Part 2. Biotechnology and genetics

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VALIDATION OF ANTHRAX SPECIFIC *pagA* QUANTITATIVE PCR FOR DETECTION OF *BACILLUS ANTHRACIS* pXO1 PLASMID

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Summary. This paper represents qPCR validation results for the detection of *Bacillus anthracis pagA* pXO1 plasmid marker. The aim of the work was to transfer, implement and validate anthrax specific *pagA* qPCR assay for the detection of *pagA*, the genetic marker of the pXO1 plasmid of *Bacillus anthracis*. qPCR was conducted using the Applied Biosystems Fast 7500 Real-time PCR system including Applied Biosystem specific reagents (AmpliTaq Gold). Anthrax pXO1 *pagA* primers (pagA_forward, pagA_reverse) and TaqMan *pagA* probe. Data analysis and statistical calculations were performed using Microsoft Excel. The limit of detection (probit analysis) was calculated using the Statgraphics software. Robustness of qPCR was adjusted by optimization of amplification parameters (annealing temperature) and concentration of reaction components ($MgCl_2$, primers, probe and Taq polymerase). In order to test the repeatability and precision of the qPCR assay after optimization, the variation within the experiment (Intra-assay variability) and between several independent experiments (Inter-assay variability) was evaluated. Probit analysis with serial dilutions of positive control with five replicates per dilution was carried out to define the 95% limit of detection (LOD). To determine if the CT value correlates with the amount of template DNA, the linearity of qPCR was analyzed. The standard curve was generated and the linear regression line and the coefficient of correlation (R^2) were calculated. To define the ability to detect sequence of interest (sensitivity), we tested mixed panel of *Bacillus anthracis* DNAs. As the result, *pagA* marker could be detected in all tested strains . To find out the specificity of our assay, we also tested DNA of various strains of *B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. globigii* (potential cross-reacting organisms) as well as DNA samples of various pathogenic bacteria and viruses which cause similar clinical symptoms as anthrax (differential diagnosis relevant organisms).

Keywords: anthrax, plasmid, validation, quantitative PCR

Introduction. Anthrax is particularly dangerous zoonotic disease caused by bacterium *Bacillus anthracis* — Gram-positive, spore-forming facultative anaerobic rod (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). Spores of this pathogen are able to remain viable in soil for 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 host organism, which protects bacterial cell from phagocytosis, is key virulence factor 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 *B. anthracis* 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. Herewith, it is necessary to take into account that, according to ISO/IEC 17025, prior to the implementation of any analytic method to laboratory practice, is has to pass the validation procedure — the set of studies for evaluation of specificity, sensitivity, accuracy and repeatability of any method (ISO, 2017; OIE, 2013; Antonov, 2002).

The **goal of this study** was to carry out validation of qPCR method for the detection of *pagA*, specific pXO1 plasmid marker of *Bacillus anthracis*.

Materials and methods. To carry out these studies, we used *pagA* TZ57 R/T recombinant positive control, which had been prepared before using TA-cloning method (Biloivan et al, 2018). To reduce the risk of contamination in the laboratory when working with plasmid DNA, we obtained *pagA* insert from it using classical PCR with *M13 forward* and *M13 reverse* primers (Chandra and Wikel, 2005). This insert was purified using Monarch™ commercial kit (New England, USA). Concentration of DNA was measured with Nanodrop DS-11 spectrophotometer by DeNovix. Taking into account the measured concentration, we prepared serial dilutions of purified product (from 10^4 to 1 copies of DNA per 1 µl)

and studied them via qPCR. Anthrax specific pXO1 *pagA* primers (*pagA_forward* GTACAAGTGCTGGACCTACG, *pagA_reverse* CACTGTACGGATCAGAAGCC), and *pagA* probe (FAM-ACCGTGACAATGATGGAATCCCTGA-BBQ) by MolBiol (Germany), as well as reagents by Applied Biosystems (AmpliTaq Gold) were used. The reaction was conducted using the Applied Biosystems Fast 7500 Real-time PCR system. Following parameters were used for amplification: activation of HotStart *AmpliTaq Gold* DNA polymerase (Applied Biosystems, 5 U/μl, 5′–3′) — 95 °C, 5 min; denaturation — 95 °C, 15 s; annealing — 60 °C, 20 s; elongation — 72 °C, 40 s; final elongation — 72 °C, 1 min; number of cycles — 40.

