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Isolation of Central Asian Subspecies of Tularemia Agent in the Altai Territory

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Within the frames of activities attributed to the Reference Center for tularemia monitoring at SRC AMB, genetically identified are 4 isolates of *Francisella tularensis*, isolated in 2011 in the Altai Territory. These bacteria prove to be virulent for BALB/c mice, DCL being lower than 10 CFU. Using single-primer PCR-typing and MLVA assay distinguished have been the subspecies of the isolates. Three of them refer to the Central Asian subspecies, one – to the Holarctic, the former being isolated in the territory of the Russian Federation for the first time ever.

Key words: *Francisella tularensis*, subspecies mediasiatica, Altai Territory.

Tularemia is a zoogenous natural focal infection. Tularemia agent, *Francisella tularensis,* infects more than 250 species of animals and can be passed to humans by multiple means – through a direct contact with infected animal, eating and drinking contaminated water and food, through aerogenic transmission, as well as through bites of ticks, flies and mosquitos.

Tularemia agent belongs to *Francisellaceae* family. *Francisellaceae* family includes among single genus *Francisella,* symbiotic bacteria of *Wolbachia* genus, isolated from *Argas arboreus* ticks, as well as *Francisella*-similar endosymbionts, distinguished from ticks of *Amblyomma maculatum, Ornithodoros pornicus, Ornithodoros moubata* and *Dermacentor* (*D. variabilis, D. andersoni, D. hunteri, D. nitens, D. occidentalis* and *D. albipictus*) species.

Currently 2 species belong to *Francisella* genus: *F. tularensis* and *F. philomiragia.* Recently a number of publications were issued however which describe cases of infections of various animal species infected by new species of Francisella: *Francisella hispaniensis, Francisella asiatica* and *Francisella noatunensis* (syn. *Francisella piscicida*) [7, 9, 10]. RNA 16S gene sequence of this infectious agents has up to 99 % homology with RNA 16S gene sequence of *Francisella tularensis* and up to 98 % homology with RNA 16S gene sequence of *Francisella philomiragia.* Inside of the species *F. tularensis* 4 subspecies are distinguished: *F. tularensis* subsp. *Tularensis, F. tularensis* subsp. *Holarctica, F. tularensis* subsp. *mediasiatica* and *F. tularensis* subsp. *novicida* [5]. It shall be noted that strains of *mediasiatica* subspecies were isolated in Middle Asia only [4].

High phenotypic conservatism is characteristic for *F. tularensis* bacteria (antigen and biochemical homogeneity, low biochemical activity etc.) [4], therefore detailed investigation of disease outbreaks to determine source of infection is difficult in present time without use of a complex of molecular biological methods, capable to classify isolates to a certain subspecies and inside of subspecies to certain genetic cluster, which is ultimately important in the light of requirements of modern public health protection and real threat of bio-terrorism. To resolve this issue, we proposed PCR diagnostics for subspecies differentiation of tularemia microbe based on Chilf primer application [2]. At that a range of amplicons is accumulated typical both for species *F. tularensis* as a whole and for individual subspecies of tularemia microbe. This method however does not allow to classify the investigated isolate to a certain genetic cluster, which complicates determination of relation to endemic strains of tularemia microbe, and localization of spread of specific strains in tularemia endemic areas. To solve this issue, use of Multiple Loci VNTR Analysis (MLVA) is most prospective. MLVA in 25 loci is in common use for tularemia microbe with the purpose of intra-specie subtyping of *F. tularensis* strains [8].

In the territory of former Soviet Union strains of *F. tularensis* subsp. *holartica* are mainly detected, but individual specificities of genomes of circulating strains are studied insufficiently. Analysis of 352 strains of all sub-species of tularemia agent by means of variable number of tandem repeats (VNTR) in four loci allowed to determine 61 genotype and to demonstrate genotypic heterogenicity of Russian strains [3].

