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PHENOTYPIC, BIOCHEMICAL AND MOLECULAR ANALYSIS OF *Bacillus anthracis* STRAINS ISOLATED DURING THE OUTBREAKS OF ANTHRAX IN THE RUSSIAN FEDERATION, 2014-2016

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Abstract

In 2014 to 2016, despite effective measures to prevent an introduction and transmission of Anthrax in the Russian Federation, there were seven outbreaks of Anthrax in Volgograd, Rostov, Belgorod, Saratov regions, the Republic of Tatarstan, and also six outbreaks in reindeer population in two districts of Yamal-Nenets Autonomous Okrug where 2657 reindeers died. In this article we present some results of comprehensive characterization of genetic, biological features and phylogenetic relationship of *Bacillus anthracis* strains isolated during the outbreaks in Volgograd region, Yamal-Nenets Autonomous Okrug and from the soils of burial in Chuvash Republic during last 3 years. Here, we differentiated 11 strains as followed from growth morphology, mobility, Gram stain procedure, capsule in vivo and in vitro formation, sporulation, proteolytic, hemolytic, lecithinase, phosphatase, glycolytic activity, protocatechuic acid production, Congo red sorption from the medium, phage sensitivity, toxicity in vitro, plasmid profile, sensitivity to antibiotics recommended for use in veterinary medicine, virulence for mice. MLVA-typing of the anthrax strains was performed for 20 VNTR loci. It was shown that the main phenotypic and diagnostic features of anthrax strains differed insignificantly and, in general, corresponded to those of a typical *B. anthracis* strain. The most significant phenotypic differences were found in asporogenous and avirulent strain *B. anthracis* № 6017 isolated in 2016 from a Lappish reindeer dog. The *B. anthracis* strains isolated during one outbreak were grouped into separate clusters, and within the cluster some strains had insignificant differences in 1-2 loci. The strains isolated from the soils of burials in the Republic of Chuvashia and from the Lappish reindeer dog during the Yamal outbreak formed separate clusters. *B. anthracis* strains showed high epizootic risk due to pathogenicity factors expressed in vitro. The tests identified the presence of capsula and toxins, high hemolytic and proteolytic activity, protocatechuic acid synthesis, and high virulence for laboratory mice (at 6-1000 spores). These results confirm the necessity of continuous monitoring and evaluation of epizootic caution of anthrax burials and case sites (frost fields), and specific preventive anti-anthrax measures.

Keywords: *Bacillus anthracis*, anthrax, strains, phenotypic properties, genotypic properties, virulence, MLVA

Single cases of anthrax in animals are annually registered in the Russian Federation, except for 2016, despite effective and quite wide measures to prevent this infection [1-4]. According to the information of analytic center of the Federal State Veterinary and Phytosanitary Surveillance (http://www.fsvps.ru/fsvps/iac/mes-

sages), seven anthrax foci were in 2014-2016 in Volgograd, Rostov, Belgorod, Saratov regions, Republic of Tatarstan, and six outbreaks were in reindeer populations at the territory of two regions of Yamal-Nenets Autonomous District (in the latter case, 2657 deer died).

Identification of new infectious strains during monitoring of animal and human diseases considering phenotype, biochemical and molecular-genetic characterization of isolates gives knowledge of spreading clones of the disease agent and their origin [5-8]. Biological properties characterize immunological identity of bacterial strains circulating in animals and vaccine strains, diagnostic features of bacterial pathogens, and the ways for emergence of resistance to medicines and biocides of various chemical classes among pathogens [9-12]. These are basic for epidemiological and epizootic passports reflecting the degree of epidemiological/epizootic danger of strains and must be used to improve effectiveness of specific and nonspecific prevention and eradication of animal and human infections [13, 14].

In present paper we have described properties and phylogenetic relations between the isolates of anthrax agents identified in the burial soil and in dead animals during disease outbreaks within Russia in 2014-2016, including reindeer epizooty which was the largest for the few past decades.