The robustness of the method was adjusted by the optimization of primer annealing temperature (several experiments were carried out with 57, 59, 60, and 62 °C annealing temperatures), as well as concentrations of reaction mix components ($MgCl_2$, primers, probe and *Taq*-polymerase).

For this purpose, we conducted titration of these components (several different concentrations of each component were analyzed and the most optimal results were chosen).

Thus, $MgCl_2$ was titrated in concentrations of 0.5–5 μM; primers — 0.1FW/0.1RV–0.6FW/0.6RV μM; probe — 0.1–1 μM and polymerase — 0.13–0.35 μl. Data was statistically analyzed using Microsoft Excel.

In order to test the repeatability and precision of qPCR method after optimization, we determined coefficients of variation (CV) within single and several independent experiments. Herewith, CV values should not be higher than 3% for single and 15% for several experiments (Moens et al., 2009).

Analytical sensitivity was determined using probit analysis, as well as measuring of linearity. We carried out probit analysis with serial dilutions in five replicates in order to determine 95% limit of detection (LOD), which was calculated using Statgraphics software.

To determine if the CT value correlates with the amount of template DNA, the linearity of qPCR was analyzed. Therefore, the fluorescence response to a standard dilution series was evaluated. The standard curve was generated and the linear regression line and the coefficient of correlation ($R^2 \geq 0.98$) were calculated (Rodríguez-Lázaro and Hernández, 2013).

To determine diagnostical sensitivity of the method (the ability to detect sequence of interest), we studied DNA mixed panel of various *Bacillus anthracis* strains (Table 1).

To determine the specificity, we also analyzed DNA of various strains of closely related bacteria: *B. cereus, B. thuringiensis, B. mycoides*, and *B. globigii* (potentially cross-reactive microorganisms), as well as DNA samples of other pathogens causing diseases with similar to anthrax clinical signs (differential diagnostics relevant organisms, Table 2).

Table 1 — Mixed DNA panel of *Bacillus anthracis* strains used for the determination of method's sensitivity

Strain	Strain number	Dilution
6282 Tirol	916	1:100
Wien A5	2610	undiluted (10 ng/μl)
BGA Nr. 2 A7	2612	1:100
2844-9IZSVE	3007	undiluted (10 ng/μl)
4-IZSLT	3008	1:10
3-IZSLT	3009	1:10
CARBOSAP	3010	undiluted (10 ng/μl)
FARMER CUTE	3011	undiluted (10 ng/μl)
FERARRA	3012	undiluted (10 ng/μl)
CEB95-002	3013	1:15
CEB94-033	3015	undiluted (10 ng/μl)
Vollum	3017	1:10
Ames	3018	undiluted (10 ng/μl)
BUL 16	3165	undiluted (10 ng/μl)
BUL 17	3166	undiluted (10 ng/μl)
BUL 28	3177	undiluted (10 ng/μl)
BUL 39	3187	1:10
BUL 41	3189	undiluted (10 ng/μl)

Table 2 — Heterological DNA panel of infectious pathogens causing diseases with similar to anthrax clinical signs

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*	N/A
10	*Citrobacter freundii*	B22
11	*Clostridium perfringens*	B888
12	*Coxiella burnetii*	Nine Mile
13	*Eikenella corrodens*	N/A
14	*Enterobacter aerogenes*	B16
15	*Enterococcus faecalis*	B871
16	*Escherichia coli*	B893
17	*Francisella tularensis holarctica*	F49
18	*Haemophilus influenzae*	B895
19	*Klebsiella pneumoniae*	B896
20	*Legionella pneumophila*	IMB 072813
21	*Listeria monozytogenes*	B435
22	*Moraxella catarrhalis*	B433
23	*Neisseria meningitidis*	B1232
24	*Propionibacterium acnes*	B438
25	*Proteus mirabilis*	B23
26	*Pseudomonas aeruginosa*	N/A

