EXPERIMENTAL WORKS

The Use of Loop-Mediated Isothermal DNA Amplification for the Detection and Identification of the Anthrax Pathogen

I. Yu. Shchit[a], K. B. Ignatov[b, c], T. Yu. Kudryavtseva[a], N. A. Shishkova[a], R. I. Mironova[a], L. I. Marinin[a], A. N. Mokrievich[a, *], V. M. Kramarov[b, c], S. F. Biketov[a], and I. A. Dyatlov[a]

[a]*State Research Center of Applied Microbiology and Biotechnology, Federal Service for the Protection of Customer Rights, Obolensk, 142279 Russia*
[b]*Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow, 117971 Russia*
[c]*All-Russian Research Institute of Agricultural Biotechnology, Russian Academy of Sciences, Moscow, 127422 Russia*
***e-mail: mokrievich@obolensk.ru**
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Abstract—The results of detection and identification of *Bacillus anthracis* strains in loop-mediated isothermal DNA amplification (LAMP) reaction performed under optimized conditions with original primers and thermostable DNA polymerase are presented. Reproducible LAMP-based detection of chromosomal and plasmid DNA targets specific for *B. anthracis* strains has been demonstrated. No cross reactions with DNA from bacterial strains of other species of the *B. cereus* group were detected. The development of tests for anthrax-pathogen detection based on the optimized reaction of loop isothermal DNA amplification is planned. These tests will be convenient for clinical studies and field diagnostics due to the absence of requirements for sophisticated equipment.

INTRODUCTION

B. anthracis, the causative agent of anthrax, belongs to the taxonomical group of *B. cereus*, which includes six closely related species, namely, *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis. B. anthracis* is an obligate pathogen of humans and herbivorous mammals, in contrast to all other species in the group. Identification of individual species from the *B. cereus* group is a complex task, since the genomic sequences of *B. anthracis*, *B. cereus*, and *B. thuringiensis* show virtually no differences when analyzed using a wide range of methods. Analysis of polymorphisms in 16S–23S rRNA and multilocus sequence typing [1, 2] revealed only minor differences between the genomes of these bacteria.

Certain chromosomal mutations and the presence of two virulence plasmids distinguish *B. anthracis* from other members of the group (importantly, plasmid-free strains lose virulence), and these features are used for the design of primers for amplification-based detection of the causative agent of anthrax. The genome of virulent *B. anthracis* strains is made up of a 5.23-Mb chromosome and two multicopy virulence plasmids pXO1 and pXO2. The pXO1 plasmid (184 kbp) contains genes that encode a toxin composed by a protective antigen (*pag*), a lethal factor

(*lef*), and an edema factor (*cya*). The pXO2 plasmid (97 kbp) harbors structural (*cap*) genes involved in the synthesis of the antiphagocytic poly-D-glutamine capsular antigen. Intraspecies differentiation of strains of the anthrax pathogen can only be performed if single nucleotide polymorphisms (SNPs) are analyzed, since *B. anthracis* strains that show the greatest genomic differences are still characterized by more than 99.99% identity between the nucleotide sequences [3, 4]. Plasmids with a similar genetic structure have been found in other species of the genus *Bacillus*, including species that do not belong to the *B. cereus* group, such as *B. circulans* and a strain similar to *B. luciferensis* [5].

Reports of the presence of complete sets of virulence genes that encode a three-component toxin and a polyglutamic-acid capsule in *B. cereus* and *B. thuringiensis* can partly explain the existence of "anthraxlike" diseases of human beings and animals reported in the recent decades. These diseases are caused by virulent strains of *B. cereus* (G9241, 03BB102, 3BB108, 03BB87, E33L, B17, 3a, and CI) and *B. thuringiensis* (97−27) [6−10]. Natural genetic exchange of virulence plasmids between *B. anthracis* and other bacilli can underlie the emergence of virulent *B. cereus* and *B. thuringiensis* strains. The experimental data available to date are not sufficient to prove such exchange, but the implementation of plasmid transfer in the lab-

oratory does not present any serious difficulties, since mobility genes and genes with a conjugative function have been found on the pXO2 plasmid [11−13]. The isolation of monoplasmid, diplasmid, and plasmid-free *B. anthracis* strains from the environment and the possibility of construction of such strains in the laboratory also provide indirect evidence for the hypothesis mentioned above.

In view of the above, it is necessary to develop and implement new methods that could distinguish between *B. anthracis*, on one the hand, and atypical strains and other species of saprophytic bacilli, as well as "anthracellar" human- and animal-disease pathogens (that have a much lower epidemic significance than the anthrax microbe), on the other hand. These methods are highly suited for epidemiological investigations of outbreaks and laboratory diagnosis of infectious diseases with clinical symptoms similar to those of anthrax.

