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ВЕСТНИК

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POPULATIONS OF THE MAJOR CARRIER *RHOMBOMYS OPIMUS*, VECTORS OF *XENOPSYLLA* FLEAS AND THE CAUSATIVE AGENT OF *YERSINIA PESTIS* IN THE CENTRAL ASIAN DESERT NATURAL FOCUS OF PLAGUE

Abstract. In the Central Asia desert natural focus of plague, the major carrier of the *Yersinia pestis* agent is the great gerbil *Rhombomys opimus*, and its vectors include fleas of the *Xenopsylla* genus. Phenotypical and genotypical properties of the *R. opimus* populations, *Xenopsylla* fleas and *Yersinia pestis* strains have been studied in the Central Asia desert natural focus of plague. Phenotypic distinctions and population discreteness have been identified in *R. opimus* on the *cytochrome b* gene of the mitochondrial genome from three autonomous plague foci: Pre-Balkhash, Betpakdala and Pre-Ustyurt. Phenotypic distinctions have been found in *Xenopsylla* fleas in the Central Asia desert natural focus of plague, and the genotype of *X. gerbilli minax* fleas on the Cox2 gene of the mitochondrial DNA; these had been captured in the Betpakdala autonomous focus. The repertoire diversity in phenotypical properties of *Y. pestis* strains from different natural foci of plague has been demonstrated, and population discreteness of *Y. pestis* strains has been determined using the next-generation sequencing method for single nucleotide polymorphism genes. Results of the study suggest that geographical and environmental isolation and natural selection have led to heterogeneity in the three populations of the great gerbil, vector fleas and *Y. pestis*.

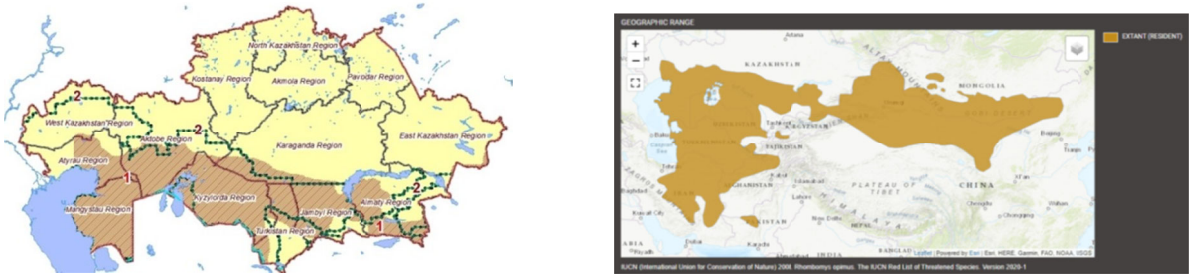
Keywords: plague, natural focus, *Yersinia Pestis*, carrier, *Rhombomys Opimus*, vector, *Xenopsylla*.

1. Introduction

Plague is a zoonotic natural focus based infectious disease. Its causative agent, *Yersinia pestis*, belongs to the *Enterobacteriaceae* family. *Y. pestis* explicably exists in the nature in deserts, steppes and mountain landscapes, within the carrier-vector-agent system. In natural foci, *Y. pestis* carriers include various species of rodents. The role of vectors is played by the rodents' fleas. Stability and sizes of natural foci, which normally correspond to the area of species of the major carrier rodent, demonstrate specificity of the *Y. pestis* ecological niche, the level of systemic interaction between the agent and its natural carriers, and the impact of the selection and selective mechanisms which ensure a dynamic equilibrium between the carrier populations and *Y. pestis* within the focus. In Kazakhstan, natural plague foci cover an area of more than 39% of the total country's area, or approximately 1.1 million square kilometers (figure 1).

Figure 1 - Natural plague foci in Kazakhstan:
I – Central Asian desert natural focus; II – Tien Shan natural focus; III – Volga-Ural sand natural focus; IV – Volga-Ural steppe natural focus; V – Dzungarian mountain natural focus; VI – Talas natural focus; VII – Ural-Oiyl steppe natural focus

In the Central Asia desert natural focus of plague, the major carrier of the *Yersinia pestis* agent is the great gerbil *Rhombomys opimus* Lichtenstein, 1823 (Fig. 2, A) [1, 2]. The roles of secondary and accidental carriers are played by other species of gerbils. There are a total of 14 autonomous foci in Kazakhstan, differing in the landscape diversity, spatial and biocenotic structure [3]. *R. opimus* is also common in desert areas of the Northern Hemisphere [4] (Figure 2, B), including countries with expansive natural plague foci.



