*. Secondary vectors are *Pulex irritans, Xenopsylla cheopis, Frontopsylla luculenta, Neopsylla abagaitui.*

The *Ordos (Gansu-Ningxia-Hui, Ordos-Shaanxi, Loess Plateau) focus* (15) was discovered in 1956. Its historical boundaries include a loess plateau in the bend of the Yellow (Huang He) River. The area of this focus is около 3000 km^2. The strains of the main subspecies (2.MED3) were isolated in the focus. The main carrier is the Alashan ground squirrel (*Spermophilus alashanicus*). The secondary carriers are the tolai hare (*Lepus tolai*), the Daurian pika (*Ochotona dauurica*), the corsac fox (*Vulpes corsac*). The main vector is *Citellophilus tesquorum.*

The *Qinling focus* (16) was discovered at the beginning of the last century and has not been epizootically active since the middle of the last century. Its historical boundaries included Shaanxi, Shanxi and northern Henan provinces. The area of this focus is at least 50 000 km^2. The strains of the main subspecies (2.ANT3) were isolated in the focus. The main carrier is the Daurian ground squirrels (*Spermophilus dauricus*). The main vector is *Citellophilus tesquorum.*

The natural foci of the tarbagan's type

The *Khulun-Buir (Hulun Buir, Barga) focus* (1) was discovered in 1863. Its historical boundaries include the northeastern part of the Inner Mongolia Autonomous Region. The area of this focus is 35,200 km^2. This focus is part of a transboundary centre that includes the Transbaikal steppe focus (Russia) and the Khoit-Kherlen focus (Mongolia). The strains of the main subspecies

(2.ANT3) were isolated in the focus. The main carrier is the tarbagan marmot (*Marmota sibirica*). The main vector is *Oropsylla silantiewi*.

The *Boro-Khorin (Focus B4) focus* (6) was discovered in 1956. Its historical boundaries include the Xinjiang Uygur Autonomous Region. The area of this focus is 5,000 km^2. This focus, along with the Bogda Shan focus, is considered by some researchers as part of the unified East Tian Shan focus. The strains of the main subspecies (0.ANT1 and 0.ANT2) were isolated in the focus. Main carriers are the gray marmot (*Marmota baibacina*) and the long-tailed ground squirrel (*Spermophilus undulatus*). Main vectors are *Oropsylla silantiewi* and *Citellophilus tesquorum*.

The *Irene-Khabirgin (Focus B3) focus* (7) was discovered in 1901. Its historical boundaries include the Xinjiang Uygur Autonomous Region. The area of this focus is 15,000 km^2. The strains of the main subspecies (0.ANT1) were isolated in the focus. The main carrier is the gray marmot (*Marmota baibacina*). Main vectors are *Oropsylla silantiewi* and *Callopsylla dolabris*.

The *Bogda Shan (Focus B2) focus* (8) was discovered in the middle of the last century. Its historical boundaries include the Bogda Shan Range. The area of this focus is 10,000 km^2. This focus, along with the Boro-Khorin focus, is considered by some researchers as part of the unified East Tian Shan focus. The strains of the main subspecies (0.ANT1 and 0.ANT3) were isolated in the focus. Main carriers are the gray marmot (*Marmota baibacina*) and the long-tailed ground squirrel (*Spermophilus undulatus*). Main vectors are *Oropsylla silantiewi* and *Citellophilus tesquorum*.

The *Tian Shan (Focus B1) focus* (9) was discovered in the middle of the last century. Its historical boundaries include the Xinjiang Uygur Autonomous Region. The area of this focus is 17,400 km^2. The strains of the main subspecies (0.ANT3 and 2.MED2) were isolated in the focus. The main carrier is the gray marmot (*Marmota baibacina*). The main vector is *Oropsylla silantiewi.*

The *Pamir (Focus A) focus* (10) was discovered in 1956. Its historical boundaries include the Kizilsu Kyrgyz Autonomous Prefecture (Akto and Ulugchat counties). The area of this focus is 18,400 km^2. The strains of the main subspecies (2.MED2) were isolated in the focus. The main carrier is the long-tailed marmot (*Marmota caudata*). Main vectors are *Oropsylla silantiewi* and *Rhadinopsylla vi ventricosa.*

The *Karangutag (West Kunlun, Focus K-1) focus* (11) was discovered in the late 19th century. Its historical boundaries include the Karangutag Range. The area of this focus is 10 000 km^2. The strains of the main subspecies (1.IN2, 2.ANT2 and 2.MED2) were isolated in the focus. The main carrier is the Himalayan marmot (*Marmota himalayana*). The main vector is *Oropsylla silantiewi.*

The *Altyn-Tagh (East Kunlun, Focus K-2) focus* (12) was discovered in 1911. Its historical boundaries include the Altyn-Tagh Range. The area of this focus is 102,600 km². The strains of the main subspecies (2.MED2) were isolated in the focus. The main carrier is the Himalayan marmot (*Marmota himalayana*). The main vector is *Oropsylla silantiewi.* The secondary vector is *Rhadinopsylla vi ventricosa.*

The *Nan Shan (Focus D) focus* (14) was discovered in 1956. Its historical boundaries include the area bounded by the Qiliangshan Range to the north-east and the Qaidam Basin to the south-west. The area of this focus is 10,000 km². The strains of the main subspecies (2.ANT2) were isolated in the focus. The main carrier is the Himalayan marmot (*Marmota himalayana*). The main vector is *Oropsylla silantiewi.*

The *Qinghai–Tibet (Focus C) focus* (17) was discovered in 1956. Its historical boundaries include much of the Sino-Tibetan Mountains, the centre of which, the Tanggula (Tanglha) Mountains, is the site of the greatest number of outbreaks. The area of this focus is 1 million km². Probably consists of a large number of independent foci[1]. The strains classified as Tibetan (0.PE7) and the strains of the main subspecies (0.ANT2, 0.ANT3, 1.IN1, 1.IN2, 2.MED2, 2.MED3) were isolated in the focus. In 2009, the first case of a dog infected with the primary pneumonic form of plague was registered in the territory of the focus. The main carrier is the Himalayan marmot (*Marmota himalayana*). The secondary carriers are the plateau pika (*Ochotona curzoniae*), the house mouse (*Mus musculus*), the steppe polecat (*Mustella eversmanni*), the dog (*Canis familiaris*), the Tibetan fox (*Vulpes ferrilata*), the European badger (*Meles meles*), the cat (*Felis catus*), the Eurasian lynx (*Lynx lynx*), the domestic sheep (*Ovis aries*), the Tibetan gazelle (*Procarpa picticauda*). Main vectors are *Oropsylla silantiewi* and *Callopsylla dolabris.* Secondary vectors are *Rhadinopsylla li ventricosa, Pullex irritans, Frontopsylla wagneri*; as well as ticks *Ixodes crenulatus, Rhipicephalus sanguineus, Dermacentor spp.* and sucking louse *Linognathoides palearcticus.*

The *Arkatag-Burhan-Buddai focus* (13) was discovered in 1954. Its historical boundaries include Qinghai province. The area of this focus is 40,000 km². This focus is considered by most researchers to be the northern part of the Qinghai–Tibet focus. The strains of the main subspecies (0.ANT1, 1.IN1 and 3.ANT1; since 1996, 1.IN2) were isolated in the focus. The main carrier is the Himalayan marmot (*Marmota himalayana*). Main vectors are *Oropsylla silantiewi* and *Callopsylla dolabris.*

The *Himalayas (Focus G) focus* (18) was discovered in the middle of the last century. Its historical boundaries include most of the Himalayas Mountains

[1] This idea is supported by the fact that even until recently the Karangutag focus (11) and the Altyn-Tagh focus (12) were included in the Qinghai-Tibet focus.

with adjacent ranges. The area of this focus is 250,000 km². Probably consists of several independent foci. The strains of the main subspecies (2.ANT1) were isolated in the focus. The main carrier is the Himalayan marmot (*Marmota himalayana*). Main vectors are *Oropsylla silantiewi* and *Callopsylla dolabris.*