Loop isothermal DNA amplification (LAMP) is a promising method of microorganism detection and differentiation. The reaction involves chain replacement and requires a DNA polymerase (Bst-polymerase in most cases) and a constant temperature of 60−65°C [14, 15]. Therefore, isothermal amplification does not require a thermocycler, in contrast to polymerase chain reaction (PCR), which involves successive cycles of temperature change. The tube with the reaction mixture is incubated at a temperature of 60−65°C in a conventional water bath or a solid thermostat block. Synthesis of the target sequence requires two or three pairs of primers. The forward (F3) and reverse (B3) outer primers are used when the chain is replaced. The forward (FIP) and reverse (BIP) internal primers have a more complex structure and participate in the formation of loop structures [16]. A number of LAMP-based tests for the detection of microbial and viral pathogens of infectious diseases, including tuberculosis [17, 18], leptospirosis [19], *Staphylococcus aureus* [20], listeriosis [21], cholera [22], swine influenza virus H1N1 [23], hepatitis B virus [24], and anthrax [25−29] have been developed in recent years.

The possibility of LAMP-based detection of *B. anthracis* spores was tested in [25]. Three sets of six pairs of primers were designed for the study of targeted fragments of the pXO1 and pXO2 plasmids (pag and capB, respectively) and a fragment of the chromosome. Thirteen *B. anthracis* strains were identified by LAMP with these primers (a positive result was obtained with at least two of three primer sets), and the analysis of 33 heterologous strains yielded negative results. Notably, the primers that targeted a chromosomal fragment had a less than 100% specificity and yielded positive results with some other *Bacillus* species, and this presented a risk of false positive results.

DNA fragments of plasmids pXO1 (*pag*) and pXO2 (*cap*B) were used as targets in another study of LAMP-based detection of strains of the anthrax pathogen

[26]. Three *B. anthracis* strains, three *B. cereus* strains, one *B. thuringiensis* strain, and one *B. subtilis* strain were used to test the specificity of primer sets [26].

We chose novel chromosomal targets [30] in order to avoid false positive signals from the DNA of bacilli of the *B. cereus* group upon the detection of *B. anthracis* and, thus, to distinguish *B. anthracis* from closely related species. The selection of primers for chromosomal and plasmid DNA targets was performed with rigid requirements imposed on the stability of the 3' end of the F2 region and the 5' end of the F1c region of the FIP primer, as well as on the stability of the 3' end of the B2 region and the 5' end of the B1c region of the BIP primer.

It should be noted that LAMP has great potential for the design of tests for field use, since the reaction is isothermal and fast. Such tests are highly appropriate for a number of epidemiological tasks, such as the identification of pathogens in the environment.

The aim of the present work consisted in the development of a LAMP-based method for species-specific detection of DNA of *B. anthracis* strains with original primers and a thermostable DNA polymerase.

MATERIAL AND METHODS

Bacterial strains. Strains of various bacterial species used in the present study were obtained from the microorganism collection of the Obolensk Research Center of Applied Microbiology and Biotechnology (Russia). Characteristics of the strains, including the presence of specific genes, are listed in Table 1.

Cultivation. Ampoules with *B. anthracis* cultures were opened, and 0.9% sodium-chloride solution was added at 0.5 mL per ampoule. Suspensions of *B. anthracis* microbes were plated on L-agar Petri dishes with bacterial loops and incubated for 18 h at 37 ± 1°C. Suspensions in 2 mL of 0.9% sodium-chloride solution were prepared separately from each culture. Suspension turbidity corresponded to 10 turbidity units of the industry standard (OSO 42-28-59-86P according to Tarasevich GISK), this being equivalent to 1×10^8 million *B. anthracis* cells/mL.

Thermolysate preparation. The suspensions of 18-h agar cultures were incubated in a thermostat at 100°C for 30 min, cooled on ice for 10 min, and centrifuged. The supernatant was used as the template.

DNA isolation. A commercial DNK-sorb-B kit (OAO InterLabServis) was used for DNA isolation. Safety requirements[1, 2] were adhered to during these operations.

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[1] SP 1.3.1285-03 Safety of working with microorganisms of pathogenicity (hazard) groups I−II.

[2] Organization of laboratory work on nucleic acid amplification from materials that contain microorganisms of pathogenicity groups I−IV: methodological guidelines MU 1.3.2569-09—Moscow, 2009.

