BioNanoScience (2018) 8:434–440
https://doi.org/10.1007/s12668-017-0477-0

Indication and Identification of *Bacillus anthracis* Isolates from the Middle Volga Region by Multi-Primer PCR

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Published online: 6 December 2017

Abstract
The article describes the results of improved preparation of various object sampling for indication, identification and genotyping of *Bacillus anthracis* isolates with molecular genetic methods based on multilocus sequence typing using specific primers. Multi-primer PCR used enabled the detection and differentiation of *B. anthracis* isolates. The used primers allowed to differentiate isolates by their belonging to the genus Bacillus and *B. anthracis* species by the pXO1 plasmid presence. The detection of the pX02 plasmid encoding for capsule formation indicates pathogenicity of the isolates studied. Using multi-primer PCR allowed to determine the epizootic situation of anthrax in the Middle Volga Region and to establish that two genotypic groups of the pathogen *B. anthracis* circulate in the region.

Keywords *Bacillus anthracis* · Anthrax · Genotyping · Multi-primer PCR · Russia

1 Introduction

Anthrax is an especially dangerous infection that occurs from time to time as a potential hazard for human and animal health. Anthrax outbreaks are regularly reported in various countries, including relatively recent ones in China [1], India [2], Bangladesh [3], Bhutan [4], Georgia [5], Germany [6] and other countries. There were 43 confirmed outbreaks in the northern region of Ghana which resulted in 131 cattle, 44 sheep, 15 goat, 562 pig and 6 human deaths [7]. In 2011, an anthrax outbreak in the district of Chama, Zambia, caused 85 hippopotamus deaths and infection of 511 people including lethal ones [8].

The widespread use of live vaccines in veterinary and human medicine requires genotyping of vaccinal and pathogenic strains of a causative agent and isolates obtained from sick animals. Current bacteriological and serological methods do not provide quick and reliable strain characterization of isolated biological pathogens. This can be done by means of molecular and genetic methods, including those for field screening [9].

Scientific studies of the genetic apparatus of bacteria enable decoding of the genome nucleotide sequence, which underlies establishing the molecular mechanisms of their antigenicity, immunogenicity and pathogenicity. At the same time, not only

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B. anthracis genomics, but also its proteomics has been researched [10]. The *B. anthracis* virulence is associated with the pXO2 plasmid, which carries the genes essential for the antiphagocytic capsule biosynthesis and degradation [11]. The pXO1 plasmid contains the genes that encode for a lethal factor, an edema factor and a protective antigen [12] present in both pathogenic and vaccinal strains. Recent studies with the use of genome data for pXO1 and pXO2 identified virulent properties of the Brazilian vaccinal *B. anthracis* strain [13]. The pXO1 plasmid encodes the synthesis of the protein Hfq3, which interacts with RNAs involved in essential functions of cell formation and growth [14]. In addition to encoding for the protective antigen, the *pag* gene of pXO1 is also involved in modulating the *B. anthracis* capsule biosynthesis [15]. A positive correlation between the number of *atxA* copies and the expression level of the *pagA* gene, encoding for a virulence factor such as the *B. anthracis* protective antigen has been found [16].

The contamination reference in a laboratory by *B. anthracis* strain, which occurred in 1981, was unexpectedly detected and confirmed with methods of next-generation sequencing and subsequent phylogenetic analyses more than 30 years after [17]. The identification of marker genes plays an important role in the detection and genotyping of new strains. For example, two new Bacillus strains—*B. cereus* E41 and *B. anthracis* F34—have been recently identified in Algeria [18]. Whole genome genotyping of historical *B. anthracis* strains has been conducted in Georgia [19] and Austria [20]. Multi-primer PCR improved the identification of *B. anthracis* typical and atypical strains and differentiation between closely related bacilli [21] and for polymorphism analysis [22].

This research was aimed to determine the effectiveness of the use of multi-primer PCR for the identification of *B. anthracis* pathogen isolates and their epidemiological status through genotyping in comparison with the historical strains.