The natural foci of the vole's type

The *Xilingol (Xilingol meadow, Seljigol, Prihingan, Focus J) focus* (3) was discovered in 1970. Its historical boundaries include the uplands of the north-eastern part of the Inner Mongolia Autonomous Region. The area of this focus is 66,400 km². Microtus biovar strains (xilingolensis and qinghaiensis) were isolated in the focus. The main carrier is the Brandt's vole (*Lasiopodomys brandti*). The secondary carriers are the Daurian ground squirrels (*Spermophilus dauricus*), the tarbagan marmot (*Marmota sibirica*), the Chinese striped hamster (*Cricetulus barabensis*), the Campbell's dwarf hamster (*Phodopus campbelli*), the house mouse (*Mus musculus*), the brown rat (*Rattus norvegicus*), the isabelline wheatear (*Oenanthe isabellina*). The main vector is *Amphipsylla primaris*. Secondary vectors are *Neopsyllus laeviceps* and *Leptopsylla pavlovskii.*

The *Qinghai-Sichuan (Qinghai-Tibet plateau, Focus I) focus* (19) was discovered in 1997. Its historical boundaries include the southeastern part of Qinghai Province and the northwestern part of Sichuan Province. The area of this focus is 20,000 km². The strains classified as Altai strains (0.PE4a) and microtus biovar strains (xilingolensis and qinghaiensis) were isolated in the focus. The main carrier is the Qinghai vole (*Microtus fuscus*). The secondary carriers are the dog (*Canis familiaris*), the cat (*Felis catus*), the domestic sheep (*Ovis aries*). Main vectors are *Callopsylla sparsilis, Amphipsylla tutua tutua, Rhadinopsylla dahurica vicina.*

The natural foci of the Gerbillinae's type

The *Erlian (Erenhot, Mongolian Plateau) focus* (4) was discovered in 1954. Its historical boundaries include the central part of the Inner Mongolia Autonomous Region. The area of this focus is 134,800 km². This focus is part of a transboundary focus that includes the Dzamyn-Ud focus (Mongolia). The strains of the main subspecies (2.MED3) were isolated in the focus. The main carrier is the Mongolian gerbil (*Meriones unguiculatus*). The secondary carriers are the tolai hare (*Lepus tolai*), the Daurian pika (*Ochotona dauurica*), the thick-tailed three-toed jerboa (*Stylodipus erythrogenys*), the red-cheeked ground squirrel (*Spermophilus dauricus*), the five-toed pygmy jerboa (*Cardiocranius paradoxus*), the Roborovski dwarf hamster (*Rhodopus roborovskii*). Main vectors are *Nosopsyllus laeviceps, Xenopsylla conformis, Neopsylla pleskei*. Secondary vectors are *Neopsylla galea, Coptopsylla lamellifer,* as well as *Haemophisalis verticalis hard ticks* and *Eulaelaps cricetuli* gamasid mites.

The *Dzungar sandy (Focus H) focus* (5) was discovered in 2005. Its historical boundaries include a small part of the Xinjiang Uygur Autonomous Region. The area of this focus is 160,000 km^2. The strains of the main subspecies (2.MED1) were isolated in the focus. The main carrier is the great gerbil (*Rhombomys opimus*). The main vector is *Xenopsylla skrjabini.*

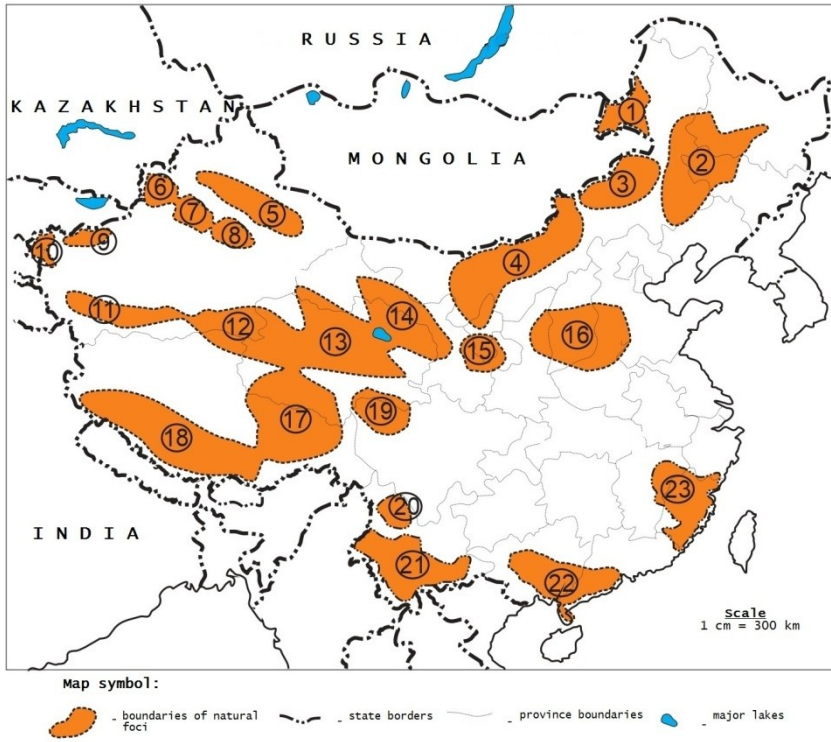
The remaining natural foci (20-23) are grouped by many researchers into one Yunnan-Guangdong-Fu Jiang focus**.**

The *Yunnan mountainous (Focus E) focus* (20) was discovered in 1974. Its historical boundaries include the central part of the Yunnan Plateau. The area of this focus is 700 km^2. The strains of the main subspecies (1.IN3 and 1.ORI3) were isolated in the focus. Main carriers are the Chevrier's field mouse (*Apodemus chevrieri*), *Eutenomys miletus*. Main vectors are *Neopsylla specialis specialis* and *Ctenophthalmus quadratus.*

The *Yunnan plains (Focus L) focus* (21) was discovered in 1956. Its historical boundaries include the southern part of Yunnan province. The area of this focus is 200,000 km^2. The strains of the main subspecies (1.ORI2) were isolated in the focus, which are subdivided into subline 1 (1.ORI2.2.1), which appeared during the 1952-1956 epidemic cycle, and subline 2 (1.ORI2.2.3.1, 1.ORI2.2.3.2, 1.ORI2.2.3.3, 1.ORI2.2.3.4), which appeared during the 1982-2006 epidemic cycle, after more than 30 years of quiescence. The main carrier is the tanezumi rat (*Rattus tanezumi*). The main vector is *Xenopsylla cheopis.*

The *Guangdong (Focus L) focus* (22) was discovered in 1894. Its historical boundaries include the southern part of Guangdong province. The area of this focus is 100,000 km^2. The strains of the main subspecies (1.ORI2) were isolated in the focus. The main carrier is the tanezumi rat (*Rattus tanezumi*). The main vector is *Xenopsylla cheopis.*

The *Fu Jiang (Focus L) focus* (23) was discovered in 1894. Its historical boundaries include the southern part of Fu Jiang. The area of this focus is 100 000 km^2. The strains of the main subspecies (1.ORI2) were isolated in the focus. The main carrier is the tanezumi rat (*Rattus tanezumi*). The main vector is *Xenopsylla cheopis.*



Overview map of natural plague foci of China and Tibet (by Вержуцкий Д. Б., 2022)

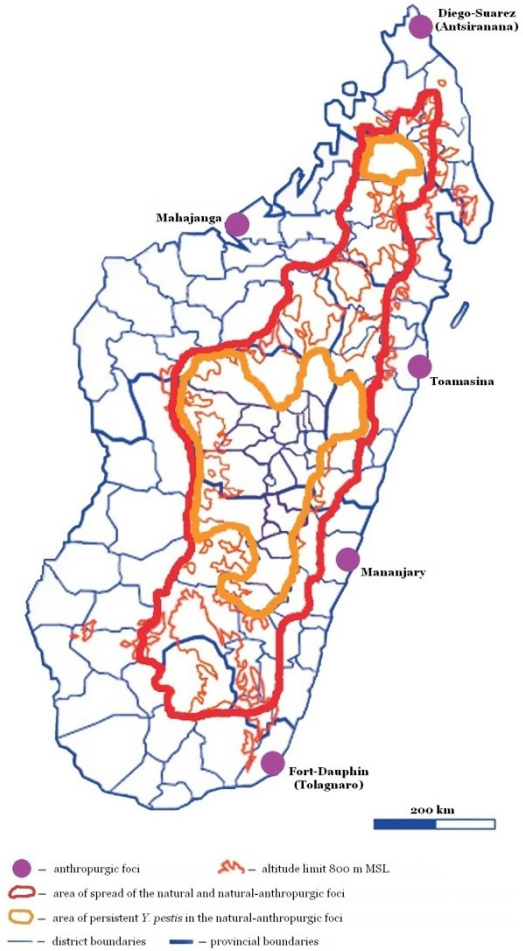
Brief information on natural foci of other countries

