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Infection of an Individual with Plague in the Gorno-Altaisk High-Mountain Natural Focus in 2014. Communication 2. Peculiarities of Laboratory Diagnostics and Molecular-Genetic Characterization of the Isolated Strains

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Laboratory diagnostics of plague was carried out in compliance with valid operational guidelines and regulations. But its peculiarity consisted in the performance of diagnostic investigations secondary to antimicrobial therapy with application of preparations characterized by the expressed activity towards gram-negative microorganisms, including the agent of plague (ceftriaxone, ciprolet, and amikacin). The studies revealed that under antibiotic treatment during the early phase of infection the most effective method for the laboratory plague diagnostics was PCR. Based on the results of the assay it was possible to establish not only provisional, but also the final diagnosis in a patient. Obtained was genetic characteristics of the strains isolated from the patient and the marmot, withdrawn at the patient's place, using techniques of molecular-genetic analysis, in particular PCR, multilocus VNTR, and multilocus and genome-wide sequencing. Thereupon the strains were attributed to antique biovar of the main subspecies of plague agent. In addition, close relation to *Y. pestis* of the main subspecies isolated in the same focus in 2012 and to the strains from Mongolian Altai and Tuvinian mountain focus was determined based on phylogenetic analysis of the isolates.

Key words: plague, plague agent, main and non-main subspecies of *Y. pestis*, laboratory diagnostics, molecular-genetic analysis.

In 2014 a case of human plague was registered in the territory of Altai mountain natural plague focus in Kosh-Agach county of Republic of Altai.

Due to instruction of the Head of Federal Service for Surveillance in the Field of Consumer's Rights Protection and Human Welfare, advisory methodological and practical assistance tasks for performance of plague laboratory tests, as well as research of isolated strains (including their genome-wide sequencing) were imposed on Plague Reference Center of Russian Research Anti-Plague Institute "Microbe".

On September 12, 2014 infectious disease physician of Kosh-Agach Central county hospital made a preliminary diagnosis for a patient A as "axillary lymphadenitis of unclear etiology on the left. Tularemia, bubonic form?" [6]. Sampling of patient's material, such as blood, bubo content, sputum, urine was carried out for laboratory tests for agents of highly dangerous infectious diseases in presence of a specialist from Altai plague control station with compliance to requirements of normative and methodological documents. Sampling was performed at the background of anti-bacterial therapy with a broad-spectrum anti-microbe preparation possessing express activity towards gram-negative bacteria, including plague agent (ceftriaxone was prescribed from 09.04.14, ciprolet – from 11.09.14 and amikacin – from 12.09.14). At sampling it was determined that no pus is in bubo content, urine is transparent.

Material was delivered to laboratory of Tashanta epidemiological unit. According to existing normative and methodical documents governing laboratory diagnostics of plague and tularemia samples under research were inoculated on solid and liquid media (nutrient medium for plague microbe culture (PNM) and Hottinger broth (pH 7,2) with addition of laky blood, FT-agar); smear bacterioscopy, gram-stained, inoculation of bio-test animals (2 white mice for each sample of material s/c and i/p); serological reactions were arranged for plague and tularemia microbes antigens (IHT/AbNT) and antibodies against plague and tularemia microbes (IHT/AgNT) [9].

Negative result was obtained at performing of serological tests with use of antigen and immunoglobulin erythrocyte diagnostic sets. No cells with morphology typical for plague and tularemia microbes were detected in smears. Growth of colonies specific for plague and tularemia was not detected at inspection of native material cultures on agar plates in 24 and 48 hours. All tested animals remained alive.

In this situation the results obtained are completely explainable. Early prescription of antibiotics changes complex of symptoms attributable to plague and complicates further not only clinical, but also bacteriological analysis. Treatment with antibiotics leads to fast reduction of infectious process and liberation of organism from the agent [2]. Depressive impact of anti-bacterial preparations on immune system is established. Due to literature, timely started treatment of infected animals with antibiotics prevented accumulation of agent in organism up to limit concentrations, which decreased chances of both serological and bacteriological diagnostics. No plague microbe culture was isolated from animals examined right after three-day treatment with antibiotics and in further terms, no antigen was detected in serological reactions. For performance of express diagnostics (PCR, FAT, IHT) and bioassay a part of clinical material was sent by car with accompanying persons to stationary laboratory of Altai plague control station at 7.00 on 13.09.2014. At 19.00 DNA of plague microbe was detected using PCR method in bubo content, single cells with 4+ fluorescence were detected in smear of bubo punctate stained with plague fluorescent immunoglobulins. No markers of plague microbe were detected in patient's urine, blood, sputum. Tularemia test results were negative. By the results of indication using FAT and PCR, in 2 and 6 hours respectfully a preliminary conclusion was given about presence of antigen and DNA of plague agent in the material obtained from the patient.

