ISSN 1993-6842 (on-line); ISSN 0233-7657 (print)
Biopolymers and Cell. 2023. Vol. 39. N 2. P 77–89
http://dx.doi.org/10.7124/bc.000A90

UDC 577.2:573.6:616.9

Features of PCR diagnosis of anthrax

U. M. Yanenko, H. A. Zaviriuha, T. B. Vasylieva, Z. Yu. Tkachuk
Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Akademika Zabolotnoho str., Kyiv, Ukraine, 03143
ulanayanenko@gmail.com

Under certain conditions, molecular methods of diagnosis have a significant advantage over long-term classical microbiological methods. Because of the highly pathogenic properties of *Bacillus anthracis*, it is necessary to put into practice express methods of the pathogen identification in case of a biological threat of the infection expand among vulnerable animals, people, and contamination of the territory. The identification of *B. anthracis* is difficult because of the spore and vegetative forms and the similarity to closely related species. Ukraine is a disadvantaged country for anthrax. The introduction of reliable, sensitive and specific molecular diagnostic methods is a priority in the issue of biosafety.

Keywords: *Bacillus anthracis*, diagnostics, identification, rapid tests, microbiological studies, methods of molecular microbiology.

Introduction

Anthrax was a natural disaster for centuries due to exterminating a huge number of farm animals — the state economic basis. The human infection occurs as a result of the contacts with carcasses of sick animals or products of animal origin [1]. The disease has a global distribution, but the incidence of livestock and humans varies depending on the local ecology, the implementation of control strategies and socio-cultural practices that determine the spread of infection from animals to humans [2].

Today, the issue of biosecurity of people, the defenders of Ukraine, is particularly relevant due to the hostilities in the country. Due to the presence of huge areas of uncontrolled territory — numerous regions can be considered a risk zone for the occurrence of the disease. In order to ensure a stable epizootic situation regarding anthrax, it is necessary to operate with the data on the location of stationary unhealthy points, as well as the location of ancient burials of animals that died due to anthrax.

In Ukraine 24,956 outbreaks of this disease were detected in the period 1920–2020, and there are more than 11,000 anthrax graves [3]. In view of these indicators, Ukraine is considered unfavorable in the context of anthrax.

The analysis of statistical data on the registration of anthrax outbreaks among farm animals and the monitoring of unfavorable points regarding this disease created prerequisites for the expansion of anthrax as a lethal biological weapon [4–7].

Clinical manifestations

The clinical picture of the disease is characterized by high intoxication, fever, septicemia, the appearance of edema and carbuncles, damage to the intestines and lungs [5, 8, 9]. Inhalational anthrax is the most lethal form of the disease with a mortality rate approaching 90 % from untreated infections [10]. Statistics indicate that 1.83 billion people live in the anthrax risk zones [11].

Currently, there is a belief in society that the disease anthrax has long been eliminated. This contradicts the known data on the biological characteristics of the anthrax pathogen, which testify to the persistence of the pathogen in the soil for centuries. In certain environments, under favorable conditions, bacilli can remain viable for up to 200 years. Soil infected with anthrax spores remains a source of infection for susceptible animals or humans for a time longer than a person's life [12].

Anthrax is a zoonotic disease. Its rapid diagnosis and identification is the key to timely detection and prevention of the pathogen's spread. Humans are infected by spores that infect the skin, respiratory system, and gastrointestinal tract. Infection through inhalation of anthrax spores without timely treatment is the most lethal for humans [13–15].

The causative agent

The causative agent of anthrax is *B. anthracis*, a highly pathogenic gram-positive, spore-forming bacterium that poses a serious threat to human health and all mammals due to high mortality [16–18].

The genome of *B. anthracis* consists of a chromosome with a size of 5.23 Mb and two large plasmids — pXO1 (182 kb; NC001496.1) and pXO2 (96 kb; NC002146.1) [19]. Three anthrax toxin genes are located in the separate loci pXO1 plasmids, whereas the capsule biosynthesis genes sarB, sarC and sarA, as well as the gene associated with capsule depolymerization — dep are organized in the pXO2 operon [20].

The pathogenicity and virulence of *B. anthracis* are determined by the capsule, which has a complex surface structure [21]. The bacteria closely related to anthrax, such as *B. cereus* and *B. thuringiensis*, do not produce capsules [22].