Table 1. Characteristics of microorganism strains used in the study

No.	Species	Strain name	Plasmid spectrum	LD50 (spores)*	Source/isolated from
1	*B. anthracis*	Ch-7	$pXO1^+_{(tox)}/pXO2^+$	4	Human corpse, 1970
2	*B. anthracis*	81/1	$pXO1^+_{(tox)}/pXO2^+$	7	Human patient, cutaneous form, 1972
3	*B. anthracis*	71/12	$pXO1^+_{(tox)}/pXO2^+$	32	Tsenkovskii's second vaccine
4	*B. anthracis*	STI-1	$pXO1^+_{(tox)}/pXO2^-$	1×10^6	N.N. Ginsburg vaccine strain, 1941
5	*B. anthracis*	STI-1 Rif 4	$pXO1^+_{(tox)}/pXO2^-$	1×10^9	Variant of STI-1 strain
6	*B. anthracis*	770NP1R	$pXO1^+_{(tox)}/pXO2^-$	1×10^9	CDC
7	*B. anthracis*	ΔAmes	$pXO1^-_{(tox)}/pXO2^+$	1×10^9	CDC
8	*B. anthracis*	1 (Kolomna)	$pXO1^+_{(tox)}/pXO2^+$	20	Cow carcass, Moscow oblast
9	*B. anthracis*	5 (Tula)	$pXO1^+_{(tox)}/pXO2^+$	8	Cow, Tula oblast
10	*B. anthracis*	15	$pXO1^+_{(tox)}/pXO2^+$	10	Cow carcass, Tajikistan
11	*B. anthracis*	Pasteur 2	$pXO1^+_{(tox)}/pXO2^+$	6	Institut Pasteur, France
12	*B. anthracis*	55 VNIIVViM	$pXO1^+_{(tox)}/pXO2^-$	1×10^6	VNIIVViM
13	*B. anthracis*	Ichtiman	$pXO1^+_{(tox)}/pXO2^-$	5×10^5	VNIIVViM
14	*B. anthracis*	34F2 sterne	$pXO1^+_{(tox)}/pXO2^-$	2.5×10^4	NIPChI Mikrob
15	*B. anthracis*	Lange1	$pXO1^-_{(tox)}/pXO2^-$	1×10^8	VNIIVViM
16	*B. anthracis*	513/1	$pXO1^-_{(tox)}/pXO2^-$	$>1 \times 10^8$	Human patient, Ryazan oblast, 1981
17	*B. anthracis*	13/39	$pXO1^+_{(tox)}/pXO2^-$	$>1 \times 10^3$	Human patient, Republic of Dagestan, 1962
18	*B. anthracis*	10 (38 Kaluga)	$pXO1^-_{(tox)}/pXO2^-$	$>1 \times 10^8$	Cow carcass, Kaluga oblast
19	*B. anthracis*	32 (603)	$pXO1^-_{(tox)}/pXO2^-$	$>1 \times 10^8$	Cow, Kazakhstan
20	*B. cereus*	5832	$pXO1^-_{(tox)}/pXO2^-$		GKPM-Obolensk
21	*B. cereus*	160	$pXO1^-_{(tox)}/pXO2^-$		GKPM-Obolensk
22	*B. cereus*	ATCC10702	$pXO1^-_{(tox)}/pXO2^-$		GKPM-Obolensk
23	*B. cereus*	504	$pXO1^-_{(tox)}/pXO2^-$		GKPM-Obolensk
24	*B. cereus*	Dakar	$pXO1^-_{(tox)}/pXO2^-$	$>1 \times 10^8$	VNIIVViM
25	*B. cereus*	771	$pXO1^-_{(tox)}/pXO2^-$		GKPM-Obolensk
26	*B. cereus* var. *anthracoides*	217	$pXO1^-_{(tox)}/pXO2^-$		GKPM-Obolensk
27	*B.cereus* var. *anthracoides*	6691	$pXO1^-_{(tox)}/pXO2^-$		GKPM-Obolensk
28	*B. thuringiensis*	214	$pXO1^-_{(tox)}/pXO2^-$		GKPM-Obolensk
29	*B. thuringiensis*	G7566	$pXO1^-_{(tox)}/pXO2^-$		GKPM-Obolensk
30	*Bacillus* spp.	Nach	$pXO1^-_{(tox)}/pXO2^-$		Animal burial ground, Nizhny Novgorod
31	*E. coli*	JM83			GKPM-Obolensk
32	*Y. pestis*	I3449			GKPM-Obolensk
33	*F. tularensis*	503			GKPM-Obolensk
34	*V. cholerae* O139	B6475			GKPM-Obolensk

* For subcutaneous inoculation in mice.

Primer design. Primer Explorer 3 software (http://primerexplorer.jp/elamp3.0.0/index.htmL) was used to design the primers. BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to test primer specificity. The primers were synthesized and purified by Sintol (Moscow, Russia).

LAMP implementation. The reaction mixture with Bst polymerase contained $0.8-2.4 \mu M$ of FIP and BIP primers each, $0.2 \mu M$ of F3 and B3 primers each, $1\times$ Bst polymerase reaction buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, and 0.1% Tween 20), $100 \mu M$ to 1.4 mM each dNTP, $0.2-0.8$ M betaine, 6 to 8 mM $MgSO_4$, $8-40$ U Bst polymerase, $1-5 \mu L$ sample, and water to the final reaction volume of $25 \mu L$. The reagents were mixed on ice. Optimization of the reaction involved changing the concentration of Mg^{2+} (in the range of $4-10$ mM), the amount of Bst polymerase (in the range of $4-32$ U/μL), and the incubation temperature (in the range of $50-68°C$). The samples were incubated in a Tertsik thermocycler (DNK-Tekhnologiya) for $30-60$ min. LAMP products were subjected to electrophoresis in 1.2% agarose gel in TAE buffer and visualized in an UV transilluminator at a wavelength of 260 nm.