Further tests were carried out according to the scheme of clinical material analysis for plague in accordance with methodical instructions of arrangement and carrying out of plague laboratory diagnostics in laboratories of territorial, regional and federal level. Taking into consideration peculiarities of sampling, solid and liquid nutrient media were used to increase efficiency of agent culture isolation: dry plague medium (manufactured by State Research Center of Applied Microbiology and Biotechnology, Obolensk); agar and Hottinger broth, pH 7,2 (Pharmacotherapy Research Center, St. Petersburg); medium for plague microbe culturing (Irkutsk Research Anti-Plague Institute). Laky blood was added there as growth stimulator. Solid nutrient media were additionally treated with anti-phage serum.

To decrease animal resistance in white mice at the first stage yolk was applied and hydrocortisone was applied during performance of blind passages. Step by step investigation of test animals was also used to increase efficiency of biological method.

The animals and inoculated cultures were observed on daily basis. In 48 h broths with native cultures were investigated additionally by means of immune chromatography, FAT and PCR methods. Positive result was obtained by means of PCR only. DNA of plague agent was detected in broth with bubo punctate culture. It is probably due to low concentration of the agent in sample, significantly lower than ultimate concentrations discovered by immune chromatography or FAT.

During entire investigation period PCR was applied at various stages of laboratory diagnostics: for identification while discovering of suspicious colonies, for broth cultures growth control, investigation of organ suspensions of tested animals, including performance of blind passages. Obtained results convincingly evidenced presence of plague agent markers in material from bubo only.

On 18.09.2014 on the 6th day from the beginning of investigation epidemiologists isolated plague microbe culture from accumulation medium (broth with bubo punctate culture). It shall be noted that culture was successfully isolated from bio-tested animals but on the 13th day after the third passage of white mouse organs suspension, which was infected with bubo punctate (with advanced pre-medication with hydrocortisone – 5 mg of 0,3 ml s/c to femoral area). On 18.09.2014 plague microbe culture was also isolated from mandibular lymph node from one of three marmots taken at patient's home. The marmots were taken in Serbistu mountain area. Determination of fermentative activities of cul-

tures was performed to determine their subspecies belonging.

On 19.09.2014, isolated cultures, objects with cultures from the patient and marmots, as well as re-taken patent's blood were delivered to the laboratory of Altai plague control station for extensive identification, including application of molecular-genetic analysis methods. Besides, organ suspensions of bio-test animals killed on the 6th day were prepared for analysis. The animals were infected at the laboratory of Altai plague control station by native material of bubo punctate, blood, urine, sputum.

At diagnostic analysis of this material following results were obtained.

By means of PCR method with test systems "Gen *Y. pestis* identification – HFR" (Russian Research Anti-Plague Institute "Microbe") and "AmpliSens *Y. pestis* – FL" (InterLabService LLC) DNA of plague agent was detected in bacterial suspensions of isolated cultures; in broth with bubo punctate culture; in broth with organ suspension cultures from one marmot taken from the patient; in organs suspension of killed white mouse infected subcutaneously with bubo punctate. Negative results were obtained at analysis of other samples.

For differentiation of main and Altai subspecies of plague agent by means of PCR method with experimental set of primers yp2769ms06 of Irkutsk Research Anti-Plague Institute, cultures isolated from patient and marmot were preliminary identified as main subspecies. FAT confirmed presence of capsule antigen (F1) synthesis in cultures of isolated plague agent strains. In smears of gram-stained cultures gram-negative bacillus were detected.

Tests by means of EIA and in IHT of paired sera of patient's blood taken on 12.09.2014 and 19.09.2014 established the following. Negative results were obtained with the first serum of 12.09.2014; antibodies in titers 1:200 were obtained in patient's blood serum taken on 19.09.2014 (in IHT with antigen erythrocyte diagnostic set made in Republic of Kazakhstan) and 1:80 (in EIA with test system "EIA-At-Fq *Yersinia pestis*", manufactured by Russian Research Anti-Plague Institute "Microbe"). The results indicate the presence of seroconversion and formation of immune response to plague agent.

