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# ANTHRAX: BIOLOGICAL FEATURES OF THE PATHOGEN

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**Abstract**. Anthrax (anthrax) — the disease, known since ancient times, refers to particularly dangerous infections with high mortality, which, with untimely diagnosis and the absence of etiotropic therapy, can reach 90%, and with the pulmonary form — 100%. According to WHO, between 2,000 and 20,000 cases of anthrax in humans are registered annually in the world, including fatal cases, which are more often observed in developing countries. The biological properties of the causative agent of anthrax — *Bacillus anthracis* — are unique. So, it forms a capsule and produces exotoxin exclusively in the host body, this property is used as a sign for differentiation of *B. anthracis* from other spore aerobes. The capsule of *B. anthracis* consists of a polypeptide formed by a right-rotating isomer of glutamic acid, which has biological inertia and resistance to destruction by proteases, and the exotoxin does not form an AB-structure typical for exotoxins, it includes three separate components. The presence of spores allows the pathogen to persist in the soil for decades and in natural disasters or man-made disasters to come out of hiding and cause diseases again, which indicates the relevance of anthrax and the need for constant vigilance in its relation.

**Key words**: Anthrax; *Bacillus anthracis;* biological properties; virulence factors; capsule; exotoxin; evolution of *B. anthracis.*

# СИБИРСКАЯ ЯЗВА: БИОЛОГИЧЕСКИЕ ОСОБЕННОСТИ ВОЗБУДИТЕЛЯ

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**Резюме.** Сибирская язва (антракс) — заболевание, известное с древних времен. Оно относится к особо опасным инфекциям с высокой летальностью, достигающей при несвоевременной диагностике и отсутствии этиотропной терапии 90%, а при легочной форме инфекции — 100%. По данным ВОЗ, ежегодно в мире регистрируется от 2000 до 20 000 случаев сибирской язвы (в том числе с летальным исходом), которые чаще наблюдаются в разви-

вающихся странах. Биологические свойства возбудителя сибирской язвы — *Bacillus anthracis* — являются уникальными. Так, он образует капсулу и вырабатывает экзотоксин исключительно в организме хозяина, что служит дифференциальным отличием его от других споровых аэробов. Капсула *B. anthracis* состоит из полипептида, образованного правовращающим изомером глутаминовой кислоты, обладающим биологической инертностью и устойчивостью к разрушению протеазами, а экзотоксин не образует типичную для экзотоксинов АВ-структуру, а включает три отдельных компонента. Наличие спор позволяет возбудителю десятилетиями сохраняться в почве и при природных катаклизмах или техногенных катастрофах выходить из подполья и вновь вызывать заболевания, что свидетельствует об актуальности сибирской язвы и необходимости постоянной настороженности в ее отношении.

**Ключевые слова:** сибирская язва; *Bacillus anthracis*; биологические свойства; факторы вирулентности; капсула; экзотоксин; эволюция *B. anthracis*.

## HISTORY

Anthrax is a zoonotic disease that has been widespread throughout natural history on most continents, in accordance with the habitat of herbivores. The name "*anthrax*" comes from the Greek word anthrax, meaning coal, and is associated with the appearance of a specific manifestation in the cutaneous form of anthrax, anthrax carbuncle.