2 Materials and Methods

B. anthracis 55, CH-7, 81, 71/12, F34 strains, collection isolates #22 and #36, as well as isolates extracted from soil samples and animal cadavers obtained in the Middle Volga region of Russia, were used in this research. Bacteria of heterologous species (such as *B. cereus* strain 8035) and other genera (such as *Brucella abortus* strain 19 and *Listeria monocytogenes* strain AUF) were used to control the reaction specificity. Vegetative forms of bacilli were obtained by 16-h culturing in beef-extract agar (BEA) or by 5- to 6-h culturing in 2% Hottinger's agar at 37 °C. The specificity of used primers were analyzed by BLAST NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RCP samples were prepared with thermolysis: the grown cultures were transferred with a loop into 2.0-ml centrifuge tubes with a thermal latch on the lid, followed by addition of a lysis buffer. The tubes were heated at 98 °C for 15 min in the heating/cooling dry block and then precipitated on a centrifuge at 12,000 rpm for 60 s. A DNA-containing supernatant was taken into other test tubes.

PCR was performed on a Tercyc amplifier (DNA-Technology, Russia) using a PCR kit (Syntol, Moscow, Russia) according to the manufacturer's instruction. The final volume of a 30 μl PCR mixture contained 3 μl of a 25 mM $MgCl_2$ solution, 1 μl of 10 pM of each primer solution, 3 μl of a 2.5 mM dNTP solution, 3 μl of a 10× buffer for PCR, 1 μl of Taq polymerase, 10 μl of a DNA extract and 8 μl of deionized water. The PCR program used was as follows: (I) denaturation at 95 °C for 2 min; (II) 42 cycles: 10 s at 95 °C, 10 s at 55 °C, 10 s at 72 °C; (III) final elongation at 72 °C for 1 min. The amplification results were assessed with 2% agarose gel electrophoresis.

For genotyping, the amplicon production was performed using PCR kit (Syntol, Moscow, Russia) according to the manufacturer's instruction. The final volume of 50 μl PCR mixture contained the following: 2 μl of 25 mM MgCl2 solution; 1 μl of 10 pM of each primer solution; 5 μl of 2.5 mMdNTP solution; 5 μl of 10× buffer for PCR; 1 μl of Taq polymerase; 5 μl of DNA extract and 30 μl of deionized water. Loci amplification for genotyping had the following programs: (I) denaturation at 95 °C for 2 min; (II) 42 cycles: 10 s at 95 °C, 10 s at 65 °C–55 °C (65 °C for the loci vrrA and vrrB1; 62 °C for the loci vrrC1 and vrrC2; 60 °C for the locus vrrB2; 58 °C for the CG3 locus, 57 °C for the pX01 locus, 55 °C for the pX02 locus), 10 s at 72 °C; (III) final elongation 72 °C for 1 min. After amplification, the fragments were dispersed in 1% low melting point agarose with subsequent DNA extraction using a GeneJETPCR purification kit (ThermoScientific) according to the manufacturer's instruction. Multilocus sequence typing (MLST) was performed on an automated Applied Biosystems 3500XL genetic analyzer (HITACHI/ High-Technologies Corporation, Japan). Sequence Scanner V1.0 was applied for reading and comparison.

3 Results and Discussion

3.1 Condition Optimization and Primer Construction for PCR

Three pairs of primers conditionally designated as *Bacillus*, *pXO1* and *pXO2* (Table 1) were designed and synthesized for *B. anthracis* indication experiments. The first pair of primers, *Bacillus*, flanked a DNA region common for the genus *Bacillus*, while *pXO1* and *pX02* being complementary to

Table 1 Nucleotide sequence of primers used for *B. anthracis* indication and genotyping in this research

Name	Gene Bank ID	Sequence 5′ -› 3′
vrrAF	DQ497165.1	ACAACTACCACCGATGGCA
vrrAR		GCGCGTTTCGTTTGATTC
vrrB1 F	AB597569.1	GAATAGGTGGTTTTCCGCAAGTT
vrrB1 R		TGAGTTTGATAAAGAATAGCCTGTGG
vrrB2 F	DQ497167.1	CCACAGGCTATTCTTTATCAAACTCAT
vrrB2 R		CCCAAGGTGAAGATTGTTGTTGA
vrrC1 F	DQ497168.1	GAAGCAAGAAAGTGATGTAGTGGACA
vrrC1 R		GCATTTCCTCAAGTGCTACAGGTT
vrrC2 F	DQ4971659.1	ACCAGAAGAAGTGGAACCTGTAGC
vrrC2 R		TCTTTCCATTAATCGCGCTCTATC
CG3 F	DQ497170.1	TGTCGTTTTACTTCTCTCTCCAATACTAA
CG3 R		GTCATTGTTCTGTATAAAGGGCATAAAT
pXO1 F	CP023002.1	TCTAGAATTAGTTGCTTCATAATGGCTG
pXO1 R		CAATTTATTAACGATCAGATTAAGTTCATTATT
pXO2 F	CP009542.1	TCATCCTCTTTTAAGTCTTGGGTTATATT
pXO2 R		TGTGATGAACTCCGACGACAAA
bacillus F	FJ694154.1	AAAGAGACCAGTAACCCCAAACCT
bacillus R		GGATACCAGGATGGGTCTCGAT