Upon results of identification, strains isolated from patient and marmot taken from patient's home possess cultural and morphological characteristics typical for main subspecies (*Yersinia pestis* ssp. *pestis*), are lysed by intact pseudotuberculosis, plague L413"S" and Pokrovsky bacteriophages. The strains ferment arabinose, glycerin, they do not decompose rhamnose, melibiose, lactose, sucrose, dulcite, sorbite, inositol, reduce nitrates into nitrites, do not possess urease activity, are stable. 100 % of cells sorb hemin in Jackson-Burroughs medium, possess fibrinolysin-pesticin-plasma-coagulase activity, produce capsule antigen F1. Four plasmids are established in this medium, three of which (pYP, pYV, pYT) are typical for plague agent and additional plasmid pTP33, typical for strains *Y. pestis* from Tuva mountain plague focus and areas of Mongolian Altai [1], as well as chromosome pigmentation area (ybt, pgm), which evidences virulence of strains.

The strains are sensitive to antibacterial preparations: amikacin, gentamicin, doxycycline, co-trimoxazole, ofloxacin, rifampicin, tobramycin, ceftriaxone, penicillin G, polymyxin, tetracycline, kanamycin, streptomycin, ciprofloxacin, ampicillin, cefotaxime.

Clinical diagnosis "Bubo plague" is confirmed upon results of laboratory tests based on isolation of culture from the patient and its identification as *Yersinia pestis* ssp. *pestis,* as well as presence of seroconversion.

In addition to the material from the patient, tests by means of bacteriological, serological and/or molecular genetic methods of the material taken from contact persons, bodies of marmots taken from native people, as well as rodents and ectoparasites obtained in the course of epizootiological monitoring of Serbistu, Irbistu, Kok-Ozek, Kurai ridge, Altai mountain natural plague focus [6, 10] were performed in laboratories of Altai plague control station and epidemiological unit. On 18.09.2014 at the same time with strains taken from patient and marmot culture *Y. pestis* was isolated from fleas *Paradoxopsyllus scorodumovi* (4 pieces) taken at hole entries of *Ochotona pallasi* in the course of epizootiological examination of the territory of Serbistu mountain area. The isolated strain belongs to non-main Altai subspecies (*Yersinia pestis* ssp. *altaica*) of plague microbe due to its