References to the disease similar to anthrax can be found on cuneiform tablets dating back 6–7 thousand years in Mesopotamia and Egypt. Anthrax has been known to Arab, Greek and Roman physicians since ancient times as "sacred fire" or "Persian fire". The illness was described by Homer in his poem "Iliad" under the name "sacred fire". In Russia, a similar disease was mentioned in ancient Russian chronicles under the name "fiery pimple". In 1640, there was a major anthrax epidemic in Moscow, and a decree was issued ordering that the corpses of dead animals be buried deep in the ground outside the city. The first description of anthrax in Russia was made by the St. Petersburg Academician Johann Gmelin in 1731 during his travels through Siberia. A great contribution to the study of anthrax was made by the Russian physician S.S. Andrievsky. After three years of observations in 1786–1788 during an epidemic in the Urals, he gave a detailed description of the clinical picture of the disease with the definition of three degrees of its severity and developed measures for non-specific prevention. He also, taking into account the place of observation and widespread distribution of the illness in Siberia at that time, gave it the name "Siberian ulcer", which was announced in 1788 in St. Petersburg in the report "On Siberian ulcer". This name is still used in Russia. In the same year, Andrievsky proved the possibility of human infection from animals. After self-infection, he fell ill with cutaneous anthrax with sepsis, which almost led to death, and during the illness he discribed his feelings. In 1849–1850, almost simultaneously, three scientists from different countries (A. Pollender, Germany; K. Daven, France; F.A. Brauell, Russia) described the causative agent of anthrax. However, only in 1863, Daven proved the role of these microorganisms in the development of anthrax. So, this year is considered the official date of the discovery of the anthrax bacillus. A pure culture of the etiological agent of anthrax was isolated **in 1876** by Robert Koch, who was unknown at that time. In May 1876, his article "Etiology of Anthrax" was published, and, in 1877, similar studies were conducted by Louis Pasteur. In Russia, the causative agent of anthrax was isolated for the first time in 1882 by V.K. Vysokovich. The etiologic agent of anthrax is a unique microorganism in the history of microbiology. Firstly, it was the first pathogen to be seen under a microscope (1849, A. Pollender), secondly, the first bacterium for which transmission with infected blood was shown (1850, K. Davenne), thirdly, the first microorganism isolated in pure culture and possessing spores (1876, R. Koch), and, fourthly, the first to be used to create a live attenuated vaccine (L. Pasteur, 1881).

## TAXONOMY AND BIOLOGICAL PROPERTIES OF *BACILLUS ANTHRACIS*

The causative agent of anthrax is *Bacillus anthracis*. This bacterium belongs to the family of spore-forming microorganisms *Bacillaceae*, genus *Bacillus.* In the genus *Bacillus,* which has about 100 species, the *Bacillus* cereus group is distinguished. It includes closely related *B. cereus, B. anthracis, B. thuringiensis, B. mycoides, B. pseudomycoides* and a recently recognised new species of psychrotolerant microbe *B. weihenstephanensis.*

### Morphological and tinctorial properties

Depending on the stage of culture development and environmental conditions, *Bacillus anthracis* can exist in three forms: as encapsulated rods (in the human or animal body or on blood or serum culture media), as acapsulated vegetative rods (on conventional culture media), and as a spore form. The etiologic agent of anthrax is one of the largest pathogenic bacteria, its size is 3.0–10.0 μm in length and 1.0–2.0 μm in width. It has the appearance of a straight or slightly curved rod (Fig. 1). In smears from cultures grown on media, the rods are arranged in long chains, the ends of