separate *B. anthracis* plasmid DNA loci. Eight paired primers such as *vrrA*, *vrrB1*, *vrrB2*, *vrrC1*, *vrrC2*, *CG3*, *pX01* and *pX02* were designed and synthesized for *B. anthracis* DNA MLST.

To determine whether the microorganisms studied belong to the genus Bacillus, the region of chromosomal DNA was amplified using forward and reverse primers *BacillusF* and *BacillusR*. More precise sample indication was carried out when amplifying the first and second *B. anthracis* plasmids, the sequence designated as *pXO1* and *pXO2*, respectively.

In genotyping, the annealing temperature changed depending upon a primer structure. To separate DNA strands more completely, preliminary denaturation (initialization) was carried out at 95 °C for 2 min. For genotyping the abovementioned data, eight pairs of primers were applied for different loci of the bacillus genome.

3.2 Multi-primer PCR of Strains and Isolates

Lysates of various *B. anthracis* strains and other species of the genus Bacillus as well as Brucella and Listeria were used to determine the reaction specificity with the primers selected.

When amplifying the DNA fragments, three specific fragments were synthesized on a matrix of *B. anthracis* strains CH-7, 81 (Fig. 1, lanes 7 and 17), that indicates they belong to the genus Bacillus and to a species having two plasmids in its genome. At the same time, the synthesis of two fragments (Fig. 1, lanes 5 and 12) was initiated in non-encapsulated *B. anthracis* strains 55 and F34, the primers neither bound with heterologous species or genus genomes nor initiated DNA fragment synthesis. Three amplicons with different molecular weights were synthesized on matrices of the derived isolates and collection strains and isolates; these amplicons coincided with those of known pathogenic strains upon this criterion.

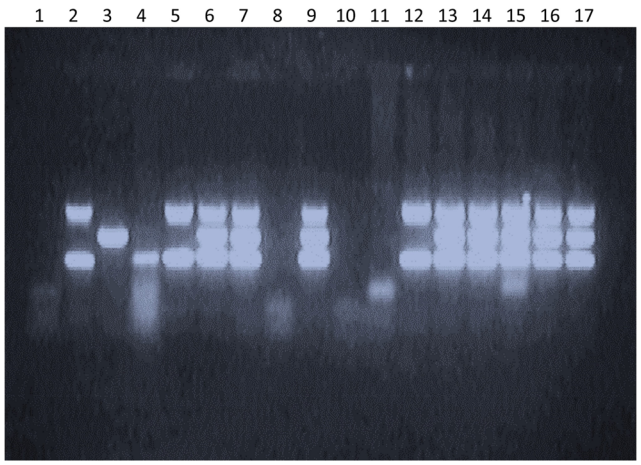

Fig. 1 Multi-primer PCR results for tested samples. Lanes: 1—negative control (TE-buffer); 2—positive control of *B. anthracis*, plasmids *chromosome* + *pXO1*; 3—positive control of *B. anthracis*, plasmid *pXO2*; 4—*B. cereus* strain 8035; 5—*B. anthracis* strain 55, vaccinal; 6—*B. anthracis* isolate Lai (derived from a cattle cadaver, Laishevo, Tatarstan, 2014); 7—*B. anthracis* strain CH-7, pathogenic; 8—*B. abortus* strain 19; 9—*B. anthracis* isolate #22, from collection (derived from a sheep cadaver, Tatarstan, 1972); 10—*L. monocytogenes* strain AUF; 11—soil sample collected on the territory of the meat packing plant, Zelenodolsk, Tatarstan, 2004; 12—*B. anthracis* strain F34; 13—isolate U2 (soil-derived, Ulyanovsk, 2004); 14—isolate U1 (derived from a pig cadaver, Ulyanovsk, 2004); 15—*B. anthracis* isolate ZEL (derived from a cattle cadaver, Zelenodolsk, 2004); 16—*B. anthracis* isolate #36, from collection (derived from a sheep cadaver, Chuvashia, 1971); 17—*B. anthracis* strain 81, pathogenic, master seed strain

The results of these experiments demonstrate that the primers detected DNA loci of microorganisms which belong to the genus *Bacillus*, while the presence of *pXO1* and *pXO2* plasmids indicated the pathogenicity of *B. anthracis* strains, i.e. the primers allowed to differentiate pathogenic and vaccinal strains as well as isolates.

