A.N.Kulichenko[1], E.I.Eremenko[1], A.G.Ryazanova[1], L.Yu.Aksenova[1], D.A.Kovalev[1], S.V.Pisarenko[1], N.G.Varfolomeeva[1], A.M.Zhirov[1], A.S.Volynkina[1], N.P.Buravtseva[1], T.M.Golovinskaya[1], E.A.Koteneva[1], O.I.Tsygankova[1], I.A.Dyatlov[2], V.S.Timofeev[2], A.G.Bogun[2], I.V.Bakhteeva[2], A.A.Kislichkina[2], R.I.Mironova[2], G.M.Titareva[2], Yu.P.Skryabin[2], Yu.O.Selyaninov[3], I.Yu.Egorova[3], D.V.Kolbasov[3]

Biological Properties and Molecular-Genetic Characteristics of *Bacillus anthracis* Strains, Isolated during the Outbreak of Anthrax in the Yamalo-Nenets Autonomous District in 2016

[1]Stavropol Research Anti-Plague Institute, Stavropol, Russian Federation; [2]State Research Center of Applied Microbiology and Biotechnology, Obolensk, Russian Federation; [3]National Research Institute of Veterinary Virology and Microbiology of the Russian Academy of Agricultural Sciences, Volginsky, Russian Federation

Objective of the study was to identify phenotypic properties and genetic peculiarities of *Bacillus anthracis* strains, isolated during the outbreak of anthrax in the territory of Yamal in 2016. **Materials and methods**. Investigated were the strains of anthrax agent, applying basic and subsequent identification tests and canSNP-, MLVA-genotyping methods and whole genome sequencing. **Results and conclusions**. The results showed the identity of the phenotypic properties, canSNP- and MLVA25-genotypes, and profiles of whole genome-sequencing, regardless of the source of the strains isolation. Confirmed was a common source of human infection. Defined were phylogenetic interrelations of the tested strains and their position in global *B. anthracis* population. For the first time ever explored was variability of the gene pattern, associated with pathogenicity, and demonstrated – the efficiency of the proposed algorithm for genetic typing.

Key words: anthrax, *Bacillus anthracis* strains, phenotypic properties, genotyping.

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Corresponding author: Alexander N. Kulichenko, e-mail: snipchi@mail.stv.ru.

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2016 was marked by the largest epizootic of anthrax among reindeer in the Yamalo-Nenets Autonomous District (YNAO), which caused the disease of 36 people with one fatal outcome. During the epidemiological investigation, strains of the anthrax pathogen were isolated in samples from sick people and deer corpses. The study of the strains was aimed at determining their biological properties, identifying genetic features, the similarity of their genotype with known variants to substantiate the epidemiological data on the source of infection of people, to get an idea of the genetic relationships of the strains, determine their place in the global population of *Bacillus anthracis*.

Materials and methods

Investigated were 10 strains of the anthrax microbe, isolated in 2016 in the YNAO. For comparison of genotypes, 10 strains from the collection of pathogenic microorganisms of the Stavropol Research Anti-Plague Institute were used, as well as literature data on the genotype of *B. anthracis* Ames Ancestor strain (Table 1).

Phenotypic properties of the strains were evaluated in accordance with the identification scheme of the anthrax microbe, given in the guidelines MUK 4.2.2413-08 "Laboratory diagnosis and detection of anthrax pathogen".

Molecular genetic typing was performed using different resolution methods. Analysis of "canonical" single nucleotide polymorphisms (canSNP typing) was performed according to the scheme [1], using allele-specific PCR amplification with LNA-modified probes and registration of the results in real-time format using a modified Rotor-Gene Q amplifier (QIAGEN, Germany). MLVA typing was performed on 25 VNTR loci with PCR primers described by F. Lista *et al.* [2], by sequencing each of the loci in an automated ABI 3500 Genetic Analyzer DNA analyzer (Applied Biosystems, USA). Whole genome sequenc-

ing (WGS) was performed using an Ion Torrent PGM sequencer, Ion 316 Chips Kit V2 chips (Life Technologies, USA) and Ion Xpress TM Plus Fragment Library Kit (Life Technologies, USA) according to the manufacturer's protocol. The analysis of the WGS data was performed using Wombac 2.0 programs (VBC (Monash University, Australia), SplitsTree 4 (version 4.14.4) (Universität Tübingen, Germany), CLC Sequence Viewer Version 7.0 (QIAGEN, Germany, CLCbio, Aarhus A/S).