Purpose of this research is comprehensive characterization and certification of the anthrax strains isolated in the Russian Federation.

Techniques. Strains (11 *Bacillus anthracis* cultures) were isolated in 2014-2016 from bovine animals, Lappish reindeer dog, and reindeer, as well as from burial soils during the anthrax outbreaks in Volgograd Region and Yamal-Nenets Autonomous District, including burial soils in the Chuvash Republic. All strains are deposited in the State Collection of Microorganisms causing dangerous and extremely dangerous animal diseases, including zooanthroponoses and diseases not found in Russia (State Collection of Microorganisms, Federal Research Center of Virology and Microbiology (GKM-FICViM)).

Diagnostic traits were studied according to methodological guidelines MUK 4.2.2413-08 (Moscow, 2009). Phenotypes associated with pathogenicity (proteolytic, hemolytic, lecithinase, phosphatase, glycolytic activities, synthesis of protocatechuic acid, absorption of Congo Red dye from the medium) were identified according to recommendations [15].

Growth and differential media were medium for anthrax microbe isolation and culture (State Research Center of Applied Microbiology and Biotechnology, Russia); nutritious semi-liquid agar (BioCompas LLC, Russia); Hottinger agar based medium for determination of capsule and toxin formation on (RF Patent No 2204607); casein agar [16]; medium based on 10 % emulsion of chicken yolk in physiological solution; L-agar with 25 μg/ml Congo Red; two-layer blood agar (RF Patent No 2238316); Mueller Hinton Agar (HiMedia Laboratories Pvt. Ltd, India). Spores of anthrax cultures were produced on potato agar according to methodological guidelines MU 3.5.2435-09 (Moscow, 2009) and stored in 30 % glycerol.

Sensitivity of *B. anthracis* strains to antibacterial agents was assessed according to MUK 4.2.1890-04 (Moscow, 2004) using Mueller Hinton Agar and disks for veterinary laboratories (Scientific Research Center of Pharmacology, Saint Petersburg).

Capsule and toxin formation of *B. anthracis* strains in vitro was assessed by presence of mucous colonies and precipitation under 10 % CO_2 in air. For in vivo identification of capsule, 2 mice of 18-20 g in weight were intraperitoneally infected with 0.5 $cm^3$1-day broth culture of each strain. In case the mice did not

died within 10 days they were subjected to CO_2 euthanasia. To confirm capsule formation, imprint smears were made from mucous colonies and organs of dead mice. Preparations were fixed by alcohol:ester mixture (1:1) during 30 minutes and stained with methylene blue by Loeffler or Romanovsky-Giemsa according to guidelines for use of dyes. Preparations were examined under microscope at magnification of ×900. Rose color capsules around cells testified production of the capsular polypeptide. Test-system for Bacillus and allied species Microgen® Bacillus-ID (MID-66) (Microgen Bioproducts, United Kingdom) was used to identify biochemically the isolates.

LD_{100} and LD_{50} of the strains were assessed in mouse virulence tests using clinically healthy white outbreeds (18-20 g mice of both sexes). Animal feeding and keeping were subject to the accepted regulations. Animals were used according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986). For each isolate tested, 5 groups of 6 mice per each were subcutaneously injected with 0.5 cm^3 of sporous suspension (2×10^8, 4×10^7, 8×10^6, 1.6×10^6, and 3.2×10^5 spores/cm^3). Survival and death of mice were recorded during 10 days. LD_{50} values were calculated by Kerber formula modified by I.P. Ashmarin and A.A. Vorobyev [17]. Minimum number of spores causing 100 % death of mice was referred to as LD_{100}.

DNA-sorb-S.M variant 50 (Central Research Institute of Epidemiology, Moscow) was used for total (chromosome and plasmid) DNA extraction.