The most plague-endemic region of the planet is the island of **Madagascar**, to which *Y. pestis* was introduced during the third pandemic from India. The first outbreak was officially recorded in 1898 in the harbour city of Toamasina (Tamatave), and the last outbreak in August 2021 in Arivonimamo Region and city of Ambalavao. The historical boundaries of the natural foci include the territories of the foothills, mountains and highlands of the Central Plateau. However, it is generally accepted that, having "merged" with anthropurgic foci, they formed a single *complex natural-anthropurgic plague* focus occupying the territory of the central, northern and southern parts of the Central Plateau, which is characterised by constant epidemic activity with zones of high activity in the provinces of Mahajanga, Fianarantsoa, Toamasina and Antananarivo, including the capital of the same name. The areas with constant epidemic activity are characterised by high population density (more than 100 people per 1 km^2). The strains of the main subspecies (1.ORI3), representing the youngest branch of the pathogen evolution, were identified in the territory of the complex focus. The Madagascar strains fall into 1.ORI.3.k cluster and its derivative 1.ORI.3.d. The former existed already at the time of isolation of *Y. pestis* strain EV76 (vaccine strain), i.e. by 1926, and is widespread, whereas the second one most likely emerged only in 1933-1939 and is restricted to a small area of the highlands of Fianarantsoa Province.

In 1995, *Y. pestis* strain 17/95 which was resistant to streptomycin, ampicillin, kanamycin, spectinomycin, sulphonamide, tetracycline, minocycline and chloramphenicol (sensitive to co-trimoxazole) was isolated from a 16-year-old boy with symptoms of bubonic plague in town of Ambalavao, Fianarantsoa Province. the strains resistant to streptomycin (1996, 2013) and doxycycline (1998) have also been isolated in subsequent years. The main carrier is the black rat (*Rattus rattus*), but it is believed that the subspecies *Rattus rattus rattus* (coastal forests) and *Ruttus rattus alexandinus* (high plateau) support the natural focality. The main carrier in Mahajanga Province is probably the brown rat (*Rattus norvegicus*). In Ambositra District (east coast), *Y. pestis* is also isolated from the highland streaked tenrec (*Hemicentetes nigriceps*). The main vector is *Xenopsylla cheopis* (in urban areas) and *Synopsyllus fonquerniei* (in rural areas). Secondary vectors are *Synopsyllus estradei* and *Dinopsyllus brachypecten*.

The African continent lies almost entirely in the zona pestica. In the northern part, within the Sahara Desert, since the first plague pandemic, natural foci have been established in the so-called Arab Maghreb, mainly in Morocco, Algeria and Libya. The distinctive feature of these foci is their location in the coastal zone at

a distance of less than 3 km from the Mediterranean coast, and in close proximity to salt lakes, where *Y. pestis* is found in L forms. It is suggested that *Y. pestis* persists in the soil and is involved in epizootic processes with a secondary role of vectors (i.e. susceptible animals are infected directly from the soil). **Algeria** was long thought to have no natural foci until a major outbreak occurred in 2003 (50 years after the last imported case) in the region of the city of Oran, caused, according to molecular genetic testing, by a local strain of the main subspecies (1.ORI) introduced from India during the third plague pandemic. The most recent outbreak occurred in 2008 in a Bedouin camp in Thait El Maa in the Laghouat area, 550 km southwest of Algiers. Both outbreaks are caused by strains imported from India during the third plague pandemic. The strains (1.ORI2iii) isolated in foci of **Morocco** are of the same origin, while foci of **Libya** has strains of genotype 1.ANT, introduced during the first pandemic. And the last outbreak in Libya occurred in 2009 near the city of Tobruk. The carriers are the Shaw's jird (*Meriones shawi*), the fat sand rat (*Psammomys obesus*), the lesser Egyptian gerbil (*Gerbillus gerbillus*), the greater Egyptian gerbil (*Gerbillus pyramidum*), the Balochistan gerbil (*Gerbillus nanus*), the lesser Egyptian jerboa (*Jaculus jaculus*), the brown rat (*Rattus norvegicus*; Northern Morocco and harbour cities), the

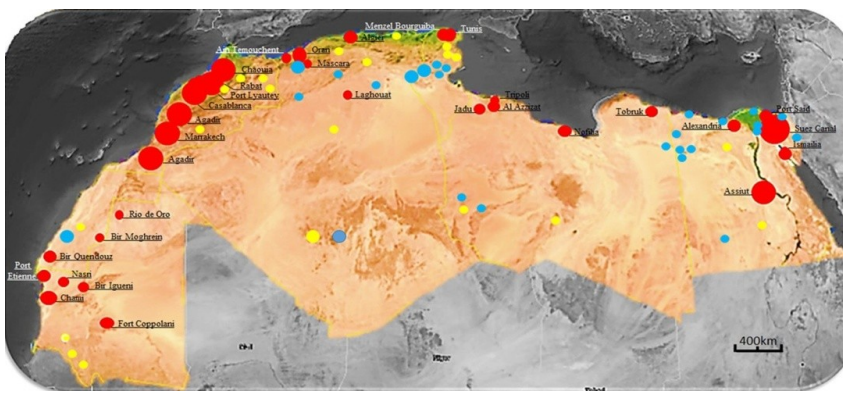


Overview map of natural plague foci of Madagascar (by B. B. Кутырев, 2011)

black rat (*Rattus rattus rattus* in northern Morocco and *Rattus rattus alexandrinus* in southern Morocco), the house mouse (*Mus musculus*), the Algerian mouse (*Mus spretus*), the wood mouse (*Apodemus sylvaticus*), the greater white-toothed shrew (*Crocidura russula*), the Libyan jird (*Meriones libycus*). The vectors are *Xenopsylla cheopis, Synosternus cleopatrae, Xenopsylla ramesis* (mainly in Algeria), *Leptopsylla segnis, Nosopsyllus fasciatus, Xenopsylla taractes, Pulex irritans*.

In North-West Africa, natural foci occur in the historic territory **Río de Oro** and **Mauritania**, as evidenced by spontaneous epizootics in rodents and camels. As in the case of North Africa, foci are located in the coastal zone, where *Y. pestis* is found as L-forms. The carriers are the lesser Egyptian gerbil (*Gerbillus gerbillus*), the greater Egyptian gerbil (*Gerbillus pyramidum*), the Balochistan gerbil (*Gerbillus nanus*). The vectors are *Xenopsylla ramesis, Xenopsylla nubica, Synosternüs cleopatrae, Xenopsylla cheopis* (in coastal cities).

In West Africa, natural foci are located in **Senegal**, where *Y. pestis* was introduced in April 1914 on a ship from Casablanca (Morocco), and in **Ghana** and **Nigeria**, where it entered from Senegal. The strains of the main subspecies (1.ORI2iii) are isolated. The carriers are the Natal multimammate mouse (*Mastomys natalensis*), the African grass rat (*Arvicanthis niloticus*), the Gambian pouched rat (*Cricetomys gambianus*), the Griselda's lemniscomys (*Lemniscomys griselda*). Rarely, epizootics involve the striped ground squirrel (*Euxerus erythropus*) and the African giant shrew (*Crocidura olivieri*). The vectors are *Xenopsylla cheopis, Xenopsylla astia, Xenopsylla brasiliensis*.