Analysis of structural and regulatory genes, pathogenicity factors (*pag*A, *lef, cya, cap*A, *cap*B, *cap*C, *cap*D, *cap*E, *abr*B, *acp*A, *acp*B, *atx*A, *pag*R), according to a rough assembly of full-genome sequencing of strains, were performed *in silico*, comparing with sequences of genes *B. anthracis* Ames Ancestor strain using the NCBI BLASTn resource (www.blast.ncbi.nlm.nih.gov).

SNR analysis for several genetic regions was performed using the primers described by L.J. Kenefic *et al.* [3].

Phylogenetic analysis was performed in the FYLOViZ 2.0 program (www.phyloviz.net) using the UPGMA and Neighbor-Joining algorithms.

Results and discussion

All strains isolated in the YNAO had biological properties characteristic of typical *B. anthracis* strains: typical morphology when Gram-stained, formed colonies in R-form on dense nutrient media and bottom growth with preservation of the transparency of the medium in the nutrient broth, had the ability to sporulation. The strains did not display phosphatase, lecithinase, hemolytic activity on blood agar, they showed hemolytic activity on special media, produced proteolytic enzymes and protocatechic acid, had the ability to toxin formation *in vitro*, capsule formation *in vitro* and *in vivo*, gave positive Ascoli reaction. PCR with DNA

Table 1

The origin and canSNP-genotypes of *B. anthracis* strains

Strain *B. anthracis*	Source of appearance	Place of appearance	Year of appearance	Loci canSNP													can SNP genotype
				A.Br.001	A.Br.002	A.Br.003	A.Br.004	A.Br.006	A.Br.007	A.Br.008	A.Br.009	B.Br.001	B.Br.002	B.Br.003	B.Br.004	A.Br.001	
11	Ill person	YNAO, Salehard	2016	T	G	A	T	C	T	T	A	T	T	A	T	A	B.Br.001/002
12	Ill person	YNAO, Salehard	2016	T	G	A	T	C	T	T	A	T	T	A	T	A	B.Br.001/002
23	Ill person	YNAO, Salehard	2016	T	G	A	T	C	T	T	A	T	T	A	T	A	B.Br.001/002
24	Corpse of deer	YNAO, territory of the lake Picieto	2016	T	G	A	T	C	T	T	A	T	T	A	T	A	B.Br.001/002
25	Corpse of deer	YNAO, territory of the lake Picieto	2016	T	G	A	T	C	T	T	A	T	T	A	T	A	B.Br.001/002
26	Corpse of deer	YNAO, territory of the lake Picieto	2016	T	G	A	T	C	T	T	A	T	T	A	T	A	B.Br.001/002
27	Corpse of deer	Yamal district, Novoportovskaya tundra	2016	T	G	A	T	C	T	T	A	T	T	A	T	A	B.Br.001/002
28	Corpse of deer	YNAO, territory of the lake Picieto	2016	T	G	A	T	C	T	T	A	T	T	A	T	A	B.Br.001/002
29	Ash residue from the place of burning deer	YNAO, territory of the river Evyaha	2016	T	G	A	T	C	T	T	A	T	T	A	T	A	B.Br.001/002
5876	Corpse of deer	YNAO, territory of the lake Picieto	2016	T	G	A	T	C	T	T	A	T	T	A	T	A	B.Br.001/002
1051/35	Corpse of horse	Ufa	1935	T	G	A	T	C	T	T	A	T	T	A	T	A	B.Br.001/002
14/41	The contents of the ulcer of patient	Dagestan	1963	T	G	A	T	C	T	T	A	T	T	A	T	A	B.Br.001/002
1284	Ravioli "Osobye"	Omsk	2010	T	G	A	T	C	T	T	A	T	T	A	T	A	B.Br.001/002
140P	Soil of burial ground	Tver Region	1979	T	G	A	T	C	T	T	A	T	T	A	T	A	B.Br.001/002
81/1	The contents of the carbuncle of patient	Stavropol Territory	1969	T	G	A	T	A	T	G	A	T	G	G	T	A	A.Br.008/009
1266	Soil of the monastery	Stavropol Territory	2006	T	G	A	T	A	T	G	A	T	G	G	T	A	A.Br.008/009
1269	Spinal liquid of the patient	RNO-A	2007	T	G	A	T	A	T	G	A	T	G	G	T	A	A.Br.008/009
1(CO)	Material from cattle	RNO-A	1968	T	G	A	T	A	T	G	A	T	G	G	T	A	A.Br.008/009
1307	Patient ulcer scab	Stavropol Territory	2013	T	G	A	T	A	T	G	A	T	G	G	T	A	A.Br.008/009
1322	Meat of sheep	Stavropol Territory	2013	T	G	A	T	A	T	G	A	T	G	G	T	A	A.Br.008/009
I-271	Soil from the place of death of livestock	Yakut Autonomous Soviet Socialist Republic	1980	T	A	G	C	A	T	T	A	T	G	G	T	A	A.Br 001/002
Ames Ancestor	Corpse of cow	USA, Texas	1981	C	A	G	C	A	T	T	A	T	G	G	T	A	A.Br.Ames