The lower amplicon is genus-specific, that is, this locus is found in all species of the genus Bacillus, for example, in *B. cereus* (Fig. 1, lane 4). However, these species have no *B. anthracis*-specific plasmids; therefore, two upper amplicons are not detectable in PCR. On the contrary, pathogenic *B. anthracis* strains have both plasmids, *pX01* and *pX02*; therefore, two more amplicons are synthesized on their matrices; they are larger in molecular weight than the Bacillus-specific amplicon.

We examined four isolates such as isolates ZEL and Lai from Tatarstan and isolates U1 and U2 from the Ulyanovsk region. The isolates Lai and ZEL were derived from cattle cadavers (Laishevo and Zelenodolsk, Tatarstan, respectively). Soil samples were collected on the territory of a meat packing plant (Zelenodolsk, Tatarstan). The isolates Lai and ZEL were found to be pathogenic *B. anthracis* strains (Fig. 1, lanes 6 and 15), with no *B. anthracis* detected in the soil samples (Fig. 1, lane 11).

The isolate U1 was obtained from a pig cadaver, while U2 derived from a soil sample collected on a farm in the Ulyanovsk region where the pig died. Pathogenic *B. anthracis* strains with a full set of plasmids were identified (Fig. 1, lanes 13 and 14). To validate the PCR results, the known pathogenic *B. anthracis* isolates obtained in Tatarstan (#22) and Chuvashia (#36) in 1972 and 1971, respectively, were examined in parallel (Fig. 1, lanes 9 and 16).

3.3 Sequencing of Derived Isolates

To clarify the origin of derived isolates ZEL, Lai, U1 and U2, they were genetically typed using amplicon sequencing at eight marker loci such as *vrrA* (310 bp), *vrrB1* (240 bp), *vrrB2* (170 bp), *vrrC1* (600 bp), *vrrC2* (530-600 bp), *CG3* (160 bp), *pX01* (120 bp) and *pX02* (140 bp) (Table 2).

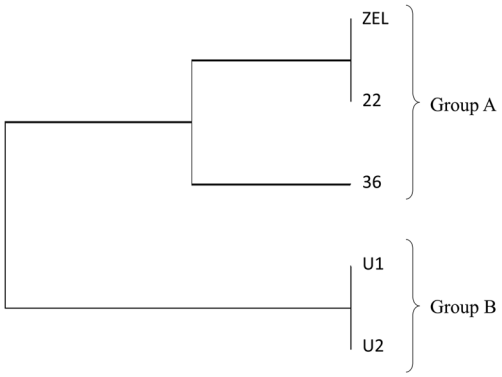

Fig. 2 Distribution of *B. anthracis* isolates according to the degree of genetic homology. Group A: isolate ZEL (derived from a cattle cadaver, Zelenodolsk, 2004); isolate #22, from collection (derived from a sheep cadaver, Tatarstan, 1972); isolate Lai (derived from a cattle cadaver, Laishevo, 2014); isolate #36, from collection (derived from a sheep cadaver, Chuvashia, 1971); Group B: isolate U1 (derived from a pig cadaver, Ulyanovsk, 2004); isolate U2 (soil-derived, Ulyanovsk, 2004)

Six isolates and one strain of *B. anthracis* were examined; they are isolates ZEL and Lai, both derived from cattle cadavers (Tatarstan), the collection isolate #22, derived from a sheep cadaver (Tatarstan), the collection isolate #36, derived from a sheep cadaver (Chuvashia); the isolate U1, derived from a pig cadaver (Ulyanovsk), the soil-derived isolate U2 (Ulyanovsk), a vaccine strain 55, which is non-encapsulated and has no pX02 plasmid.

Using complementary primers to the marker sites studied oligonucleotides (amplicons) were synthesized on a DNA matrix which were later sequenced. The analysis performed demonstrated the genetic relationship of the strains and isolates studied (Fig. 2). The vaccine strain 55 cannot be included into a phylogenetic tree as it lacks one marker. A special software was used to analyze a structural composition of sequences (Table 3).