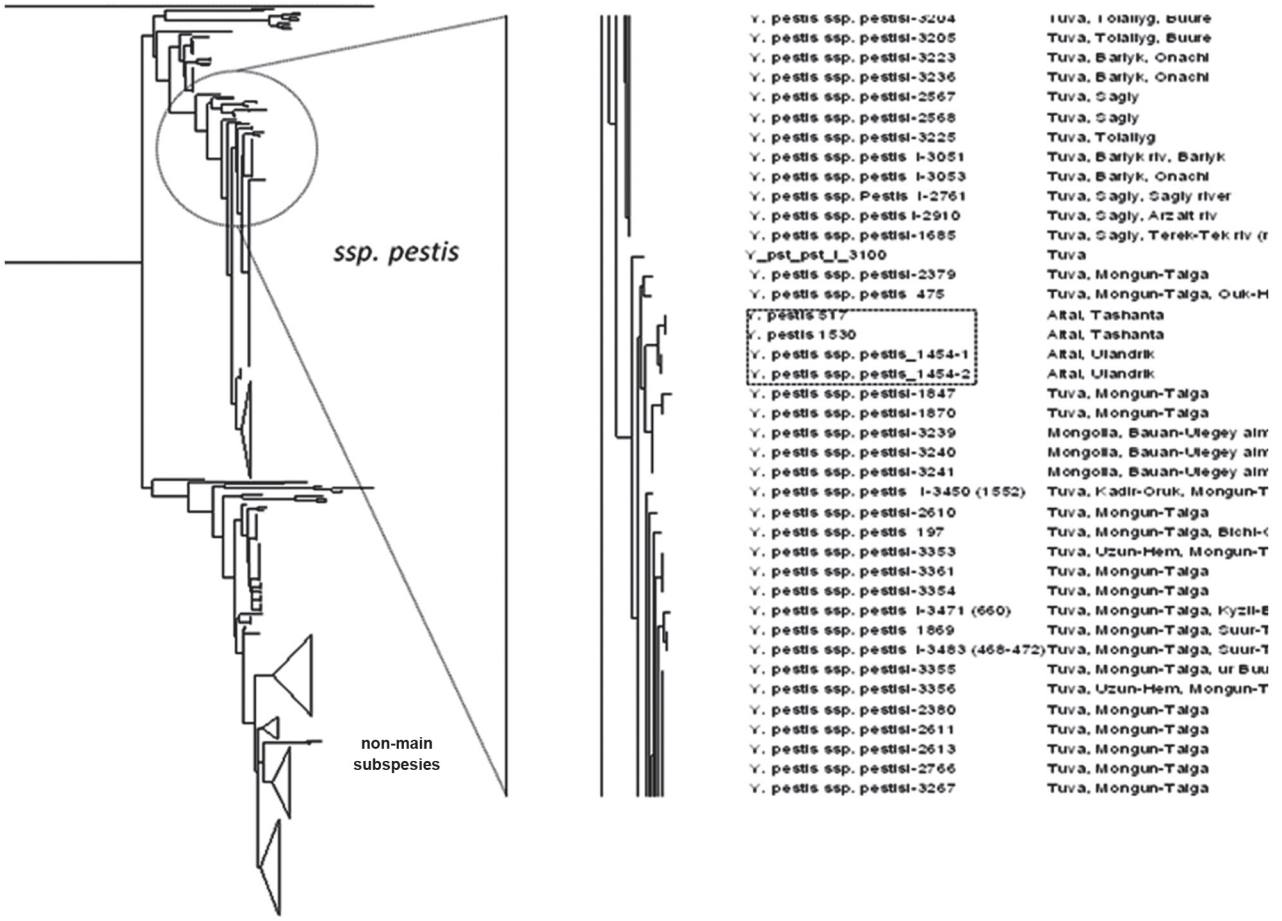

Fig. 1. Phylogenetic relationship of *Y. pestis* strains of main subspecies (in the frame), isolated in 2012 and 2014 in Altai mountain natural plague focus with the strains from Tuva mountain focus and Mongolia based on results of $VNTR_{25}$ analysis.

cultural and morphological features and biochemical activity: it does not ferment arabinose, lactose, sucrose, dulcite, sorbite, inositol, it does not reduce nitrates into nitrites, ferments glycerin, rhamnose, xylose, melibiose, glucose, maltose, mannite, does not possess urease activity. Due to results of molecular-genetic test, the strain has three plasmids (pYP, pVV, pYT). Plague agent culture from *Ochotona pallasi* fleas was also identified as Altai subspecies by means of PCR method with experimental set of primers yp2769ms06 from Irkutsk Research Anti-Plague Institute.

The results obtained, and tests performed allow to conclude that the most efficient method of plague laboratory tests is PCR in conditions of therapy with antibiotics, and final diagnosis can be stated in certain conditions (presence of characteristic clinical signs, epidemiological anamnesis) and based on this method. The last requires solution of the issue about respective amendments in normative and methodical regulations, governing plague laboratory diagnostics.

Use of registered PCR test systems in laboratory plague diagnostics already at the stage of indication allows to determine species belonging of the agent, but does not differentiate subspecies, which occurred to be important in specific conditions. Therefore, elaboration and implementation into practice of simple and durable diagnostic preparations is necessary for subspecies differentiation of plague agent which may be used in laboratories of regional level (anti-plague stations) already at the indication stage.

The following stage of research was molecular-genetic analysis of *Y. pestis* strains isolated in Altai high mountain natural plague focus in 2014. The strains isolated from patient and marmot were numbered as *Y. pestis* 517 and *Y. pestis* 1530 respectively, the strain from fleas *P. scorodumovi – Y. pestis* 1313.

Digital VTNR-profiles of strains were obtained by 25 VTNR multi-locus analysis, based on which their phylogenetic research was performed by means of data base BioNumerics (Applied Maths) (fig. 1).

It is established that strains *Y. pestis* 517 and *Y. pestis* 1530 have fully identical VNTR-profiles which significantly differ from that of *Y. pestis* 1313, isolated in the same period in focus from fleas *P. scorodumovi,* as well as from *Y. pestis* typical strains, isolated in Altai mountain natural plague focus since 1961 until recent time.

The strains *Y. pestis* 517 and *Y. pestis* 1530 as well as the strain of main subspecies 1454 isolated in 2012 in this natural focus belong to the same group with the strains isolated in the end of 80th in Huuh-Serh-Munh-Hairhaan natural focus of plague (Deluun-somon of Bayan-Ulgiy aimag in Mongolia) which evidences for close phylogenetic relationship of these strains (fig. 1).

Further molecular-genetic analysis of the strains *Y. pestis* 517, 1530 and 1313 was performed in Reference Center for monitoring of plague agent and other hazardous infections at Russian Research Anti-Plague Institute "Microbe".

Molecular-genetic analysis of isolated Y. pestis strains by means of PCR and multi-locus sequencing methods

Subspecies belonging of isolated strains *Y. pestis* was determined according to standard algorithm of molecular typing of plague agent [4]. Experimental set of primers complementary to nucleotide sequences of genes *terC, ilvN, inv, rhaS, araC, ssuA, melB, metB, glpD, napA* was used for analysis [3, 4, 5, 7, 8].