The purpose of study was determination of subspecies classification of strains received by the reference center for tularemia monitoring at SRC AMB from Center of Hygiene and Epidemiology in Altai territory using single primer PCR-typing and their genotyping by means of MLVA in 25 VNTR loci.

Materials and methods

F. tularensis strains used in the study: 4 strains isolated in Altai territory, 12 strains of various geographic origin from State collection of pathogenic microorganisms and cell cultures (SCPMCC-Obolensk) and 12 strains typical for Siberian tularemia foci received from collection of Irkutsk Research Anti-Plague Institute of Siberia and Far East (table).

F. tularensis bacteria were cultivated at temperature 37 °C on FT-agar (SRC AMB) with addition of polymyxin B (Pm). To determine sensitivity to erythromycin the cultures were seeded on media containing erythromycin 50 mkg/ml.

DNA isolation from biomass of daily agar culture was performed by means of nucleo-sorption method on silica-gel in presence of GuSCN using commercial set "Ribo-sorb" (InterLabService).

BALB/c mice were used in work. DCL was determined using Kerber method in modification of I.P. Ashmarin and A.A. Vorobyev [1]. Mice (5 in a group, males and females, 6–8 weeks old, mass 18–20 g) were infected by subcutaneous injection of bacterial suspension in doses $1 \cdot 10^1$, $1 \cdot 10^2$ and $1 \cdot 10^3$ CFU/mouse and observed within 21 days.

25 couples of primers were used for VNTR analysis (Ft-M1 – Ft-M25), their sequences were described in articles [6, 8, 11]. PCR conditions were calculated by means of Vector NT1 software package. Sizes of amplicons were determined due to their electrophoretic mobility in 3 % agar gel in relation to mobility of molecular markers (pace 20 bp, BIO-RAD, USA) by means of PhotoCaptMw program. The gels were photographed by means of gel-documenting system Vilber Lourmat (France). During analysis of Ft-M1 and Ft-M25 loci nucleotide sequence of amplicons was determined.

Results and discussion

For initial characteristic of features of four *F. tularensis* cultures isolated in different counties of Altai territory in summer-autumn 2011 and supplied by Center of Hygiene and Epidemiology in Altai territory, virulence for mice and sensitivity

***F. tularensis* strains used in the study**

Sub-specie	Strain	Place of isolation	Source of isolation	Year of isolation
Natural isolate	A-554	Altai territory, Eltsovka county, Martynovo village	Ticks *H. concinna*	2011
Natural isolate	A-678	Altai territory, Pervomayskoye county, Pokrovka village	Ticks *Ix. persulcatis*	2011
Natural isolate	A-823	Altai territory, Shelabolikha county, Molokovo village	Clethrionomys rutilos	2011
Natural isolate	A-1045	Altai territory, Aleisk county, Druzhba settlement	Ticks *D. reticulatus*	2011
tularensis	Schu	USA	Human being	1941
tularensis	B399 A-Cole	USA	Human being	1972
tularensis	8859	USA	Foal	1958
holarctica	9	Moscow territory	Microtus arvalis	1948
holarctica	21/400	Moscow territory	Arvicola terrestris	1949
holarctica	15/10	Alma-Ata	Arvicola terrestris	1942
holarctica	503	Moscow territory	Ticks *D. pictus*	1949
holarctica bv. japonica	Miura	Japan	Human being	1975
holarctica bv. japonica	Jasoe	Japan	Human being	1974
mediasiatica	117	Kazakhstan	Ticks *Hyalomma sp.*	1960
mediasiatica	120	Middle Asia	Human being	1968
novicida	112	Utah, USA	Water	1955
holarctica	I-282	Chita territory	Hamster	1971
holarctica	I-305	Chita territory	Ticks *D. nuttalli*	1972
holarctica	I-329	Republic of Buryatia	Microtus fortis	1973
holarctica	I-346	Republic of Buryatia	Ondatra ziberthicus	1977
holarctica	I-347	Republic of Buryatia	Ondatra ziberthicus	1978
holarctica	I-349	Chita territory	Microtus brandti	1977
holarctica	I-365	Republic of Buryatia	Ondatra ziberthicus	1988
holarctica	I-367	Krasnoyarsk region	Microtus arvalis	1989
holarctica	I-373	Chita territory	Ochotona daurica	1989
holarctica	I-382	Republic of Buryatia	Sorex	1991
holarctica	I-387	Novosibirsk territory	Sorex	2010
holarctica	I-388	Novosibirsk territory	Water	2011