A B

Figure 2 - Natural habitat of *R. opimus* in Kazakhstan and worldwide

In individual parts of the natural focus, *R. opimus* populations differ in their phenotypical properties, including the scaffold and dimensions of the skull [5]. Apparently, geographical isolation and different landscapes have determined heterogeneity of the three great gerbil populations in question [6]. To the best of our knowledge, the genome of plague carrier rodents in Central Asian plague foci has not yet been properly investigated while genomes of *R. opimus*, inhabiting areas in Iran and northern China, have been sequenced [7, 8. 9].

Major vectors of plague in the desert focus of Central Asia are *R. opimus* fleas of the *Xenopsylla* genus [3, 10, 11, 12]. A few species of *Xenopsylla* fleas prevail in autonomous foci of the Central Asian natural focus: in Pre-Balkhash - *X. hirtipes*; in Betpakdala - *X. gerbilli minax*, and in Pre-Ustyurt - *X. skrjabini* (figure 3).

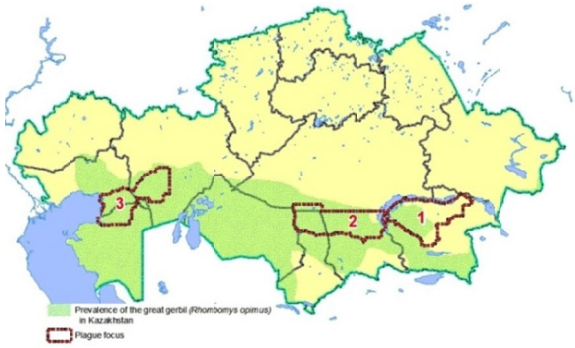


Figure 3 - Habitats of *Xenopsylla* fleas in autonomous foci of the Central Asian natural desert plague focus:
1 - *X. hirtipes* (Pre-Balkhash autonomous focus); 2 - *X. gerbilli minax* (Betpakdala autonomous focus);
3 - *X. skrjabini* (Pre-Ustyurt autonomous focus)

In Kazakhstan, anti-plague stations (NSCEDI branches), located within natural foci, carry out continuous epizootological monitoring of enzootic areas. Only isolated sporadic cases have been recorded in Kazakhstan over the last years, with no epidemic outspread. The last four plague cases were recorded in 2003.

The study of *Y. pestis* strains from some other natural foci of Asia and Europe suggests phenotypical and genotypical diversity of ecological variants of *Y. pestis*. [13, 14]. Different limited areas of the Central Asian natural focus show a steady long-standing circulation of *Y. pestis* strains atypical in the need in amino acids as growth factors [15]. An analysis of *Y. pestis* strains from the Central Asian desert and the Tien Shan mountain natural plague foci with various environmental conditions shows that the *Y. pestis* strains in question belong to three biovars: Antiqua, Mediaevalis and Orientalis [14]. The *Y. pestis* genome has been sequenced, and strains from numerous plague foci have been genotyped [16].

2. Materials and methods

Study materials included: 1) samples of mitochondrial RNA isolated from the liver of *R. opimus;* 2) samples of mitochondrial RNA of *Xenopsylla* fleas; 3) DNA of *Y. pestis* strains from the NSCEDI collection isolated in the Central Asian plague focus and other foci.

All manipulations with *R. Opimus* organs, fleas and *Y. pestis* strains followed appropriate biosafety standards and pathogen handling techniques [17].

Isolation of the *R. opimus* mitochondrial RNA. A total of 88 great gerbil samples had been captured from three independent population groups. Extraction of mitochondrial RNA from the liver of *R. opimus* utilized the QIAamp DNA Mini Kit (Qiagen, USA) [18]. A fragment of *cytB* with the length of 578 bps (without primers) was amplified using UNFOR403 and UNREV1025 primers [19].

The nucleic acid sequence of the D-loop of *R. opimus* DNA was amplified using Thr-L15926 and DL-H16340 primers [20]. PCR product purification involved the enzyme-based method using Exonuclease I (Fermentas) and alkaline phosphatase (Shrimp Alkaline Phosphatase, Fermentas) [21]. Sequencing assay utilized BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The phylogenetic analysis used MEGA 7.0 software, Tamura 3-parameter model, discrete gamma distribution, and Bootstrap 1000 [22].