Overview map of natural plague foci of North and North-West Africa (by M. A. Malek, 2017): saltwater zones are marked in blue, freshwater zones in yellow, and epizootic zones in red

Y. pestis entered Central Africa in two ways, from Egypt during the first pandemic and from South Africa during the third pandemic. The crossroads of these routes was **Zambia**, where strains of the main subspecies of both 1.ANT group and 1.ORI2iv and 1.ORI2vii groups have been isolated in foci. In addition to Zambia, the enzootic zone includes Zaire, Uganda, Kenya, Tanzania and Malawi, extending 2,200 km from south to north and 1,000 km from east to west. The largest focus is located in Uganda, in the north-west of the country, in the so-called West Nile region, where, in addition to "mouse's" foci, there are "rat's" foci, which emerged during the construction of the Uganda railway in 1896-1908. It is likely that strains of 1.ORI2 group (which then entered Zambia) were imported at the same time, but failed to establish, so only strains of genovariant 1.ANT are currently isolated. The carriers are the Natal multimammate mouse (*Mastomys natalensis*), the black rat (*Rattus rattus*). The vectors are *Xenopsylla cheopis, Xenopsylla brasiliensis*. In **Kenya**, natural foci are confined to savannahs. The carriers include the Angoni vlei rat (*Otomys angoniensis*), the African grass rat (*Arvicanthis abyssinicus*), the four-striped grass mouse (*Rhabdomys pumilio*), the creek groove-toothed swamp rat (*Pelomys fallax*), the typical striped grass mouse (*Lemniscomys stratus*), *Tatera robusta*. The vectors are *Dinopsyllus lypusus, Xenopsylla brasiliensis, Xenopsylla cheopis*. In **Zaire**, natural foci are located on the border with Uganda near Lake Albert and Lake Edward. The situation is similar in **Tanzania**, where the foci are located near Lake Victoria and on the slopes of Mount Kilimanjaro and Pare Mountains. The carriers here are the Natal multimammate mouse (*Mastomys natalensis*), the African grass rat (*Arvicanthis abyssinicus*), the Gambian pouched rat (*Cricetomys gambianus*), the woodland thicket rat (*Grammomys dolichurus*), the forest thicket rat (*Grammomus dryas*). The vectors are *Dinopsyllus lypusus, Xenopsylla brasiliensis, Xenopsylla cheopis*.

Y. pestis was introduced to South Africa in 1900, when the Second Anglo-Boer War forced the importation of large quantities of fodder from South American ports to the city of Cape Town. From there, *Y. pestis* spread to south coast ports and further inland, establishing itself between the Congo and Zambezi river basins, where strains of the main subspecies (1.ORI2iv and 1.ORI2vii) are found, although their epizootic activity is minimal. The total area of the enzootic zone is 3.6-4 million km^2. Foci in the Kalahari Desert, areas south of the Limpopo River and between the Kwanza (Cuanza) River and Orange River are relatively active. In **RSA**, plague foci are located in the Cape Province, Orange Free State (province) and former province of Transvaal. There are four plague foci in **Angola**: in the north (inactive since 1934), Benguela Province (inactive since 1950), a transboundary one from the Okavango River to Ovamboland (Namibia), and in the south (east of the Okavango River). It is believed that this is where the "Angola strain" (0.PE3), characterised by the absence of capsule formation (in the presence of the pFra plasmid), was first discovered. In addition to

the designated transboundary focus, **Namibia** has foci on the Central Plateau of Damaraland and on the right bank of the lower Orange River. In **Zambia**, the foci are located in the western and eastern parts of the country. Interestingly, although plague has been known in this country since 1937, to date no cultures of the pathogen have been isolated (all diagnoses have been made by PCR). Four mesofoci are known in northern **Botswana**: two in the area of the dried-up Lake Ngami and west of the lower Okavango (Ngamiland), two between the channel connecting Lake Makgadikgadi to the Okavango basin and the eastern and north-eastern edges of the Okavango Delta. The carriers are *Tatera schinzi*, the Cape short-eared gerbil (*Desmodillus auricularis*), the southern multimammate mouse (*Mastomys coucha*), the Natal multimammate mouse (*Mastomys natalensis*), the four-striped grass mouse (*Rhabdomys pumilio*), the Angoni vlei rat (*Otomys irroratus*). The vectors are *Xenopsylla piriei, Xenopsylla philoxera, Xenopsylla brasiliensis, Dinopsyllus ellobius.*

In the Middle East (Near East), plague first appeared in **Palestine** at the end of World War I, after which it was not reported from 1925 to 1940. Only in 1941 and 1947 did it reappear in the city of Haifa, probably as a result of importation from the Suez Canal Zone. Further epizootic activity was not registered, from which it can be concluded that *Y. pestis* has not established itself in this territory, which is not the case with **Saudi Arabia** and **Yemen**, where the transboundary *Saudi-Yemen desert (highland plain) focus* is located. Its historical territory includes the south-western part of the Arabian Peninsula (El Asir Province and Hayran District). The strains of the main subspecies (2.MED1) were isolated in the focus. The carriers are the king jird (*Meriones rex*), the black-tufted gerbil (*Gerbillus famulus*), the Algerian gerbil (*Gerbillus garamantis*), the house mouse (*Mus musculus*), the black rat (*Rattus rattus*). No vector data available (probably *Xenopsylla cheopis, Xenopsylla piriei*).

The *Syrian-Mesopotamian (plateau-levelled) focus* is located in **Syria**, **Iran** and, probably, **Kuwait**. Its historical territory extends to the Tigris and Euphrates plains. The strains of the main subspecies (2.MED1) were isolated in the focus. The carriers are the Indian gerbil (*Tatera indica*) and the Libyan jird (*Meriones lybicus*). The vector is *Pulex irritans*.

There are two transboundary foci in **Iran**. The *Iran-Afghan lowland (desert) focus* includes the eastern part of Iran and **Afghanistan**. The strains of the main subspecies (2.MED1) were isolated in the focus. The main carriers are the great gerbil (*Rhombomys opimas*), the Libyan jird (*Meriones lybicus*), the Persian jird (*Meriones persicus*). The secondary carriers are the Vinogradov's jird (*Meriones vinogradovi*) and the Tristram's jird (*Meriones tristrami*). The vectors are *Xenopsylla buxtoni, Stenoponia tripectinata*. The *Iran-Kurdistan (Kurdo-Caspian) mountain-steppe focus* includes the western part of the Iranian Plateau, south-eastern **Turkey** and north-eastern **Iraq**. It is likely that this focus is related to the

Transcaucasian foci of the CIS countries. The strains of the main subspecies (2.MED1) were isolated in the focus. The main carriers are the Persian jird (*Meriones persicus*) and the Qazvin vole (*Microtus qazvinensis*). The carriers are the Libyan jird (*Tatera lybicus*), the Vinogradov's jird (*Meriones vinogradovi*), the Tristram's jird (*Meriones blackleri*), the Indian gerbil (*Tatera indica*). The vectors are *Xenopsylla buxtoni, Stenoponia tripectinata insperata*.

Strains introduced during the first and third pandemics are circulating in South Asia. In **India**, there are three focus in the south, on the Deccan Plateau (Andhara Pradesh, Mysuru, Chennai, Maharashtra), one focus in the central part of the country (Madhya Pradesh), and three more in the north, at the foot of the Himalayas (Punjab, Uttar Pradesh, Bihar). The strains of the main subspecies (1.ORI1, 1.ORI2 and 1.ANT1) have been isolated in the territory of foci. And 1.ANT1 group is found only in the north of the country. The "first" carriers in the central part and in the north are the Indian gerbil (*Tatera indica*) and the soft-furred rat (*Millardia meltada*); the "second" carriers are the brown rat (*Rattus norvegicus*) and the black rat (*Rattus rattus*). A distinctive feature of these foci is the double chain of carriers, i.e. infection of humans occurs after sequential infection of carriers and rats (for example: carrier – vector – rat – vector – human). The main carriers in the southern part are the lesser bandicoot rat (*Bandicota bengalensis*), the greater bandicoot rat (*Bandicota indica*). The secondary carrier are the Indian gerbil (*Tatera indica*), the brown rat (*Rattus norvegicus*), the black rat (*Rattus rattus*). The vectors are *Xenopsylla cheopis, Xenopsylla astia, Xenopsylla brasiliensis, Nosopsyllus fasciatus*.

There are no natural foci in **Europe**, except for those described earlier, located in the European part of the CIS, because after three pandemics the pathogen has not become established.