strains was positive with primers for the *pag, cya, cap*A genes and the *prophage_03* chromosomal region. The strains are sensitive to the anthrax bacteriophages Fah-VNIIVV and R/D-ph-6, Gamma A26, a wide range of antibacterial drugs of the penicillin, tetracycline groups, fluoroquinolones, rifampin, aminoglycosides, levomycetin, and are resistant to polimixin. The value of LD50 for white outbred mice was 5÷23 spores, for guinea pigs – 237÷830 (subcutaneous injection), which indicates a high virulence of the strains.

SNP typing showed that all strains isolated in 2016 in the YNAO, regardless of the source and location of the isolation, have the same canSNP genotype B.Br.001/002. A similar genotype is characteristic of strains of *B. anthracis* 1284, *B. anthracis* 1051/35, *B. anthracis* 14/41 and *B. anthracis* 140P. The strains *B. anthracis* 81/1, *B. anthracis* 1266, *B. anthracis* 1307 and *B. anthracis* 1322, had the genotype A.Br.008/009, and the *B. anthracis* Ames Ancestor strain – A.Br.Ames (Table 1).

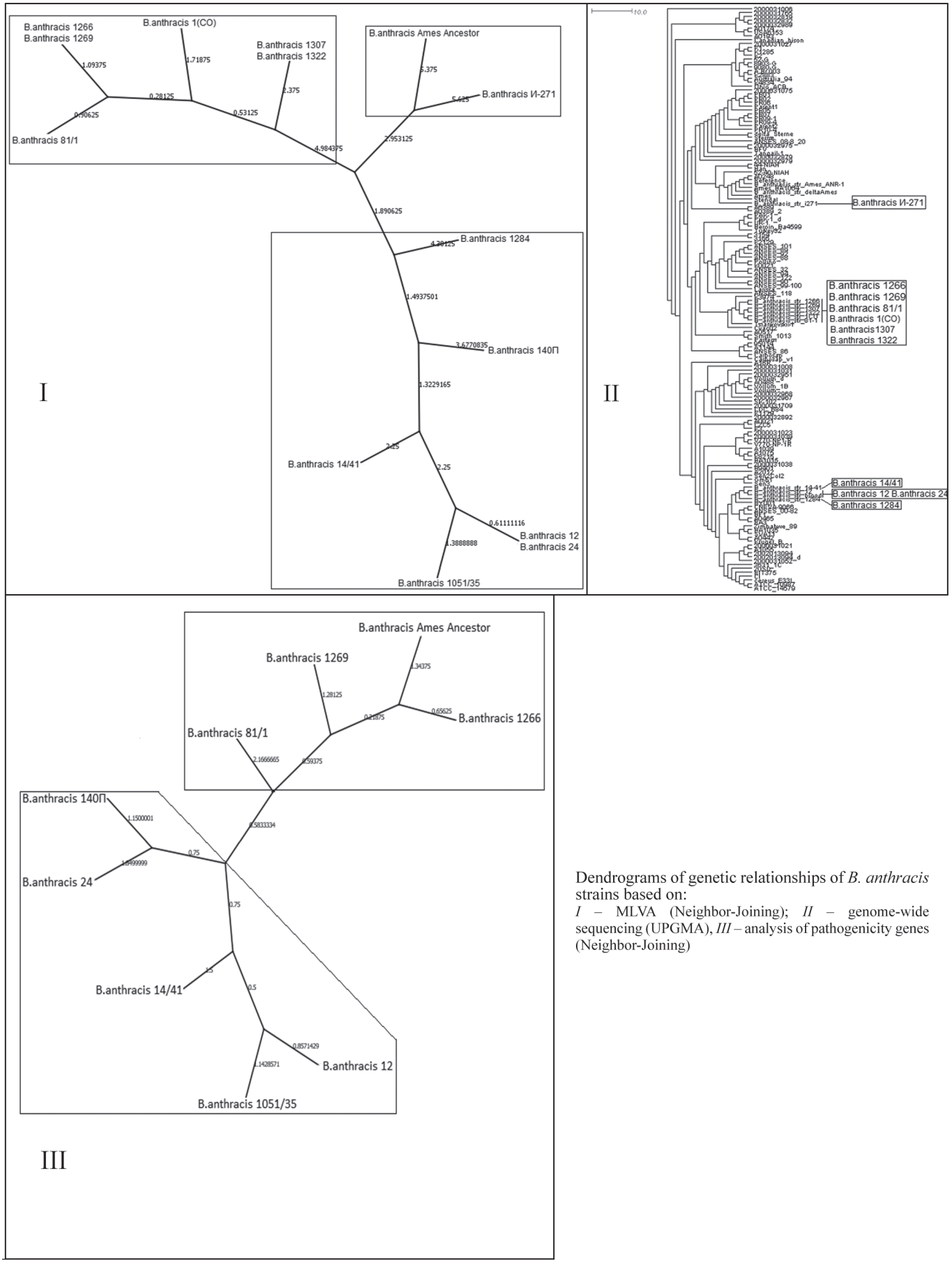
These data became the first evidence of the identity of the strains from the outbreak in the YNAO to a single source and the proximity of their genotype to the genotypes of the strains *B. anthracis* 1051/35, *B. anthracis* 1284, *B. anthracis* 14/41 and *B. anthracis* 140P.