No.	Pathogen	Strain number
27	*Salmonella typhi*	20-3267
28	*Serratia marcescens*	B14
29	*Shigella dysenteriae*	B476
30	*Staphylococcus aureus/SEB*	B946
31	*Staphylococcus epidermidis*	B26
32	*Stenotrophomonas maltophilia*	B918
33	*Streptococcus pneumoniae*	B847
34	*Streptococcus pyogenes*	N/A
35	*Streptococcus pyogenes*	B846
36	*Vibrio cholerae*	B962
37	*Yersinia enterocolitica*	Y105
38	*Yersinia pestis*	02. Apr
39	*Clostridium sporogenes*	DSMZ795
40	Affenpocken Virus	MSF-6
41	Vaccinia Virus	VACV-0273/2004
42	Varicella-zoster-Virus	N/A

Samples of DNA used for these studies were given by Bundeswehr Institute of Microbiology (Munich, Germany) in frames of German Partnership Programme for Excellence in Biological and Health Security.

In order to visualize obtained results, gel-electrophoresis of amplified in 1.5% agarose gel was conducted after qPCR.

Results. Optimization of qPCR parameters was the first step of validation process. PCR product which we obtained after purification of *pagA* TZ57 R/T recombinant plasmid using M13 primers, was diluted from 10^4 down to 1 copy of DNA in 1 µl. The dilution which contained 10^3 copied of DNA and had Ct value of 27 cycles was used for further experiments as template DNA (Fig. 1).

As a result of primer annealing temperature optimization, we chose the temperature of 62 °C for further experiments (Fig. 2 and Fig. 3).

Titration of $MgCl_2$ has shown that the most optimal reaction results can be obtained with its concentration of 3 µM. The best concentration of primers and probe (*pagA*_forward, *pagA*_reverse and *pagA*_probe) is 0.3 µM. As a result of *AmpliTaq Gold* DNA polymerase, we found that it works the most specifically with the concentration of 0.2 µl per reaction.

As a result of repeatability evaluation, we obtained coefficients of variability of 1.89% for the Intra-assay (Table 3) and 5.23% for the Inter-assay (Table 4), indicating that the variability within the *pagA* qPCR assay is low resulting in repeatable results.

As a result of probit analysis, we obtained a LOD of 4.26 copies, indicating that in 95% cases 4 copies can be detected, demonstrating that our qPCR assay is highly sensitive (Fig. 4).

Based on the linearity results, the standard curve was generated, and the linear regression line and the coefficient of correlation (R^2) were calculated. The obtained R^2 values of 0.9995 for *Bacillus anthracis* sample 3014 and 0.9994 for *B. anthracis* sample 3189 show that fluorescence signal is direct proportional to the administrated templated DNA and that the efficiency of the qPCR is high and consistent at varying concentrations (Fig. 5).

The results of *Bacillus anthracis* mixed panel (Table 2), have shown that genetic marker *pagA* is present in all tested samples (Table 5, Fig. 6).