B. anthracis produces a tripartite toxin AB, which consists of the receptor-binding subunit, protective antigen (PA) and two enzymatic subunits, lethal factor and edema (LF and EF). These subunits together can form two active toxins: a lethal toxin (PA + LF) and an edematous toxin (PA + EF), the components of which are structurally organized into two separate domains — effector and receptor [20, 23].

Plasmid pXO2 (96 kb) carries the capsule biosynthesis genes and is a necessary component for the classical manifestation of anthrax disease [19]. The vaccine strains of *B. anthracis* do not have pXO2, but they contain the

pXO1 plasmid with its toxins, and are used to make a live anthrax vaccine. The antigenic protein, the gene of which is present in this plasmid, causes a strong immune response that can prevent the disease occurring. Its function as a transporter of toxins can be blocked by specific antibodies. Nevertheless, one should not forget about some genes that are candidates the for virulence genes in the composition of the bacterial chromosome, because the chromosomal background, even in the presence of plasmids, can serve as a distinguishing factor between an opportunistic pathogen and those that cause a fatal disease with global distribution. The plasmids pXO1 and pXO2 are not self-transmissible, these and other plasmids can be transmitted by the conjugative plasmids derived from *B. thuringiensis*.

The protective antigen (PA) binds to a specific receptor on the surface of mammalian cells (M.m. 83 kDa), after binding it is cut into 2 fragments, while the smaller fragment (20 kDa) is released into the environment, and the larger one (63 kDa) remains attached to the receptor. This fragment can bind to EF or LF, and the complex is transported to the inner surface of the membrane by receptor-linked endocytosis. The heptamer of the PA-LF or PA-EF complex forms a pore in the cell membrane, through which the toxic protein passes into the cell. Both LF and EF act on intracellular targets [24]. The virulence of *B. anthracis* is vital for survival and is an inherent characteristic of the bacterium. The acquisition of two virulence plasmids occurred during the genesis of this species and they have survived to our time [25].

The high phenotypic and genetic similarity of *B. anthracis* with *B. cereus* and other closely related species of bacilli creates difficulties in the identification of the bacterium. The similarity with *B. cereus* is great, which gives reason to researchers to consider *B. anthracis* as a pathogenic variant of *B. cereus* [26, 27]. The *B. cereus* group (*B. cereus* sensu lato) consists of 6 genetically related species: *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. weihanstephanensis*, and *B. pseudomycoides* [28–30].

There is a high degree of genomic homology between *B. cereus*, *B. anthracis* and *B. thuringiensis*, which some authors consider genetically one species. The ability to transfer parts or the very plasmids of *B. anthracis* virulence has been established, with the exception of the most divergent *B.cytotoxicus* group, the genomes of species of the *B. cereus* group are highly conserved [31–33]. There are *B. cereus* bacteria that contain anthrax-specific pXO-like plasmids. *B. cereus* D–17, *B. cereus* 43881, and *Bacillus thuringiensis* 33679 were found to contain sequences similar to more than half of the pXO1 ORF sequences examined. Most of the DNA fragments that were amplified by PCR from these organisms had DNA sequences between 80 and 98 % similar to those of pXO1 [34].

B. cereus G9241 was isolated from the sputum and blood of a patient with life-threatening pneumonia whose history, clinical features, and laboratory findings were consistent with 10 patients with bioterrorism-related inhalational anthrax in 2001 [34]. The *B. cereus* G9241 isolate was indeed found to be a *B. cereus* isolate containing the pXO1 genes, including genes encoding anthrax toxins [30]. *B. cereus* G9241 contains two virulence plasmids, pBCXO1 and pBC210, as well as the

linear pBClin29. Plasmid pBCXO1 is highly similar to pXO1 and contains the toxin genes pagA, lef, and cya, which encode toxin proteins with amino acid sequences that are 96 % or more identical to their counterparts in *B. anthracis* [35].

B. anthracis bacteria reproduce exclusively during short periods of infection, which are terminated by the death of the host or elimination of the bacteria by the immune system or by therapeutic agents. The genetic evolution of *B. anthracis* is limited to short vegetative periods from infection to the death of the host. Unlike most other bacteria with a similar generation time, the pathogen develops very slowly, which is the reason for the extraordinary genetic and phenotypic homogeneity of *B. anthracis* [25]. At the current stage of research, the only source of molecular variation among known strains of *B. anthracis* is a sequence with a variable number of tandem repeats (VNTR), with chromosomal localization, 5 different allelic states have been established [36]. *B. anthracis* isolates are differentiated by determining single nucleotide polymorphisms (SNPs) and variable number of tandem repeats (VNTRs) [37].