The sequencing results demonstrate that the derived isolate ZEL is the same strain as the collection isolate #22 which was obtained in Tatarstan in 1972 and is different from the collection strain #36 obtained in Chuvashia (Fig. 2). At the same time the derived isolate Lai is the same strain as the collection isolate #36. However, their similarity facilitated combining all

Table 2 Size of amplified *B. anthracis* strain and isolate loci, basepairs

Strain/isolate	Locus							
	VrrA	*VrrB1*	*VrrB2*	*VrrC1*	*VrrC2*	*CG3*	*pXO1*	*pXO2*
ZEL	313	229	162	617	604	152	135	137
#36	313	229	154	617	604	152	126	137
#22	313	229	162	617	604	152	135	137
Lai	313	229	154	617	604	152	126	137
U1	301	256	162	581	532	157	132	137
U2	301	256	162	581	532	157	132	137
55	313	229	162	617	604	152	129	–

Table 3 Nucleotide sequence of the resulting amplicons

Marker name	Amplicon molecular weight, bp	*Nucleotide sequence 5′ -› 3′
vrrA	301	gcgcgtttcgtttgattcatacatttgaggagggatcatttgctcgggatgatgctgcatgtatggttggtattgctgtgagtattgttgcgggtattgttgttgatattgttgt ttgatattgttgttgatattgctgtcgatattggggtggcggttgctgttgttggtgcatcattggcggtggtccttccatttgtcgataaggcggaaccatttgcatgaa aggttcggttggatcgtgttttttaaagagtttagcaaggaaccctttctttttttgtgccatcggtggtagttgt
	313	gcgcgtttcgtttgattcatacatttgaggagggatcatttgctcgggatgatgctgcatgtatggttggtattgctgtgagtattgttgcgggtattgttgttgatattgttg ttgatattgttgttgatattg**ttgttgatattg**ctgtcgatattggggtggcggttgctgttgttggtgcatcattggcggtggtccttccatttgtcgataaggcggaa ccatttgcatgaaaggttcggttggatcgtgttttttaaagagtttagcaaggaaccctttctttttttgtgccatcggtggtagttgt
vrrB1	229	gaataggtggttttccgcaagttattcatggtcatcatggacatcaccaccatcaaggtcaccaaggtcaccatggtcatcaaggccaccatcatcaccaaggtcac cacggtcatcatgggcatcatcatcaccaaggtcaccacggtcatcatgggcatcatcaacaacaagtacatcaccacggccatcatcatatacatccacaggc tattctttatcaaactca
	256	gaataggtggttttccgcaagttattcatggtcatcatggacatcaccaccatcaaggtcacca**cggtcatcatggacatcaccaccatca**aggtcaccacggtc atcatggacatcaccaccatcaaggtcaccacggtcatcaaggccaccatcatcaccaaggtcaccacggtcatcatgggcatcatcaacaacaagtacatca ccacggccatcatcatatacatccacaggctattctttatcaaactca
vrrB2	154	ccacaggctattctttatcaaactcatcaaggtcaccaaggccaccatgatcatcacggtcaccacggtcatcaaggccaccacggtcaacacagtcaacagcac caacagcaccaacaatatcaacaatatcaacaacaatcttcaccttggg
	163	ccacaggctattctttatcaaactcatcaaggtcaccaaggccaccatgatcatcacggtcaccacggtcatcaaggccaccacggtcaacacagtcaacagcac caacagcaccaacaatatcaacaatatcaacaa**tatcaacaa**caatcttcaccttggg
vrrC1	581	gaagcaagaaagtgatgtagtggacaaaaacattctacaagaggaattagtagattctaaagtagagcacgaggatacaatattatcagaagaaattaaacgtagta cagaaatagaacaacctactattgaagtagaaaaacaagcaccagaagaatcagtgattgtcaaagcagaagaaaaacttgaagaaacaattgttgtagaaata ccagaagaagtggaagtaattgcagaagcggaagaaccagaagaagtagaagtaattgcggaaacagaagagtcagaagaagtagaagtaattgcggaaa cagaagagtcagaagaagtagaagtaattgcagaaacagaagagttagaagaagtggaagtaactgcagaaacagaagaattagaagaagtggaagtagttg cagaaacagaagagttagaagaagtggaagtaattgcagaaacagaaaaattagaagaattagaagaagtggaagtaattgcagaaacagaagagtcagaa gaagtagaagtaattgcagaaacagaagcaccagaagaagtggaacctgtagcacttgaggaaatgc