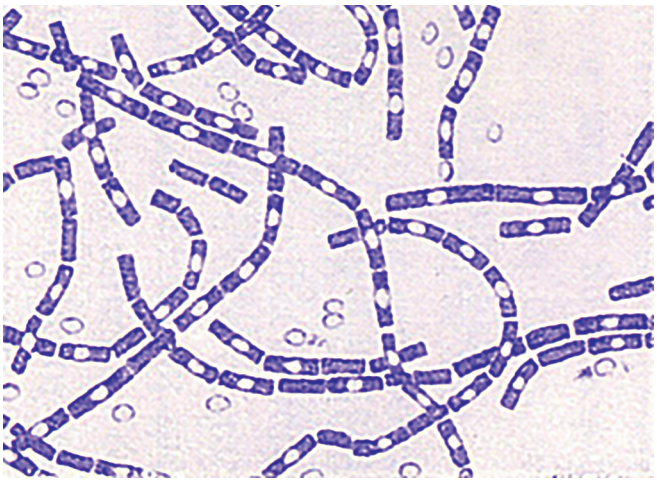

**Fig. 1. The causative agent of anthrax**

the microbes in stained preparations are cut off and the chain resembles a bamboo cane with knee joints. In smears from pathological material, the rods are arranged singly, in pairs, or in short chains, surrounded by a well-defined capsule surrounding the entire chain. The capsule is formed only in a living organism or in culture media with blood or serum and at an elevated $CO_2$ concentration. Capsules are more resistant to the action of putrefactive bacteria than vegetative cells, and in cadaveric material one can see so-called "shadows" of bacteria, empty capsules without a cell inside. Capsule synthesis is encoded by genes located on a 60 kDa plasmid. Unlike other representatives of the genus, *B. anthracis* is immobile. The absence of flagella is one of the important differential features that distinguishes it from other bacilli. In unfavorable environmental conditions, the causative agent of anthrax forms spores. When spores enter favorable conditions (humidity, aeration, temperature, the presence of necessary nutrients), vegetative cells are quickly formed from them. Biologically valid spores germinate in 100% of cases. Spore formation, as in all prokaryotes, is not a reproduction method, but means of protection from unfavorable environmental conditions, and performs the function of conservation of the species. Spores are able to preserve genetic material for a long time and ensure the transfer of basic properties to offspring in subsequent generations. Vegetative cells accept aniline dyes well and are Gram-positive. The capsule and spores do not accept dyes well; special methods are used to stain them. To identify the capsule, staining methods according to Rebiger, Mikhin, Romanovsky–Giemsa are used, and the best staining is Rebiger's stain. Rebiger's solution (a solution of gentian violet in 40 % formalin) is applied to the surface of a fixed smear and left for 30–60 seconds, washed off with water and dried. In smears from fresh pathological material, the capsule around the microbe is stained pink or red-purple, while the body of the microbial cell is dark purple. When methanol or ethanol are used for fixation of smears, significant compression of the capsule occurs, as a result of which clearly defined unstained halos can be seen in smears instead of a capsule around the microbes. Spores are detected using Ziehl-Neelsen and Orzeszko stains.

*Cultural properties*

The etiological agent of anthrax is an aerobe or facultative anaerobe, and is not demanding to culture media. It can grow in the temperature range from 12 to 45 °C, the optimum temperature is 34–37 °C. The microorganism grows well in simple media. In liquid culture media, it forms whitish flakes, which after 16–24 hours sink to the bottom, producing a flocculent precipitate in the form of a lump of cotton wool, which is difficult to break up when shaken. The broth remains clear. In solid culture media, after 17–24 hours, it forms characteristic large grayish-matte rough colonies from 1 to 5 mm in diameter with a flat or slightly convex "shagreen" surface, uneven edges and wavy processes extending from them. When viewed under low magnification on a microscope, the colonies resemble the Medusa head or a lion's mane, with threads extending from the center (Fig. 2). The colonies are firmly attached to the agar. These are R-forms, which are virulent, unlike S-forms, that form round, smooth colonies with smooth edges and uniform turbidity in the broth. When *B. anthracis* is grown in culture media with 0,05–0,5 U/ml benzylpenicillin, after 3 hours of incubation due to cell wall destruction and formation of protoplasts the so-called "pearl necklace" (or "beads") is formed, which are chains of round cells. They can be seen during microscopy of smears from the colonies. This feature is characteristic of the causative agent of anthrax, distinguishes it from other aerobic spore-forming bacteria and is used as a "pearl necklace" test (Fig. 3).

*Antigenic properties*

The antigenic structure of the etiologic agent of anthrax is complex and depends on its morphological form. *B. anthracis*

