

UDC 579.62: 577.213.3

REVIEW AND ANALYSIS OF ANTHRAX DIAGNOSTIC TOOLS ON THE TERRITORY OF EURASIAN ECONOMIC UNION

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ABSTRACT

This review examines anthrax diagnostic tools registered in the national Veterinary Drugs Registers and Medicines and Medical Devices Registers of the Eurasian Economic Union (EAEU). Actually, throughout the entire territory of the EAEU, there are natural soil foci of anthrax, which causes annual cases of infection of animals and people. Continuous monitoring of the epidemiological situation in the EAEU countries should be ensured by modern diagnostic tools. All diagnostic tools used for epidemiological surveillance must be registered in the national registers of the EAEU countries. For all test systems and diagnostic kits included in the registers of the EAEU countries, the authors provide comparative characteristics that reflect the sensitivity and specificity of diagnostic tools. An analysis of various methods for identifying the anthrax pathogen, focused on modern molecular diagnostic technologies, is presented. The authors discuss the most important problems associated with improving the efficiency of modern methods for detecting the bacterium *Bacillus anthracis*. Some considerations about the shortcomings in certain areas of early diagnosis of the anthrax microbe are also provided. The authors present a number of proposals that should improve the efficiency of the currently available anthrax monitoring system.

Keywords: *Bacillus anthracis*, diagnostic methods, biosafety, anthrax, Eurasian Economic Union, test systems

INTRODUCTION

The Eurasian Economic Union (EAEU) [1] includes five countries, four of which are unfavorable for anthrax (Kazakhstan, Kyrgyzstan, Armenia and Russia). Only in Belarus, the epidemiological situation with anthrax has remained stable since 1995 – over the past 28 years, no cases of disease have been recorded among people [2]. Nevertheless, the threat of anthrax is also relevant for Belarus, since anthrax cattle burial grounds are located on its territory, at least in the Vitebsk region, and cases of animal diseases are sporadically recorded [2].

Severe situation in terms of the anthrax spread is on the territory of the Republic of Kazakhstan, where 1778 permanently unfavorable for anthrax settlements, 2433 epizootic and 2249 soil foci of anthrax are registered [3]. According to the "Scientific and Practical Center for Sanitary and Epidemiological Expertise and Monitoring" of the Ministry of Health of Kazakhstan (<https://rk-ncph.kz/>), the average anthrax cases since 2009 is 6 people per year.

At present, anthrax continues to pose a serious problem for the Russian Federation, where more than 35,000 stationary anthrax sites (SNPs) are registered and 3,193 anthrax animal burial grounds are registered [4]. According to statistics over the past twenty years in Russia, the average incidence of anthrax is about ten people per year [5, 6].

In the Republic of Kyrgyzstan, the average anthrax cases since 2005 is about 19 people per year [7, 8]. At the same time, according to the National Statistical Committee of the Kyrgyz Republic, from 2011 to 2022, the average case number is about 13 people per year [9]. In Kyrgyzstan, 1,445 soil foci were identified, and in 2012, 1,235 soil foci were officially registered [8].

The Republic of Armenia does not maintain epidemiological statistics for anthrax. From separate sources [5, 10],

it was possible to establish the number of cases in different years (2012-2014, 2019, 2021); on average, over these years, the incidence was about 9-10 people per year.

Currently, there are no effective and environmentally friendly methods and means for anthrax foci elimination [11]. *B. anthracis* cells are able to live and multiply in soil amoebas and plant rhizosphere [12, 13] and are spread from the anthrax focus by earthworms [14], which makes possible the constant presence of anthrax in the affected areas. Thus, a well-established system for the early diagnosis of anthrax is necessary throughout the EAEU.

Anthrax diagnostic tools registered in EAEU countries

In the EAEU countries, a number of diagnostic tools and test systems for the anthrax detection are currently registered. It should be noted that in all EAEU countries, except for Armenia, such test systems and reagents can only be used after they are registered in the Veterinary Drugs Register or Medicines and Medical Devices Register, depending on the subject of diagnosis [15]. In Armenia, according to national legislation, the import, sale and use of medical equipment and medical devices (which includes diagnostic tools) is allowed without state registration. However, in connection with Armenia's accession to the EAEU, the national legislation is being harmonized. Thus, an updated version of the law «On Medical Assistance and Services to the Population» and a number of related laws regarding the process of registration and circulation of medical devices have already been adopted. But the Register of Medical Devices of Armenia is still under development. Table 1 provides information on anthrax diagnostic tools included in the registers of the EAEU countries.