In **Nepal**, the disease was first reported in 1967-1968 and 1971 in former Setli Zone (now part of the Sudurpashchim Province), but the focus has not been studied to date. *Y. pestis* strain Nepal516 isolated in 1967 during an outbreak of pneumonic plague was non-pigmented and belonged to the major subspecies (1.ANT1). The main carrier is probably the Himalayan marmot (*Marmota himalayana*).

Y. pestis was introduced into Southeast Asia during the third pandemic from China, and therefore mainly strains of 1.ORI2ii group are isolated. Imported through ports into urban areas, the plague became established in the local rodent population, forming foci. In **Thailand**, they are located in the southern and northern parts of the country. The main carrier is the greater bandicoot rat (*Suncus murinus*). **In Indonesia**, the foci are located in the central part of the island of Java. The carriers here are the wild-living Polynesian rat (*Rattus exulans*), the ricefield rat (*Rattus argentiventer*) and *Rattus rattus diardiine*.

Within **Myanmar**, foci are located in the central and northern parts of the country, where the carriers are the brown rat (*Rattus norvegicus*), the black rat (*Rattus rattus*), the Polynesian rat (*Rattus exulans*), the Himalayan field rat (*Rattus nitidus*), the Savile's bandicoot rat (*Bandicota savilei*). The same carriers are present in foci in southern **Vietnam**, where *Y. pestis* has not been isolated since 2005 (it is quite possible that it was the extermination of rats in human settlements that led to the elimination of anthropurgic foci)[1]. The capsular antigen was isolated from the Asian red-cheeked squirrel (*Dremomys rufigenis*), the Berdmore's ground squirrel (*Menetes berdmorei*), the Pallas's squirrel (*Callosciurus erythraeus*), the Himalayan striped squirre (*Tamiops mcclellandii*), the Bower's white-toothed rat (*Berylmys bowersi*), the Edwards's long-tailed giant rat (*Leopoldamys edwardsi*), the flat-haired mouse (*Mus platythrix*) and the greater bandicoot rat (*Bandicota indica*). The vector (probably the only one) is *Xenopsylla cheopis.* In the foci of both countries, the strains of 1.ORI2ix group have been isolated.

Y. pestis came to South America in 1899, when a ship with Indian rice arrived in the Uruguayan capital of Montevideo from Rotterdam, The Netherlands (the first outbreak occurred in 1901). The cargo then travelled by steamship to the Argentine capital of Buenos Aires and ports of La Plata, and from there to the Paraguayan capital of Asuncion. Thus, the strains of 1.ORI2viii group appeared in Latin America. In addition to this gruop, the strains of 1.ORI2v group are also currently isolated. **Argentina** and **Peru** have foci in the central and northern parts of the country, **Bolivia** in east of the central massif of the Central Andes, **Brazil** in the north-eastern parts of the country, **Ecuador** in the central, southern and western parts, and **Venezuela** in the northern part of the country. The main carriers are the Brazilian guinea pig (*Cavia aperea*), the southern mountain cavy (*Microcavia australis*), the common yellow-toothed cavy (*Galea musteloides*), the gray leaf-eared mouse (*Phyllotis griseoflavus*), the gray leaf-eared mouse (*Lagidium viscacia*), the gray short-tailed opossum (*Monodelphis domestica*); rice rats *Oryzomys andinas* (in Peru) and *Oryzomys subflavus* (in Brazil). The secondary carriers are small hamsters of genus *Akodon* (*Akodon mollis* in Ecuador), *Holochilus, Eligmodontia, Phyllotis*, as well as the common tapeti (*Sylvilagus brasiliensis*) and the European hare (*Lepus europaeus*). The vectors are *Polygenis platensis cisandinus, Polygenis byturus, Panallius galeanus, Delostichus talis, Hectopsylla eskeyi* (in Peru).

Y. pestis was brought to North America from the city of Hong Kong (China) on a ship that in 1899 arrived in the city of Honolulu (Hawaii), and from there to the port of San Francisco (USA). In **Hawaii**, the epidemic raged for about ten

[1] For this reason, some researchers have disputed the existence of natural foci in Vietnam.

years, spreading to all islands. Epizootics continued until 1949. Most researchers agree that due to high rainfall and a tropical trade wind climate, no natural foci have formed here, which is not the case in the **USA**, where natural foci have formed in 15 states (west of 98° W). The strains of the main subspecies (1.ORI1) were isolated in the foci. In the Pacific zone, semi-deserts and deserts of the Midwest, the main carriers are the Richardson's ground squirrel (*Spermophilus richardsoni*), the Columbian ground squirrel (*Spermophilus columbianus*), the Uinta ground squirrel (*Spermophilus armatus*), the Belding's ground squirrel (*Spermophilus beldingi*), the California ground squirrel (*Otospermophilus beecheyi*). In the dry steppes of the Midwest, semi-deserts and deserts of the central and southeastern parts of the Intermountain Plateau, the main carriers are Prairie dogs of the genus *Cynomys* and rodents of the genus *Neotoma*. In the California focus, the main carriers are the California vole (*Microtus californicus*) and rodents of the genus *Peromyscus*. The secondary carriers are the least chipmunk (*Neotamias minimus*), the Colorado chipmunk (*Neotamias quadrivittatus*), the Townsend's chipmunk (*Neotamias townsendii*), the northern flying squirrel (*Glaucomys sabrinus*), the yellow-bellied marmot (*Marmota flaviventris*), the black-tailed jackrabbit (*Lepus californicus*), as well as the cat (*Felis catus*) and the dog (*Canis familiaris*). The vectors are *Diamanus montanus, Oropsylla idahoensis, Thrassis bacchi, Thrassis pandorae, Hoplopsyllus anomalus, Opisocrostis hirsutus, Orchopeas sexdentatus, Malaraeus telchinus, Hystrichopsylla linsdalei, Megabothris clantoni*.

Y. pestis was expected to arrive in **Canada** in 1938, so a survey of wild rodents was conducted in the western part of the country, but no *Y. pestis* was detected. It was not until the following year that an epizootic was recorded in the steppe zone of southeastern part of province of Alberta, and by 1946 the enzootic zones had spread to the province of Saskatchewan. The carriers are the Richardson's ground squirrel (*Citellus richardsoni*) and the yellow-bellied marmot (*Marmota flaviventris*).

In **Mexico**, the foci are located in the desert part of the country (state of Coahuila), where the carriers are the *Cynomys mexicanas* (*Cynomys mexicanus*).

Japan, as Kitasato Shibasaburō writes, "*did not know about plague until the disease was first brought on a merchant ship in 1896*". It is about the importation to the island of Formosa (now Taiwan), which as a result of the First Sino-Japanese War (1894-1895) was given to the Japanese (the infection did not spread beyond the island). Then, in 1899, *Y. pestis* was brought to the city of Kobe (it is reported that the source of infection was imported cotton, which infected harbour rats), which started the first epidemic in the history of Japan[1]. The second outbreak

[1] It has been shown many times before how *Y. pestis* has influenced Japanese culture (think of the plague Jinja-shrine, the yōkai Amabie, etc.), which contradicts what Kitasato

occurred in 1902 (city of Yokohama) and the third in 1905 (Tokyo City). Subsequent outbreaks were reported in the port city of Kigo, city of Osaka, Mie Prefecture and Kanagawa Prefecture. No cases have been reported since 1929.

First page of the manuscript by Kitasato Shibasaburō ►
「日本におけるペストの蔓延及び撲滅」
("Plague outbreaks and eradication in Japan").
Courtesy of the 学校法人北里研究所北里柴三郎記念博物館

Shibasaburō says. The fact is that traditionally infections from Japanese legends are associated with plague, but it can be seen that, for example, the "aquatic" origin of Amabie points more to cholera (moreover, the "cult" of this yōkai eventually spread to it). All *Y. pestis* strains isolated to date in the Japanese islands belong to bv. orientalis, so there is no reliable evidence of the presence of *Y. pestis* before the third pandemic, but some experts, based on the data of historical chronicles, do not exclude the possibility of its earlier introduction, for example, from the Korean peninsula as a result of trade relations.