The reaction mix with Sd-polymerase had a total volume of $50 \mu L$ and contained $1\times$ SD buffer (Bioron, Germany), 40 units SD polymerase, 3.5 mM $MgCl_2$, 0.5 mM each dNTP, four primers ($0.2 \mu M$ F3, $0.2 \mu M$ B3, $0.8 \mu M$ BIP, and $0.8 \mu M$ FIP), and $5 \mu L$ template. The reaction was performed at 60°C for 60 min after 2 min of incubation at 92°C in a Tertsik thermocycler (DNK-Tekhnologiya). LAMP products were subjected to electrophoresis in 1.2% agarose gel in TAE buffer and visualized in an UV transilluminator at a wavelength of 260 nm.

Implementation of polymerase chain reaction. The reaction mix ($25 \mu L$) for the synthesis of a 204-bp chromosomal fragment (dhp73.019) contained $1\times$ reaction buffer, 1.5 mM $MgCl_2$, 0.25 mM each dNTP, $0.2 \mu M$ primers chBA20F3 and chBA20B3, and $5 \mu L$ template. The reaction was performed in a Tertsik thermocycler (DNK-Tekhnologiya) according to the following program: initial denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 15 s, primer annealing at 54°C for 20 s, and elongation at 72°C for 20 s), and the final stage of amplification for 2 min at 72°C. PCR products were subjected to electrophoresis in 1.2% agarose gel in TAE buffer and visualized in a UV transilluminator at a wavelength of 260 nm.

Reproducibility. All experiments, including the cultivation of *B. anthracis* strains, DNA isolation, PCR, and LAMP, were repeated at least three times.

RESULTS AND DISCUSSION

Target selection and primer design. A large number of primers that target chromosomal loci of the pathogen DNA are currently available for PCR detection and identification of *B. anthracis*. Wide use of specific primers is usually followed by the discovery of new strains that lack the target sequences or contain single nucleotide polymorphisms that have a significant effect on the properties of the target sequence. Sometimes the target sequences are detected in the strains of closely related species, and this necessitates additional analysis for the identification of more specific loci. We combined bioinformatic analysis with subtractive hybridization data presented in [30] to select 28 species-specific DNA fragments of *B. anthracis*. Two of these sequences (dhp73.019 and dhp73.017) were selected as chromosomal targets, and two pairs of primers (F3-B3 and FIP-BIP) for LAMP amplification of DNA were designed for each sequence.

The *pagA* gene on the pXO1 plasmid and the *Cap*ABC gene on the pXO2 plasmid were selected as the plasmid targets, and two pairs of LAMP primers were designed for these targets as well.

Optimization of the loop isothermal amplification procedure. Bst or Bsm polymerases used in LAMP can elongate a new DNA strand and displace the previous one, in contrast to the enzymes used in conventional PCR [31, 32]. SD polymerase, a recently constructed mutant form of Taq polymerase, is also capable of strand displacement, but has a higher thermostability than Bst and Bsm polymerases, and, therefore, the initial high-temperature denaturation of DNA that increases reaction efficiency can be performed when this enzyme is used [33]. Bst and SD polymerases were used in the present study in order to identify the most suitable enzyme for LAMP. Bst polymerase was used at first. Regardless of the numerous changes of amplification parameters, such as the concentrations of Mg^{2+}, Bst polymerase, and dNTPs, primer ratio, the presence of betaine, and reaction temperature (in the range of $50-68°C$), there were no positive results (characteristic "ladder" patterns on the electrophoregram). PCR with the F3-B3 external primer pair and Taq polymerase yielded a fragment of the expected size. LAMP with Bst polymerase and this amplicon (instead of native DNA) as template yielded a characteristic ladder of bands (data not shown) on the electrophoregram. Replacement of Bst polymerase by a more thermostable SD polymerase for LAMP allowed for preheating of the reaction mixture at 92°C that provided for efficient amplification of both chromosomal and plasmid fragments of the native *B. anthracis* DNA. SD polymerase was used in the further experiments that included incubation of the reaction mix at 92°C for 2 min.

