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DEVELOPMENT OF IN-HOUSE DIAGNOSTIC TOOL FOR THE DETECTION OF ANTHRAX GENETIC MATERIAL IN REAL-TIME PCR

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Summary. This paper represents preliminary trials of the 'Anthrax-DNA-test', diagnostical tool for the detection of anthrax DNA. It includes recombinant positive controls *p-pagA-TZ57R/T* and *p-capC-TZ57R/T* for the detection of anthrax plasmid markers, as well as *p-dhp61-CR2.1-TOPO*, positive control for the detection of *Bacillus anthracis* chromosomal marker. Besides, three mixtures of primers and probes for the detection of each genetic marker (*dhp61, pagA*, and *capC*) and ready-to-use 'RT-PCR MasterMix' PCR diluent were also included. Concentrations of $MgCl_2$ and Taq-polymerase obtained during qPCR validation procedure were considered when preparing the diluent. To determine specificity, qPCR was conducted with heterological panel of DNA of pathogenic bacteria and viruses causing diseases with similar to anthrax clinical signs. To determine repeatability of the results when using 'Anthrax-DNA-test' PCR test kit, samples were studied twice. The sensibility of the kit was analyzed by serial dilutions of *p-dhp61-CR2.1-TOPO*, *p-pagA-TZ57R/T* and *p-capC-TZ57R/T* plasmid DNAs containing fragments of anthrax chromosome and plasmids. To compare the tool's ability to identify anthrax DNA, classical PCR was carried out using ANT-PA_F/R and ANT-CAP_F/R primers recommended by OIE for the detection of pXO1 and pXO2 plasmid DNA. Sensitivity testing has shown that the test kit is able to identify all positive samples. It has been found that the diagnostics tool detects anthrax DNA in recombinant positive control samples containing *B. anthracis* chromosomal and plasmid DNA fragments in serial dilutions from 1:100 to 1:1,000 with Ct values of 25.29–34.70. The specificity of this diagnostic tool is proved by the absence of Ct in heterological samples. Besides, repeatability of trial results has been found, which is proved by complete congruence in duplicates with each of the tested sample

Keywords: *Bacillus anthracis*, plasmid, validation

Introduction. Anthrax is a zoonotic disease to which mainly grazing herbivores, but also omnivores, carnivores and human are susceptible. It is caused by Gram-positive, spore-forming facultative anaerobic rod *Bacillus anthracis* (Purcell, Worsham and Freidlander, 2007; Hoffmaster et al., 2002; Keim et al., 2004). Depending on the way of transmission, it can cause cutaneous, gastro-enteritic of pulmonary forms of anthrax (WHO, FAO and OIE, 2008). Under adverse conditions the causative pathogen *B. anthracis* is able to form spores that may remain viable in the environment, especially in soil, for many decades (Martin, Christopher and Eitzen, 2007). When spores penetrate to host organism, they turn to vegetative form, reproduce and therefore cause the disease. The ability to produce toxins and form capsule in hosts organism, which protects bacterial cell from phagocytosis, are key virulence factors of *B. anthracis.* Genes responsible for capsule formation are located on pXO2 plasmid, while pXO1 plasmid genes encode synthesis of toxins. Both these plasmids together with chromosome form anthrax genome (Mock and Fouet, 2001).

Together with classical bacteriological and serological methods, classical polymerase chain reaction and real-time PCR (quantitative PCR, qPCR) are commonly used for express diagnostics of anthrax. Usually, the diagnosis of anthrax is performed following a specific PCR test to detect gene fragments which are specific to plasmids pXO1 and pXO2, respectively (Janzen et al., 2015). However, due to the high degree of homology between *B. anthracis*, *B. cereus* and *B. thuringiensis* (Helgason et al., 2000), the detection of only plasmid markers is insufficient for the diagnosis of anthrax. It should also be noted that plasmids pXO1 and pXO2 can be lost by microorganisms or may also be present in closely related bacteria of the *B. cereus* group (Hurtle et al., 2004; Pannucci et al., 2002), which serves as an additional factor complicating the diagnosis of anthrax.