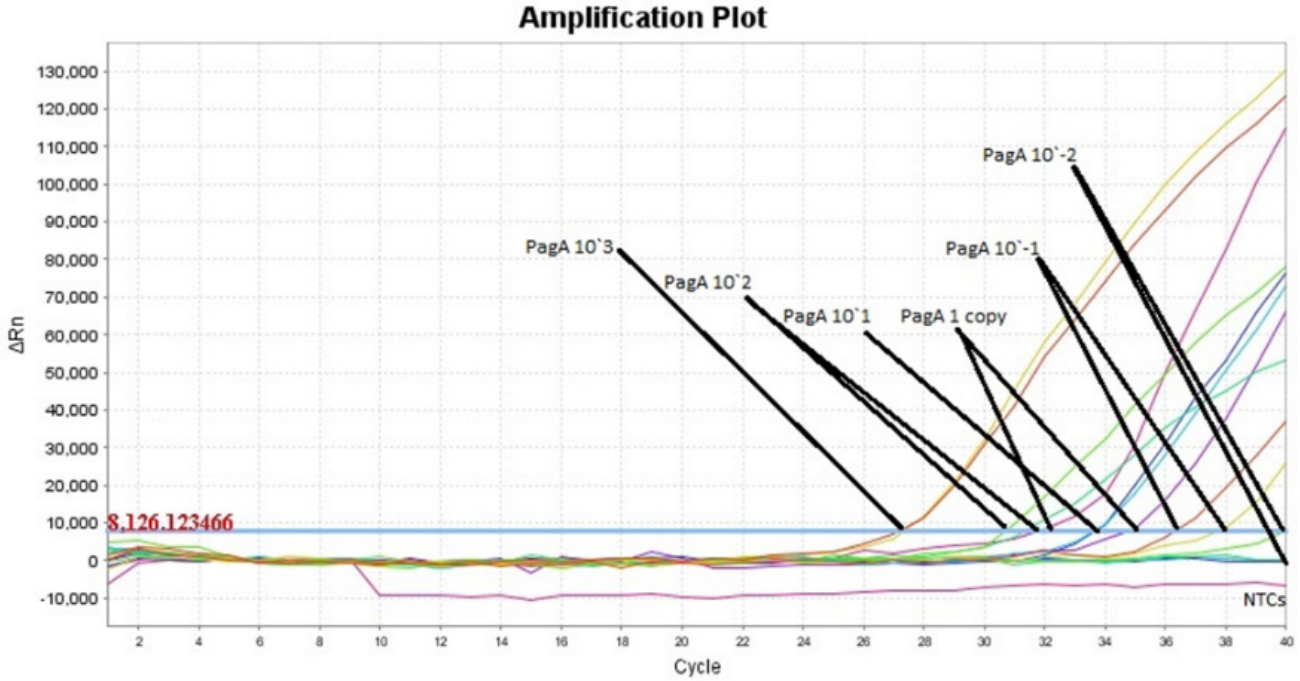

Figure 1. Results of qPCR with *pagA* DNA dilution series obtained from *pagA* TZ57 R/T plasmid