	617	gaagcaagaaagtgatgtagtggacaaaaacattctacaagaggaattagtagattctaaagtagagcacgaggatacaatattatcagaagaaattaaacgtagta cagaaatagaacaacctactattgaagtagaaaaacaagcaccagaagaatcagtgattgtcaaagcagaagaaaaacttgaagaaacaattgttgtagaaata ccagaagaagtggaagtaattgcagaagcggaagaaccagaagaagtagaagtaattgcggaaacagaagagtcagaagaagtagaagtaattgcggaaa cagaagagtcagaagaagtagaagtaattgcagaaacagaagagtcagaagaagtagaagtaattgcagaaacagaagagttagaagaagtggaagtaact gcagaaacagaagaattagaagaagtggaagtagttgcagaaacagaagagttagaagaagtggaagtaattgcagaaacagaaaaattagaaga**attaga agaagtggaagtaattgcagaaacagaaga**gtcagaagaagtagaagtaattgcagaaacagaagcaccagaagaagtggaacctgtagcacttgagg aaatgc
vrrC2	532	accagaagaagtggaacctgtagcacttgaggaaatgcaacaagaaatggtgttaaatgaagcaattgaacaaaagaatgaattcatacatgttgctgtggctgatg aacaaacgaaaaaagatgttcaaagctttgcggatgttttaattgcggaggaacaatcagttgtagaagaaacaccgattgtagaagaacaaccagttgcagaa gaagcaccggtcgtagaagaacaatcagttgtagaagaaacaccgattgtagaagaagcaccggtcgtagaaggacaatcagttgtagaagaagcaccgatt gcagaagaacaaccagttgcagaggaaacatcggtcgtagaagaacaaccagttgcagaggaaacatcgatcgtagaagaacaaccagttgcagaggaag caccagtcgtagaagaacaaccagttgtacaaaaagaagaaccaaaacgtgagaaaaagcgtcacgtaccatttaatgttgttatgttgaaacaagatagagcg cgattaatggaaaga
	604	accagaagaagtggaacctgtagcacttgaggaaatgcaacaagaaatggtgttaaatgaagcaattgaacaaaagaatgaattcatacatgttgctgtggctgatg aacaaacgaaaaaagatgttcaaagctttgcggatgttttaattgcggaggaacaatcagttgtagaagaaacaccgattgtagaagaacaaccagttgcagaa gaagcaccggtcgtagaagaacaatcagttgtagaagaaacaccgatt**gtagaagaacaaccagctgcagaagaagcaccggtcgtagaagaacaat cagttgtagaagaaacaccgatt**gtagaagaagcaccggtcgtagaaggacaatcagttgtagaagaagcaccgattgcagaagaacaaccagttgcaga ggaaacatcggtcgtagaagaacaaccagttgcagaggaaacatcgatcgtagaagaacaaccagttgcagaggaagcaccagtcgtagaagaacaacca gttgtacaaaaagaagaaccaaaacgtgagaaaaagcgtcacgtaccatttaatgttgttatgttgaaacaagatagagcgcgattaatggaaaga
CG3	152	tgtcgttttacttctctctccaatactaaaatgtatgtatttttagtatagcctatttacataattagtaaaagtaaaatatgcatgtaaaatcttacaaatatgaaaataatatcac caaataaatttatgccctttatacagaacaatgac
	157	tgtcgttttacttctctctccaatactaaaatgtatgtatttttagtatagcctatttacataattagtaaaagtaaaatatgcatgtaaaatcttacaaatatgaaaataatat**aatat** caccaaataaatttatgccctttatacagaacaatgac
pXO1	126	tctagaattagttgcttcataatggctggtcccgttattattattattattattaaatccatttgaaaatttttaatacattacttatgagttaataatgaacttaatctgatcgttaataaattg
	129	tctagaattagttgcttcataatggctggtcccgttattattattattattatt**att**aaatccatttgaaaatttttaatacattacttatga gttaataatgaacttaatctgatcgttaataaattg
	132	tctagaattagttgcttcataatggctggtcccgttattattattattattatt**attatt**aaatccatttgaaaatttttaatacattacttatgagttaataatgaact taatctgatcgttaataaattg
	135	tctagaattagttgcttcataatggctggtcccgttattattattattattatt**attattatt**aaatccatttgaaaatttttaatacattacttatgagttaataatga acttaatctgatcgttaataaattg
pXO2	140	tcatcctcttttaagtcttgggttatatttatctacctttgcggtatgtgaaattatatatatatatatatatcaaaagaaaaaaggaaggggaatacccccctcctttacttgta ctttgtcgtcggagttcatcaca

*Polymorphism sites are in bold

four strains into conditional group A. The isolated strains U1 and U2 were different from the four abovementioned isolated strains by genotype, which indicates their separate grouping (group B).