Currently, using PCR test kits allows to make diagnostic analyses in a laboratory significantly simpler and faster. However, there is no effective domestic PCR diagnostic tool for the detection of *B. anthracis* genome in Ukraine. Besides, using foreign analogs is quite expensive due to the high cost price of reagents and expenses for their transportation. Also, it is necessary to note that the transportation issue has become especially acute in Ukraine due to the lack of airborne routes and complicated logistics caused by the Russian military aggression.

Therefore, the key task of our work was to create a domestic diagnostic tool designed to detect not only plasmids pXO1 and pXO2 in the tested material but also a highly specific region of the *B. anthracis* genome present only in the chromosome of this pathogen.

Materials and methods. Testing of the diagnostic tool was conducted using previously-developed recombinant positive controls, namely *p-pagA-TZ57R/T* and *p-capC-TZ57R/T* in various dilutions (Fig. 1) (Biloivan et al., 2018). The positive control sample *p-dhp61-CR2.1-TOPO* for detecting of anthrax *dhp61* specific chromosomal marker, developed by Antwerpen et al. (2008) and kindly provided by colleagues from the Bundeswehr Institute of Microbiology (Munich, Germany), was also included as the component of this test kit. In this process, plasmid DNA *p-dhp61-CR2.1-TOPO*, *p-pagA-TZ57R/T*, and *p-capC-TZ57R/T* were used at various dilutions (1:100, 1:1,000, 1:10,000), as well as positive and negative control samples.