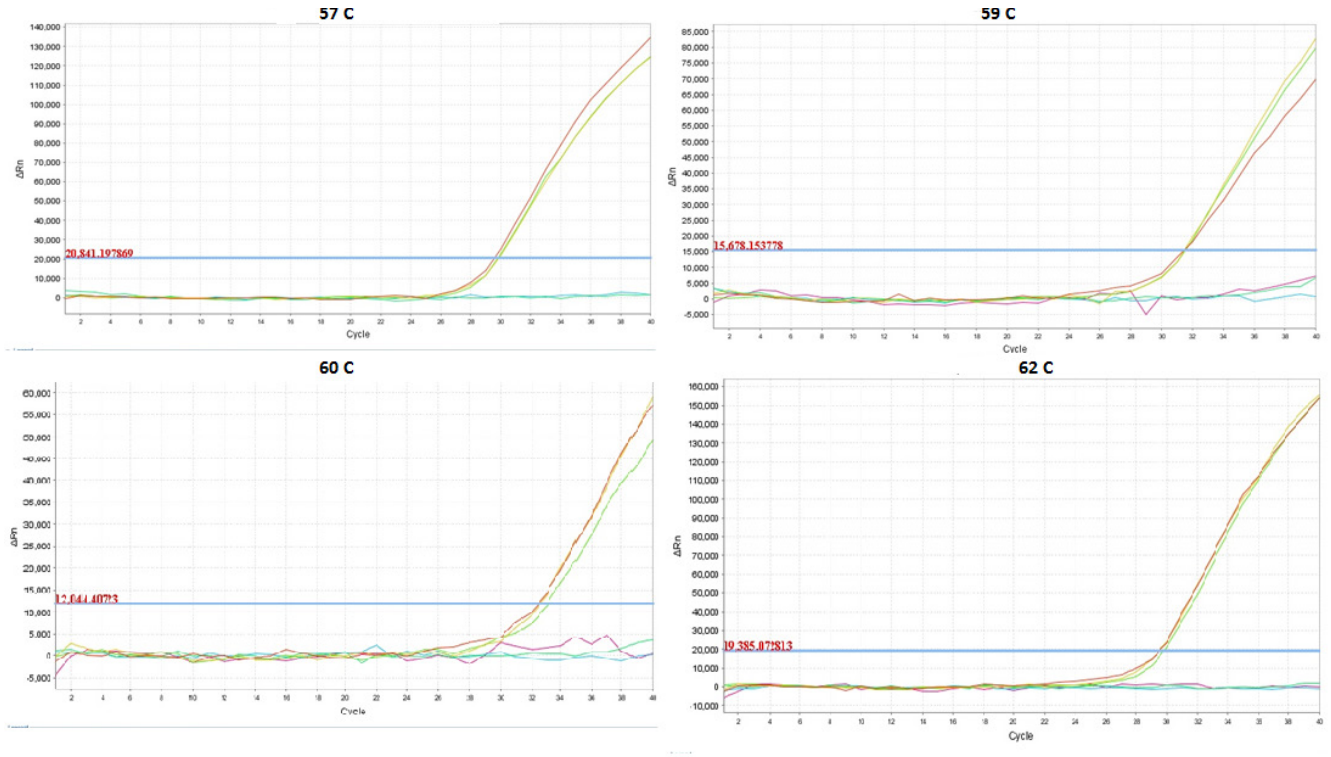

Figure 2. Results of qPCR with *pagA* DNA obtained using different primer annealing temperatures

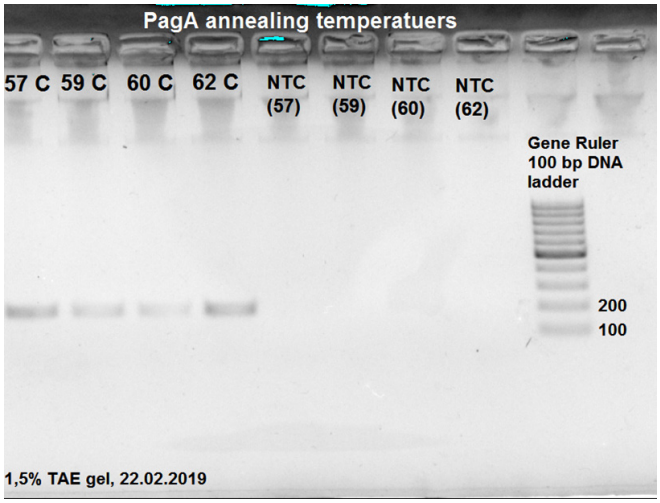

Figure 3. Gel picture of *pagA* qPCR amplification products obtained with different primer annealing temperatures. Negative controls are marked as 'NTC'

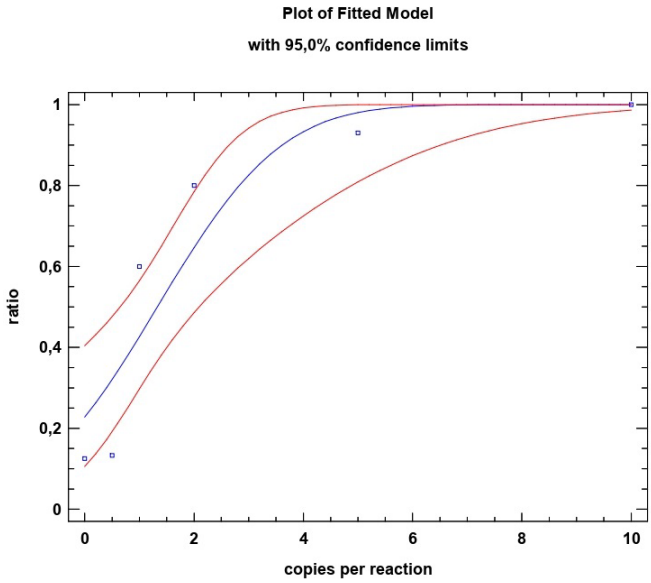

Figure 4. Graphic model based on the results of probit analysis

Table 3 — Ct values obtained after Intra-assay variability test, as well as calculated mean values, standard deviations and CVs

Sample	Ct value			Mean	Standard deviation	CV, %
	Replicate 1	Replicate 2	Replicate 3			
B. anthracis 3012 (1:4) (positive sample)	18.94	18.32	18.35	18.54	0.35	1.89
B. anthracis 3189 (1:50000) (weakly positive sample)	30.00	30.06	29.99	30.02	0.04	0.14
Negative sample	Negative	Negative	Negative	—	—	0