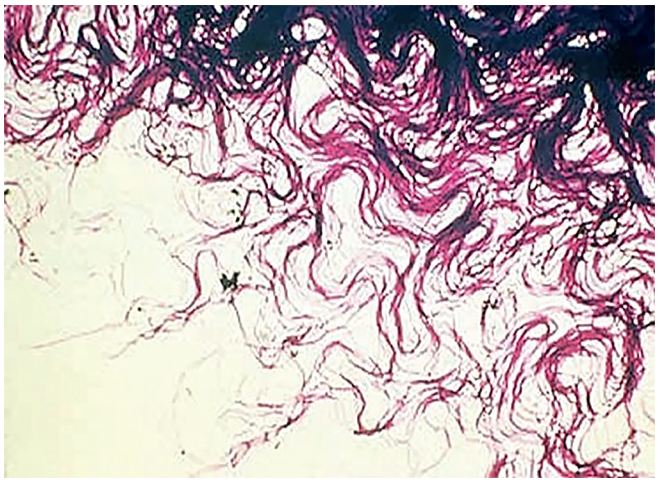

**Fig. 2. The edge of *B. anthracis* colony at low magnification on microscope**

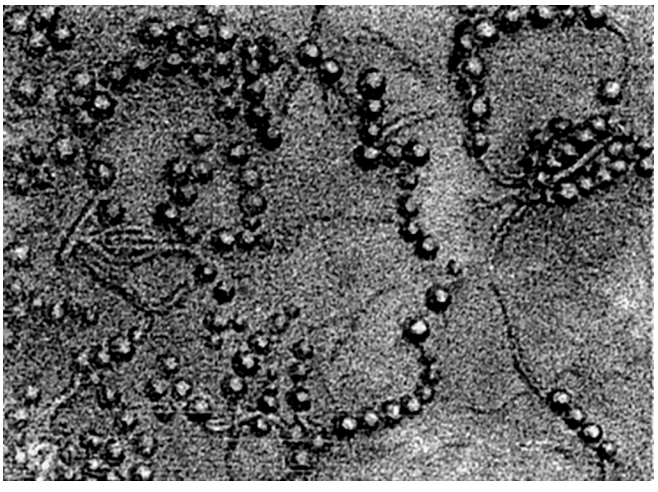

**Fig. 3. The "pearl necklace" test**

antigens can be divided into two groups. Antigens of the first group are closely associated with the structural components of the cell, antigens of the second are represented by the products of its metabolism (exotoxins). Cellular antigens include somatic and capsular. The somatic cell surface antigen, a component of the bacillus cell wall, is primarily of a polysaccharide nature. Its structure involves the S-layer, homologous to the surface area of human red blood cells, which can be the cause of the immunological areactivity of the macroorganism [5, 11]. The somatic antigen is heat-stable, it is detected in express methods for indicating the pathogen in various materials (Ascoli's thermo precipitation test). It is group-specific and is found in other closely related species. The capsular antigen is found only in virulent strains and has weak immunogenic properties. The antigenic composition of the spore form differs somewhat from the antigenic structure of vegetative cells, especially in terms of heat-stable antigens, which are characterized by high intraspecies specificity. In particular, the spore surface contains BclA Glycoprotein, which plays a role in the immune response.

In the second group of *B. anthracis* antigens, representing the products of its metabolism, the most interesting is the protein protective antigen, which has high immunogenic properties and causes the body to develop specific immunity. The protective antigen is synthesized by both virulent and vaccine strains, and is part of the subunit and combined vaccines for immunising people against anthrax.

*Virulence factors and genetic regulation of their synthesis*

The causative agent of anthrax has almost universal pathogenicity for mammals, such as humans, farm animals, wild animals and laboratory animals [4, 7, 8]. The genome of *B. anthracis* consists of a ring chromosome of about 5200–5500 thousand nucleotide base pairs or kilobases (kb) in size and two plasmids: pXO1 with a molecular weight of 110 kDa and pXO2 [4, 6, 10] with a molecular weight of 60 kDa. The first plasmid encodes synthesis of the toxin, and the second is responsible for capsule synthesis. Loss of one or both plasmids results in a loss of virulence. Plasmid pXO1 contains a 44.8-kb "pathogenicity island" which includes three toxin structural genes: *pag A (protective antigen), lef (lethal factor) and cya (edema factor*). In addition to the toxin structural genes, "pathogenicity island" also contains regulatory genes (including *atxA*, the main regulator of the virulence gene), *bslA* encoding an adhesion protein located in the S-layer of vegetative cells [10, 11] and three spore germination operons. Plasmid *pXO2* contains structural and regulatory genes determining capsule synthesis. Multilocus variable number tandem-repeat analysis (MLVA) and whole-genome single nucleotide polymorphism analysis are used for genotyping of *B. anthracis* strains [2, 3, 10]. According to the MLVA method, *B. anthracis* strains are divided into three lineages: A, B, and C. The most significant of these is lineage A, which has a global distribution and to which 90% of all strains belong.