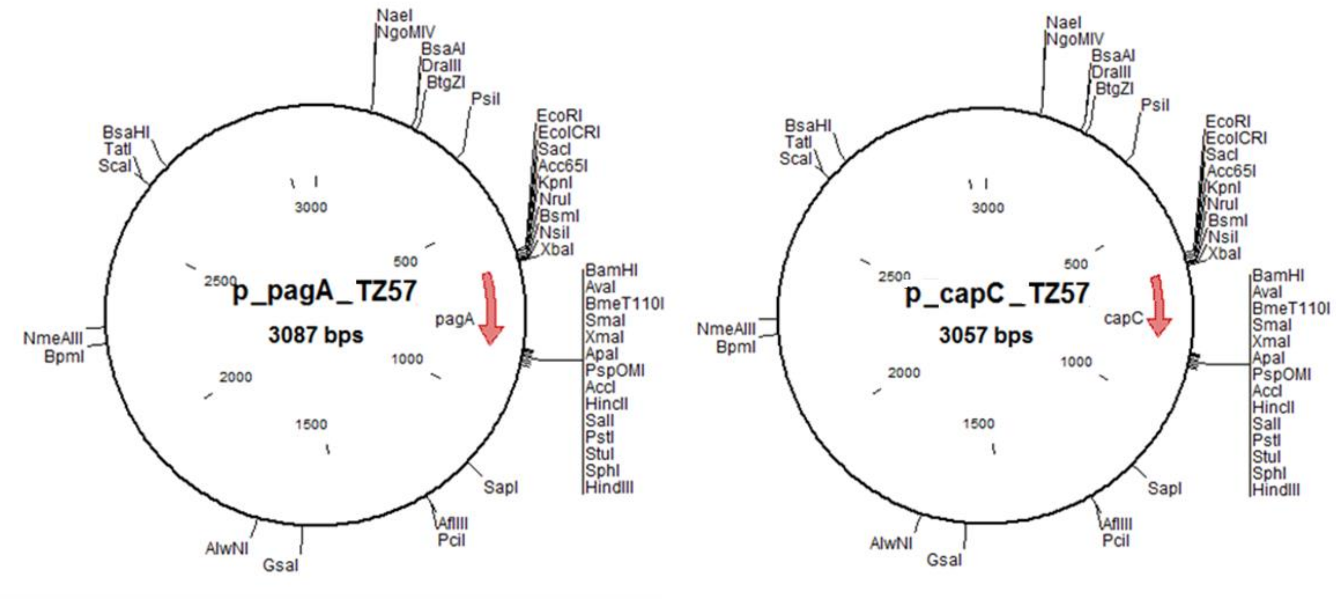

Figure 1. Plasmid maps of recombinant positive control assays *p-pagA-TZ57R/T* (left) and *p-capC-TZ57R/T* (right) for the detection of pXO1 and pXO2 anthrax plasmids, respectively.

The reaction mix was prepared using *TaqMan* reagents and concentrations of its components ($MgCl_2$ and *Taq*-polymerase) were optimized at previous validation stages of our studies (Beloyvan et al., 2019; Biloivan et al., 2019).

The working mixture for real-time PCR was prepared based on the number of tested samples plus one sample, according to the following scheme presented in Table 1.

Table 1 — The reaction mix prepared for the RT-PCR

Component	Final concentration	1× per reaction, µl
RT-PCR MasterMix	1×	18.5
The mixture of primers and probe for the detection of *dhp61*, *pagA* or *capC* markers	200 nM	1.5
DNA (sample or control)		5

Species specificity was identified using heterological DNA samples of various pathogens with cause infectious diseases with similar to anthrax clinical signs (Table 2).

In order to assess the reproducibility of the results obtained when using the developed test kit, the sample analysis was conducted twice.

Table 2 — Heterologous panel used to determine the species specificity of the diagnostic tool for the detection of anthrax DNA

No.	Pathogen	Strain number
1	*Acinetobacter baumanii*	B431
2	*Brucella spp*	03-0391
3	*Burkholderia cepacia*	P112
4	*Burkholderia mallei*	05-0580
5	*Burkholderia pseudomallei*	Jun 88
6	*Burkholderia thailandensis*	P412
7	*Campylobacter jejuni*	B1229
8	*Candida albicans*	B885
9	*Chlamydophila pneumoniae*	No data
10	*Citrobacter freundii*	B22
11	*Clostridium perfringens*	B888
12	*Coxiella burnetii*	Nine Mile
13	*Enterobacter aerogenes*	B16
14	*Enterococcus faecalis*	B871
15	*Escherichia coli*	B893
16	*Francisella tularensis holarctica*	F49
17	*Haemophilus influenzae*	B895
18	*Klebsiella pneumoniae*	B896
19	*Legionella pneumophila*	IMB 072813
20	*Listeria monozytogenes*	B435
21	*Moraxella catarrhalis*	B433
22	*Mycobacterium tuberculosis*	No data

Table 2 — continuation

No.	Pathogen	Strain number
23	*Neisseria meningitidis*	B1232
24	*Propionibacterium acnes*	B438
25	*Proteus mirabilis*	B23
26	*Salmonella typhi*	20-3267
27	*Serratia marcescens*	B14
28	*Shigella dysenteriae*	B476
29	*Staphylococcus aureus/SEB*	B946
30	*Staphylococcus epidermidis*	B26
31	*Stenotrophomonas maltophilia*	B918
32	*Streptococcus pneumoniae*	B847
33	*Streptococcus pyogenes*	B846
34	*Vibrio cholerae*	B962
35	*Yersinia enterocolitica*	Y105
36	*Yersinia pestis*	02. Apr
37	*Clostridium sporogenes*	DSMZ795
38	*Monkey pox virus*	MSF-6
39	*Cowpox virus*	VACV-0273/2004
40	*Chickenpox virus*	No data

The real-time amplification reaction was set up according to the protocol outlined in Table 3.