All strains isolated in 2016 in the YNAO have the same MLVA genotype (Table 2). This confirmed the preliminary conclusion on the basis of SNP-genotyping about their common origin, association of human disease with anthrax epizootics in the YNAO. On dendrograms based on MLVA results, the separation of strains into three clusters is clearly visible (figure, II). The first cluster includes all Yamal strains, as well as strains 1051/35, 1284, 14/41 and 140P; the second, strains 81/1, 1 (CO), 1266, 1269, 1307 and 1322; the third is strains I-271 and Ames Ancestor. A feature of the Yamal strains, as well as other strains from the first cluster, is the lack of amplification with primers to the Bams34 locus. The genotype of strain 1051/35, which differed only in two loci, and then strain 14/41 (differences in five loci) was the closest to the strains from Yamal. The uniqueness of the he MLVA25 genotype of the Yamal strains was established through a comparative analysis of the genotypes of 1713 isolates represented in the database from the MLVAbank for Bacterial Genotyping resource (http://mlva.upsud.fr/mlvav4/genotyping). The identity of the genotypes of the Yamal strains isolated in three distant foci (Lake Petieso area, Novoportovskaya tundra, Evyaha river area) may evidence of past epizootic spills caused by one type of *B. anthracis*. Due to the identity of the genotypes of all the strains from the outbreak, *B. anthracis* 12 (isolated from humans) and *B. anthracis* 24 (isolated from deer) were selected for further