to erythromycin was determined. All strains possessed EryS phenotype and were highly virulent (DCL<10 CFU).

For molecular-genetic confirmation of belonging of strains investigated to *F. tularensis* specie PCR analysis was performed using primers specific to genes *fopA* (5'-GCAAATCTAGCAGGTCAAGCAACAGGT-3' and 5'-CATCACCATTTATTGTATAGCACGCGAC-3') and *iglC* (5'-ACAGGTAACAAGTGGCGAGACC-3' and 5'-AAACACCCATAAGTTCTGTTGGCTC-3') of *F. tularensis* bacterium. Electrophoregram of PCR products obtained using primer Chilf and lysates of bacterial samples is represented at the fig. 1.

In tracks of all isolates there are lines with dimensions of ~280 and ~830 bp, common for bacteria of *F. tularensis* species which confirms belonging of strains investigated to *Francisella tularensis* specie. A-1045 culture having a fragment with dimensions of ~570 bp characteristic for Holarctic sub-specie evidences its belonging to *holarctica* sub-specie. Tracks of samples obtained from lysates of strains A-554, A-678, A-823 contain amplicon with dimension of ~950 bp specific for sub-species *mediasiatica* and *tularensis* which allows to classify the isolates investigated to *mediasiatica* subspecies.

For determination of phylogenetic relations of strains investigated with strains deposited to SCPMCC-Obolensk including strains of Siberian tularemia foci received from Irkutsk Anti-Plague Institute VNTR-analysis was performed due to scheme proposed by A. Johanson and co-authors [8]. According to obtained data a phylogenetic tree was drawn up on the basis of Unweighted Pair Group Method with Arithmetic Mean (UPGMA) which is considered to be the most suitable for phylogenetic drawings (fig. 2).

The analysis confirmed subspecies belonging of tularemia microbe strains established by one primer typing: A-1045 strain belongs to Holarctic and A-554, A-678 and A-823 strains to mediasiatica sub-specie.

At that A-1045 strain is in the same cluster with the strains typical for Siberian tularemia foci with a small difference from them as for its VNTR profile. Geographical place of isolation of A-1045 strain (fig. 3) is at the border of natural habitat of analyzed strains, typical for Siberia tularemia foci. Territorial and genetic similarity of these strains in our opinion allows distinguishing separate species of Siberian tularemia agent of *holarctica* subspecies.

A-554 and A-678 strains are genetically identical in 25 VNTR loci. A-823 strain differs from them in length of three loci (Ft-M3, Ft-M7 and Ft-M3), including hyper-variable locus Ft-M3 which allows to determine differences between close-related strains [8]. At that, differences in length of VNTR loci between strains A-574, A-678 and A-823 are less than between those of two other strains of middle Asian subspecies of tularemia microbe represented in collection of SRC AMB (117 and 120). This observation demonstrates presence in Altai territory of a genetic isolated population of *F. tularensis* subsp. *mediasiatica,* natural habitat of which is territorially covered with western part of habitat of Siberian strains of holarctic race of tularemia microbe (fig. 3).