Table 3 — The real-time PCR amplification protocol for the detection of *B. anthracis* genetic material

Stage	Amplification mode	Number of cycles
Activation	95 °C — 5 min	1
Denaturation Annealing Elongation	95 °C — 15 sec 60 °C — 20 sec 72 °C — 40 sec	40
Final elongation	72 °C — 1 min	1

The specificity of the test kit was determined by comparing to primers recommended by OIE for the detection of anthrax plasmid DNA fragments: PA5/8 (*pag* gene of pXO1 plasmid) and 1234/1301 (*cap* gene of pXO2 plasmid) (WOAH, 2023a, 2023b; Hutson et al., 1993; Beyer et al., 1995; WHO, FAO and OIE, 2008).

The primer pair PA5/8 flanks a 596-base pair region of the *pag* gene of pXO1 plasmid:

PA5, 5'-TCCTAACACTAACGAAGTCG-3';

PA8, 5'-GAGGTAGAAGGATATACGGT-3'.

The primer pair 1234/1301 flanks an 846-base pair region of the *cap* gene of pXO2 plasmid:

1234, 5'-CTGAGCCATTAATCGATATG-3';

1301, 5'-TCCCACTTACGTAATCTGAG-3'.

The results of conventional PCR using PA5/8 and 1234/1301 primers were recorded by horizontal gel electrophoresis. A sample was considered positive for the presence of anthrax pXO1 and pXO2 plasmid DNA if yellow-hot 596 bp (for the *pag* gene of pXO1 plasmid) and 846 bp (for the *cap* gene of pXO2 plasmid) bands were visible on the gel and negative if they were absent, respectively.

Results. The developed test kit includes the following components:

(1) 'RT-PCR MasterMix' — 1 (2) tubes of 1 ml each;

(2) primer and probe solutions for detecting of *dhp6*, *pagA* and *capC* markers (10 pmol/μL) — 1 tube each, 0.03 (0.06) ml (each);

(3) deionized water — 1 (2) tubes of 0.5 ml each;

(4) positive control samples for detecting of *dhp61* chromosomal marker as well as *pagA* and *capC* plasmid markers (for 5 or 10 reactions) — 1 tube each, 0.1 (0.2) ml (each).

The 'RT-PCR MasterMix' solution includes $MgCl_2$, deoxyribonucleotide triphosphates (dNTPs), and Taq-polymerase. The concentrations of $MgCl_2$ and Taq-polymerase were optimized during a previous stage of our studies (Beloyvan et al., 2019; Biloivan et al., 2019).

For convenience in a diagnostic laboratory setting, primer and probe mixtures were prepared in equal volumes, with three mixtures in total for each of the genetic markers (*dhp61*, *pagA*, and *capC*). Additionally, the test kit includes positive control samples for detecting the plasmid markers of *B. anthracis* (*pagA* and *capC*), which were prepared previously (Biloivan et al., 2018).

The positive control sample *p-dhp61-CR2.1-TOPO* for detecting the chromosomal marker of the anthrax agent, developed by Antwerpen et al. (2008) and kindly provided by colleagues from the Bundeswehr Institute of Microbiology, is also included in the test kit. Each component is aliquoted into tubes in quantities sufficient for conducting 50 analyses.

It has been found that the test kit is able to detect anthrax DNA in plasmid DNA samples *p-dhp61-CR2.1-TOPO*, *p-pagA-TZ57R/T* and *p-capC-TZ57R/T* which contain fragments of DNA markers of the chromosome and plasmids of *B. anthracis*, respectively, in dilutions ranging from 1:100 to 1:10,000 with Ct values between 25.29 and 34.70 (Table 4).

At the same time, no amplification product was observed in heterologous samples (Ct values were absent), demonstrating the specificity of this test system. Furthermore, the repeatability and reproducibility of the results have been established, as evidenced by their complete agreement in two replicates with each tested sample.

Thus, the preliminary testing of the developed 'Anthrax-DNA-test' has demonstrated that it meets the OIE requirements in terms of specificity, sensitivity, and reproducibility (WOAH, 2023a, 2023b).