Immunobiological diagnostic tools

Among the veterinary diagnostics, diagnostic sets designed to detect specific antibodies to *B. anthracis* in the blood of animals using the indirect hemagglutination reac-

tion (IHA) prevail. These drugs include anthrax precipitating serum, which is registered in Armenia (paragraph 1.1 of Table 1), Kazakhstan, Russian Federation and Kyrgyzstan (paragraph 1.2 of Table 1). The IHA method makes it possible to

detect specific antibodies against anthrax in the blood of sick animals; the reaction of IHA must be placed in parallel with the reaction of indirect hemagglutination inhibition to confirm the specificity of antibodies [16]. To do this, anthrax bacterial

Table 1 – Anthrax diagnostic tools included in the registers of the EAEU countries.

№	Name of the diagnostic tool	Manufacturer	Country of registration
1. Veterinary purpose			
1.1	Precipitating serum against anthrax	«Antigen» LLC, Kazakhstan	Armenia
1.2	Precipitating anthrax serum from horse blood	FCE «Orlovskaya bio factory», Russia	Kazakhstan Russia Kyrgyzstan
1.3	Standard bacterial anthrax antigen	«Antigen» LLC, Kazakhstan	Kazakhstan
1.4	Standard bacterial anthrax antigen	FCE «Orlovskaya bio factory», Russia	Russia
1.5	Test system for <i>Bacillus anthracis</i> species identification by polymerase chain reaction	«Vetbiohim» LLC, Russia	Armenia
1.6	Set of reagents «PCR-F-anthrax-factor»	«VetFaktor» LTD, Russia	Russia
2. Medical purpose			
2.1	Fluorescent immunoglobulin for anthrax diagnostic, non-adsorbed, dry, lyophilized (for diagnostic purposes)	«Medgamal» Branch of FSBI «RIE named after N.F. Gamalei», Russia	Russia
2.2	A set of reagents for the immunochromatographic rapid detection and identification of spores of the anthrax pathogen (IC-test <i>B. anthracis</i>)	FBUN SSC PMB, Obolensk village, RF	Russia
2.3	Anthrax antigen erythrocytic diagnosticum, dry	RSE on the REM “National Scientific Center for Especially Dangerous Infections named after M. Aikimbaev”, Ministry of Health of Kazakhstan	Kazakhstan
2.4	Anthrax immunoglobulin erythrocytic diagnosticum, dry	RSE on the REM “National Scientific Center for Especially Dangerous Infections named after M. Aikimbaev”, Ministry of Health of Kazakhstan	Kazakhstan
2.5	Diagnostic phage-test-kit for identification of anthrax pathogen (Phage-test-kit «Obolensk R1»)	FBUN SSC PMB, Obolensk village, RF	Russia
2.6	Diagnostic bacteriophage «A» against anthrax; dry and liquid	RSE on the REM “National Scientific Center for Especially Dangerous Infections named after M. Aikimbaev”, Ministry of Health of Kazakhstan	Kazakhstan
2.7	Test system for <i>B. anthracis</i> pXO1+ DNA detection by PCR (GenSib)	FKUZ RosNIPCHI «Microbe», RF	Russia
2.8	A set of reagents for <i>Bacillus anthracis</i> DNA detection in biological material and environmental objects by polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection «AmpliSense <i>Bacillus anthracis</i> -FRT»	FKUZ RosNIPCHI «Microbe», RF	Russia
2.9	A set of reagents for <i>Bacillus anthracis</i> DNA (pXO1 and pXO2) detection in biological material and environmental objects by real-time PCR «7R Bio® <i>Bacillus anthracis</i> »	«7R Bio», Kazakhstan	Kazakhstan
2.10	Kit «CAMOMILE- <i>Bacillus anthracis</i> -PCR» for <i>Bacillus anthracis</i> DNA (pXO1 and pXO2) detection in biological material and environmental objects by real-time PCR	«Diamed Asia Test», Kazakhstan	Kazakhstan
2.11	A set of reagents for the detection of DNA pathogens of plague, anthrax and tularemia by real-time PCR «MULTI-FLU»	FBUN SSC PMB, Obolensk village, RF	Russia

antigen, which is registered in Kazakhstan (paragraph 1.3 of Table 1) and the Russian Federation (paragraph 1.4 of Table 1), is introduced into the reaction. Both reactions are carried out in special round-bottom polystyrene plates, the total reaction time from serum preparation to analysis of the results is about five hours. The main disadvantages of this method are its low sensitivity and cross-specificity with other species of the genus *Bacillus* [17]. Also, the disadvantages of the method include the lack of numerical data and a subjective assessment when analyzing the results of precipitation, since at low antibody titers it is almost impossible to find the difference between “agglutinated” and “non-agglutinated” wells.