日本ニ於ケル「ペスト」ノ蔓延及撲滅

東京傳染病研究所長
醫學博士　北里柴三郎

日本ニハ古來「ペスト」ヲ知ラサリシガ千八百九十六年始メテ一商船ノ香港ヨリ病毒ヲ齎ラセシ以來本病菌ノ侵入セルコト已ニ三十六回ニ達セリ然レモ其多數ニ於テハ輸入港ニ於テ直チニコレヲ撲滅セルヲ以テ著レキ流行ヲ起セルモノハ僅カニ三回ナリトス、

第一回ノ流行ハ千八百九十九年十月神戸市ニ起リ次ヲ大阪市ニ蔓延シ和歌山縣下湯淺町ノ流行ヲ終リトシ千九百一年四月ニ至リテ止メリ此流行ニ於テ患者二三一、死亡二〇八、有菌鼠二六二ヲ出セリ、

起テ千九百二年三月長崎市ニ於テ二名ノ「ペスト」ヲ出タセルモ流行

Prescriptions (formulations) of some nutrient media used for work with *Y. pestis*

Cary – Blair Medium (transport)

Dissolve 1.1 g sodium hydroorthophosphate, 1.5 g sodium thioglycolate, 5 g sodium chloride and 5 g agar in 1000 ml distilled water, then heat until dissolved and cool to 50°C / 122°F. Add 9 ml of 1% aqueous calcium chloride solution; if necessary, bring the pH to 8.4. Pour into small bottles with screw cap to prevent drying out.

The low redox potential of the medium favours the long-term survival of the bacteria. *Y. pestis* survives for 75 days.

Preservative of Berlin and Bascheva (transport)

Mix petroleum jelly and paraffin in the ratio of 3:1 or pure petroleum jelly, vaselin oil and anhydrous lanolin in the ratio of 1:2:1.

Y. pestis survives for 30 days.

Brquet's fluid for storing organ slices

Dissolve 20 ml of glycerine and 2 g calcium carbonate in 80 ml distilled water. Pour into 5-10 ml containers.

Pieces of organs and fragments of femur bone are preserved for several days.

Hottinger's broth

Clean beef meat from fat, tendons and films, cut into pieces about 1 cm thick and 3-4 cm long, put into boiling water in the proportion of 1 kg meat per 2 litres of water. Boil for 20 minutes, after which take out the pieces of meat and pass through a meat grinder. Then put into water cooled to 50°C / 122°F (in which boiled meat) and add 3 ml/L sodium carbonate, 20-30 ml chloroform and 100 g pancreatic gland, pre-cleaned of films and fat and passed through a meat grinder. All ingredients should be prepared in advance, so that the water does not get cold as a result of the manipulations performed. Cover the vessel with heavy paper to avoid evaporation of chloroform and keep for a day and a half at 45-50°C / 113-122°F, when instead of meat at the bottom will remain only a small slime-like sediment (digestion is over), and above it - a liquid of yellow colour. Digest, stirring well, every 24 hours at 37°C / 98.6°F for 7-8 days with daily determination of amine nitrogen. End the manipulation when the amine nitrogen content stops rising. Then filter and add 20-30 ml/L chloroform. Dilute

with distilled water to 100 mg% amine nitrogen, add 5 g/L sodium chloride and boil for 10-20 minutes. Filter and autoclave for half an hour at 110°C / 230°F.

Hottinger Agar

Boil Hottinger's broth, then add 2.3% pre-soaked agar and 1.2 g/L sodium hydroorthophosphate until clarified[1]. Autoclave for 40 minutes at 115°C / 239°F, then leave for 18-20 hours at 60-70°C / 140-158°F for precipitation. Filter the supernatant, after which autoclave for one and a half hours at 100°C / 212°F.

To grow *Y. pestis*, add the required amino acids to broth or agar (see **Table 30.1** and **Table 30.2**). To obtain a culture with more a capsule antigen, add 199 medium (3 ml of nutrient supplement per 100 ml of medium).

Martin's broth

Wash the outside of uncut fresh stomachs cleaned of fat and membranes with water. Then cut open, clean the inside without removing the mucus from the inner wall (be careful not to remove the pepsin). Cut into large pieces and grind. In a litre of water heated to 54°C / 129.2°F, put 200 g ground stomachs and add 10 ml of hydrochloric acid. Mix well and leave for 24 hours at 45-50°C / 113-122°F (stirring occasionally). After 24 hours, when at the bottom instead of meat will remain only a small slime-like sediment (digestion is over), autoclave 5 minutes at 80-85°C / 176-185°F (stir carefully to avoid disturbing the sediment). Then cool, filter, heat to 80°C / 176°F and add 20% sodium hydroxide solution (to pH 7), then boil for 10 minutes, filter and mix with an equal volume of filtered meat water. Then boil for 30-40 minutes and add 20% sodium hydroxide solution (to pH7.8). Autoclave for 20 minutes at 120°C / 248°F (pH may decrease slightly).

Note: some formulations call for the addition of a 1% sodium chloride solution, but this is prohibited when working with *Y. pestis*!

Martin Agar

Add 20% sodium hydroxide solution (to pH 8) and 2.3% agar soaked for 10-15 minutes to Martin's broth. Autoclave for 20 minutes at 120°C / 248°F or boil for 30 minutes after boiling. After filling into vials, autoclave for another 20 minutes at 115°C / 239°F.

To grow *Y. pestis*, add the required amino acids to broth or agar (see **Table 30.1** and **Table 30.2**).

Sheep blood agar (SBA)

[1] Brighten can be egg white, for which cooked agar cooled to 50°C / 122°F and pour into it, stirring, egg white, equally mixed with distilled water (20-30 ml per 1 litre).

Dissolve 14 g pancreatic digest of casein, 4.5 g neutralised peptone, 4.5 g yeast extract, 6 g sodium chloride, 50 ml defibrinated sheep blood and 12.5 g agar in 1000 ml distilled water. Store at 4-8°C / 39.2-46.4°F.

Lysogeny broth (LB)

Dissolve 10 g tryptone (i.e. a mixture of peptides obtained by hydrolysis of casein by trypsin protease), 5 g yeast extract and sodium chloride (0.5 g by Luria, 5 g by Lennox, 10 g by Miller) in 800 ml distilled water, then add distilled water to a final volume of 1000 ml. Add 15 g agar to obtain a solid medium. Autoclave for 15 minutes at 121°C / 249.8°F, cool to 45-50°C / 113-122°F and mix thoroughly.

199 medium (nutrient supplement)

To Hanks' Balanced Salt solution (HBSS), add M199 medium, i.e., dilute 80 mg sodium chloride, 4 mg potassium chloride, 1 mg magnesium sulfate-7-water, 1 mg magnesium chloride-6-water, 0.6 mg sodium dihydrophosphate (can be substituted with 0, 75 mg 2-water or 1.5 mg 12-water), 0.6 mg potassium dihydroorthophosphate, 10 mg glucose, 1.4 mg calcium chloride (may be replaced by 1.85 mg 1-water or 2.76 6-water), then bring to 1000 ml with distilled water and add 25 mg L-alanine, 70 mg L-arginine chloride, 30 mg asparagine acid, 0.0987 mg L-cysteine chloride, 66.82 mg L-glutamic acid, 100 mg L-glutamine, 0.05 mg glutathione, 50 mg glycine, 21.88 mg L-histidine chloride-1-water, 10 mg L-hydroxyproline, 20 mg L-isoleucine, 60 mg L-leucine, 70 mg L-lysine chloride, 15 mg L-methionine, 25 mg L-phenylalanine, 40 mg L-proline, 25 mg L-serine, 30 mg L-threonine, 10 mg L-tryptophan, 49.72 mg L-tyrosine disodium salt, 25 mg L-valine, 0.05 mg L-ascorbic acid, 0.01 mg biotin, 0.1 mg calciferol, 0.01 mg D-calcium pantothenate, 0.5 mg choline chloride, 0.01 mg folic acid, 0.05 mg inositol, 0.019 mg menaphthon sodium-3-water bisulfate, 0.025 mg nicotinic acid, 0.025 mg nicotinamide, 0.05 mg para-amino-benzoic acid, 0.025 mg pyridoxal chloride, 0.025 mg pyridoxine chloride, 0.01 mg riboflavin, 0, 01 mg thiamine chloride, 0.01 mg D-L-tocopherol phosphate disodium salt, 0.1147 mg vitamin A acetate, 185.5 mg calcium bicarbonate chloride, 400 mg potassium chloride, 60 mg potassium dehydroorthophosphate, 200 mg magnesium sulfate-7-water, 8000 mg sodium chloride, 47.5 mg sodium hydroorthophosphate, 10 mg adenine sulfate, 0.2 mg 5-AMP, 10 mg ATP disodium salt, 0.2 mg cholesterol, 0.5 mg 2-deoxyribose, 1000 mg D-glucose, 0.3 mg guanine chloride, 0.3 mg hypoxanthine, 0.5 mg D-ribose, 36.71 mg sodium acetate, 0.3 mg thymine, 5 mg tween-80, 0.3 mg uracil and 0.3 mg xanthine. Filter (after filtration the final pH may decrease by 0.1-0.3).