Table 4 — Trial results of the ‘Anthrax-DNA-test’ diagnostic tool

No.	Assay	PA5/8 and 1234/1301 primer pairs	The first replicate, Ct	The second replicate, Ct
1	*p-dhp61-CR2.1-TOPO* plasmid DNA diluted 1:100	positive	28.90	29.10
2	*p-dhp61-CR2.1-TOPO* plasmid DNA diluted 1:1,000		31.30	31.40
3	*p-dhp61-CR2.1-TOPO* plasmid DNA diluted 1:10,000		34.70	34.40
4	*p-pagA-TZ57R/T* plasmid DNA diluted 1:100		27.28	27.39
5	*p-pagA-TZ57R/T* plasmid DNA diluted 1:1,000		30.73	31.46
6	*p-pagA-TZ57R/T* plasmid DNA diluted 1:1,000		33.69	33.70
7	*p-capC-TZ57R/T* plasmid DNA diluted 1:100		26.06	25.29
8	*p-capC-TZ57R/T* plasmid DNA diluted 1:1,000		29.14	28.96
9	*p-capC-TZ57R/T* plasmid DNA diluted 1:1,000		32.86	32.36
10	DNA of *Acinetobacter baumanii*	negative	Ct is absent	Ct is absent
11	DNA of *Brucella* spp.			
12	DNA of *Burkholderia cepacia*			
13	DNA of *Burkholderia mallei*			
14	DNA of *Burkholderia pseudomallei*			
15	DNA of *Burkholderia thailandensis*			
16	DNA of *Campylobacter jejuni*			
17	DNA of *Candida albicans*			
18	DNA of *Chlamydophila pneumoniae*			
19	DNA of *Citrobacter freundii*			
20	DNA of *Clostridium perfringens*			
21	DNA of *Coxiella burnetii*			
22	DNA of *Enterobacter aerogenes*			
23	DNA of *Enterococcus faecalis*			
24	DNA of *Escherichia coli*			
25	DNA of *Francisella tularensis holarctica*			
26	DNA of *Haemophilus influenzae*			
27	DNA of *Klebsiella pneumoniae*			
28	DNA of *Legionella pneumophila*			
29	DNA of *Listeria monozytogenes*			
30	DNA of *Moraxella catarrhalis*			
31	DNA of *Mycobacterium tuberculosis*			
32	DNA of *Neisseria meningitidis*			
33	DNA of *Propionibacterium acnes*			
34	DNA of *Proteus mirabilis*			
35	DNA of *Salmonella typhi*			
36	DNA of *Serratia marcescens*			
37	DNA of *Shigella dysenteriae*			
38	DNA of *Staphylococcus aureus/SEB*			
39	DNA of *Staphylococcus epidermidis*			
40	DNA of *Stenotrophomonas maltophilia*			
41	DNA of *Streptococcus pneumoniae*			
42	DNA of *Streptococcus pyogenes*			
43	DNA of *Vibrio cholerae*			
44	DNA of *Yersinia enterocolitica*			
45	DNA of *Yersinia pestis*			
46	DNA of *Clostridium sporogenes*			
47	Monkey pox virus DNA			
48	Cowpox virus DNA			
49	Chickenpox virus DNA			

Conclusions. The key objective of our work was to create a in-house diagnostic tool designed to detect the anthrax genetic material. One of the distinguishing features of the developed 'Anthrax-DNA-test' kit is its capability to detect not only the pXO1 and pXO2 plasmids but also the chromosomal marker *dhp61*, which is highly specific and present only in the *Bacillus anthracis* chromosome. This feature is of great importance when distinguishing the anthrax pathogen from other closely related bacteria. The developed test kit, in terms of specificity, sensitivity, and reproducibility, is comparable to the method recommended by the OIE and, after the completion of the registration process, will be used for the express diagnosis of anthrax in Ukraine.

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