Thus, the distribution of *B. anthracis* isolates according to the degree of genetic homology based on the sequencing results suggests that the derived isolates were local strains surviving for a long time in the environment rather than those brought from other regions of Russia.

4 Conclusion

Our research has shown that multi-primer PCR can in general be used to clarify the epidemic situation with anthrax. Multi-primer PCR has sufficient sensitivity and specificity that defines it as an effective method for *B. anthracis* detection in laboratory diagnostics. The used primers allow to differentiate isolates by their belonging to the genus Bacillus and as *B. anthracis* species by the presence of pXO1 plasmid. The detection of the pX02 plasmid encoding for capsule formation indicates pathogenicity of the isolate studied. Using multi-primer PCR allowed to determine the epizootic situation of anthrax in the Middle Volga Region and to establish that two genotypic groups of the pathogen *B. anthracis* circulate in the region.

Funding This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Compliance with Ethical Standards Animals were not used in this study.

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Huang, Y., Du, Y., Wang, Y., Wang, N., Bai, J., Chen, H., He, H., Xu, J., Wu, Y., Luo, Y., Li, X., & Liang, G. (2016). An outbreak of cutaneous anthrax in Yunnan, China. *Emerging Microbes and Infections, 5*, e64. https://doi.org/10.1038/emi.2016.65.
2. Mondal, T. K., Ghosh, S., Dasgupta, S., & Sarkar, A. P. (2015). Suspected anthrax outbreak: Investigation in a rural block of west Bengal and public health response. *Indian Journal of Public Health, 59*(4), 302–305. https://doi.org/10.4103/0019-557X.169662.
3. Siddiqui, M. A., Khan, M. A., Ahmed, S. S., Anwar, K. S., Akhtaruzzaman, S. M., & Salam, M. A. (2012). Recent outbreak of cutaneous anthrax in Bangladesh: Clinico-demographic profile and treatment outcome of cases attended at Rajshahi medical college hospital. *BMC Research Notes, 5*, 464. https://doi.org/10.1186/1756-0500-5-464.
4. Thapa, N. K., Tenzin, W. K., Dorji, T., Migma, D. J., Marston, C. K., & Hoffmaster, A. R. (2014). Investigation and control of anthrax outbreak at the human-animal interface, Bhutan, 2010. *Emerging Infectious Diseases, 20*(9), 1524–1526. https://doi.org/10.3201/eid2009.140181.
5. Kracalik, I., Malania, L., Tsertsvadze, N., Manvelyan, J., Bakanidze, L., Imnadze, P., Tsanava, S., & Blackburn, J. K. (2014). Human cutaneous anthrax, Georgia 2010-2012. *Emerging Infectious Diseases, 20*(2), 261–264. https://doi.org/10.3201/eid2002.130522.
6. Antwerpen, M., Elschner, M., Gaede, W., Schliephake, A., Grass, G., Tomaso, H. (2016) Genome sequence of *Bacillus anthracis* strain Stendal, isolated from an anthrax outbreak in cattle in Germany. *Genome Announcements, 4*(2), e00219-16. https://doi.org/10.1128/genomeA.00219-16.
7. Nsoh, A. E., Kenu, E., Forson, E. K., Afari, E., Sackey, S., Nyarko, K. M., & Yebuah, N. (2016). Mapping as a tool for predicting the risk of anthrax outbreaks in northern region of Ghana. *The Pan African Medical Journal, 25*(Suppl 1), 14. 10.11604/pamj.supp.2016.25.1.6205.
8. Lehman, M. W., Craig, A. S., Malama, C., Kapina-Kany'anga, M., Malenga, P., MunsakaF, M. S., Shadomy, S., & Marx, M. A. (2017). Role of food insecurity in outbreak of anthrax infections among humans and hippopotamuses living in a game reserve area, rural Zambia. *Emerging Infectious Diseases, 23*(9), 1471–1477. https://doi.org/10.3201/eid2309.161597.
9. Ozanich, R. M., Colburn, H. A., Victry, K. D., Bartholomew, R. A., Arce, J. S., Heredia-Langner, A., Jarman, K., Kreuzer, H. W., & Bruckner-Lea, C. J. (2017). Evaluation of PCR systems for field screening of bacillus anthracis. *Health Security, 15*(1), 70–80. https://doi.org/10.1089/hs.2016.0043.
10. Terry, C., Jiang, S., Radford, D. S., Wan, Q., Tzokov, S., Moir, A., & Bullough, P. A. (2017). Molecular tiling on the surface of a bacterial spore – The exosporium of the bacillus anthracis/cereus/thuringiensis group. *Molecular Microbiology, 104*(4), 539–552. https://doi.org/10.1111/mmi.13650.
11. Makino, S., Uchida, I., Terakado, N., Sasakawa, C., & Yoshikawa, M. (1989). Molecular characterization and protein analysis of the cap region, which is essential for encapsulation in bacillus anthracis. *Journal of Bacteriology, 171*(2), 722–730.
12. Koehler, T. M. (2002). Bacillus anthracis genetics and virulence gene regulation. *Current Topics in Microbiology and Immunology, 271*, 143–164 **Review**.
13. Siqueira, FM., Cibulski, SP., Mayer, FQ., Driemeier, D., Pavarini, SP., Vargas, APC. (2017) Genome sequencing of two bacillus anthracis strains: A virulent strain and a vaccinal strain. *Brazilian Journal of Microbiology*. https://doi.org/10.1016/j.bjm.2017.04.007.
14. Keefer, A. B., Asare, E. K., Pomerantsev, A. P., Moayeri, M., Martens, C., Porcella, S. F., Gottesman, S., Leppla, S. H., & Vrentas, C. E. (2017). In vivo characterization of an Hfq protein encoded by the bacillus anthracis virulence plasmid pXO1. *BMC Microbiology, 17*(1), 63. https://doi.org/10.1186/s12866-017-0973-y.
15. Liang, X., Zhu, J., Zhao, Z., Zheng, F., Zhang, H., Wei, J., Ji, Y., & Ji, Y. (2017). Thepag gene of pXO1 is involved in capsule biosynthesis of bacillus anthracis Pasteur II strain. *Frontiers in Cellular and Infection Microbiology, 7*, 203. https://doi.org/10.3389/fcimb.2017.00203.
16. Pomerantsev, A. P., McCall, R. M., Chahoud, M., Hepler, N. K., Fattah, R., & Leppla, S. H. (2017). Genome engineering in bacillus anthracis using tyrosine site-specific recombinases. *PLoS One, 12*(8), e0183346. https://doi.org/10.1371/journal.pone.0183346.
17. Antwerpen, MH., Sahl, JW., Birdsell, D., Pearson, T., Pearce, MJ., Redmond, C., Meyer, H., Keim, PS. (2017) Unexpected relations of historical Anthrax strain. *MBio, 8*(2), e00440-17. https://doi.org/10.1128/mBio.00440-17.
18. Daas, MS., Rosana, ARR., Acedo, JZ., Nateche, F., Kebbouche-Gana, S., Vederas, JC., Case, RJ. (2017) Draft genome sequences of *Bacillus cereus* E41 and *Bacillus anthracis* F34 isolated from