Medium with blood and gentian violet (by Victor M. Tumansky)

To 100 ml pre-melted and cooled to 45°C / 113°F Hottinger agar or Martin agar add 1 ml lysed blood and 0.5-1 ml alcohol-water solution of gentian violet. The prepared agar medium should have a pale violet colour.

Medium with sodium sulphite and gentian violet (by Galina N. Lenskaya)

To 100 ml pre-melted and cooled to 45°C / 113°F Hottinger agar or Martin agar add 1 ml freshly prepared 2.5% sodium sulfite solution and 0.5-1 ml alcohol-water solution of gentian violet. The prepared agar medium should have a faint violet colour, lighter than that of agar with blood and gentian violet, because sodium sulphite slightly reduces gentian violet.

Colour differential medium (by L. A. Timofeeva)

Dissolve 8 g pancreatic digest of casein, 3 g sodium chloride, 0.69 g sodium dihydrophosphate, 0.69 g glucose, 9.6 g lactose, 4.8 g urea, 0.09 g fuchsine acidic, 0.09 g bromthymol blue and 8.65 g agar in 1000 ml distilled water, bring to a boil and boil for about 3-5 minutes until the agar is completely dissolved. Then filter and pour into test tubes so that the surface of the bevelled part is twice the height of the column. Autoclave with flowing steam (i.e. at 100°C / 212°F with the vent valve open) for 30 minutes three times.

Medium for mobility studying

Dissolve 4 g pancreatic digest of casein, 4 g sodium chloride, 1 g sodium dihydrophosphate and 4 g agar in 1000 ml distilled water, then bring to the boil and boil until dissolved. Then filter and autoclave for 20 minutes at 121°C / 249.8°F.

Medium for the determination of pesticinogenic activity

Add a solution of gentian violet (0.2 ml per 100 ml medium, i.e. 1:500000) to 1.5 % Hottinger agar (pH 7.2). Pour no more than 20 ml into dish.

HmsD medium (by E. G.Boolgakova with colleagues)

Dissolve 0.75 g Casamino acids vitamin free (Difco Laboratories), 0.15 g sodium sulphite, 1.35 g agar (Difco Laboratories), 1 μg thiamine bromide, 3 mg congo red in 1000 ml distilled water. Pour into containers and autoclave for 15 minutes at 121°C / 249.8°F. Then pour into dishes and dry for 35-45 minutes after solidification. Store at 2-6°C / 35.6-42.8°F for a maximum of 3 days.

$HmsD_{pstbc}$ medium (by E. G.Boolgakova with colleagues)

Dissolve 0.75 g Casamino acids vitamin free (Difco Laboratories), 0.35 g sodium sulphite, 0.075 g agar (Difco Laboratories), 1 μg thiamine bromide, 3 mg congo red in 1000 ml distilled water. Pour into containers and autoclave for 15

minutes at 121°C / 249.8°F. Then pour into dishes and dry for 35-45 minutes after solidification. Store at 2-6°C / 35.6-42.8°F for a maximum of 3 days.

Medium for determining calcium ion requirements (by Irkutsk Antiplague Research Institute of Siberia and Far East)

Dissolve 12 g pancreatic digest of casein, 1.2 g fermentative hydrolysate of fodder yeast, 1.5 g D-glucose, 1 g sodium citrate, 4 g magnesium chloride, 2, 5 g potassium dihyde-roorthophosphate, 7.5 g potassium hydro-orthophosphate, 0.5 g ammonium molybdate, 0.0025 g gentian violet and 6-8 g agar in 1000 ml distilled water, then bring to a boil with constant stirring. Boil 3-5 minutes until completely dissolved, cool to 40-50°C / 104-122°F, shake thoroughly and pour 25 ml into dishes.

To remove condensation, dishes should be ajar and incubated for 35-45 minutes at 19-24°C / 66.2-75.2°F.

Rabbit plasma for the study of fibrinolytic and coagulase activity

In a tube with 1 ml 5% sodium citrate solution add 9 ml rabbit blood (access from the marginal ear vein), then centrifuge for 15 minutes (3000 rpm) or place in the refrigerator for 18-20 hours. The resulting clear supernatant is sucked off and stored in the refrigerator for up to 10 days (at -25°C / -13°F for up to six months).

Gissa's medium

Dissolve 20 g dry fermentative peptone, 5 g sodium chloride, 1 g carbohydrate of interest in 1000 ml distilled water. Add 15 ml of Andrade's indicator, which is prepared by dissolve 0.5 g fuchsine in 100 ml distilled water and add 15 ml sodium hydroxide. When after 24 hours the colour of the solution changes from red to brown, sodium hydroxide is added dropwise to a final pH of 7.1-7.2 (the colour of the solution becomes straw-yellow). Pour the obtained nutrient medium into vials with hermetically sealed packaging and autoclave for 15 minutes at 112°C / 233.6°F.

Lactose Sucrose agar

(Russell Double Sugar Agar)

Dissolve 2.5 g peptic digest of animal tissue, 7.5 g casein hydrolysate, 3 g meat extract, 10 g lactose, 1 g glucose, 5 g sodium chloride, 0.025 g phenol red[1] and 15 g agar in 1000 ml distilled water. Stir thoroughly and boil until dissolved, then autoclave for 15 minutes at 118-121°C / 244.4-149.8°F. Cool in an inclined position to form a bevelled portion ("jamb"); pH 7.3±0.2. Store at 2-8°C / 35.6-46.4°F.

[1] The original formulation specifies litmus.

Cystine tryptic agar (semisolid)

Dissolve 20 g pancreatic digest of casein, 5 g sodium chloride, 0.5 g L-cysteine, 0.5 g Na-sodium sulphite, 0.017 g phenol red and 3.5 g agar in 1000 ml distilled water. Stir thoroughly and boil until dissolved, then autoclave for 15 minutes at 121°C / 249.8°F. Cool to 50°C / 122°F and add the required carbohydrate; pH 7.3 ± 0.2.

To prepare carbohydrate in 100 ml distilled water dissolve 1 g carbohydrate (in the case of inositol 0.5 g) and add a drop of bromothymol blue until the green colour is established, then autoclave for 15 minutes at 121°C / 249.8°F. After cooling, the solution should acquire a swamp-green colour (the presence of blue colouring indicates a violation of proportions).

ONPG Broth

Dissolve 13.8 g Disodium phosphate in 50 mL water warmed to 37°C / 98.6°F in a 100 mL measuring bottle. In a fume cupboard slowly add approx. 15 mL sodium hydroxide, reaching pH 7, and top up with distilled water to 100 mL. Add 0.6 g OMPG and sterilise by filtration. This solution remains stable for 3 months under sterile darkened conditions, at 2-8°C / 35.6-46.4°F.

To prepare broth to 25 ml obtained solution add 75 ml peptone water obtained by dissolving 1 g peptone in 1000 ml distilled water (autoclaved for 15 minutes at 121°C / 249.8°F). The broth remains stable at -20°C / -4°F for a month.

Gelatin medium

Dissolve 120 g gelatine in 1000 ml distilled water heated to 50°C / 122°F. Add 3 g meat extract and 5 g peptone and heat to 50°C / 122°F. Bring the pH to 6.8-7.0. Pour into 4-5 ml tubes in "column", then autoclave for 15 min at 121°C / 249.8°F. Cool and store at 2-8°C / 35.6-46.4°F.