A number of parameters were varied in order to identify optimal conditions for LAMP. The primer ratio that yielded the best results was 10 pmol of external primers B3 and F3 and 40 pmol of internal primers FIP and BIP. The addition of 0.8 M betaine necessary
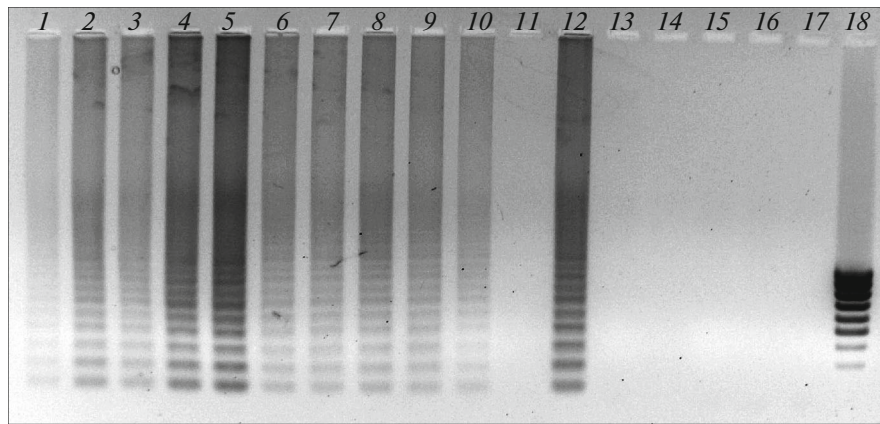
Fig. 1. Isothermal amplification (LAMP) of a fragment of *B. anthracis* chromosomal region dhp73.019 with the chBA20a primer set and SD polymerase. (*1*) Lysate of *B. anthracis* ΔAmes, (*2*) lysate of *B. anthracis* STI RIF-4, (*3*) lysate of *B. anthracis* 71/12, (*4*) DNA of *B. anthracis* 71/12, (*5*) DNA of *B. anthracis* STI-1, (*6*) DNA of *B. anthracis* 1, (*7*) DNA of *B. anthracis* 5, (*8*) DNA of *B. anthracis* Ch-7, (*9*) DNA of *B. anthracis* 81/1, (*10*) DNA of *B. anthracis* 770NP1R, (*11*) DNA of *B. cereus* 5832, (*12*) DNA of *B. anthracis* 15, (*13*) DNA of *B. cereus* 160, (*14*) DNA of *B. thuringiensis* G 7566, (*15*) lysate of *B. cereus* 10702, (*16*) lysate of *B. thuringiensis* 214, (*17*) negative control for PCR (water), and (*18*) molecular-weight markers (100−1000 bp).

for the stabilization of AT and GC pairs had a positive effect on amplification quality as well.

LAMP of chromosomal DNA. Detection of chromosomal DNA of the anthrax pathogen was performed with *B. anthracis* strains that varied with regard to the degree of pathogenicity and plasmid composition. Diplasmid strains (pXO1$^+$/pXO2$^+$), monoplasmid strains (pXO1$^-$/pXO2$^+$ and pXO1$^+$/pXO2$^-$), and plasmid-free strains (pXO1$^-$/pXO2$^-$) were used. Strains of closely related species of bacilli were used in the experiments as well (Table 1).

A fragment of *B. anthracis* chromosomal sequence dhp73.019 was amplified with the chBA20 set of four primers, and a fragment of the dhp73.017 chromosomal sequence was amplified with the chBA188 primer set. Both primer sets detected chromosomal fragments with both purified DNA and DNA from thermolysates of specific *B. anthracis* strain cultures as templates. Amplification with both primer sets was successfully used to differentiate between *B. anthracis* and other species of bacilli (*B. cereus* and *B. thuringiensis*, Fig. 1).

PCR amplification of chromosomal DNA fragments from *B. anthracis*. The results of isothermal amplification of chromosomal DNA fragments from bacilli were compared to the results of conventional PCR. Specific PCR with the external F3-B3 chBA20 primer pair and Taq polymerase yielded a fragment of the expected length (204 bp, Fig. 2).

This specific fragment was only formed in samples that contained DNA from the strains of the species *B. anthracis*, and all other samples either contained no amplicons or contained non-specific amplification products with a length that differed from the expected value.

As shown in Fig. 2, a positive signal was obtained with DNA samples of plasmid-free strains *B. anthracis*

STI RI F4, Lange1, and 513/1. Plasmid-free *B. anthracis* strains would not be detected if only plasmid-specific primers were used. Notably, primers suggested for chromosomal DNA in [25] yielded positive results with DNA from *B. mycoides* and *B. cereus* strains (F3502/73 and 421-4, respectively). Our experiments did not yield any positive results with strains that did not belong to the *B. anthracis* species, this being indicative of a high diagnostic value of primers that we designed for targets on *B. anthracis* chromosome.

Studies of strains 10 and 32 performed earlier in our laboratory addressed the character of growth of the microbes in liquid and solid nutrient media, virulence for mice, and sensitivity to three anthrax-specific bacteriophages. However, the results of these studies were insufficient for unambiguous assignment of these strains to a species. The present study did not yield positive amplification results with primers that targeted the chromosomal loci of the anthrax pathogen, this being in agreement with the published results of single-primer PCR and VNTR typing [34, 35] that did not support the assignment of strains 10 and 32 to the *B. anthracis* species.