Table 4 — Mean Ct values, standard deviations and CVs obtained after several independent experiments (Inter-assay variability)

Sample	Day 1 mean	Day 2 mean	Day 3 mean	Mean (days 1–3)	Standard deviation (days 1–3)	CV, %
B. anthracis 3012 (1:4)	18.54	20.45	20.17	19.72	1.03	5.23
B. anthracis 3189 (1:50000)	30.02	30.27	30.09	30.13	0.13	0.44
Negative sample	0	0	0	0	0	0

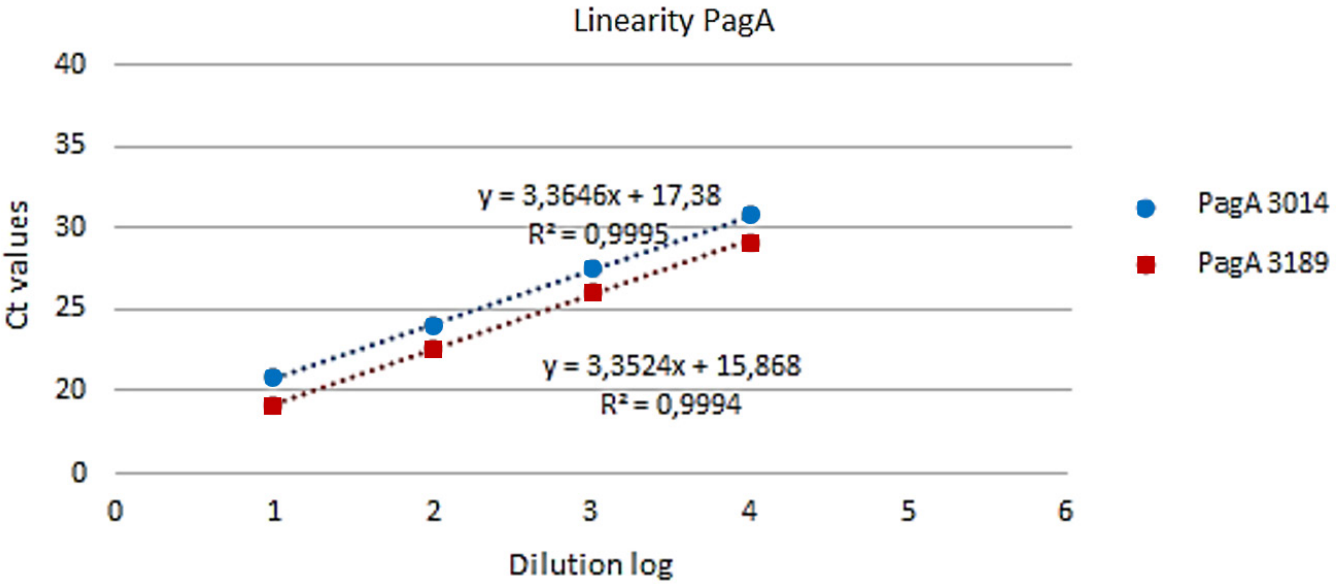

Figure 5. Linearity of *pagA* qPCR: linear regression lines and coefficients of correlation for tested $\log_{10}$ dilution series of samples 3014 and 3189. Samples were diluted from 101 to 103 (3014) and from 101 to 104 (3189)

Table 5 — *pagA* qPCR results of mixed DNA panel of *Bacillus anthracis* for evaluation of method's sensitivity. Positive control (*pagA* template DNA) is marked as 'PC *pagA* 10^3'

No.	Sample	Strain number	Ct values
1	6282 Tirol	916	29.68
2	Wien A5	2610	23.43
3	BGA Nr. 2 A7	2612	27.08
4	2844-9IZSVE	3007	17.37
5	4-IZSLT	3008	27.56
6	3-IZSLT	3009	26.63
7	FARMER CUTE	3011	20.10
8	FERARRA	3012	20.37
9	CEB95-002	3013	28.97
10	CEB94-033	3015	20.15
11	Vollum	3017	30.47
12	Ames	3018	26.68
13	BUL 16	3165	22.35
14	BUL 17	3166	20.01
15	BUL 28	3177	22.08
16	BUL 37	3185	34.75
17	BUL 39	3187	27.96
18	BUL 41	3189	22.06
19	PC *pagA* 10^3	—	32.02

Testing of homological DNA panel of closely-related *Bacillus* bacteria has shown that only one out of six *Bacillus cereus* strains was positive for the presence of *pagA* pXO1 genetic marker (Table 6, Fig. 7). As expected, this sample had pXO1 plasmid in its genome, and therefore *pagA* genetic marker located on it.