Test system AmpliSens® Bacillus anthracis-FRT (Central Research Institute of Epidemiology, Moscow) was used in PCR to identify genetic determinants of capsular polypeptide and toxin, the main pathogenies factors. Multilocus variable number tandem repeat analysis (MLVA) was carried out for 20 chromosomal and plasmid VNTR (variable number tandem repeat) loci with PCR primers [18] of a reagent kit for genetic typing anthrax strains by fragment analysis (OM-Anthrax-Genotype, Sintol LLC, Moscow). PCR and MLVA protocols were as per guidelines for test systems (an amplifier C1000 Touch Thermal Cycler with module for PCRq CFX96 Real-Time System, Bio-Rad, USA). At performance of MLVA [18], sequencing of each locus was done in an 8-capillary automated genetic analyzer NANOFOR 05 (Trial Plant of Science Instrument Engineering RAS, Chernogolovka).

UPGMA was used to plot MLVA data based dendrogram.

Results. Geographic origin of the studied isolates are given in Table 1.

1. Strains of *Bacillus anthracis* isolated in Russia 2014-2016

Strain	Inventory No.	Origin
		Volgograd Region
		(substracted in Volgograd Regional Veterinary Laboratory, 2014)
6246	370	Bovine spleen
3158/317-318	371	Soil
3184/410	372	Biomaterial (Dubtsovsky District)
		Chuvash Republic
		(substracted in Chuvash Republican Veterinary Laboratory, 2016)
5833	373	Burial soil
		Yamal-Nenets Autonomous District
		(substracted in All-Russia R&D Institute of Veterinary Virusology and Microbiology at Russian Academy of Agricultural Sciences, 2016)
5875	374	Reindeer ear
5885	375	Reindeer ear
5886	376	Reindeer ear
6017	377	Discharges from nose of Lappish reindeer dog
6019	378	Reindeer ear
6063	379	Reindeer ear
6064	380	Reindeer ear

2. Manifestation of phenotype, serological, and biological properties associated with pathogenicity in strains of anthrax agents *Bacillus anthracis* substracted at the Russian territory in 2014-2016

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
6246	T	R	–	+	+	+	+	+	+	–	+++	+	+++	α	+	±	+
3158/317-318	T	R	–	+	+	+	+	+	+	–	+++	+	+++	α	+	±	+
3184/410	T	R	–	+	+	+	+	+	+	–	+++	+	+++	α	+	±	+
5875	T	R	–	+	+	+	+	+	+	–	+++	–	+++	α	+	+	+
5885	T	R	–	+	+	+	–	+	+	–	+++	–	+++	α	+	+	+
5886	T	R	–	+	+	+	–	+	+	–	+++	–	+++	α	+	+	+
6019	T	R	–	+	+	+	+	+	+	–	+++	–	+++	α	+	+	+
6063	T	R	–	+	+	+	–	+	+	–	+++	–	+++	α	+	+	+
6064	T	R	–	+	+	+	–	+	+	–	+++	–	+++	α	+	+	+
6017	T	R	–	–	+	–	–	–	–	–	+++	+	+++	α	+	+	+
5833	T	R	–	+	+	+	+	+	+	–	+++	+	+++	α	+	+	+

N o t e. 1 — cell morphology, 2 — colony morphology, 3 — phosphatase activity, 4 — spore formation, 5 — presence of plasmid pXO1, 6 — presence of plasmid pxo2, 7 — toxin production in vitro, 8 — capsulation in vitro, 9 — capsulation in vivo, 10 — lecithinase activity, 11 — protease expression, 12 — synthesis of protocatechuic acid, 13 — hemolysine expression, 14 — hemolysis type, 15 — absorption of Congo Red, 16 — lysis by phage Fah-VNIIVViM, 17 — lysis by phage RD-ph-6. T — standard morphology, R — R type colonies (rough); «+/–» — manifestation of the trait is positive/negative, «±» — weak sensitivity to phage.