By means of multi-locus PCR with primers for genes *terC, ilvN* and *inv Y. pestis* strains were differentiated upon their belonging to main and non-main subspecies of plague agent. As a result, *Y. pestis* strains 517 and 1530 obtained from patient and marmot were attributed to main subspecies *Yersinia pestis* ssp. *pestis* (fig. 2.A). Deletion of 89 bp is present in *terC* gene (PCR fragment's size is 300 bp) and deletion of 45 bp is in gene *ilvN* (PCR fragment's size is 515 bp). The both deletions are marker ones for *Y. pestis* strains of main subspecies. These mutations are absent in DNA sample of the strain *Y. pestis* 1313 isolated from *Ochotona pallasi* fleas, whose genes *terC* and *ilvN* are in intact condition. DNA fragment sizes of *Y. pestis* strain 1313 are following: *terC*- 389 bp; *ilvN* – 560 bp. Based on obtained results *Y. pestis* strain isolated from *Ochotona pallasi* fleas is attributed to non-main subspecies. All three DNA samples were different from DNA of *Y. pseudotuberculosis* bacterium closely relative to plague agent in presence of insertional sequence IS*1541* in gene *inv* (PCR fragment's size of *Y. pestis* strains is 877 bp and that of *Y. pseudotuberculosis* is 169 bp).

In mono-locus PCR with primers for inter-gene area *YPO3333-YPO3332* it was established that *Y. pestis* strain 1313 from *Ochotona pallasi* fleas belongs to Altai (non-main) subspecies, because the 122 bp deletion was detected in this inter-gene area which is a marker deletion for strains of Altai subspecies and is absent in *Y. pestis* strains of other subspecies (fig. 2, B). PCR fragment's size of *Y. pestis* 1313 strain (like that of other strains of Altai subspecies) is 90 bp and all other subspecies have 212 bp.

To confirm the belonging of isolated strains *Y. pestis* 517 and 1530 to the main subspecies and strain 1313 to Altai subspecies their genotypes were determined based on multi-locus sequencing with use of variability of nucleotide sequence of genes of differential biochemical signs *rhaS, araC, glpD, napA* [4]. As a result, is was established that strains 517 and 1530 from patient and marmot have genotype rhS1-arC1-npl-gpD1, characteristic for strains of

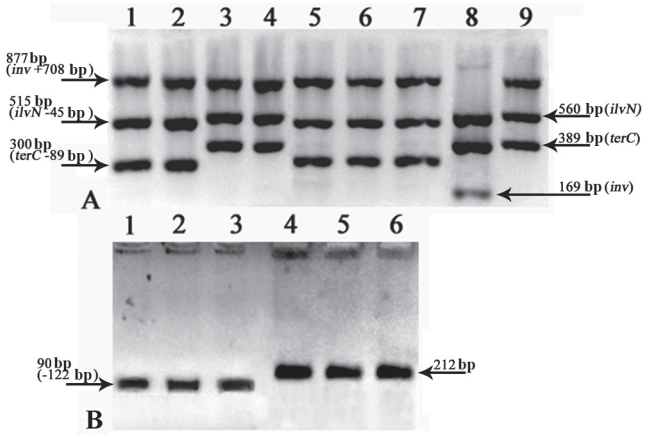

Fig. 2. PCR analysis of *Y. pestis* strains isolated in Altai mountain natural plague focus in 2014:

A – differentiation of main subspecies *Y. pestis* from non-main subspecies and from *Y. pseudotuberculosis.* The strains *Y. pestis,* main subspecies: *1* – I-3358 (Tuva mountain focus), *2* – KM932 (I-3223) (Tuva mountain), *5* – 1530 (Altai mountain), *7* – I-3244 (Bayan-Ulgiy aimag, Mongolia); Altai subspecies (Altai mountain): *3* – KM683 (I-2359), *4* – 1313, *9* – I-2998, 8 – *Y. pseudotuberculosis* IV. ***B*** – PCR-detection of *Y. pestis* Altai subspecies strains. Altai subspecies: *1* – KM638 (I-2359), *2* – 1313, *3* – I-2998; main subspecies: *4* – 1530, 5 – 517, *6* – KM932 (I-3223)

Table 1

Genetic base of phenotypical differences between strains of main and Altai subspecies of plague agent