Also in Kazakhstan is registered “Anthrax antigen erythrocytic diagnosticum” (paragraph 2.3 of Table 1), which includes both anthrax precipitating serum and anthrax bacterial antigen. This diagnosticum is intended both for IHA and IHA-inhibition performance in the human serum. Also, immunobiological diagnostic products for medical purposes include «Anthrax immunoglobulin erythrocytic diagnosticum» (item 2.4 of Table 1), registered in Kazakhstan, and registered in the Russian Federation «A set of reagents for the immunochromatographic rapid detection and identification of spores of the anthrax pathogen (IC-test *B. anthracis*)» (item 2.2 of Table 1) and «Fluorescent immunoglobulin for anthrax diagnostic» (item 2.1 of Table 1).

«Anthrax immunoglobulin erythrocytic diagnosticum» is intended for the detection of *B. anthracis* specific antigens, both in blood and in tissues, or in pathological material [16]. For this diagnosticum, the reactions of IHA and IHA-inhibition are also put in parallel to assess the specificity of the antibodies used; agglutinating serum is additionally added when IHA-inhibition is performed. This method is very specific, but has low sensitivity [18, 19]. At the same time, the search for bacterial antigens can be carried out in virtually any type of material (including pathological), and not only in serum.

«Fluorescent immunoglobulin for anthrax diagnostic» is used to set up the method of fluorescent antibodies (MFA) of smears from a bacterial suspension, materials from corpses, smears-imprints from animal and human organs. The active substance is an immunoglobulin fraction of anthrax serum labeled with fluorescein-5-isothiocyanate (FITC) [20]. Smears are microscopied in a fluorescent microscope with a system of selected light filters and an immersion lens. Spores and vegetative cells of *B. anthracis*, stained with luminescent immunoglobulins, give a bright glow to the cell periphery. Such luminescence is called specific, in contrast to nonspecific luminescence, which is characterized by a uniform, dim luminescence of the entire cell surface [21]. The time of setting up the method to obtain the result takes from two to six hours. It should be taken into account that the specificity of this method is no more than 70% due to the presence of cross-reacting antigens in representatives of the *B. cereus* group [22]. It should also be noted that the sensitivity of this method is low [23] and in diagnosing anthrax, it is used only as an additional one.

The last of the immunobiological diagnostic kits registered in the EAEU is “A set of reagents for the immunochromatographic rapid detection and identification of spores of the anthrax pathogen (IC-test *B. anthracis*)”. A specific target, the presence of which in the sample is detected by this test, is the *B. anthracis* spore antigen. *B. anthracis* spores present in

the sample interact with gold-labeled antibodies, after which the stained antigen-antibody complex accumulates in the test zone of the membrane [24]. This kit produces results after 15-20 minutes of incubation, that is, the total time for setting up the analysis does not exceed half an hour. The sensitivity of the kit is low (10^8 spores / ml), which allows usage of this diagnosticum only as an addition to bacteriological methods for identifying anthrax, it was also shown that this IC-test shows the presence of cross-reactions with closely related saprophytes [19].

Diagnostic bacteriophages

There are two registered diagnostic bacteriophages – Phage-test-kit «Obolensk R1» (item 2.5 of Table 1), registered in Russian Federation, and Diagnostic bacteriophage «A» against anthrax (item 2.6 of Table 1), registered in Kazakhstan. The anthrax bacteriophage susceptibility test is one of the key tests in obtaining the final results of a complete laboratory analysis [19, 21]. Bacteriophages used to identify *B. anthracis* cultures principally differ in the spectrum of anthrax strains they lyse, as well as in the specificity of their action [19, 25]. Most often, the results of determined lytic activity of phages in various studies are not always unambiguous and depend on the microorganism panels used [19].

Thus, Gamma A-26 bacteriophage (“Bacteriophage diagnostic anthrax “A”) in one study [26] showed lytic activity against 97.9% of *B. anthracis* strains out of 47 studied, and a specificity of 95.8% (69 out of 72 strains heterologous microorganisms were resistant to bacteriophage). According to the results of another study [25], both Gamma A-26 and R/D-Ph-6 (bacteriophage of the “Obolensk R1” test kit) showed a lytic effect in 99.1% of cases (test panel of 114 strains of *B. anthracis*). Golovinskaya *et al.* [27] evaluated the specificity of bacteriophages with 74 strains of genus *Bacillus* that do not belong to the species *B. anthracis*. It was shown that R/D-Ph-6 had 100% specificity, and for Gamma A-26 non-specific lysis was observed in 5 strains, thus, its specificity was 93.2%.

The average time to obtain the result of specific lytic activity against an isolated suspicious *Bacillus* strain ranges from 5 (preliminary results are possible) to 18 hours [26]. It should also be noted that this method requires dishes with a monoculture of a bacterial strain isolated from the test sample, which adds at least two days to the total analysis time. In addition to the duration of the analysis, the lack of 100% specificity and sensitivity should be taken into account. For this reason, combinations of various bacteriophages are often used [25].