Table 6 — Homological DNA panel of closely related *Bacillus* bacteria used for evaluation of specificity (cross-reactivity) of the method

Microorganism	Ct values	Expected result
B. cereus E33L (diluted 1:15)	negative	negative
B. cereus 03BB102	19.60	positive
B. cereus B792	32.62	positive
B. cereus ATCC10987 (BCE-0696)	33.03	positive
B. thuringiensis spp. israeliensis WIS493 (diluted 1:15)	35.10	positive
B. mycoides B298 (diluted 1:15)	35.60	positive
B. globigii WIS399	36.44	positive
B. weihenstephanensis B498 (diluted 1:15)	38.24	positive
B. cereus var. anthracis CI (3265)	19.35	positive
B. cereus var. anthracis CA (3266)	20.52	positive

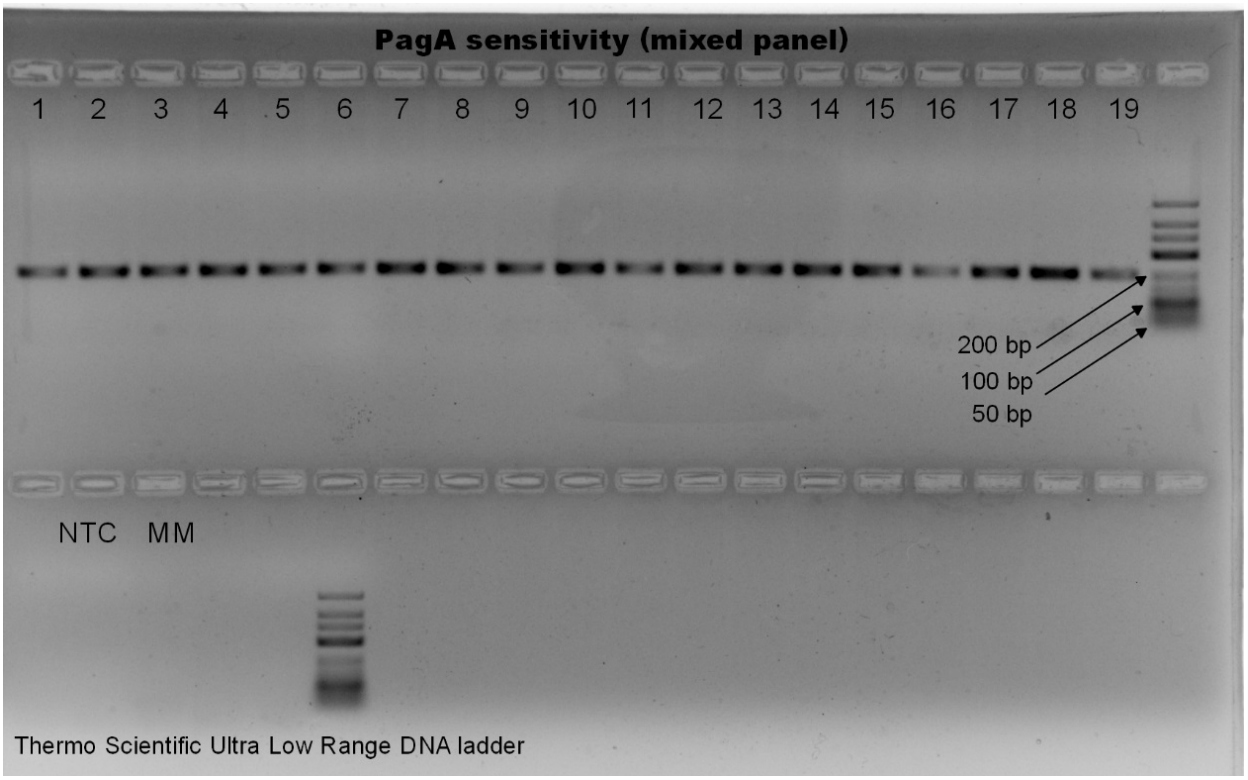

Figure 6. Gel picture of *pagA* qPCR results with mixed DNA panel of *Bacillus anthracis* samples for evaluation of method's sensitivity