Y. pestis strain	Mutations in genes				
	rhaS (671*)	*araC* (773*)	*melB* (1231*)	*ssuA* (302*)	*metB* (988*)
517 (main subspecies), from patient	A	–	+ IS*285*	–	– G
1530 (main subspecies), from marmot	A	–	+ IS*285*	–	– G
1313 (main subspecies), *P. scorodumovi* fleas	G	+ G	–	+ G	–

* Position of variable nucleotide in gene.

main subspecies. Genes have intact structure except of *rhaS* gene, in position 671 of which nucleotide replacement G→A is present, which is a marker for strains of main subspecies and which is absent in strains of non-main subspecies and *Y. pseudotuberculosis*. Additionally, belonging of strains 517 and 1530 of main subspecies to antique biovar of plague agent was established based on intactness of genes *glpD* and *napA*. *Y. pestis* 1313 strain from *Ochotona pallasi* fleas has genotype rhS3-arC2-np3-gpD1 characteristic for Altai subspecies and contains inserted single nucleotide G after 773rd nucleotide from the beginning of the gene *araC* and intactness of other genes *araC, glpD* and *napA*. Therefore, belonging of strains *Y. pestis* 517 and 1530 to main subspecies and strain *Y. pestis* 1313 to Altai subspecies was confirmed by means of multi-locus sequencing.

Diversity in genes encoding other phenotypic differences was detected in isolated strains *Y. pestis* 517 and 1530 in relation to the strain 1313 (melibiose fermentation, nitrate reduction, nutrient demand for methionine amino-acid) (table 1) [3, 8].

To establish belonging of strains 517 and 530 isolated from patient and marmot to specific phylogenetic line of plague agent main subspecies, a set of experimental primers was used which detect single nucleotide replacements which are marker ones for main phylogenetic lines of plague agent. As a result of analysis, a marker mutation was determined characteristic for phylogenetic line 4.ANT – replacement of single nucleotide G→A in position 124 of *YPO*1418 gene. It evidences belonging of isolated strains *Y. pestis* 517 and 530 to the line 4.ANT, which strains circulate in Tuva mountain focus and an area in Mongolia at the borderline with Altai mountain natural plague focus.

Thus, based on complex genetic analysis of housekeeping genes – sugar (*rhaS, araC, melB*) and glycerin fermentation (*glpD*), nitrate reduction (*napA, ssuA*) and biosynthesis of methionine amino-acid (*metB*), as well as marker mutations of various phylogenetic lines of plague agent it is established that *Y. pestis* 517 and 1530 strains isolated from patient and marmot belong to the strains of antique biovar of main subspecies of 4.ANT line. The strain *Y. pestis* 313 from *Ochotona pallasi* fleas belongs to Altai subspecies.

Genome-wide DNA sequencing of Y. pestis 517, 1530, 1313 strains and bioinformation analysis of obtained data

Genome DNA sequencing was performed on Ion PGM platform (Life Technologies) using set of reagents, allowing to obtain reading length of single DNA fragment of approx. 400 bp. Further, using the Newbler v.2.6 software (454 Life Sciences, Roche Diagnostics), single readings are collected to contigs and annotated using Prokka software (http://www.vicbioinformatics.com/software.prokka.shtml).

Whole genome sequence analysis established that all three samples investigated have chromosome pigmentation area (*pgm*), including *hms* operon and high pathogenicity island HPI with *ybt* locus, genes *pla* and *pst* (pPst plasmid), *caf* and *ymt* (pFra), *lcrV; yopM* and *yopN* (pCad). Thus, *Y. pestis* strains 517, 1530 and 1313 have a set of main genes associated with virulence of plague agent.

To reconstruct phylogenetic relations, nucleotide DNA sequences of full genomes of strains isolated from infected person (*Y. pestis* 517) and marmot (*Y. pestis* 1530) and strains isolated from *Ochotona pallasi* fleas (*Y. pestis* 1313) were compared with genomes of strains *Y. pestis* earlier sequenced by us, isolated in areas of natural plague foci of the Russian Federation and bordering countries as well as genomes of plague agent represented in international database NGBI GenBank, isolated in Mongolia, China, Nepal and USA.