Thus, the *B. anthracis* species can be distinguished from *B. cereus* and *B. thuringiensis* and, in particular, from the highly homologous strains of *B. cereus* Dakar and *B. cereus* var anthracoides 217 and 6691, by LAMP with primers that target chromosomal DNA fragments (chBA20-B3 and chBA20-F3). Therefore, these primers can be regarded as very promising components of LAMP-based test kits for the identification of the anthrax pathogen.

Isothermal amplification of plasmid DNA fragments. *Plasmid pXO1.* The results of comparative analysis of DNA samples of diplasmid, monoplasmid, and plasmid-free *B. anthracis* strains and saprophytic representatives of the genus *Bacillus* by loop isothermal
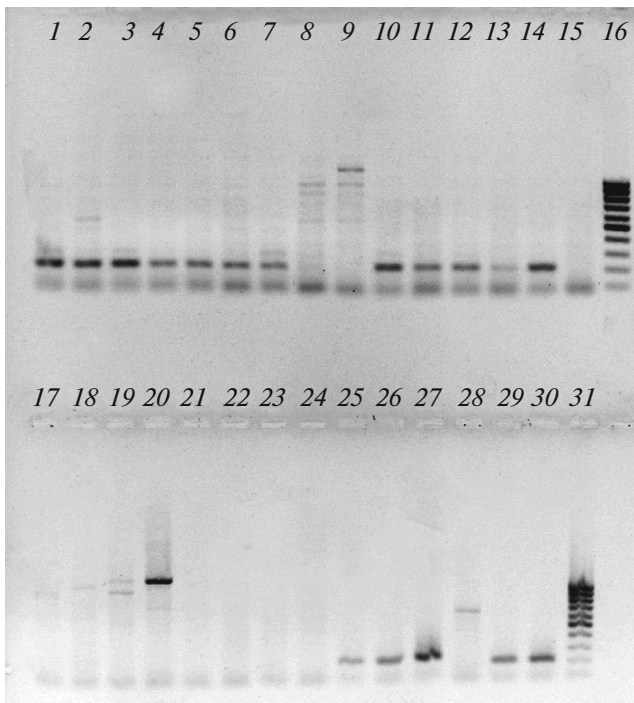
Fig. 2. PCR detection of a fragment of *B. anthracis* chromosomal region dhp73.019 with F3-B3 chBa20 primers. (*1*) DNA of *B. anthracis* Ch-7, (*2*) DNA of *B. anthracis* 81/1, (*3*) DNA of *B. anthracis* ΔAmes, (*4*) DNA of *B. anthracis* 770 NP1R, (*5*) DNA of *B. anthracis* 71/12, (*6*) DNA of *B. anthracis* STI-1, (*7*) DNA of *B. anthracis* STI RIF-4, (*8*) DNA of *B. cereus* var. *anthracoides* 217, (*9*) DNA of *B. cereus* 771, (*10*) DNA of *B. anthracis* Pasteur 2, (*11*) DNA of *B. anthracis* 55, (*12*) DNA of *B. anthracis* Ichtiman, (*13*) DNA of *B. anthracis* 34F2 Sterne, (*14*) DNA of *B. anthracis* Lange1, (*15*) negative control for PCR (water), (*16*) molecular-weight markers (100−1000 bp), (*17*) DNA of *Bacillus* spp., Nach, (*18*) DNA of *B. cereus* var. *anthracoides* 6691, (*19*) DNA of *B. cereus* Dakar, (*20*) DNA of *B. cereus* 5832, (*21*) DNA of *B. thuringiensis* 214, (*22*) DNA of *B. thuringiensis* G 7566, (*23*) DNA of *B. anthracis* 10, (*24*) DNA of *B. anthracis* 32, (*25*) DNA of *B. anthracis* 1, (*26*) DNA of *B. anthracis* 5, (*27*) DNA of *B. anthracis* 15, (*28*) DNA of *B. cereus* 160, (*29*) DNA of *B. anthracis* 513/1, (*30*) DNA of *B. anthracis* 13/39, and (*31*) molecular-weight markers (100−1000 bp).

amplification reaction with primers to DNA fragments localized on the plasmid pXO1 are shown in Fig. 3.