Molecular biological methods based on PCR

The most sensitive and specific of the currently available anthrax diagnostic tools are test kits based on the polymerase chain reaction (PCR) principle [23]. Less sensitive and more labor-intensive test systems based on the classical (by the final product) PCR are presented in the registries of the EAEU countries in three sets – two for veterinary use and one for medical use. Diagnostic kits for veterinary purposes include «Test system for *Bacillus anthracis* species identification by polymerase chain reaction» (item 1.5 of Table 1), registered in Armenia, and «PCR-F-anthrax factor» (item 1.6 of Table 1) registered in Russian Federation. Diagnostic kit for medical purposes – «Test system for *B. anthracis* pXO1+ DNA de-

tection by PCR (GenSib)» (item 2.7 of Table 1) is registered in Russian Federation.

The GenSib test system is based on the detection of the *pagA* gene fragment (plasmid pXO1) and is one of the first test systems for anthrax PCR diagnosis that has passed acceptance tests and was approved for use [28]. This test system uses two pairs of primers, BA1-BA2 (outer), flanking a 409 nt region of the *pagA* gene located on the pXO1 plasmid, and VAZ-BA4 (internal), providing amplification of a 154 nt DNA fragment [29]. The product of the PCR reaction is analyzed in a 1.5-2.5% agarose gel. For this test system, 100% specificity was shown, and sensitivity was defined as 10 - 1000×10⁶ cell/ml (on pure *B. anthracis* cultures) and 100 - 1000×10⁶ cell/ml (on biological material and environmental objects artificially infected with *B. anthracis*). Since the test system was developed and approved for use at the dawn of PCR diagnostics era (acceptance tests, examination and approval lasted from 1998 to 2004) [29], it lacks both the detection of reference genes and internal control. Sensitivity is also determined in units of «millions of cells per milliliter», which is dubious for modern molecular biological methods.

The “PCR-F-anthrax-factor” kit allows specific amplification of a fragment of the *Bacillus anthracis* genome (200 nt PCR product) and internal positive control DNA (500 nt PCR product) in a multiplex polymerase chain reaction. Detection of amplification products occurs in agarose gel. The kit includes an Internal Control (Bacteriophage T4 DNA), positive and negative controls. The sensitivity for this test system is not specified, as well as the bacterial target gene [30].

The last classic PCR kit registered for veterinary usage in EAEU is the «Test system for the identification of the species *Bacillus anthracis* by polymerase chain reaction» manufactured by Vetbiohim (Moscow). This test system consists of three kits: for DNA extraction, for detection of *B. anthracis* DNA by PCR, and for electrophoresis [31]. In this test system, *B. anthracis* DNA is identified by detection of the capsular formation gene (*CapB*), that is, the presence of the pXO2 plasmid in the analyzed material is determined. The kit contains positive (plasmid) and negative controls; the size of the fragment detected after PCR is 300 nt. Neither the sensitivity nor the specificity of the test system is specified.

The registries of EAEU countries include four test systems for *B. anthracis* DNA detection using real-time PCR (qPCR), two of which are registered in Russian Federation (items 2.8 and 2.11 of Table 1) and two in Kazakhstan (items 2.9 and 2.10 of Table 1). Of all currently used tools for detecting the anthrax microbe, test systems based on qPCR are the most sensitive and specific [23, 32], but also the most expensive.

The “AmpliSense *Bacillus anthracis*-FRT” reagent kit (item 2.8 of Table 1) is designed to detect *B. anthracis* DNA in biological material and environmental objects by PCR with hybridization-fluorescence detection in the «real time» mode [33]. This kit is used to determine the plasmid composition of *Bacillus anthracis* by detecting the *pagA* gene (plasmid pXO1) and the *capA* gene (plasmid pXO2). The diagnostic kit includes an internal control that is added to the samples prior to DNA extraction and serves as a marker of the quality of the extraction procedure and the presence of PCR inhibitors. Thus, this multiplex test system detects three targets – two *B. anthracis* plasmid genes (fluorescent probes labeled

with FAM and JOE) and an internal control gene (fluorescent probe labeled with ROX). The sensitivity of this test system in the study of pure cultures of *B. anthracis* was 1 × 10³ spores/ml [34]. At the same time, field trials of “AmpliSense *Bacillus anthracis*-FRT” revealed a number of shortcomings of the kit [23] – the target *pagA* gene was detected only in 32% of positive samples (confirmed by bacteriology), while the *capA* gene was detected in 89%. The obtained results contradicted the phenotypic properties of the cultures, since all of them were typical virulent strains, so the sensitivity of the test system for determining the plasmid composition of *B. anthracis* is insufficient.