Algerian Salt Lakes. *Genome Announcements, 5*(20), e00383-17. https://doi.org/10.1128/genomeA.00383-17.

19. Khmaladze, E., Dzavashvili, G., Chanturia, G., Nikolich, MP., Chain, PSG., Johnson, SL., Imnadze, P. (2017) Ten genome sequences of human and livestock isolates of *Bacillus anthracis* from the country of Georgia. *Genome Announcements, 5*(19), e00256-17. https://doi.org/10.1128/genomeA.00256-17.
20. Antwerpen M, Wölfel R, Grass G (2017) Genome sequence of historical *Bacillus anthracis* strain Tyrol 4675 isolated from a bovine Anthrax case in Austria. *Genome Announcements, 5*(10), e00002-17. https://doi.org/10.1128/genomeA.00002-17.
21. Riazanova, A. G., Eremenko, E. I., Tsygankova, O. I., & Tsygankova, E. A. (2009). Improvement of methods for identification of atypical anthrax strains and their differentiation from closely related bacilli. *Zhurnal Mikrobiologii, Epidemiologii, i Immunobiologii, 3*, 76–80.
22. Dubiley, S., Kirillov, E., & Mirzabekov, A. (1999). Polymorphism analysis and gene detection by minisequencing on an array of gel-immobilized primers. *Nucleic Acids Research, 27*(18), e19.