Phenetic studies of fleas. The material included 681 *Xenopsylla* fleas from autonomous foci and from the collection of the NSCEDI zoological and parasitological museum. Data were processed in free statistical environment R version 4.0.0, with RStudio graphic environment [23]. Head and head bristle measuring was based on pictures of fleas using ImageJ software [24]. The analysis used meristic features [10].

Isolation of mitochondrial DNA and genotyping of *Xenopsylla* fleas. 22 *Xenopsylla* flea samples were selected for the assay. DNA isolation used the established protocol [25]. The *CoxII* nucleic acid sequence fragment was amplified using Insect-A-LEU and Insect-B-T primers [26]. PCR product purification involved used of exonuclease I (Thermo Scientific) and alkaline phosphatase (Thermo Scientific) [21]. Sequencing utilized cycle sequencing kit BigDye® Terminator v3.1 (Applide Biosystems) and primers, for PCR amplification. The phylogenetic analysis used MEGA 7.0 software, with the maximum likelihood method, Tamura 3-parameter model, discrete gamma distribution, and Bootstrap 1000 [22].

***Y. pestis* DNA genotyping. *Y. pestis* DNA genotyping method.**

Isolation of *Y. pestis* strain DNA utilized QIAamp DNA Mini Kit (Qiagen, USA) [25]. Genotyping of 31 *Y. pests* strains was based on the whole-genome sequencing method. Assessment of preliminary sequencing data used FastQC v0.11.7 and Multiqc v1.8 software. The phylogenetic tree derivation used BioNumerics v8.0 software (Applied Maths, Belgium).

3. Results

The epizootic status of plague in the Central Asian natural focus

Over the last decade (2010-2019) of monitoring in the Central Asian desert focus, active plague epizootics have been registered; 1024 *Y. pestis* strains have been isolated and studied. During the study of *R. opimus* and *Xenopsylla* fleas in three autonomous foci, the following numbers have been isolated: in Pre-Balkhash - 264; in Betpakdala – 60, and in Pre-Ustyurt - 20 *Y. pestis* strains.

***R. opimus* and *Xenopsylla* flea populations in the Central Asian natural plague focus.** All three regions in study are in the northern desert area, where special impact on vegetation is made by trends in changing precipitation amounts and frequencies. An analysis of climatic and geographical features has found synchronization degrees of the air temperature and precipitation trends [6]. Intensive growth of the yearly average temperature has been noted in the surface air since the mid-1970s. Therefore, irregularities of climatic, geographical and geobotanical conditions in the three autonomous foci in question have resulted in genetically isolated *R. opimus* population.

A morphometric study was carried out for 681 samples of *Xenopsylla* fleas (*X. hirtipes, X. skrjabini* and *X. g. minax*), captured in the Pre-Balkhash, Betpakdala and Pre-Ustyurt autonomous foci. A number of significant distinctions have been found in *Xenopsylla* fleas regarding their morphometric parameters: position of parietal bristle and the back edge bristle. *X. hirtipes* fleas from the Pre-Balkhash and Betpakdala foci show statistically significant difference from fleas from other foci, regarding the distance between the ocular and parietal bristle (p = 0,00126 и 0,00025, respectively), as well as the distance between the parietal and angular bristle (p = 0,00138 and 0,00402, respectively). This may suggest formation of an independent population of *X. hirtipes* fleas in the Pre-Balkhash and Betpakdala plague foci.

Sequencing of mitochondrial RNA of *R. opimus*. Genotyping of *R. opimus* from the Pre-Balkhash, Betpakdala and Pre-Ustyurt foci has been carried out. Based on a review of publications [7, 9], a conclusion has been made that genotyping on the *CytB* gene sequence should be performed to study population differences in great gerbils.

A total of 19 unique haplotypes have been identified on the fragment of nucleic acid sequence of *CytB* gene. Out of 578 analyzed bases, the share of transitions was 37, and transversions - four. Eight of the polymorphisms analyzed result in amino acid replacement. 88 samples clustered into seven haplogroups. 25 haplotypes were established using *D-loop* nucleic acid sequence. 63 of 468 bases were variable. Of them, 59 were transitions, and three – transversions; also, adenine insertion was found in three samples from I-B15, I-B16, I-B17 samples captured in southern Pre-Balkhash.