Two test systems registered in Kazakhstan – «7R Bio® *Bacillus anthracis*» and «CAMOMILE-*Bacillus anthracis*-PCR», are designed to detect *B. anthracis* DNA (pXO1 and pXO2) in biological material and environmental objects by real-time PCR time. These sets are in fact the same set; in addition, both sets are produced at the same production site and are represented by one authorized representative (Diamed LLP) [35, 36]. In both sets, *B. anthracis* is identified by the *pagA* gene (pXO1) and *capA* gene (pXO2). The amplification reaction is carried out in the reaction mixture in the presence of an internal control, since it is part of the PCR mix of the kit. Thus, the internal control in this test system serves only to determine the presence of inhibitors in the analyzed sample. To detect the fluorescence signal DNA probes labeled with fluorophores HEX (for IC), FAM (for *pagA*), and ROX (for *capA*) are used. For both test systems, the sensitivity is indicated as 1000 GE/ml for pXO1 and pXO2 DNA in standard samples of manufacturer; the specificity is 100% also according to the company’s standard panel of negative DNA extracts. These references to standard samples of the enterprise, when indicating sensitivity and specificity, show the need for unification and standardization of units of measurement. The only difference between these test systems is their positive controls – “CAMOMILE-*Bacillus anthracis*-PCR” has a single positive control (PC) containing specific pXO1 and pXO2 DNA fragments, while “7R Bio® *Bacillus anthracis*” has two separate positive control samples «PC pXO1» and «PC pXO2».

The last of the registered test systems is a set of reagents for the detection of DNA of the causative agents of plague, anthrax and tularemia by real-time PCR «MULTI-FLU» (item 2.11 of Table 1). This kit defines four targets: one gene from each pathogen and one internal control [37]. Fluorophore-labeled DNA probes detect IC (by Cy5), a fragment of the *Y. pestis* methyltransferase gene (by ROX), a fragment of the ISFtu5 insertion element gene of *F. tularensis* (by HEX), and a fragment of the *sspE* gene of the *B. anthracis* chromosome (by FAM). The kit contains a positive control sample (PC), which is the same for all three types of bacteria, a negative control sample (NC), and negative inner control for sample isolation (NIC). The PC contains fragments of the target genes of *Y. pestis*, *F. tularensis*, *B. anthracis*, and the NIC contains an internal control sequence, which is also added to the sample of negative isolation control during isolation. Thus, during real-time PCR, the fluorescent signal through the Cy5 channel is detected only in NC and NIC, which makes it possible to evaluate the performance of the test system components (by NC) and the quality of the DNA extraction procedure

(by NIC). The set has a sensitivity of 1×10^4 m.c. / ml (millions of cells) of each of the bacteria and diagnostic sensitivity – not less than 85%. Only diagnostic specificity is indicated (the proportion of correct negative results in clinical trials) – at least 90%.

DISCUSSION AND CONCLUSION

Such is a brief overview of the diagnostic tools included in the registers of the EAEU countries. Analyzing the information above, attention is drawn to the disproportion in the availability of diagnostic tools for veterinary and medical sciences – for the needs of veterinary, six positions have been entered in the registers of the EAEU countries, and for the needs of medicine – eleven. Moreover, out of six diagnostic tools registered for veterinary use, four are sera and antigens for IHA, and two are test systems based on classical PCR. Not a single kit based on real-time PCR (the most specific and sensitive diagnostic method at the moment) has been registered for the needs of veterinary. Such diagnostic support is clearly not enough, since it is predominantly animals that are infected – they can become infected directly from soil anthrax foci and, therefore, are the primary and most numerous sources of infection for humans. Of course, diagnostic tools based on IHA are the cheapest and do not require complex and expensive equipment, but they are also the least specific and sensitive. The development and registration of a separate qPCR test system for the needs of veterinary will entail serious labor and financial costs, and will also take a long time. We propose to consider at the legislative level the possibility of using registered medical diagnostic tools for the needs of veterinary without any additional research and checks. Since these test systems have already passed all the tests when they were registered as medical devices, their use as certified diagnostic kits for veterinary is fully justified. The cost of real-time PCR test systems makes it impossible for them to be widely used for routine veterinary checks, but in the analysis of animal products (meat, milk, skin) for which infection is suspected, or in the case of epidemic outbreaks, when speed and accuracy of analysis are required, these test systems should be used first.

In addition to the “veterinary-medicine” disproportion, there is also a strong bias in relation to individual state registries, that is, in some EAEU countries; there is a lack of registered diagnostic tools in case of epidemic outbreaks. Thus, in the registries of Armenia and Kyrgyzstan there are no test systems for medical purposes and in the veterinary registries of Kazakhstan and Kyrgyzstan there are no sensitive PCR test systems. Since the harmonization of the legislative bases of the EAEU countries continues and the “Unified Register of Medical Devices Registered within the EAEU” (<https://portal.eaeunion.org/>) is currently being created, it is likely that after all approvals are completed, the registration of a diagnostic tool in one of EAEU states will mean the possibility of its application in all other states.