Phylogenetic analysis by means of comparison of Single Nucleotide Polymorphism

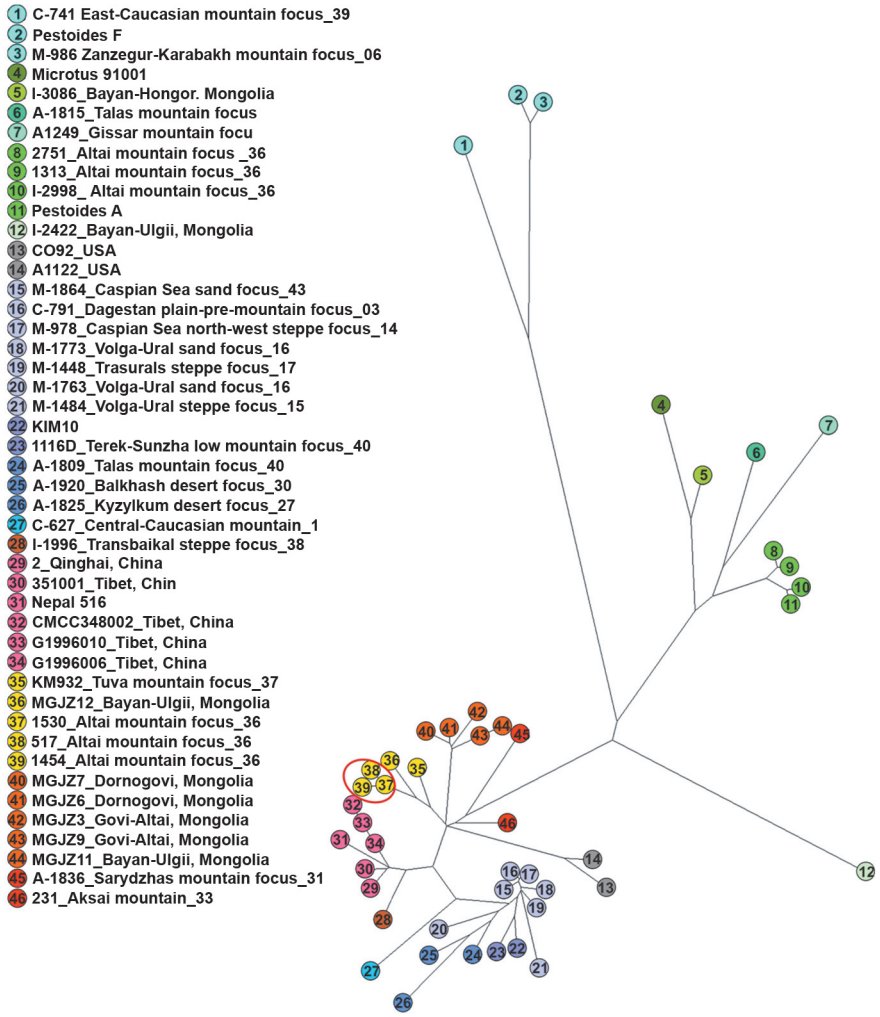

Fig. 3. Phylogenetic relationship of *Y. pestis* strains isolated in Altai mountain natural plague focus in 2014 with the strains isolated from other natural plague foci based on SNPs analysis

(SNP) of draft genomes was performed with Wombac software (https://github.com/Victorian-Bioinformatics-Consortium/wombac). The essence of phylogenetic analysis was in determination of Single Nucleotide Polymorphism Matrix of high reliability grade from 46 genomes of *Y. pestis* strains, in relation to core part of genome of reference strain *Y. pestis* CO92. Number of variable positions (SNPs) in this matrix was 2102. Obtained SNPs matrix was clustered with BioNumercis v. 7.1 software (Applied Maths) with use of Maximum parsimony tree algorithm.

The analysis showed that both nucleotide DNA sequences of full genomes in *Y. pestis* strains 1530 and 517 have maximum coincidence in SNPs matrix (99.9 %) both with each other and with nucleotide DNA sequence of *Y. pestis* strain 1454 isolated in Altai high mountain natural plague focus in Bolshie Sary-Gobo natural boundary in 2012 (fig. 3, group N 37, 38 and 39 accordingly).

Nucleotide sequences of these strains showed similarity grade in 98.5 % in SNPs matrix with the strain of main subspecies *Y. pestis* MGJZ12 (NCBI GenBank), isolated in Saylungem natural plague focus (area of Dayan Nur and Khalgashin Nur area) of Bayan-Ulgiy aymag in Mongolia [13] which borders with Altai mountain natural plague focus (fig. 3, N 36).

Besides, nucleotide sequences of these strains showed similarity grade of 98.1 % in SNPs matrix with DNA sequence of *Y. pestis* strain KM932 of main subspecies from Tuva mountain natural plague focus, which borders with Altai mountain natural plague focus in north-east (fig. 3, N 35).

Y. pestis 1313 strain (Altai subspecies, isolated from *Ochotona pallasi* fleas in 2014) has maximum coincidence in SNPs matrix (99,7 %) with the strain of Altai subspecies *Y. pestis* 2751-55 (2012) from Altai mountain natural plague focus (fig. 3, N 9 and 8 accordingly).