Diagnostics

Microbiological research methods are essential for a final diagnosis of anthrax, and are considered as a "gold standard". According to the Instructions for the laboratory diagnosis of anthrax in humans, in raw materials of animal origin and environmental objects, the identification of the causative agent of anthrax is carried out by the following features: the morphology of the microbe, including the presence of capsules in smears from the researched material; cultural properties; lack of hemolysis, immobility; sensitivity to penicillin; sensitivity to the specific phages; pathogenicity for laboratory animals [38].

The specified Instruction is valid but needs some revision, in particular, it lacks recommendations on the use of molecular methods of anthrax diagnosis. After all, there are much more specific and sensitive tests for the detection of the anthrax pathogen, which are outlined in the fourth edition of the "Recommendations for surveillance and control of anthrax in humans and animals" 2008, Geneva [4].

In 2014, the Ministry of Agrarian Policy and Food of Ukraine approved scientific and methodological recommendations "Laboratory diagnosis of animal anthrax, indication of the pathogen from pathological and biological material, raw materials of animal origin and environmental objects" for the work of laboratory specialists and scientific research institutions of veterinary medicine, teachers and students of the faculties of veterinary medicine of universities. This update covers all possible methods of anthrax diagnosis, including the lecithinase test, As-coli reactions, immunofluorescence, and polymerase chain reaction [39].

According to the Methodological recommendations, the test sample must be identified through a smear microscope; the study of cultural properties on nutrient media; carrying out tests for sporulation, motility and capsule formation, as well as detection of hemolysis. To speed up the pathogen identification, it is suggested to use PCR with primers for the genes with plasmid and chromosomal localization

The above-mentioned documents indicate the terms of examination of the experimental

material by the microscopic method — on the day of receipt of the material; bacteriological — up to 3 days; biological method — up to 10 days. The use of conventional microbiological methods requires the cultivation and processing of live microorganisms, which is always associated with the risk of laboratory infections. Because of this, the anthrax research must be conducted in laboratory facilities with biosafety level (BSL) 2+ or, ideally, level 3. Not all laboratories satisfy such criteria [40].

Diagnostics of anthrax by PCR has some difficulties due to the characteristics of the pathogen, since *B. anthracis* can be in a vegetative form in a living organism and in a spore form in the environment. Therefore, the applied research option directly depends on the type of a sample — clinical or from the environment.

The diagnosis of anthrax is definitively confirmed by a specific PCR test [32]. The essence of the method consists in the identification of the pXO1 plasmid and the pXO2 plasmid, as well as the pag and cap genes, which are specific for these plasmids [41, 42].

An important feature is that different strains of *B. anthracis* can have different variants of the plasmid composition. Depending on the presence of pXO1 and pXO2 plasmids, anthrax strains can be differentiated by virulence. According to the degree of virulence associated with the presence or absence of a capsule, four types of pathogens are distinguished: a virulent strain of *B. anthracis* (capx+ tocx+), containing plasmids pXO1 and pXO2, pathogenic for humans and animals; the vaccine strain of *B. anthracis* (capx– tocx+) includes the pXO1 plasmid in the absence of the pXO2 plasmid; an avirulent strain of *B. anthracis* (capx+ tocx–) containing the pXO2 plasmid, in the absence of the pXO1 plasmid. There is also a distinction between a pathogenic for laboratory animals and a non-pathogenic for humans strain of *B. anthracis* (capx– tocx–), which lacks both plasmids and, accordingly, lacks virulence.

PCR systems for the detection of *B. anthracis* were developed as early as in the 1990s [18, 43–45], but it probably takes some time before they become fully autonomous and widely available for use in the lay laboratory. This method does not involve the isolation of DNA and therefore a positive result is now rarely accepted separately for clinical samples and simple environmental samples such as tap water and air samples. If the reason for the negative PCR result is the presence of inhibitors of the polymerase reaction, it is necessary to conduct additional bacteriological studies. Clinical samples and more “complex” samples in terms of microbiome composition, such as feces, turbid water or soil, usually require a DNA isolation step, and also require prior application of microbiological methods of sample preparation to isolate and analyze a pure culture of bacteria to confirm positive or negative results.