The next important point related specifically to PCR test systems (both classical and real-time PCR) is the lack of common units for measuring their sensitivity. So, at present, different test systems use such sensitivity units as GE/ml (genome equivalent), spores/ml, m.c. /ml (millions of cells per milliliter) and copies/reaction. First of all, spores and millions of cells per milliliter are standard units for bacteriology, but

for molecular biological methods they are not accurate due to the presence of a nucleic acid isolation step. Depending on the extraction method and on the isolation kit, the amount of DNA extracted from the same number of cells will change (sometimes even multifold), so these units of measurement cannot be universal.

As for genomic equivalents and copies per reaction, these units of measurement are virtually identical if only one copy of detectable gene is presented in the genome. However, this does not apply to the PCR test systems we have considered, except for MULTI-FLU, since only in MULTI-FLU the target is the *spsE* chromosomal gene, which is present in a single copy on a single chromosome of *B. anthracis*. This bacterium, like the entire *B. cereus sensu lato* group, is a monoploid species [38], but monoploidy (or haploidy, as is customary for eukaryotic species) extends only to the genetic material of the chromosome, since the virulent plasmids pXO1 and pXO2 are present in the bacterial cell in multiple copies. Moreover, the number of copies of plasmids is different for different strains of *B. anthracis*. Thus, in the most complete study of 412 strains [39], the average number of their copies per cell was found to be 3.86 and 2.29, respectively, for plasmids pXO1 and pXO2. Since *B. anthracis* in all PCR kits included in the registries of the EAEU countries (except for “MULTI-FLU”) is detected by one or more genes of the pXO1 and/or pXO2 plasmids, the number of genomic equivalents will differ greatly for different strains. At the same time, such a unit of measurement as “copy/reaction” is universal, since it absolutely unambiguously determines the minimal number of target copies that can be detected in one PCR reaction. And it concerns neither the volume of the introduced sample, nor the number of cells from which the sample was extracted. In many foreign test systems for *B. anthracis* detection, sensitivity is indicated precisely in copies per reaction – «RealArt™ *B. anthracis* PCR» (Roche), «ViPrimePLUS *Bacillus anthracis* qPCR Kit» (VivanTechnologies), «*Bacillus anthracis* genesig Advanced Kit» (Primerdesign Ltd). In addition, the transition to sensitivity in copies per reaction is relatively simple, since all test systems are provided with plasmid positive controls with known sequences (and hence molecular weights), it will not be difficult to calculate the copy number and build a calibration curve from dilutions in copies.

Analysis of the PCR test systems presented on the international market and those included in the EAEU registers revealed the need to develop a test system based on real-time PCR, which would detect one target gene of both virulent plasmids pXO1 and pXO2, a chromosomal target and reference gene or internal control sequence. Such a complex system is necessary because of the high homology between *B. cereus sensu lato* group, which includes *Bacillus anthracis*. It is known that there are strains of *B. anthracis* lacking one or both virulent plasmids [40] and isolates of *B. thuringiensis* and *B. cereus* containing plasmids or fragments of plasmids pXO1 [41] or pXO2 [42]. Thus, the need to determine the “chromosomal signature” of *B. anthracis* is obvious, but at the same time, the virulence of strains directly depends on the presence of plasmids, so the determination of plasmid target genes is also necessary. At the same time, for such a test system, it is necessary to have control over the course of the amplification and assess the quality of nucleic acid extraction

– for samples obtained from cells of eukaryotic or prokaryotic organisms, it would be logical to determine the reference gene, and for environmental samples – inner control [43].

Work in this direction was carried out at the Stavropol Anti-Plague Institute – multiplex qPCR «*Bacillus anthracis* multiplex 3 FRT» was developed there to determine the gene of edema factor *cya* (pXO1), the encapsulation gene *capC* (pXO2), the chromosomal *sap* gene and the internal control sample, which is a modified fragment of the lambda phage genome [44]. Apparently, the experimental test system is still under development, since in the latest available publication [23], the developers changed the chromosomal *sap* gene to the prophage region_03 (the name of the test system was shortened to “*Bacillus anthracis* 3 FRT”). The Central Reference Laboratory of Kazakhstan is also developing a multiplex qPCR test system «Anthrax Screener 18S». In this system, the *RNA18S1* gene region, which is highly conservative in 18S rRNA of all vertebrates [43], is detected as a reference gene; two genes *BA_5358* and *BA_5361* of the lambda_Ba03 prophage [45], as well as the *cya* gene of the pXO1 plasmid and gene *14* of the plasmid pXO2.

In addition to molecular biological methods for diagnosing the anthrax pathogen, immunological methods, or rather their extreme one-pointedness, also attract attention. The most common, and in the veterinary field, the only immunological test systems are IHA-diagnosticums. Their widespread usage is explained by their low cost and minimal requirements for laboratory equipment. Not a single ELISA test system has been registered in any of the EAEU countries, although ELISA test systems have both higher sensitivity and higher specificity compared to erythrocyte diagnosticums [32]. Unlike IHA-diagnosticums, ELISA test systems require a special reading device – a plate-reader, but at the same time they provide numerical data and not a subjective difference «by eye». If, when developing an ELISA test system, modern molecular biological methods are applied, in particular, obtaining *B. anthracis* antigens using genetic engineering, then the specificity and sensitivity of such test systems will increase multi-fold compared to IHA-diagnosticums.