The phylogenetic analysis with nucleic acid sequences of 19 haplotypes, established in *R. opimus* from fovi in Kazakhstan, and sequences of *CytB* gene of *R. opimus* captured in Iran and China [7, 8], formed three major clusters (figure 4).

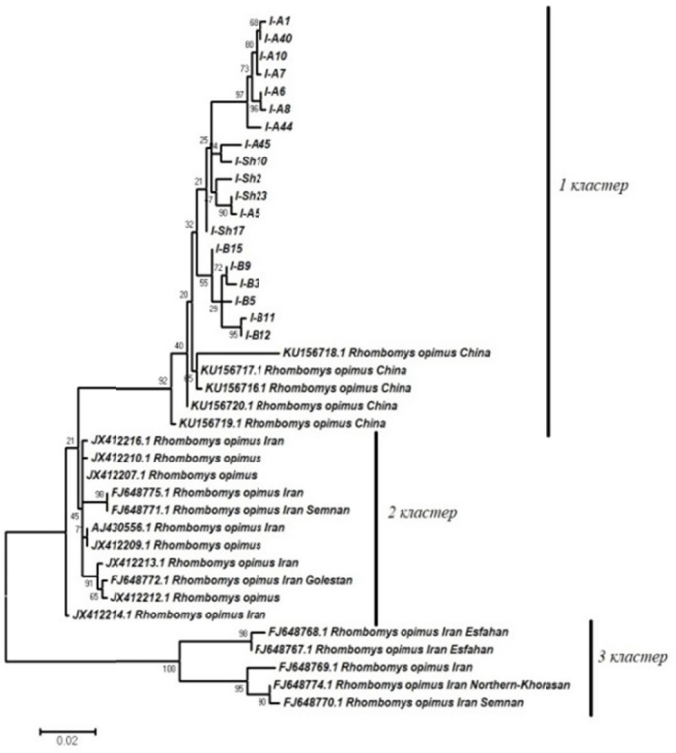


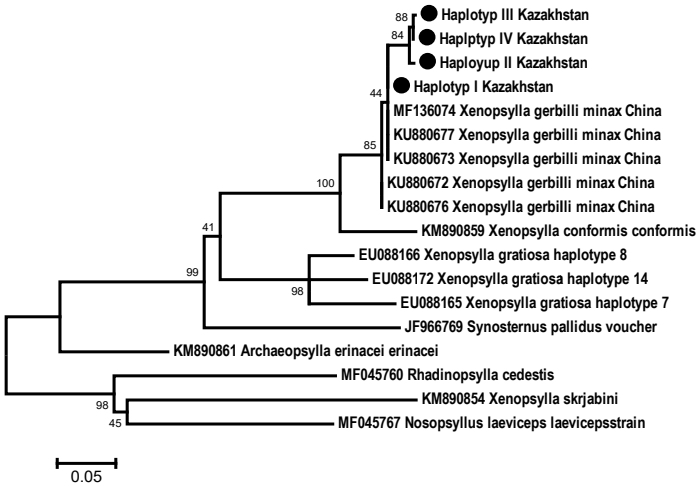
Figure 4 –
The phylogenetic tree based on the analysis of nucleic acid sequence of *CytB* gene of *R. opimus*, captured in Kazakhstan, Iran and China:
B - samples collected in southern Pre-Balkhash;
Sh - samples collected in western Betpakdala;
A - samples collected in eastern Pre-Ustyurt

The first cluster includes sequences of *R. opimus* captured in Kazakhstan and China; concurrently, the great gerbil captured in Kazakhstan is a separate clade. The second and third cluster includes sequences of the great gerbil captured in Iran.

Genotyping, looking for population diversity and genomic features of the *Xenopsylla* genus from the Central Asian desert natural plague focus

A total of 743 bps in 22 *Xenopsylla* samples have been sequenced. The resulting sequences include the whole protein coding sequence *COII* (cytochrome oxidase subunit II) and a fragment of sequence tRNA-Lys. A total of 4 haplotypes have been found in 22 *Xenopsylla* samples. The largest genotype includes 17 sequences from fleas from the southern Pre-Balkhash and western Betpakdala. Flea samples from the eastern Pre-Ustyurt represent a separate clade, are more genetically diverse, and include 3 haplotypes (figure 5, A). An analysis including sequences of the *Xenopsylla* genus allowed to cluster haplotype I with sequences *X. gerbilli minax* collected in China's Xinjiang Uyghur Autonomous Region (figure 5, B). Haplotypes II-IV are unique for Kazakhstan and represent a separate clade.