Fragments of the *pagA* gene from *B. anthracis* plasmid pXO1 were amplified using pagBA272 (Fig. 3) and pagBA997 (data not shown) primer sets. The target fragment of the *pagA* gene was amplified in all samples from strains that harbored the pXO1 plasmid, whereas no amplification occurred if DNA from strains that lacked the pXO1 plasmid was used. A weak signal of the product was observed in some experiments with the pagBA997 primer set and DNA from *B. cereus* var. *anthracoides* 217 as the template, this probably being indicative of a lower specificity of this primer set relatively to the pagBA272 primer set.
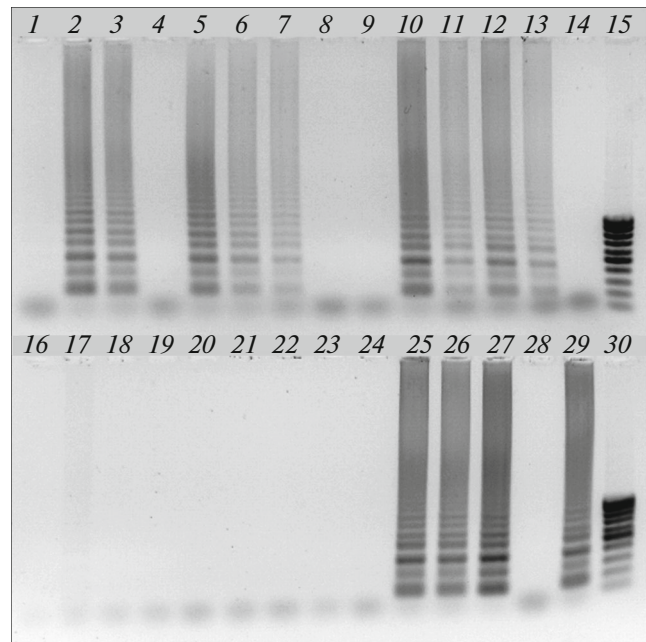

Fig. 3. Isothermal amplification (LAMP) of a fragment of *pagA* (pXO1) gene of *B. anthracis* with the pagBA272 primer set and SD polymerase. (*1*) DNA of *B. cereus* var. *anthracoides* 217, (*2*) DNA of *B. anthracis* Ch-7, (*3*) DNA of *B. anthracis* 81/1, (*4*) DNA of *B. anthracis* ΔAmes, (*5*) DNA of *B. anthracis* 770 NP1R, (*6*) DNA of *B. anthracis* 71/12, (*7*) DNA of *B. anthracis* STI-1, (*8*) DNA of *B. anthracis* STI RIF-4, (*9*) DNA of *B. cereus* 771, (*10*) DNA of *B. anthracis* Pasteur 2, (*11*) DNA of *B. anthracis* 55, (*12*) DNA of *B. anthracis* Ichtiman, (*13*) DNA of *B. anthracis* 34F2 Sterne, (*14*) negative control for PCR (water), (*15*) molecular-weight markers (100−1000 bp), (*16*) DNA of *B. anthracis* Lange1, (*17*) DNA of *Bacillus* spp., Nach, (*18*) DNA of *B. cereus* var. *anthracoides* 6691, (*19*) DNA of *B. cereus* Dakar, (*20*) DNA of *B. cereus* 5832, (*21*) DNA of *B. thuringiensis* 214, (*22*) DNA of *B. thuringiensis* G7566, (*23*) DNA of *B. anthracis* 10, (*24*) DNA of *B. anthracis* 32, (*25*) DNA of *B. anthracis* 1, (*26*) DNA of *B. anthracis* 5, (*27*) DNA of *B. anthracis* 15, (*28*) DNA of *B. anthracis* 513/1, (*29*) DNA of *B. anthracis* 13/39, and (*30*) molecular-weight markers (100−1000 bp).

Plasmid pXO2. Primer sets capBA194 and capBA31 were used to amplify fragments of the *Cap*ABC gene from the pXO2 plasmid of *B. anthracis* (data not shown). LAMP products appeared on the electrophoregram if DNA from strains that carried the pXO2 plasmid was used, in contrast to the results of amplification of DNA from strains that lacked the plasmid.

The results of amplification of DNA from the strains of *B. anthracis* and other representatives of the genus *Bacillus* with the primer set capBA194 that targeted the *Cap*ABC gene from the pXO2 plasmid are shown in Fig. 4. PCR with the capBA31 primer set yielded similar results.

Thus, both primer sets (capBA194 and capBA31) allowed for reproducible synthesis of the characteristic LAMP product when DNA from di- and monoplas-

mid strains that carried the pXO2 plasmid was analyzed.

Assessment of the sensitivity of chBA20 primers for LAMP and PCR detection of *B. anthracis*. The sensitivity of F3-B3 chBA20 primers for PCR detection of *B. anthracis* chromosome fragment dhp73.019 in DNA samples from *B. anthracis* STI-1 was sufficient for amplification of the target fragment from 6 ng, 600, 60, and 6 pg of total DNA. The sensitivity of LAMP with the chBA20 set of primers was similar (data not shown). Thus, the sensitivity of LAMP with primers for the detection of *B. anthracis* chromosomal region dhp73.019 was comparable to that of PCR.