Table 2

MLVA25- and canSNP-genotypes of *B. anthracis* strains

Strain, No	vrrA	vrrB1	vrrB2	vrrC1	vrrC2	CG3	pX-O1aat	pXO2at	Bams01	Bams03	Bams05	Bams13	Bams15	Bams21	Bams22	Bams23	Bams24	Bams25	Bams28	Bams30	Bams31	Bams34	Bams44	Bams51	Bams53
11, 12, 23, 24, 25, 26, 27, 28, 29, 5876	9	19	7	53	17	2	9	7	14	27	7	27	45	10	15	10	11	13	12	17	85	-1	8	6	6
1051/35	9	19	7	53	17	2	7	7	14	27	7	27	45	10	15	10	11	27	12	17	85	-1	8	6	6
14/41	9	19	7	53	17	2	8	8	14	27	7	27	45	10	15	10	11	13	13	17	84	-1	8	8	6
1284	9	19	9	53	17	2	9	8	14	26	7	7	45	10	15	9	11	13	14	17	84	-1	8	6	8
140P	9	19	7	53	17	2	8	8	13	27	7	27	24	10	17	10	11	13	12	17	30	-1	8	9	8
81/1	10	16	7	57	21	1	11	8	13	27	7	30	45	10	15	9	11	13	14	76	65	9	8	9	8
1266	11	16	7	57	21	1	11	8	13	30	7	30	45	10	15	9	11	13	14	76	65	9	8	9	8
1269	11	16	7	57	21	1	11	8	13	30	7	30	45	10	15	9	11	13	14	76	65	9	8	9	8
1(CO)	10	16	7	57	21	1	11	8	13	30	7	33	45	10	15	9	11	13	14	76	40	9	8	9	8
1307	10	16	7	57	21	1	11	8	14	30	7	30	45	10	15	10	11	13	14	76	65	9	8	9	6
1322	10	16	7	57	21	1	11	8	14	30	7	30	45	10	15	10	11	13	14	76	65	9	8	9	6
I-271	10	16	7	53	17	2	7	11	16	28	7	5	45	10	16	11	13	7,3	57	64	8	11	8	9	8
Ames Ancestor	10	16	6	53	17	2	7	10	16	26	5	70	24	10	16	11	11	13	14	57	64	11	8	9	8



Dendrograms of genetic relationships of *B. anthracis* strains based on:
I – MLVA (Neighbor-Joining); *II* – genome-wide sequencing (UPGMA), *III* – analysis of pathogenicity genes (Neighbor-Joining)

study, as well as comparison strains *B. anthracis* 1284, 14/41, 140P, 81/1, 1(CO), 1266, 1269, 1051/35, 1307, 1322 and Ames Ancestor.

The study of genome-wide nucleotide se-

quences of 149 strains of *B. anthracis* and one strain of *Bacillus cereus* provided an insight into the genetic relationships of the strains and their place in the global population of *B. anthracis*. From the dendrogram, built on the results of the analysis, it is clear that the strains *B. anthracis* 12 and 24, isolated in the YNAO, are grouped together with the strains *B. anthracis* 14/41, *B. anthracis* 1284, and also with the strain *B. anthracis* HYU01 (isolated in Korea in 2009) (figure, II). Comparison *B. anthracis* strains 1(CO), 81/1, 1266, 1269, 1307 and 1322 are grouped separately. The strain *B. anthracis* I-237 is a separate branch associated with the Ames group. This pattern of distribution of strains corresponds to the data of canSNP genotyping and MLVA (Figure, I, II).

The study of the genetic variability of 9 strains under study (figure, III) was carried out on the basis of the data of the draft assembly, provided that each of the genes was located within one of the assembly contigs. Based on this criterion, the *pag*A gene in *B. anthracis* 12 and *B. anthracis* 24 strains and the *lef, acp*B and *ath* genes in *B. anthracis* 1284 strain were excluded from the study. The *B. anthracis* 12 and 24 strains differed from the reference strain in five genes and an insert of nine bases in *acp*A. The latter is also found in strains of *B. anthracis* 140P and 141/41, in six strains *B. anthracis* from the NCBI database, one of which (*B. anthracis* HYU01, Korea), according to WGS, is close to *B. anthracis* 12 and 24. Changes in the *acpB* gene in *B. anthracis* strains 12 and 24 distinguish them from all the others strains. Among themselves, these strains differed in genes *cya, acpB* and *atxA*. Taken together, the differences in the studied genes allowed identifying from 4 to 7 types of the *lef, cya, acp*A, *cap*A and *acp*B genes and three types of the *atxA* genes. In the *pag*R, *cap*B, *cap*C, *cap*D, and *cap*E genes, the variability was not observed.