A



B

Figure 5 - The phylogenetic tree based on nucleic acid sequence of *COII* and fragment tRNA-Lys

Genotyping of *Y. pestis* strain DNA.

Whole-genome data of 31 штамма *Y. pestis* strains have been received. The strains form four phylogenetic branches: 0.PE4, 1.ORI3, 2.MED0 и 2.MED1 (figure 6).

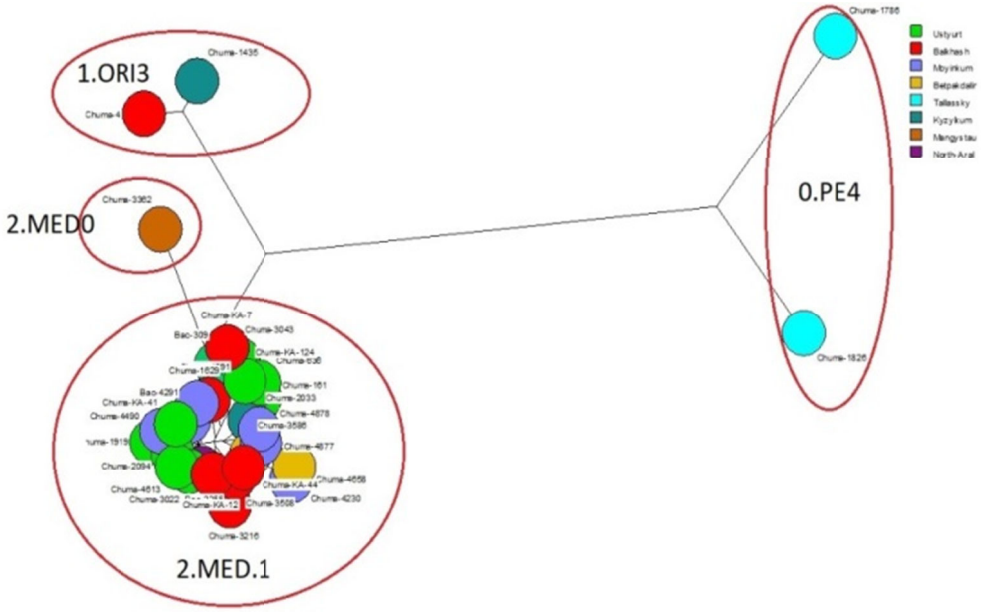


Figure 6 - Clusterization of 31 *Y. pestis* strains

The remaining 26 strains clustered into medieval biovar of the phylogenetic branch 2.MED1. Two strains, Chuma-1435 and Chuma-4, went to the phylogenetic branch 1.ORI3; the strains were isolated from the great gerbil from the Kyzylkum plague focus and from a person infected with plague in the Pre-Balkhash autonomous focus, respectively. Strain Chuma-3362 was phylogenetically identified as 2.MED0; it was isolated from a sick camel in the Mangistau autonomous focus.

4. Conclusions

The results of the studies are as follows: a) Identification of the genetic structure of mitochondrial DNA of *R. opimus* captured in different geographically remote areas in the Central Asian desert natural focus; b) Finding of genetic differences in *Xenopsylla g. minax* fleas regarding the nucleic acid sequence *Cox2* of the mitochondrial DNA gene; c) The studied populations of *Y. pestis* strains are genetically diverse and belong to four phylogenetic branches: 0.PE4, 1.ORI3, 2.MED0 and 2.MED1.

Geographic isolation, different climatic conditions and landscapes, and natural selection determined heterogeneity of three populations of the great gerbil, *Xenopsylla* fleas and *Y. pestis* strains in autonomous foci of the Central Asian desert natural plague focus.

The statistically significant differences of phenotypical features in the great gerbil R. opimus and some species of Xenopsylla fleas suggest formation of distinct populations of rodent carriers and flea vectors in natural plague foci. The studies that have been carried out exemplify comprehensive research of genomic variability regarding co-members of the plague enzootic triade (carrier-vector-agent), and an attempt to find co-evolution of biological species of the plague biocenosis participants, with environmental isolation.