In particular, it is known that a powerful humoral immune response occurs in the body in response to anthrax toxins: PA (protective antigen), LF (lethal factor), and EF (edematous factor) [46]. ELISA test systems based on the detection of antibodies (IgG) to the protective antigen – «Anthrax Protective Antigen IgG ELISA» (Sigma-Aldrich, Abnova, Creative Diagnostics) are currently presented on the international market. However, it is known that the immune response to LF in patients [47] is stronger and occurs faster compared to the response to PA. In addition, PA is the main component of anthrax vaccines, which makes it difficult to differentiate cases of infection from vaccination. Therefore, the development of an ELISA test system aimed at detecting antibodies to a lethal factor is a promising direction in the diagnosis of *B. anthracis*. Based on the logic of the development of epidemiological diseases diagnostics, ELISA kits should first become an alternative, and eventually completely replace IHA-diagnosticums in the field of veterinary. It is in veterinary medicine that ELISA kits will be more in demand compared to PCR kits. This is primarily due to the simplicity of sample preparation for ELISA, the lower cost of both the test system itself and the necessary

accompanying reagents. Thus, it is advisable to develop and implement a domestic (for the entire EAEU) ELISA test system for detecting antibodies to *B. anthracis*.

The last factor that we would like to discuss in our analysis is the fact that *B. anthracis* not only belongs to the pathogens of group II (group A according to the CDC standards), but is also a successfully used bioweapon [46, 48]. As the anthrax spore attacks in the United States in 2001 showed, the speed of diagnosing this biological threat is insufficient. With covert and massive aerosolization of spores, the initial diagnosis of anthrax in the United States would have been a complete failure. This is because the initial symptoms of inhaled anthrax mimic those of the flu, so by the time a correct diagnosis is made it is too late to do anything. In addition, even after establishing the fact of a biological attack, at the initial stage it is difficult to diagnose which of the possible biological agents was used. In particular, the pulmonary form of anthrax is extremely similar symptomatically to the pulmonary form of plague, tularemia, as well as melioidosis and legionellosis [48].

Thus, it becomes necessary to create a diagnostic system in which all biological agents that give similar symptoms by military (or terrorist) use would be simultaneously determined. The possibilities of qPCR (the most sensitive method) are limited by the number of detectable fluorescent dyes – a maximum of 4-5 simultaneously, while at least three target genes are required for the full detection of anthrax alone. The only solution to this problem at the moment is the technology of microarrays (DNA-microarray) or as they are also called DNA-chips or biochips. This approach, in addition to good sensitivity and high specificity [49], makes it possible to simultaneously detect several thousand targets. For example, Applied Biosystems' «GeneChip *E. coli* Genome 2.0 Array» DNA-chip detects 10,000 targets. Currently, this technology is used mainly for analysis of the expression of complete genomes (transcriptomes), microRNA profiles, or for genotyping. With such an excess number of recognizable targets, each pathogen can be detected by several hundred specific DNA sequences. In addition to pathogen detection and identification, microarrays are ideal for characterizing genetic differences between isolates of the same species at the strain level [50]. For example, Zwick *et al.* [50] designed and applied a custom analysis panel for 56 *B. anthracis* strains. The overall time to obtain results was much faster with DNA-chip compared to conventional sequencing methods, and typing results were comparable.

Thus, based on the foregoing, we made the following general conclusions on the availability of diagnostic tools for detecting anthrax on the territory of the EAEU:

1) insufficient provision of veterinary with diagnostic kits. It is necessary to be able to use kits registered for medical use in veterinary needs. The disproportion between the EAEU states in registered diagnostic tools needs to be leveled.

2) the need to determine the common sensitivity measurement units to all PCR test systems. Since plasmid controls are used in all PCR test systems, it is possible to proceed to the determination of sensitivity in copies per reaction for test systems already included in the registers. This will also enable the EAEU test systems to enter the international market, as a significant part of imported PCR kits indicate the sensitivity

in copies per reaction.

3) the need to develop and add to the registries a qPCR test system in which both chromosomal and plasmid genes would be determined simultaneously as target genes, along with the mandatory determination of a reference gene or an internal control gene.

4) immunological diagnostic methods are represented mainly by IHA-diagnosticums; there are no ELISA test systems in the registries of the EAEU countries. ELISA test systems for the determination of anthrax antibodies in the blood of infected animals should become the basis for epidemiological surveillance in the field of veterinary.