The primary virulence factors of B. anthracis are the capsule and exotoxin [4, 9, 10, 12]. The capsule and toxin are synthesized under the influence of the environment as a response to an increase in carbon dioxide and bicarbonate ion concentration, so the pathogen forms them only in an infected macroorganism or when grown on synthetic culture media under special conditions [1, 5, 12]. This ability is one of the leading features that allows us to distinguish the etiological agent from the group of spore-forming aerobes [6, 12]. The capsule is a polypeptide formed by alternating lambda and gamma chains of the D-isomer of glutamic acid. The presence of polysaccharides in the capsular substance is also possible. The capsule is important in the initial stages of the infection process, since it ensures the adhesion of vegetative forms of the bacterial pathogen to the cells of the body, while spores do not have adhesive properties. Other functions of the capsule include protecting the pathogen from phagocytosis by preventing its capture and intracellular destruction, reducing the bactericidal activity of serum and opsonization, and metabolic disruption in the body's cells [5, 10, 12]. The capsular polypeptide has very low immunogenicity and, possibly, immunosuppressive action [5]. Non-capsular strains are avirulent.

Based on their ability to synthesise a capsule, *B. anthracis* strains can be divided into three groups. Type I includes virulent strains, which form a capsule at an elevated $CO_2$ concentration. The next group, type II, contains B. anthracis strains with reduced virulence or avirulent, which form a capsule regardless of $CO_2$ concentration. Type III is represented by avirulent strains, which are unable to form a capsule.

**The exotoxin** is produced in the infected organism and plays a leading role in the pathogenesis of the disease. It belongs to the AB-type protein toxins and consists of three separate protein components with different biological activities, two of which perform an effector function (subunit A), one — an acceptor

and internalizing function (subunit B). **The first component (factor I)** is an edema inflammatory factor (EF), which is responsible for the development of edema. This is a calmodulin-dependent adenylate cyclase, which is activated only inside the cell in the presence of calcium ions upon contact with the intracellular protein calmodulin, which is absent in bacteria, and increases intracellular levels of cyclic AMP (cAMP), which disrupts water homeostasis, leads to cellular dehydration and causes severe edema. **The second component (factor II)** is a protective antigen (PA), is an immunogen, is responsible for interaction with receptors on the cytoplasmic membrane (CPM) of the cell and ensures the passage of factors I and III into the cell. **The third component (factor III)** is the toxin itself, or lethal factor (LF), is a zinc-dependent metalloprotease. It cleaves NH2 fragments of cellular mitogen-activated protein kinases, which lose their enzymatic activity. It has a cytotoxic effect, causes epithelial cell dysfunction and a hyperinflammatory state of macrophages and their lysis, activates the "oxidative burst", stimulates the production of tumor necrosis factor (TNF), interleukin-1 (IL-1) and other proinflammatory cytokines by macrophages, while one toxin molecule causes the synthesis of more than $1 \times 10^5$ of each of these molecules. It is the high level of cytokines that leads to the development of toxic shock syndrome and the death of the patient. Individually, all of the above components are not toxic; the effect only occurs when they enter the body together. The protective antigen is a common subunit B for the EF and LF components, so it is currently accepted to speak of two toxins — edema (EF + PA) and lethal (LF + PA) [5, 6]. PA binds to the cellular glycoprotein receptor ATR (*anthrax toxin receptor)*, the number of which on one cell ranges from 8000 to 30 000. Then, with the help of cellular furin protease, hydrolysis of PA occurs in a unique site into two fragments, the larger of which (63 kD) remains on the cell surface and serves as a receptor for EF and LF. Calcium ions are required for PA binding to the cell and its cleavage. After cleavage, 7 PA 63 molecules are converted into a heptametric pore with a diameter of 3,6 angstroms. PA 63 binds to the EF and LF components, whereby the latter compete for its binding site, and promotes endocytosis of the formed toxin complex and its transfer to the cytosol as part of the endosome. At low pH, a channel is formed in the endosome, through which EF and LF pass into the cytoplasm, where they exert their cytotoxic effect, causing sharp disturbances of cellular metabolism and leading to degradation and death of the cell. The scheme of interaction between anthrax toxin and the cell is shown in Figure 4.