The methods based on DNA amplification have some advantages, including the absence of culturing microorganisms and the possibility of testing inactivated samples, which makes these methods safer than traditional methods, and most importantly, the result can be obtained within a few hours.

Identification of microorganisms by the methods based on DNA amplification is carried out by selecting specific genetic markers.

These *B. anthracis* detection markers are located on pXO1 and pXO2 plasmids, which are anthrax-specific virulence detection plasmids [46]. Identification is based on the genes encoding the capsule located on the pXO2 plasmid and the detection of the genes located on the pXO1 plasmid encoding the three-component anthrax toxin [47, 48]. The presence or absence of one of the factors determines the degree of virulence of the pathogen [49–51].

DNA sequencing allows detection of specific single nucleotide polymorphisms (SNPs) specific to *B. anthracis*. However, DNA sequencing is a more complex method which requires special equipment. Therefore, for the detection of specific point mutations, the use of various molecular probes, HRM ("High Resolution Melting"), as well as PDRF (restriction fragment length polymorphism) methods, was proposed; as an example, we can cite the use of PDRF marker SG-850 (in other sources SG-749 due to the size of the amplicon in *B. anthracis*, as well as RSI-PCR of the plcR marker. Both methods are considered specific, but require time-consuming manipulations after PCR [52, 53].

The use of specific probes and real-time PCR for the determination of a point mutation in the plcR gene of *B. anthracis* has become an alternative research method. However, for some strains of *B. cereus*, a positive signal can be obtained with a delay, that reduces the sensitivity of the diagnosis.

The PCR anthrax identification is quite fast, but the plasmids diverging in sequence, may be missed so it cannot reveal the full content of plasmid genes. In addition, the number of copies of the plasmid and the degree of variation in the number of copies among representatives of *B. anthracis* have not yet been clarified. For example, using quantitative PCR (qPCR), some researchers found a ratio of up to 40.5 copies of plasmid pXO1 and 5.4 copies of plasmid pXO2 per genome [54], while Pilo *et al.* reported 10.89 as the average copy number for pXO1 and 1.59 for pXO2 [25, 55, 56]. Using digital PCR (dPCR) in the analysis of three isolates, Straub *et al.* reported that there are probably 3 to 4 copies of pXO1 per cell and 1 to 2 copies of pXO2 [57]. Sequence-based studies also indicated that there are probably 2 to 3 copies of pXO1 for each copy of the chromosome. An important limitation of these previous evaluations was that they were performed with a relatively small number of isolates, which may affect the characterization of copy number variation in the population [58, 59]. Additionally, the previous studies have shown that equally virulent *B. anthracis* strains, carrying both plasmids, can differ depending on the number of plasmid copies [60]. These results highlight the need to quantify accurately the plasmid copy variation in a large collection of diverse *B. anthracis* isolates and to assess whether the plasmid copy number is a phylogenetically stable trait.

With the acquisition of new knowledge on the molecular characterization of pathogenic microorganisms — new gene sequences and their fragments in PCR databases and samples of various forms: linear TaqMan, hairpin molecular beacons, scorpion primers, LUX primers, Sunrise primers, LNA-modified primers, it was established that the use of different test systems can give non-specific results.

The use of samples in TaqMan and molecular beacon formats with the help of polymerase chain reaction in real time differenti-

ated *B. anthracis* bacteria from representatives of the *B. cereus* sensu lato group. A fragment of the *ssp* gene of chromosomal DNA was experimentally determined as a target for primers and probes.

The basis was the hexanucleotide inertia specific only for the *B. anthracis* isolates.

The effectiveness of the method was confirmed [61] by the comparison of samples in TaqMan and molecular beacon formats for the detection of *B. anthracis* bacilli and their differentiation from the closely related species *B. cereus* and *B. thuringiensis* using real-time PCR. Currently, some difficulties have arisen in Ukraine with the usage of PCR for research and detection of the anthrax causative agent.