Phylogenetic analysis shows that the studied strains are divided into 9 individual genotypes, which constitute two groups (Figure III). According to these data, the strain *B. anthracis* 12 is closest to the strain *B. anthracis* 1051/35, and the strain *B. anthracis* 24 is close to the strain 140P. For the first time, the applied sequence analysis of pathogenicity genes allowed us to obtain a separation of the genotypes of *B. anthracis* strains 12 and 24, which is unattainable with canSNP analysis and MLVA.

A study of strains of *B. anthracis* 12 and 24, isolated from human and deer corpses, respectively, showed that there are differences in the number of single repeats of adenine nucleotides in three CIS loci (CL10, CL12 and CL35): in *B. anthracis* 12 – 20, 13 and 12 repeats, respectively, in *B. anthracis* 24 – 18, 12, 11, and in the Ames Ancestor strain – 16, 15, and 15.

These differences between the two Yamal strains could be the result of repeated transmissions of infections between deers and transmission to humans during one outbreak, since the mutation rate in SNR loci is the highest and sufficient for the emergence of new genetic variants.

The study showed that all *B. anthracis* strains isolated during the anthrax outbreak in the YNAO in 2016 have the same typical morphological, biochemical, genetic properties and high virulence. The strains of *B. anthracis*, isolated from deer and sick people, have the same canSNP- and MLVA25 genotypes, the identical profile of genome-wide sequencing, which confirms the infection of people during various types of contact with sick animals. The coincidence of their genotypes can be explained by the circulation of one strain of the anthrax pathogen in the YNAO now and during spilled epizootics in the past. The closest to them is the strain *B. anthracis* 1051/35, isolated in 1935 in Ufa. The intraspecific variability of nucleotide sequences of genes associated with the pathogenicity of *B. anthracis* was detected. The strains of *B. anthracis* 12 (from a sick person) and *B. anthracis* 24 (from deer) have the type of *acp*B gene, which distinguishes them from other strains of *B. anthracis*, and the type of *cya, acp*B and *atx*A genes distinguishing them from each other. SNR analysis assumes the existence of a repeated transmission of the pathogen during anthrax outbreaks. In general, the selected algorithm and set of methods for the molecular analysis of strains – canSNP-typing, MLVA25-typing, whole genome sequencing – showed suitability for carrying out an operational molecular epidemiological investigation of anthrax outbreaks.

Conflict of interest. The authors confirm the absence of a conflict of financial/non-financial interests associated with writing an article.

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Authors:

Kulichenko A.N., Eremenko E.I., Ryazanova A.G., Aksenova L.Yu., Kovalev D.A., Pisarenko S.V., Varfolomeeva N.G., Zhirov A.M., Volynkina A.S., Buravtseva N.P., Golovinskaya T.M., Koteneva E.A., Tsygankova O.I. Stavropol Research Anti-Plague Institute. 13–15, Sovetskaya St., Stavropol, 355035, Russian Federation. E-mail: snipchi@mail.stv.ru.

Dyatlov I.A., Timofeev V.S., Bogun A.G., Bakhteeva I.V., Kislichkina A.A., Mironova R.I., Titareva G.M., Skryabin Yu.P. State Research Center for Applied Microbiology and Biotechnology. Obolensk, Moscow Region, 142279, Russian Federation. E-mail: info@obolensk.org.

Selyaninov Yu.O., Egorova I.Yu., Kolbasov D.V. National Research Institute of Veterinary Virology and Microbiology of the Russian Academy of Agricultural Sciences. 1, Akademika Bakulova St., Volginsky, Vladimir Region, 601125, Russian Federation. E-mail: VNIIVViM@niiv.petush.elcom.ru.

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