The possibility of detection of unusual *B. anthracis* strains or other bacilli that cause "anthrax-like" diseases of humans and animals, as well as the possibility of detection of saprophyte isolates with a genomic sequence highly similar (up to 99.9% identity) to the "classical" strains of anthrax pathogen should be taken into account upon laboratory diagnosis and epidemiological investigation of infectious diseases with clinical symptoms similar to anthrax. The currently used identification methods based on DNA detection require expensive devices, well-equipped laboratories, and qualified personnel. The LAMP method is suitable for use in field studies and poorly equipped laboratories due to its simplicity and high speed of analysis. Therefore, the present study involved an attempt to develop a test for the identification of the anthrax pathogen and discrimination between this pathogen and closely related species. The results of detection and identification of *B. anthracis* strains in loop isothermal DNA amplification (LAMP) reaction with original primers, optimized conditions, and thermostable DNA polymerase are presented. Reproducible detection of targets specific for chromosomal and plasmid DNA of *B. anthracis* strains has been demonstrated. No cross reaction with DNA from strains of other species of the *B. cereus* group was observed.

The sensitivity of LAMP-based detection of the anthrax pathogen is of great importance, especially for field studies. The sensitivity of LAMP with primers that targeted a fragment of *B. anthracis* chromosome was the same as that of PCR. Importantly, the amount of LAMP products is higher than that of PCR products formed in a reaction mix with the same amount of template, and therefore visualization of the reaction products in the former case can be performed without specialized equipment. Research on the visualization of LAMP products without specialized equipment is currently in progress. The introduction of an additional pair of loop primers is planned in order to increase the sensitivity of isothermal amplification. This modification may allow for an increase in product yield and a decrease in the reaction time.

The optimized reaction of loop isothermal DNA amplification is intended for use in the development of test systems for clinical and field diagnostics of the
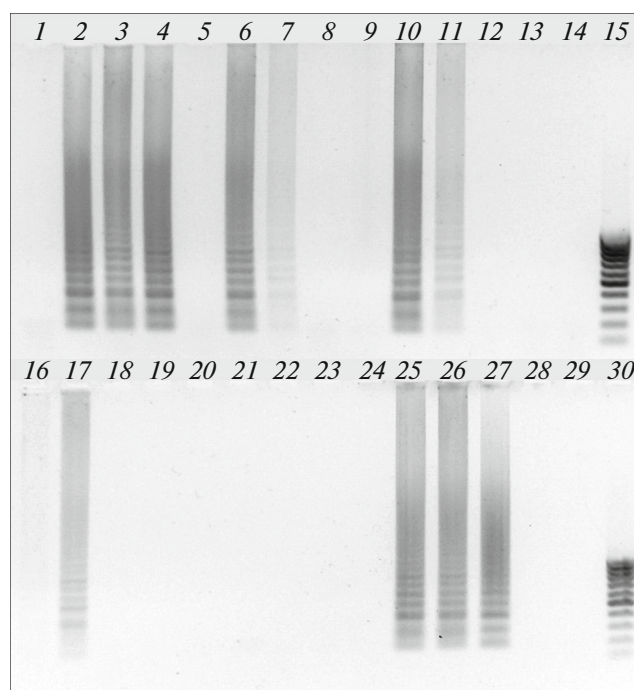

Fig. 4. Isothermal amplification of a fragment of the *Cap*ABC gene (plasmid XO2) of *B. anthracis* with the capBA194 primer set and SD polymerase. (*1*) DNA of *B. cereus* var. *anthracoides* 217, (*2*) DNA of *B. anthracis* Ch-7, (*3*) DNA of *B. anthracis* 81/1, (*4*) DNA of *B. anthracis* ΔAmes, (*5*) DNA of *B. anthracis* 770 NP1R, (*6*) DNA of *B. anthracis* 71/12, (*7*) lysate of *B. anthracis* 71/12, (*8*) DNA of *B. anthracis* STI RIF-4, (*9*) DNA of *B. cereus* 771, (*10*) DNA of *B. anthracis* Pasteur 2, (*11*) lysate of *B. anthracis* Pasteur 2, (*12*) DNA of *B. anthracis* Ichtiman, (*13*) DNA of *B. anthracis* 34F2 Sterne, (*14*) negative control for PCR (water), (*15*) molecular-weight markers (100−1000 bp), (*16*) DNA of *B. anthracis* Lange1, (*17*) lysate of *B. anthracis* 1, (*18*) DNA of *B. cereus* var. *anthracoides* 6691, (*19*) DNA of *B. cereus* Dakar, (*20*) DNA of *B. cereus* 5832, (*21*) DNA of *B. thuringiensis* 214, (*22*) DNA of *B. thuringiensis* G 7566, (*23*) DNA of *B. anthracis* 10, (*24*) DNA of *B. anthracis* 32, (*25*) DNA of *B. anthracis* 1, (*26*) DNA of *B. anthracis* 5, (*27*) DNA of *B. anthracis* 15, (*28*) DNA of *B. anthracis* 513/1, (*29*) DNA of *B. anthracis* 13/39, and (*30*) molecular-weight markers (100−1000 bp).

anthrax pathogen. These test systems will be easy to use and will not require sophisticated equipment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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