Scientific and diagnostic institutions used a kit manufactured in the Russian Federation — "AmpliSens® Bacillus anthracis–FRT" for real-time PCR (registration certificate of Roczdravnadzor FSR 2008/02417 dated March 13, 2019) for qualitative detection of DNA of vegetative and spore forms of *B. anthracis* in biological material and environmental samples and to determine the composition of the *B. anthracis* plasmid by identifying the pagA gene (plasmid pXO1) and the capA gene (plasmid pXO2) using polymerase chain reaction (PCR) with real-time hybridization–fluorescence detection.

In 2010, a domestic kit for the diagnosis of anthrax, the ANTHRAX test, was developed and registered, designed for the detection of specific DNA fragments in the studied samples using a multiplex variant of the polymerase chain reaction (PCR), which is distinguished by the fact that the authors used artificially synthesized oligonucleotide primers for PCR. (Patent Ukrainy No. 55775). It is used to control Ukrainian-produced vaccines and must be supplemented with VNTR locus analysis, since the control strain "Tsenkovsky-2" differs by the vrrC2 marker from the vaccine and endemic strains of *B. anthracis*. The set, designed for 50 samples of research material, is of high quality and used by the State Scientific and Control Institute of Biotechnology and Microorganism Strains for the characterization of the vaccine preparations.

Also in Ukraine, in commercial laboratories, PCR tests are used to identify the causative agent of anthrax, namely: Hypothetical protein Bacillus anthracis genesig Advanced Kit and Pheno Extreme — Bacillus anthracis. The institutions used these tests in food research.

If we analyze the above-mentioned kits for molecular diagnostics, then the AmpliSens® Bacillus anthracis–FRT test, RT–PCR analysis, manufactured by the Russian Federation, is designed for 50 reactions. This is an *in vitro* nucleic acid amplification test for the qualitative detection of DNA of vegetative and spore forms of *Bacillus anthracis* in biological material and environmental samples. It also determines the composition of the *Bacillus anthracis* plasmid by identifying pagA (plasmid pXO1) and capA (plasmid pXO2) using real-time hybridization-fluorescence detection of amplified products. Targets are specific fragments of the RA gene (pagA) and one of the structural genes of capsule formation (capA or capB) and does not contain a specific target for amplification of a gene fragment that has a chromosomal localization).

ANTHRAX test, analysis — CR, manufacturer — Ukraine, number of reactions — 50, detection threshold — qualitative determina-

tion of specific fragments of nucleic acid (DNA) in the tested samples using a multiplex variant of the polymerase chain reaction (PCR), which differs by using artificially synthesized oligonucleotide primers.

Hypothetical protein *Bacillus anthracis* genesig Advanced Kit test, analysis — RT–PCR, manufacturer — Primerdesign Ltd TM Concepción, Chile, number of reactions — 150 tests, MAX MIN specificity developed for quantitative determination of *B.anthracis* genomes *in vitro*. The primers present 100 % homology to more than 95 % of the NCBI reference sequence database; "Pheno Extreme — Bacillus Anthracis", assay — RT-PCR, manufacturer — Nottingham, UK, number of reactions — 100 tests, detection threshold — sensitivity: the AMD Bacillus Anthracis DNA kit is a very sensitive kit that reaches up to 2.2 copies/µl "rxn volume 25ul" according to our testing methods and devices. Specificity according to passport: The AMD Bacillus Anthracis DNA kit is highly specific to 100 % Yersinia pestis DNA according to our test methods and devices.

The PCR tests used to diagnose and differentiate anthrax are specific. If we compare Russian and domestic kits with Hypothetical protein Bacillus anthracis genesig Advanced Kit and "Feno Extreme — Bacillus Anthracis", the imported ones are quantitatively more sensitive. The primers of the above sets were tested for suitability for identification of anthrax (spore and vegetative form) in low concentrations from the studied blood samples [42–45]. Thus, with the help of the Hypothetical protein Bacillus anthracis genesig Advanced Kit and "Pheno Extreme — Bacillus Anthracis" sets, it is possible to identify vegetative forms of *B. anthracis* from blood samples at a concentration of 4×10^6 CFU per ml of blood, and spores at a concentration of 2×10^6 spores (corresponding to the insert sheet). Similar characteristics of "AmpliSens® Bacillus anthracis-FRT" (Russia) and ANTHRAX-test (Ukraine) are not indicated in patents and publications. The ANTHRAX — test is a fairly sensitive kit, but it gives false and false-positive results due to the phylogenetic similarity of *B. anthracis* with *Bacillus cereus*. Currently, no kit for the diagnosis of anthrax by the PCR method is registered or used in Ukraine.