Olkenitsky's agar

(Triple Sugar Iron Agar)

Melt 25 g of dry nutrient agar (pH 7.2-7.4) in 1 litre of distilled water and cool to 50°C / 122°F. Dissolve 0.2 g ammonium iron (II) sulfate (Mohr's salt) and 0.3 g sodium thiosulphate in a test tube. Dissolve 10 g lactose, 10 g sucrose, 1 g glucose and 10 g urea in a flask on a water bath. In another test tube dissolve 0.1 g phenol red in 25 ml distilled water (this indicator solution can be stored for up to 10 days). Add the solutions from the first tube and flask to the melted agar, stir and filter through gauze. Add the solution from the second tube (indicator) and pour into 5-6 ml tubes. Sterilise for 20 minutes at 112°C / 233.6°F and then place in an inclined position to solidify the agar in the form of a bevelled

column ("jamb") – column about 2.5 cm, bevelled part about 4 cm. The finished medium is pale pink. Stable when stored in a dark place for 2 weeks at 2-8°C / 35.6-46.4°F and for 3 days at 18-25°C / 64.4-77°F.

Interpretation of results (after 18-24 h): yellow column – bacteria ferments glucose, yellow "jamb" – bacteria ferments lactose, crimson (red) whole medium or "jamb" – bacteria ferments urea (in this situation glucose and lactose fermentation may be masked), black column – formation of hydrogen sulphide.

Kligler agar

It is prepared like Olkenitsky's medium, only without sucrose.

Clarke Medium

Dissolve 5 g monocalcium phosphate, 5 g dextrose, 3.5 g pancreatic casein hydrolysate and 3.5 g peptide digest of animal tissue in 1000 ml distilled water. Heat gently until dissolved and distribute 10 ml into a test tube. The prepared medium is light yellow in colour. Autoclave at 121°C / 249.8°F for 15 minutes.

Urea Agar Base (Christensen)

Recipe I. Dissolve 1 g peptone, 5 g sodium chloride, 1 g glucose, 2 g potassium dihydrophosphate, 20 g urea and 0.012 g phenol red in 100 ml distilled water. It is allowed to use 6 ml phenol red at a concentration of 1:500 (0.2 g phenol red per 100 ml of distilled water). Bring the pH to 6.8-6.9 and filter. Add the above to the liquid agar obtained by dissolving 15 g of agar in 900 ml of distilled water, then autoclave for 15 min at 121°C / 249.8°F and cool down to 50-55°C / 122-131°F.

Recipe II. Dissolve 1 g dry peptone, 5 g sodium chloride, 20.2 g agar and 1 g glucose in 1000 ml distilled water. Boil the mixture until the agar is completely dissolved, adding hot distilled water at boiling to the original volume, then dropwise add 20% sodium hydroxide solution (to pH 6.8). Then add 0.012 g phenol red dissolved in 1 ml of 96% ethanol solution, stir and filter. Autoclave for 20 minutes at 121°C / 249.8°F. The medium should turn orange in colour. When the agar has cooled to 50°C / 122°F, add an equal volume of a 20% solution of urea on bidistilled water (20 g of urea dissolved in bidistilled water and boiled in a water bath for half an hour after boiling). The colour of the agar pales to yellow.

Urea broth (Rustigan and Stuart)

Dissolve 20 g urea, 9.1 g potassium dihydrophosphate, 9.5 g sodium hydrophosphate, 0.1 g yeast extract, and 0.01 g phenol red in 100 ml distilled water, then filter.

Nitrate broth

To 100 ml of distilled water add 20 g Bacto Peptone and 2 g potassium nitrate, bringing the pH to 7. Pour 4 ml into test tubes and autoclave for 15 minutes at 121°C / 249.8°F.

Cole-Belcourt agar

Add 2 ml glycerol to 100 ml of pre-melted and cooled to 45°C / 113°F Hottinger agar or Martin agar, followed by 1-5 ml litmus or Andrade's reagent. Mix well and autoclave for half an hour at 110°C / 230°F.

Mueller–Hinton agar

Dissolve 300 g beef extract, 17.5 g acid casein hydrolysate, 1.5 g starch and 17 g agar in 1000 ml distilled water, then boil until dissolved. Add 20% sodium hydroxide solution (to pH 7.3) and autoclave for 15 min at 121°C / 249.8°F.

Givental'-Ved'mina's agar

Dissolve 20 g pancreatic digest of sprat (or lysate of fodder yeast clarified by enzymes), 5 g starch, 10 g sodium chloride, 1 g sodium hydroorthophosphate and 13.5 g agar in 1000 ml distilled water, then boil until dissolved. If necessary, add 20% sodium hydroxide solution (to pH 7.4) and autoclave for 20 minutes at 121°C / 249.8°F.

If the medium is used for antibiotic susceptibility testing, add 5% sterile defibrinated sterile animal blood to the dishes before pouring directly into the dishes.

Methodology for the determination of amine nitrogen

Add 18 ml distilled water to 2 ml test solution, after which 0.5% water solution of neutral red (3-amino-6-dimethylamino-2-methylphenazonium chloride) is added dropwise. Depending on the pH, the solution will become crimson red (acidic medium) or yellow (alkaline medium). By adding acid or alkali, respectively, the pH should be adjusted to 7.2 (yellowish red colour of the solution) and 2.5 ml formol mixture[1] should be added. The solution will acquire a crimson-yellow colour due to the destruction of amino groups. Then titrate with alkali (NaOH), i.e. add the alkali solution drop by drop (stirring constantly) until the colour of the solution changes from crimson-yellow to red, noting the amount of alkali consumed.

The mass fraction (content) of amine nitrogen is calculated from the amount of alkali used for titration and the fact that 1 ml of 0.1 mol/L sodium hydroxide corresponds to 1.4 mg of nitrogen, which is expressed by the formula:

$$\omega_N = \frac{V_{NaOH} \cdot 140}{V_{sol}},$$

where V_{NaOH} – quantity of 0.1 m/l of alkali (NaOH) used for titration; V_{sol} – quantity of the solution under study.

[1] Method of preparation: 50 ml of formalin solution with a mass fraction of 30% and 0.1 ml of phenolphthalein alcohol solution with a mass fraction of 1%; the mixture is brought to a weak pink colour with sodium hydroxide solution with a concentration of 0.0002 mol/mL.

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Vladimir A. Gorshkov-Cantacuzene

The Opera on the Plague

textbook

Vladimir A. Gorshkov-Cantacuzéne

Un opéra sur la peste

le manuel scolaire

緩拓然　甫慈診

（カーンタークーゼーン ・ ヴラディミル）

ペストについてのオペラ

教科書

ACT I – «Monster of the night»; ACT II – «Life»; ACT III – « The other side »

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Plague has accompanied mankind throughout its historical journey. It has ravaged cities, instilling fear in hearts, been an object of research that captivates the best minds, and a formidable weapon that corrupts dark souls. But how, appeared 20,000 years ago has it rised to its pedestal? How has it learnt to so elegantly circumvent the human immune system? About what it is, how to work with it safely and much more - in this book!

※

La peste a accompagné l'humanité tout au long de son parcours historique. Elle a ravagé des villes, faisant froid dans le dos. C'est un objet de recherche qui captive les meilleurs esprits et une arme redoutable qui corrompt les âmes sombres. La peste est apparue il y a 20 000 ans. Mais comment est-elle arrivée sur son piédestal ? Comment a-t-elle appris à contourner si élégamment le système immunitaire humain ? Découvrez qui elle est, comment travailler avec elle en toute sécurité, et bien plus encore dans ce livre!

※

ペストは歴史を通じて人類に付きまとう。
心に恐怖を植え付けながら都市を荒廃させ、優れた知性を魅了する研究の対象となり、そして闇の魂を堕落させる恐るべき武器となった。

しかし、2万年前に現れたペストはどのようにしてこのような地位に上り詰めることができたのだろう？人間の免疫系をこれほど巧妙に回避する方法をどのようにして習得したのだろう？

ペストとは何者か、安全に作業する方法、その他様々な情報がこの一冊に！