Another virulence factor of the causative agent can be considered the two siderophores bacillibactin and petrobactin,

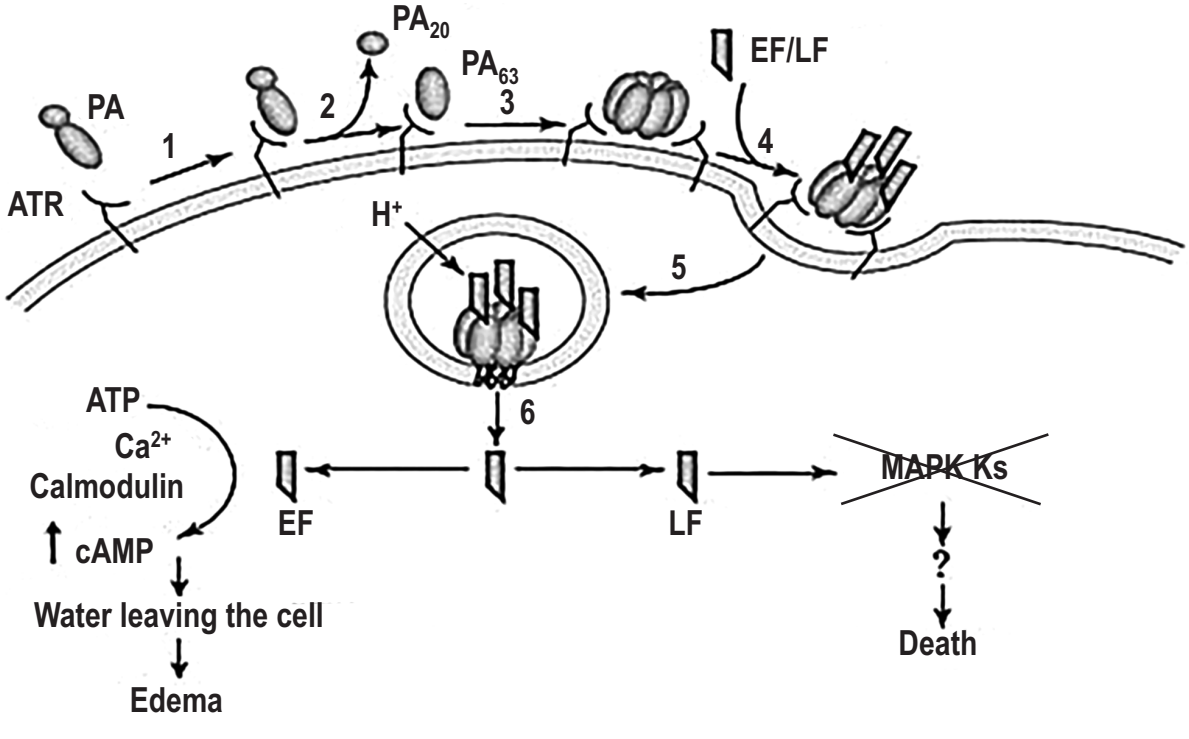


**Fig. 4. Scheme of interaction between anthrax toxin and eukaryotic cell (Petosa C. et al., 1997)**
1. Binding of protective antigen (PA 83) to the host cell receptor (ATR).
2. Calcium-dependent cleavage of PA 83 by furin and release of the PA 20 fragment.
3. Formation of the PA 63 heptamer.
4. Binding of lethal (LF) or edema factor (EF) to PA 63.
5. Calcium-dependent receptor-mediated endocytosis.
6. Calcium-dependent transfer of LF and EF into the cytosol at acidic pH with MAPKKs — mitogen-activated protein kinase kinases.

secreted by *B. anthracis* in the host organism in response to iron-limited conditions. These trivalent iron chelators bind iron with very high affinity and transport it into the bacterium via substrate-binding proteins (SBPs) and other ABC transporters. *B. anthracis* can also remove iron from heme and heme-containing proteins, including hemoglobin, using iron-regulated surface determinant (Isd) proteins [10].

The *B. anthracis* cytolysin, called anthrolysin O, has also been characterized and belongs to the family of cholesterol-dependent thiol-activated cytolysins, which also includes listeriolysin O, perfringolysin O, and streptolysin O [5].