Table 2

Unique point mutations of nucleotides of *Y. pestis* strains 517, 1530 and 1454 of main subspecies, isolated in Altai mountain natural focus

Position due to reference strain CO92, bp	Point mutation (SNP)	Name (number) of *Y. pestis* strains					SNP containing area or gene
		517	1530	1454	MGJZ12	KM932	
394234	C > A	+	+	+	-	-	before *YPO*0379
928294	G > T	+	+	+	-	-	between *YPO*0847 и *YPO*0847
1484392	C > T	+	+	+	-	-	*dacC* (*YPO*1320)
1996447	C > T	+	+	+	-	-	*YPO*1751a
2057419	C > A	+	+	+	-	-	*YPO*1811
2092484	T > G	+	+	+	-	-	*yecC* (*YPO*1811)
3270727	G > A	+	+	+	-	-	*murQ* (*YPO*2925)
3362591	A > G	+	+	+	-	-	*YPO*3009
3495476	C > T	+	+	+	-	-	before *rpmE2*
3693309	G > T	+	+	+	-	-	*YPO*3311
3718023	A > G	+	+	+	-	-	*YPO*3332
4339797	G > A	+	+	+	-	-	*wzzE (*YPO3865)

According to the results of clustering (fig. 3) analyzed genomes of 46 *Y. pestis* strains are divided into a number of groups, close to the set of single point mutations of nucleotides. A separate cluster was formed by three *Y. pestis* strains of main subspecies from Altai mountain focus – 1454, 1530, 517 and strains KM932 from Tuva mountain focus and MGJZ12 from Bayan- Ulgiy aymag in Mongolia. The closest cluster to them consists of other strains *Y. pestis* of main subspecies, isolated in Mongolia (fig. 3, N 40–44). More remote are *Y. pestis* strains of main subspecies from Tianshan mountain plague foci (N 45,46), as well as from China and Nepal (fig. 3, N 29–34). Other *Y. pestis* strains of main subspecies have also formed separate clusters represented at dendrogram (fig.3). As follows from this dendrogram, a group of *Y. pestis* strains of non-main subspecies (N 1–12) is sufficiently remote from clusters of species of non-main subspecies. *Y. pestis* strain 1313 (N 9) is in cluster of Altai subspecies strains from Altai mountain focus (N 8–11). It has maximum similarity to *Y. pestis* strain 2751-55 (N 8), isolated in this focus in 2012.

Based on genome comparison of 46 used *Y. pestis* strains, 61 unique point mutations of nucleotides in DNA sequence of strains 1530, 517, 1454, MGJZ12 and KM932 are detected which differ them from all represented 229 *Y. pestis* genomes in NCBI GenBank and *Y. pestis* genome group sequenced by us earlier. From isolated 61 mutations 12 SNPs are characteristic for *Y. pestis* strains 517, 1530 and 1454 (table 2) only; 9SNPs – for *Y. pestis* strains 517, 1530, 1454 and MGJZ12; 13 SNPs – for *Y. pestis* strains 517, 530, 1454, MGJZ12 and KM932; 14SNPs – for *Y. pestis* strains MGJZ12 and 13 SNPs – for *Y. pestis* strain KM932. The unique point mutations found can be used for identification of *Y. pestis* strains of main subspecies from Altai mountain plague focus and *Y. pestis* strains closed to it from bordering territories (table 2).

Thus, based on data obtained with PCR methods, multi-locus VNTR analysis, multilocus and genome-wide sequencing it can be concluded that *Y. pestis* strains isolated from patient and marmot (517 and 1530 respectively) in Altai mountain plague focus in 2014 belong to main subspecies and *Y. pestis,* 1313 strain from *Ochotona pallasi* fleas belongs to Altai subspecies. Phylogenetic analysis of SNPs of core parts of genomes of isolated *Y. pestis* strains 517 and 1530 of main subspecies is indicative of their maximum proximity to *Y. pestis* 1454 strain of main subspecies isolated in 2012 in the same plague focus as well as proximity to the strain of main subspecies *Y. pestis* MGJZ12 from Bayan-Ulgiy aymag in Mongolia. All these strains belong to phylogenetic line 4.ANT which does not occur in other regions of the world. Obtained molecular genetic data allow to make substantiated supposition that circulation of *Y. pestis* strains of main subspecies of antique biovar of 4.ANT line (Tuva variant) is characteristic for the entire territory of Altai mountain region.

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