3. Biochemical activity in strains of anthrax agents *Bacillus anthracis* substracted at the Russian territory 2014-2016

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
6246	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
3158/317-318	–	+	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
3184/410	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
5875	–	–	–	-	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
5885	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
5886	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	+	+
6019	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
6063	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
6064	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
6017	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+
5833	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–	–	–	–	+

N o t e. 1, 2, 3, 4, 5, 6 — fermentation of arabinose, mannitol, ramnose, saccharose, adonitol, methyl-D-glucoside; 7 — production of indole, 8 — utilization of citrate; 9, 10, 11, 12, 13, 14 — fermentation of cellobiose, mannose, salicine, tregalose, galactose, inulin; 15 — production of ONPG (ortho-nitrophenyl-β-D-galactopyranoside), 16 — Voges-Proskauer test, 17, 18, 19, 20, 21, 22 — fermentation of inositol, raffinose, sorbitol, xilose, methyl-D-mannoside, melicitose; 23 — arginine decomposition, 24 — reduction of nitrates to nitrites; «+/–» — manifestation of the trait is positive/negative.

At studying of the main identification traits of *B. anthracis* strains, it was established that working collection was represented by 10 standard virulent cultures and one strain atypical for capsule and spore formation. Comprehensive assessment of strain properties shown insignificant differences in phenotype (growth morphology in liquid and on solid nutritious mediums, motility, Gram staining, capsulation in vivo and in vitro, spore formation), biochemical activity (proteolytic, hemolytic, lecithinase, phosphatase, glycolytic activities, synthesis of protocatechuic acid, Congo Red absorption), antigen properties (Ascoli's thero precipitation test), sensitivity to anthrax bacteriophages Fah-VNIIVViM and RD-ph-6, toxin production in vitro, plasmid profile (PCR detection of fragments of genes for capsule and toxin formation), sensitivity to antibiotics recommended for veterinary use (Tables 2, 3). Except for strain No 6017 from Lappish reindeer dog, all cultures have highly expressed hemolysine and proteases, produce capsule polypeptide in vitro (mucous colonies of S, M or SM type on medium for capsulation and toxin production) and in vivo (capsulated rods in cytological preparations from organs of died mice), cause α-type hemolysis of erythrocytes, and absorb Congo Red. Distinctive property of *B. anthracis* isolates from Yamal-Nenets Autonomous District is lack or weak production of protocate-

chuic acid. Strains were visually divided into producing toxin in vitro (No 6246, 3158/317-318, 3184/410, 5833, 5875, 6019) and non-producing (No 5885, 5886, 6017, 6063, 6064). Studied cultures also differed in sensitivity to anthrax phages: all were lysed by phage RD-ph-6, however strains No 6246, 3158/317-318 and 3184/410 were poorly sensitive to phage Fah-VNIIVViM.

Strains were divided into four groups (Table 4) subject to classification (19) for LD_{50} in outbreed white mice which is deemed the most objective value.

4. Virulence of *Bacillus anthracis* strains isolated in Russia during 2014-2016 in outbreed white mice

Strain	Indicator, spores per animal		Virulence assessment
	LD_{100}	LD_{50}	
6246	5.4×10^2	8	Highly virulent
3158/317-318	6	Not titered	Highly virulent
3184/410	1.2×10^2	5	Highly virulent
5833	30	6	Highly virulent
5875	20	5	Highly virulent
5885	1.1×10^3	7	Highly virulent
5886	1.7×10^2	12	Virulent
6017	Not titered	Not titered	Avirulent
6019	4.7×10^2	19	Moderately virulent
6063	150	10	Virulent
6064	60	21	Moderately virulent

Six of the studied strains are highly virulent, two are virulent, and two are moderately virulent. *B. anthracis* isolated from Lappish reindeer dog (No 6017) do not cause death in outbreed white mice and is referred to as avirulent. LD_{50} for the studied strains was of 5 to 37 spores per animal, LD_{100} of 6 to 2000 spores per animal. Determination of LD_{50} in strain No 3158/317-318 was impossible since total value of LD_{100} was only 6 spores per animal.