Obtained data evidences of circulation of two

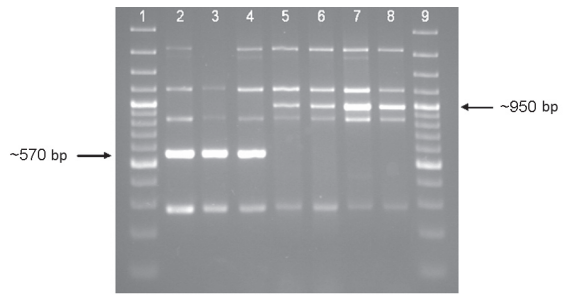

Fig. 1. Electrophoregram of amplicons obtained via PCR with Chilf primer and DNA of *F. tularensis:*

1, 9 – GeneRluer™ 100 bp Plus DNA Ladder; 2 – subsp. *holarctica* 15; *3* – subcp. *holarctica* 503; *4* – A-1045; 5 – A-554; 6 – A-678; 7 – A-823; *8* – subsp. *mediasiatica* 120

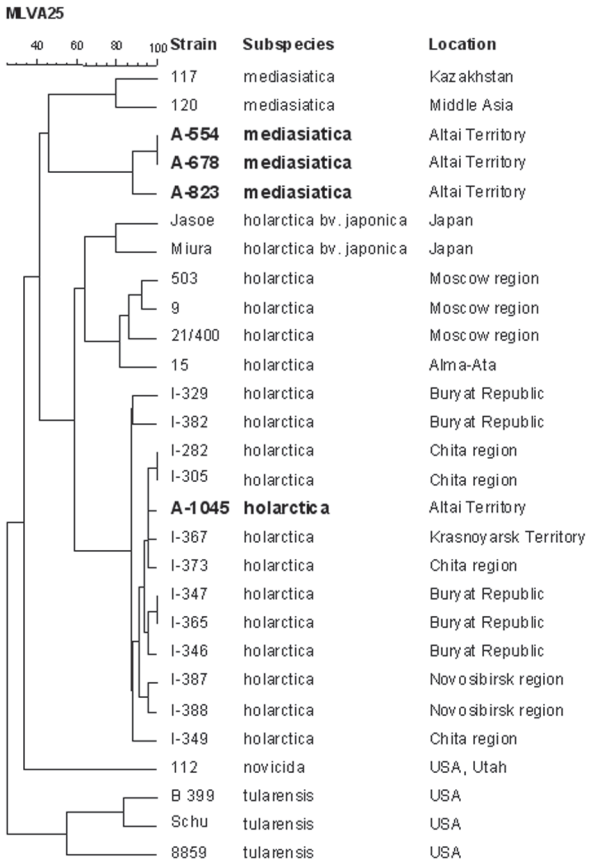

Fig. 2. Phylogenetic tree of *F. tularensis* strains

Fig. 3. *F. tularensis* spreading map, received from Center of hygiene and epidemiology in Altay territory and Irkutsk scientific and research anti-plague institute:

1 – I-346, I-347; *2* – I-382; *3* – I-365; *4* – I-329; *5* – I-349; *6* – I-282, I-305, I-373; *7* – I-388; *8* – I-387; *9* – A-823; *10* – A-678; *11* – A-1045; *12* – A-554; *13* – I-367

tularemia microbe subspecies in Altai territory. Altai isolate of *holarctica* sub-specie is genetically close to the strains typical for Siberian tularemia foci, while three isolates of *mediasiatica* subspecies differ from strains isolated earlier in the territory of Middle Asia (117 and 120). There are no data available in literature about isolation of tularemia microbe cultures of *mediasiatica* subspecies in the territory of the Russian Federation, which may indicate either a lack of knowledge on tularemia foci or drift of Middle Asian strains towards Siberia.

Use of 25-loci VNTR analysis allowed to detect 25 genotypes among 28 tularemia agent strains, which is significantly more than resolution obtained at analysis of 4 loci [3]. PCR differentiation method of *Francisella tularensis* subspecies by means of one primer [2] allowed to determine with at minimum cost sub-specie belonging of newly isolated tularemia cultures.

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