5) from the standpoint of biosafety for the detection of dangerous and especially dangerous pathogens, it is necessary to develop technologies for DNA-chips. It would be timely to develop a chip that can simultaneously identify pathogens that have similar symptoms at the initial stage of infection. For example, detection of plague, anthrax, tularemia, melioidosis and legionellosis pathogens on a single biochip.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FUNDING

The analysis was funded under the program BR10764975 of the Ministry of Agriculture of the Republic of Kazakhstan.

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ЕУРАЗИЯЛЫҚ ЭКОНОМИКАЛЫҚ ОДАҚТЫҢ АУМАҒЫНДАҒЫ СІБІР ЖАРАСЫН АНЫҚТАУҒА АРНАЛҒАН ДИАГНОСТИКАЛЫҚ ҚҰРАЛДАРДЫ ШОЛУ ЖӘНЕ ТАЛДАУ

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ТҮЙІН

Бұл шолуда Еуразиялық экономикалық одақ мемлекеттерінің (ЕАЭО) медициналық бұйымдары және ұлттық дәрі-дәрмектер тізілімі мен ветеринарлық препараттар тізіліміне кірген сібір жарасының диагностикасына арналған бұйымдар қарастырылған. Іс жүзінде ЕАЭО-ның бүкіл аумағында жануарлар мен адамдар арасында жыл сайын ауру жағдайларын тудыратын сібір жарасының табиғи топырақ ошақтары бар. ЕАЭО елдерінің эпидемиологиялық жағдайы заманауи диагностикалық құралдарымен сібір жарасына тұрақты бақылануы қажет. Эпидемиологиялық қадағалау үшін қолданылатын барлық диагностикалық құралдар ЕАЭО елдерінің ұлттық тізілімдерінде тіркелуі тиіс. ЕАЭО елдерінің тізілімдеріне енгізілген барлық тест-жүйелер мен диагностикалық жиынтықтар үшін авторлар диагностикалық құралдардың сезімталдығы мен ерекшелігін көрсететін салыстырмалы сипаттамаларды келтіреді. Қазіргі молекулярлық диагностикалық технологияларға бағытталған сібір жарасының қоздырғышын анықтаудың әртүрлі әдістерін қолданудың талдауы ұсынылады. Авторлар *Bacillus anthracis* бактериясын анықтаудың заманауи әдістерінің тиімділігін арттыруға байланысты маңызды мәселелерді талқылап, сібір жарасы қоздырғышының ерте диагностикасының жекелеген бағыттарындағы кемшіліктерді қарастыруға салады. Сондай-ақ авторлар сібір жарасының бақылау жүйесінің қазіргі кездегі тиімділігін арттыруға тиіс бірқатар ұсыныстарды келтіреді.

Түйін сөздер: *Bacillus anthracis*, диагностика әдістері, биоқауіпсіздік, сібір жарасы, Еуразиялық Экономикалық Одақ, тест-жүйелер.

ОБЗОР И АНАЛИЗ ДИАГНОСТИЧЕСКИХ СРЕДСТВ ДЛЯ ВЫЯВЛЕНИЯ СИБИРСКОЙ ЯЗВЫ НА ТЕРРИТОРИИ ЕВРАЗИЙСКОГО ЭКОНОМИЧЕСКОГО СОЮЗА

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АБСТРАКТ

В настоящем обзоре рассматриваются средства диагностики сибирской язвы, внесённые в национальные Реестры ветеринарных препаратов и Реестры лекарственных средств и медицинских изделий стран Евразийского Экономического Союза (ЕАЭС). Фактически на всей территории ЕАЭС присутствуют природные почвенные очаги сибирской язвы, что вызывает ежегодные случаи заражения животных и людей этим заболеванием. Постоянный контроль за эпидемиологической обстановкой в странах ЕАЭС должен обеспечиваться современными средствами диагностики сибирской язвы. Все диагностические средства, используемые для эпидемиологического надзора, должны быть зарегистрированы в национальных реестрах стран ЕАЭС. Для всех внесённых в реестры стран ЕАЭС тест-систем и диагностикумов авторы приводят сравнительные характеристики, отражающие чувствительность и специфичность средств диагностики. Приводится анализ использования различных методов выявления возбудителя сибирской язвы, акцентированный на современных молекулярно-диагностических технологиях. Авторами обсуждаются наиболее важные проблемы, связанные с повышением эффективности современных методов детекции бактерии *Bacillus anthracis* и выносятся на рассмотрение недоработки по определённым направлениям ранней диагностики сибиреязвенного микроба. Также авторами приводится ряд предложений, долженствующих повысить эффективность имеющейся на настоящий момент системы мониторинга сибиреязвенного микроба.

Ключевые слова: *Bacillus anthracis*, методы диагностики, биобезопасность, сибирская язва, Евразийский Экономический Союз, тест-системы