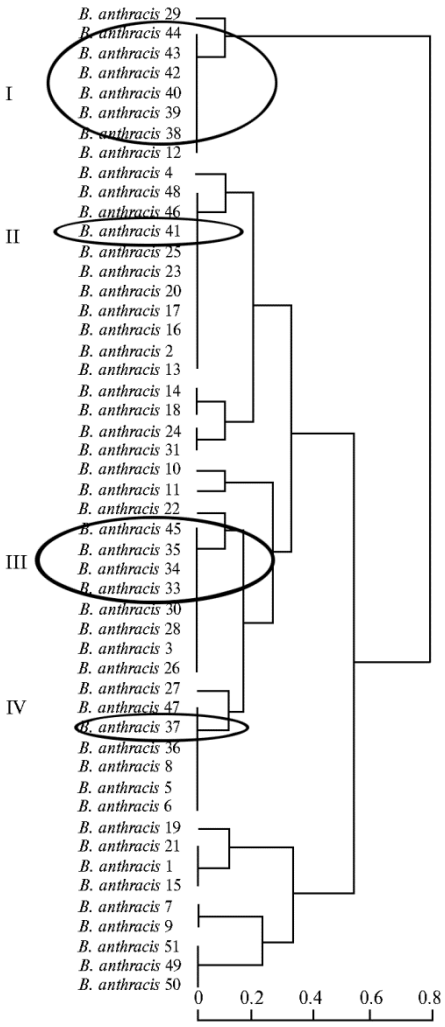
Nine of 11 studied strains are naturally resistant to polymyxin B, sensitive and highly sensitive to levomycetine, kanamycin, penicillin, tylosin, strepto-mycin, neomycin, tetracycline, ampicillin and enrofloxacin. Strain No 6246 is resistant to kanamycin and streptomycin (Table 5).

5. Antibiotic sensitivity of *Bacillus anthracis* strains isolated in Russia during 2014-2016

Strain	1	2	3	4	5	6	7	8	9	10
6246	S	S	MS	S	MS	MS	R	R	MS	R
3158/317-318	S	S	S	S	S	S	S	MS	S	R
3184/410	S	S	S	S	S	S	S	MS	S	R
5875	S	S	S	S	S	S	S	S	S	R
5885	S	S	S	S	S	MS	S	MS	S	R
5886	S	S	S	MS	MS	R	MS	MS	S	R
6019	S	S	R	S	MS	MS	MS	MS	S	R
6063	S	S	S	S	S	MS	S	MS	S	R
6064	S	MS	S	S	MS	MS	S	MS	S	R
6017	S	S	S	S	S	MS	S	S	S	R
5833	S	S	S	S	S	MS	S	S	S	R

Note. 1 — ampicillin, 2 — penicillin, 3 — neomycin, 4 — enrofloxacin, 5 — tylosin, 6 — levomycetine, 7 — streptomycin, 8 — kanamycin, 9 —tetracycline, 10 — polymixin B; R (resistance) — poorly sensitive; MS (medium sensitive) — medium sensitive; S (sensitive) — sensitive.

B. anthracis is one of the most genetically homogenous pathogens that challenges identification. Multilocus variable number tandem repeat analysis was used to tackle the problem. MVLA ensures PCR identification of bacterial DNA areas which are shortened or elongated during erroneously copying caused by slipping replication fork [20]. MLVA typing was performed for 20 VNTR loci at multiplex DNA amplification with estimation of lengths of fluorescently labeled products for each VNTR locus. Number of repeats in each locus was used to calculate the distances between strains (Fig.).



Dendrogram of phylogenetic relations of the studied *Bacillus anthracis* isolates (MLVA, UPGMA method)**:** cluster I — strains denoted as 38-40 are No 5875, 5885, 5886), 42-44 are No 6019, 6063, 6064); cluster II — strain denoted as 41 is No 6017); cluster III — strains denoted as 33-35 (are No 6264, 3158/317-318, 3184/410); cluster IV — strain denoted as 37 is No 5833 (State Collection of Microorganisms, Federal Research Center of Veterinary and Microbiology; see description of strains in Table 1).