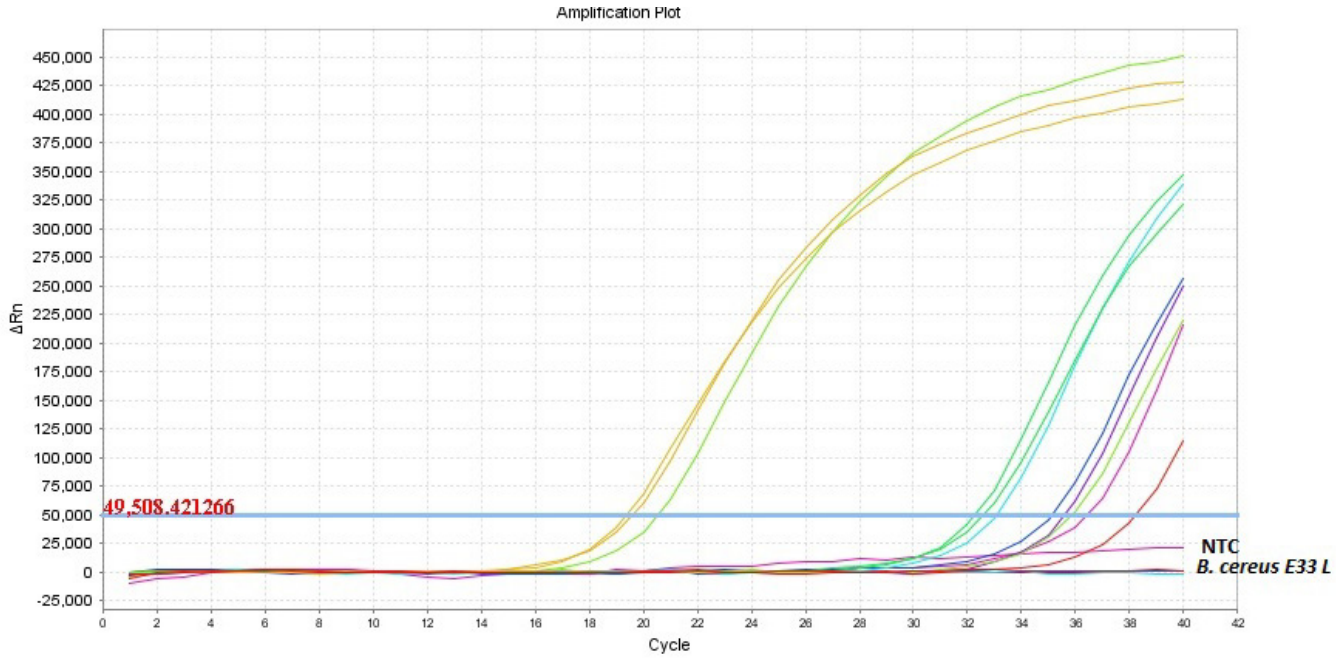

Figure 7. *pagA* qPCR results of homological DNA panel of closely related *Bacillus* bacteria used for evaluation of specificity (cross-reactivity) of the method

The result of qPCR with heterological panel did not show any samples containing *pagA* genetic marker, which is specific only for *Bacillus anthracis* and some bacteria of *Bacillus* group.

Conclusions. All conducted assays proved that qPCR test which we used for detection of *pagA*, *Bacillus anthracis* pXO1 genetic marker, is specific and trustable. In addition, this validation procedure allowed to make this test more robust, repeatable and sensitive. This method was adjusted in the laboratory of molecular diagnostics at NSC 'IECVM' for diagnostical detection of anthrax in environmental samples.

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