*Environmental resistance*

The resistance and survival rate of the pathogen, causing anthrax depends on its biological form. The resistance of vegetative forms to physical and chemical factors does not differ from that of other non-spore-forming bacteria. Unlike vegetative cells, anthrax spores are extremely resistant [4, 5, 7, 9], and their resistance depends on the conditions of their formation. Spores formed at 18–20 °C are more resistant than those formed at the higher temperature (35–38 °C). Spores are resistant to chemicals. Ethyl alcohol kills spores within 50 days, phenol 5 % solution, chloramine 5–10 % solution— within a few hours or even days, hydrogen peroxide 3 % solution — within an hour, formalin 1% solution and caustic soda 10 % solution — within two hours. The spores can withstand multiple freeze and thaw cycles, boiling for 1 hour, dry heat (120–140 °C) for up to 3 hours, 1 hour at 150 °C, and autoclaving at 110 °C for 5–10 minutes. They are preserved for a long time in wool and leather, especially tanned ones; salting and drying meat and leather helps preserve the spores. In dried agar cultures, the spores remain viable for 55 years, and in soil for tens or even hundreds of years. Moreover, under favorable conditions, they can germinate in soil and then form spores again, thus forming and maintaining a soil-borne anthrax focus. Thus, in Yamal, where a focus was recorded in July 2016, the last time an anthrax epizootic was observed in 1941, i.e. 75 years ago. In South Africa, in the Kruger National Park, 2 viable strains of *B.anthracis* were isolated from animal bones found during archaeological excavations, the age of which was approximately 200 years [4].

## EVOLUTION OF *BACILLUS ANTHRACIS*

The soil contains a large number of saprophytic aerobic bacilli, many of which are very similar to the etiologic agent of anthrax in their biological properties, including antigen properties. In particular, *B. cereus* has the ability to produce exotoxin and has a high degree of DNA homology with *B. anthracis.* Modern methods (ribosomal RNA analysis, multilocus enzyme electrophoresis, multilocus sequence typing) have shown the identity of these two microorganisms. Most of the functional differences between them are associated with the presence of plasmids, which differ in number and size.

It is logical to assume that *B. anthracis* either derived from *B. cereus* (as some authors believe), or, having lost its virulence, itself transformed into a new species (according to other authors).

The results of molecular genetic studies suggested that *B. cereus, B. anthracis* and *B. thuringiensis* derived from soil bacilli containing potential virulence genes. *B. cereus* and *B. thuringiensis* diverged earlier, while *B. anthracis* separated from *B. cereus* only 10,000–20,000 years ago, and this may be the reason for the very low level of genetic diversity (genetic monomorphism) of its strains. At the same time, the increase in virulence and the change in the host range were associated with the acquisition of two plasmids, pXO1 and pXO2, encoding the main virulence genes in *B.anthracis*. In addition, certain mutations occurred in the genome, changing the expression of some genes of the microorganism. As a result of repeated contacts with herbivores, a mutant could have arisen that formed a capsule in the host organism and subsequently began to produce a toxin.

An important factor in the formation of *B. anthracis* was a mutation in the gene encoding the biosynthesis of glutamic acid, which is part of the capsule, and its assembly. Strains with a capsule of D-isomers of glutamic acid, which are biologically inert and resistant to destruction by proteases, acquired protection from host phagocytes. The capsule of saprophytic bacilli, such as *B. cereus*, also consists of glutamic acid, but it contains L- and D-isomers.

## CONCLUSION

Therefore, unique properties of the etiologic agent of anthrax pathogen determine the severity of the diseases it causes. The presence of spores and ability to persist in soil for a long time and, under favorable conditions, even vegetate and reproduce there, increase the relevance of anthrax for Russia. The importance of this issue is confirmed by the large number of registered and unrecorded anthrax-infected soil foci in the Russian Federation. Thus, in the Yamalo-Nenets Autonomous Okrug, an outbreak of anthrax was registered in July 2016, while the last time an epizootic of this disease was there in 1941, i.e. 75 years before the current outbreak. Also, quite recently, in March 2023, several cases of anthrax in people were registered in Chuvash Republic.

## ADDITIONAL INFORMATION

**Author contribution.** Thereby, all authors made a substantial contribution to the conception of the study, acquisition, analysis, interpretation of data for the work, drafting and revising the article, final approval of the version to be published and agree to be accountable for all aspects of the study.

**Competing interests.** The authors declare that they have no competing interests.

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## ДОПОЛНИТЕЛЬНАЯ ИНФОРМАЦИЯ

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**Конфликт интересов.** Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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