Molecular diagnostics of anthrax ranks first in terms of speed and sensitivity. For example, in Slovenia, in 2015, six cows died suddenly within three days, two weeks later the number of dead animals increased to 12. Anthrax was suspected during the autopsy. The spleen tissue samples were collected (from 6/12 animals) and laboratory studies (microscopy, culture, and real-time PCR) were performed. The results of tissue staining for microscopy and culture were similar, whereas real-time PCR outperformed both methods of identification. The test of splenic tissues from all six animals was positive for anthrax in case of using real-time PCR, whereas *B. anthracis* were successfully cultured and detected by microscopy in spleens from only three animals [62]. The results suggest that molecular testing should be chosen as the first-line tool for confirming the animal anthrax outbreaks to ensure timely public health protection.

Traditional chromogenic and selective agars for the isolation of *B. anthracis* (including PLET agars) are complex and expensive for laboratories that investigate only the isolated cases of anthrax every few years [63]. Another

challenge for the laboratory diagnosis of sporadic anthrax is the use of alternative commercial methylene blue M'Fadyean stains, which often give ambiguous results and lead to the diagnostic errors [62]. On the other hand, the PCR study takes three hours and the test sample is inactivated.

An alternative approach for the detection of *B. anthracis* are immunoassays, such as: flow cytometry assays in combination with fluorescein-labeled antibodies [54] and FRET (Förster resonance energy transfer) [55, 58], ELISA [59], Luminex assay, magnetic fluorescent substance analysis, (MPFIA) [60], ABICAP immunofiltration, lateral flow analysis, biosensors, and some others [64, 65].

A possibility of apply the anthrax spores as a biological weapon emphasizes the need to develop the express diagnostic tests for the identification of pathogens that can be used as the agents of bioterrorism. The PCR-based methods are fast, specific and relatively easy to use and are ideal for accurate detection of the pathogen source (in this case, a dangerous biological agent), which will allow you to take timely measures for the localization, treatment, prevention and disinfection of the source of infection. One of the prerequisites for the timely investigation of a possible outbreak is the rapid and effective detection and confirmation of the disease on the basis of expert personnel of various specializations, from field veterinarians to pathologists and microbiologists.

Conclusions

Diagnosis of an infectious agent by molecular methods is a fast and sensitive type of identification. However, in the case of anthrax, there is a problem with obtaining false positive and false negative results due to the similarity of *B. anthracis* to the closely related species that are common in the environment. Because of this, it is recommended to carry out additional microbiological tests. This is especially true when diagnosing the infected people. After all, the further appointments and success of treatment will depend on the accuracy of the diagnosis.

The introduction of domestically produced portable test systems in the field of infectious disease diagnostics at the level of both scientific and research institutions and in city hospital and veterinary laboratory will open up the possibility of a fast response of specialists in the event of danger.

In order to eliminate the development of epizootics and to ensure biological security regarding anthrax, it is necessary to develop fast, sensitive and specific diagnostic methods. This will make it possible to localize the outbreak of anthrax and abolish the biological threat on the territory of Ukraine.

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Особливості ПЛР діагностики сибірки

У. М. Яненко, Г. А. Завірюха, Т. Б. Васильєва, З. Ю. Ткачук

Молекулярні методи діагностики за певних умов мають значну перевагу над тривалими класичними мікробіологічними методами. Через високопатогенні властивості *Bacillus anthracis* необхідно впровадити у практику швидкі методи ідентифікації збудника у разі виникнення біологічної загрози розповсюдження збудника інфекції серед сприятливих тварин, людей та зараження території. Ідентифікація *B. anthracis* є складною через спорову та вегетативну форму існування та схожість із близькоспорідненими видами. Україна є неблагополучною державою щодо сибірки. Впровадження надійних, чутливих і специфічних молекулярних методів діагностики є пріоритетним напрямком у вирішенні питання біобезпеки.

Ключові слова: *Bacillus anthracis*, діагностика, ідентифікація, експрес–тести, мікробіологічні дослідження, методи молекулярної мікробіології.

Received 05.09.2022