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ОРТАЛЫҚ АЗИЯ ШӨЛДІ ТАБИҒИ ОБА ОШАҒЫНДА ТАРАЛҒАН *YERSINIA PESTIS* КОЗДЫРҒЫШЫН ТАСЫМАЛДАУШЫ *XENOPSYLLA* ТУЫСЫНА ЖАТАТЫН БҮРГЕЛЕР МЕН *RHOMBOMYS OPIMUS* ПОПУЛЯЦИЯЛАРЫ

Аннотация. Ортаазиялық шөлді табиғи оба ошағында Yersinia pestis қоздырғышының негізгі тасымалдаушысы-үлкен rhombomys opimus гербилі, ал оның тасымалдаушылары-Xenopsylla тұқымдас бүргелер. Орталық Азия шөлдеріндегі обаның табиғи ошағында R. opimus популяциясының, Xenopsylla және Yersinia pestis бүрге штаммдарының фенотиптік және генотиптік қасиеттері зерттелді. R.opimus-тегі фенотиптік айырмашылықтар мен популяциялық дискреттілік обаның үш автономды ошақтарынан: Балхаш, Бетпақдала және Прустюртскийден митохондриялық геномға цитохромды генмен анықталды. Фенотиптік айырмашылықтар xenopsylla бүргелерінде Орталық Азия шөліндегі обаның табиғи ошағында және митохондриялық ДНҚ-ның Cox2 генінен X. gerbilli minax бүргесінің генотипінде табылды; олар Бетпақдала автономды ошағында ұсталды. Обаның әртүрлі табиғи ошақтарынан алынған Y. pestis штаммдарының фенотиптік қасиеттерінің репертуарының әртүрлілігі көрсетілген және Y. pestis штаммдарының популяциялық дискреттілігі бір нуклеотидті полиморфизм гендерінің келесі ұрпағын жүйелеу арқылы анықталған. Зерттеу нәтижелері географиялық және экологиялық оқшаулау және табиғи сұрыптау үлкен гербилдің, векторлық бүргелердің және Y. pestis-тің үш популяциясында гетерогенділікке әкелгенін көрсетеді.

Түйін сөздер: оба, табиғи ошағы, Оба қоздырғышы, тасымалдаушы, Үлкен құмтышқан, тасымалдаушы, Ксенопсилла.

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ПОПУЛЯЦИИ ОСНОВНОГО НОСИТЕЛЯ *RHOMBOMYS OPIMUS*, ПЕРЕНОСЧИКОВ БЛОХ РОДА *XENOPSYLLA* И ВОЗБУДИТЕЛЯ *YERSINIA PESTIS* В ЦЕНТРАЛЬНО-АЗИАТСКОМ ПУСТЫННОМ ПРИРОДНОМ ОЧАГЕ ЧУМЫ

Аннотация. В Среднеазиатском пустынном природном очаге чумы основным переносчиком возбудителя Yersinia pestis является большая песчанка Rhombomys opimus, а ее переносчиками являются блохи рода Xenopsylla. Изучены фенотипические и генотипические свойства популяций R. opimus, штаммов блох Xenopsylla и Yersinia pestis в природном очаге чумы в пустынях Центральной Азии. Выявлены фенотипические различия и популяционная дискретность у R. opimus по гену цитохрома в митохондриального генома из трех автономных очагов чумы: Предбалхашского, Бетпакдальского и Предустюртского. Фенотипические различия были обнаружены у блох Xenopsylla в природном очаге чумы в пустыне Центральной Азии и генотипа блох X. gerbilli minax по гену Cox2 митохондриальной ДНК; они были захвачены в автономном очаге Betpakdala. Показано разнообразие репертуара фенотипических свойств штаммов Y. pestis из различных природных очагов чумы и определена популяционная дискретность

штаммов Y. pestis методом секвенирования следующего поколения генов однонуклеотидного полиморфизма. Результаты исследования свидетельствуют о том, что географическая и экологическая изоляция и естественный отбор привели к гетерогенности в трех популяциях большой песчанки, векторных блох и Y. pestis.

Ключевые слова: чума, природный очаг, *Возбудитель чумы*, носитель, *Большая песчанка*, переносчик, *Ксенопсилла*.

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