MLVA data divide the strains into four clusters. Strains isolated from reindeer during outbreak of anthrax in Yamal-Nenets Autonomous District in 2016 form cluster I, one strain (No 6017, isolated from Lappish reindeer dog, Yamal-Nenets Autonomous District, 2016) is in cluster II, strains from Volgograd Region (2014) form cluster III, and cluster IV includes *B. anthracis* isolates from burial soils in Chuvash Republic (see Fig., Table 6).

This information confirms previously established facts that strains isolated from reindeer and humans in 2016 in Yamal-Nenets Autonomous District have one and the same MLVA genotype [21], and that different anthrax strains circulating in certain territories have geographically affiliated genotypes [22-25].

Significant differences are found in strains from clusters I and III by 1-2 loci inside cluster that may evidence on their subculture in sensitive animals.

6. MLVA-based genotyping of *Bacillus anthracis* strains isolated in Russia during 2014-2016 for 20 VNTR loci

Strain	1	2	3	4	5	6	7[a]	8	9	10	11	12[b]	13[b]	14	15	16[b]	17	18	19	20
Cluster III																				
6246	30	31	14	9	45	13	8[B]	20	13	57	78	4	20	5	20	14[B]	4	4	14	17
3158/317-318	30	31	14	9	45	13	10	20	13	57	78	4	20	5	20	12	4	4	14	17
3184/410	30	31	14	9	45	13	10	20	13	57	78	4	20	5	20	12	4	5	14	17
Cluster I																				
5875	27	27	15	10	45	14	9	16	14	53	17	3	21	3	23	9	3	5	14	15
5885	27	27	15	10	45	14	9	16	14	53	17	3	21	3	23	9	3	5	14	15
5886	27	27	15	10	45	14	9	16	14	53	17	3	21	3	23	9	3	5	14	15
6019	27	27	15	10	45	14	9	16	14	53	17	3	21	3	23	9	3	5	14	15
6063	27	27	15	10	45	14	9	16	14	53	17	3	21	3	23	9	3	5	14	15
6064	27	27	15	10	45	14	8[B]	16	14	53	17	3	21	3	23	9	3	5	14	15
Cluster II																				
6017	24	30	16	12	45	13	8	20	13	57	75	–	–	4	20	–	4	4	14	16
Cluster IV																				
5833	30	30	14	9	45	13	10	20	13	57	75	4	20	4	20	13	4	4	14	16

Note. VNTR — variable-number tandem-repeat; number of repeats in locus are indicated (from 1 to 45). Loci: 1 — Geb-Bams3, 2 — Geb-Bams13, 3 — Geb-Bams22, 4 — Geb-Bams23, 5 — Geb-Bams15, 6 — VNTR32, 7 — pXO1 aat, 8 — vrrC2, 9 — Geb-Bams1, 10 — vrrC1, 11 — Geb-Bams30, 12 — VNTR17, 13 — VNTR16, 14 — vrrA, 15 — vrrB1, 16 — CL33, 17 — VNTR23, 18 — VNTR35, 19 — vrrB2, 20 — CL12; [a] — locus located on plasmid pX01 *B. anthracis*, [b] — polymorphic loci located on plasmid pX02 *B. anthracis*; dashes mean that locus in not found.

Discovery in one anthrax outbreak (Yamal-Nenets Autonomous District, 2016) of the strains relating to two different clusters (I and II) allows assumption on presence of at least two sources of animal infection. However, this hypothesis requires further confirmation.

Thus, by main phenotypic properties and diagnostic features, anthrax strains isolated during 2014-2016 in three Russian Federation districts do not practically differ from the typical strain. Our findings indicate that these isolates belong to four MLVA20 genotypes having different geographic affiliation. The exception is *Bacillus anthracis* strain No 6017 (isolated from Lappish reindeer dog) on the position between strains from Volgograd Region and Chuvash Republic. The studied isolates possess high epizootic danger which is confirmed by the presence of pathogenic factors in vitro and high virulence in laboratory animals. This necessitates microbiological monitoring of anthrax burials and places of ani-mal death (together with improved preventive measures).

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