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BACTERIAL VIRULENCE

Virulent non-capsulate *Yersinia pestis* variants constructed by insertion mutagenesis

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Summary. Insertion mutagenesis with the help of the plasmid pFS23 was used to generate *Yersinia pestis fra* mutants. The results of pFra⁻ strain production under non-selective conditions suggested that such *Y. pestis* variants may be generated in natural plague foci at high frequency and may participate in supporting the epizootic process. Present data suggest that the reduction of virulence in Fra⁻ strains reported by the majority of investigators was connected with the use of *Y. pestis* variants carrying additional unidentified mutations. It was shown that the loss of the ability to produce capsular antigen (FI) alone or in combination with absence of murine toxin production did not lead to an increase in LD50 absolute values. Simultaneous loss of these two virulence determinants did not influence the duration of survival of the infected animals. However, absence of only FI antigen production in the infecting strain resulted in prolonged survival of the infected animals. Conversion of plague infection from acute to chronic form is probably dependent on the animal host species and the *Y. pestis* parent strain subjected to mutagenesis.

Introduction

It has been suggested that the ability of non-capsulate *Yersinia pestis* variants to cause chronic infection[1] can play an important role in supporting the epizootic process in natural plague foci.[2] The laboratory-constructed non-capsulate strains usually showed reduced virulence.[3] The known methods of construction of non-capsulate *Y. pestis* strains were based on spontaneous or induced mutant selections that were of low productivity[4] or led to many unforeseen mutations.[5] Thus, the incubation of *Y. pestis* cultures on magnesium-oxalate agar at 37°C led not only to *fra* locus deletions of the plasmid pFra[5] but also to insertions or deletions of the plasmid pCad and to *Y. pestis* chromosomal mutations.[6] However, the treatment of *Y. pestis* cells by ethidium bromide failed to achieve directed mutagenic action.[7] These mutations resulted in uncontrolled changes of the biological properties of the *fra* mutants.

Recently the plasmid-located *fra* operon encoding capsular antigen (fraction I; FI) was cloned and sequenced.[8-12] It was shown that the *fra* operon consists of four[11] or five[8] open reading frames able to encode proteins. The FI structural gene *ycaF* (*caf1*) was localised near the *Cla*I site.[8,10]

The objective of the present study was to construct *ycaF* (*caf1*) mutants and pFra⁻ variants of virulent *Y. pestis* strains with the help of insertion mutagenesis and to study their biological properties.

Materials and methods

Bacterial strains

Y. pestis strains used in the study are listed in the table.[13] *Escherichia coli* strain HB101[14] was used for genetic engineering manipulations.

Media and culture conditions

The cultures were grown on Luria Broth medium (LB; tryptone 10 g, yeast extract 5 g, NaC1 10 g) supplemented with agar 2 %, pH 7·2, at 37°C (*E. coli*; *Y. pestis* for FI immunochemical testing) and at 28°C *Y. pestis* in all the other cases). Antibiotics were used in the following concentrations: kanamycin 50 mg/L and ampicillin 100 mg/L.

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Table. *Y. pestis* strains: relevant attributes

Strain no.	Virulence determinants (Phenotype)	Source or reference
231(708)	$Fra^+Tox^+Lcr^+V^+Pst^+Fb^+Cg^+Pgm^+$*	CCIM†
231pFra/pFS23	$Fra^-Tox^+Lcr^+V^+Pst^+Fb^+Cg^+Pgm^+$	This study
231pFra$^-$	$Fra^-Tox^+Lcr^+V^+Pst^+Fb^+Cg^+Pgm^+$	This study
231pPst$^-$	$Fra^+Tox^+Lcr^+V^+Pst^-Fb^-Cg^-Pgm^+$	The author's collection
231pPst$^-$pFra/pFS23	$Fra^-Tox^+Lcr^+V^+Pst^-Fb^-Cg^-Pgm^+$	This study
231Psb$^-$	$Fra^+Tox^+Lcr^+V^+Pst^+Fb^+Cg^+Pgm^-$	[13]
231Psb$^-$pFra/pFS23	$Fra^-Tox^+Lcr^+V^+Pst^+Fb^+Cg^+Pgm^-$	This study
358	$Fra^+Tox^+Lcr^+V^+Pst^+Fb^+Cg^+Pgm^+$	CCIM
358pFra/pFS23	$Fra^-Tox^+Lcr^+V^+Pst^+Fb^+Cg^+Pgm^+$	This study
358pFra$^-$	$Fra^-Tox^+Lcr^+V^+Pst^+Fb^+Cg^+Pgm^+$	This study
358pPst$^-$	$Fra^+Tox^+Lcr^+V^+Pst^-Fb^-Cg^-Pgm^+$	The author's collection
358pPst$^-$pFra/pFS23	$Fra^-Tox^+Lcr^+V^+Pst^-Fb^-Cg^-Pgm^+$	This study

*Fra, synthesis of capsular antigen; Tox, synthesis of mouse exotoxin; Lcr, low calcium response; V, synthesis of V antigen; Pst, synthesis of pesticin; Fb, fibrinolysin activity; Cg, coagulase activity; Pgm, storage of exogenous haemin.
†CCIM, The Culture Collection of Institute "Microbe".

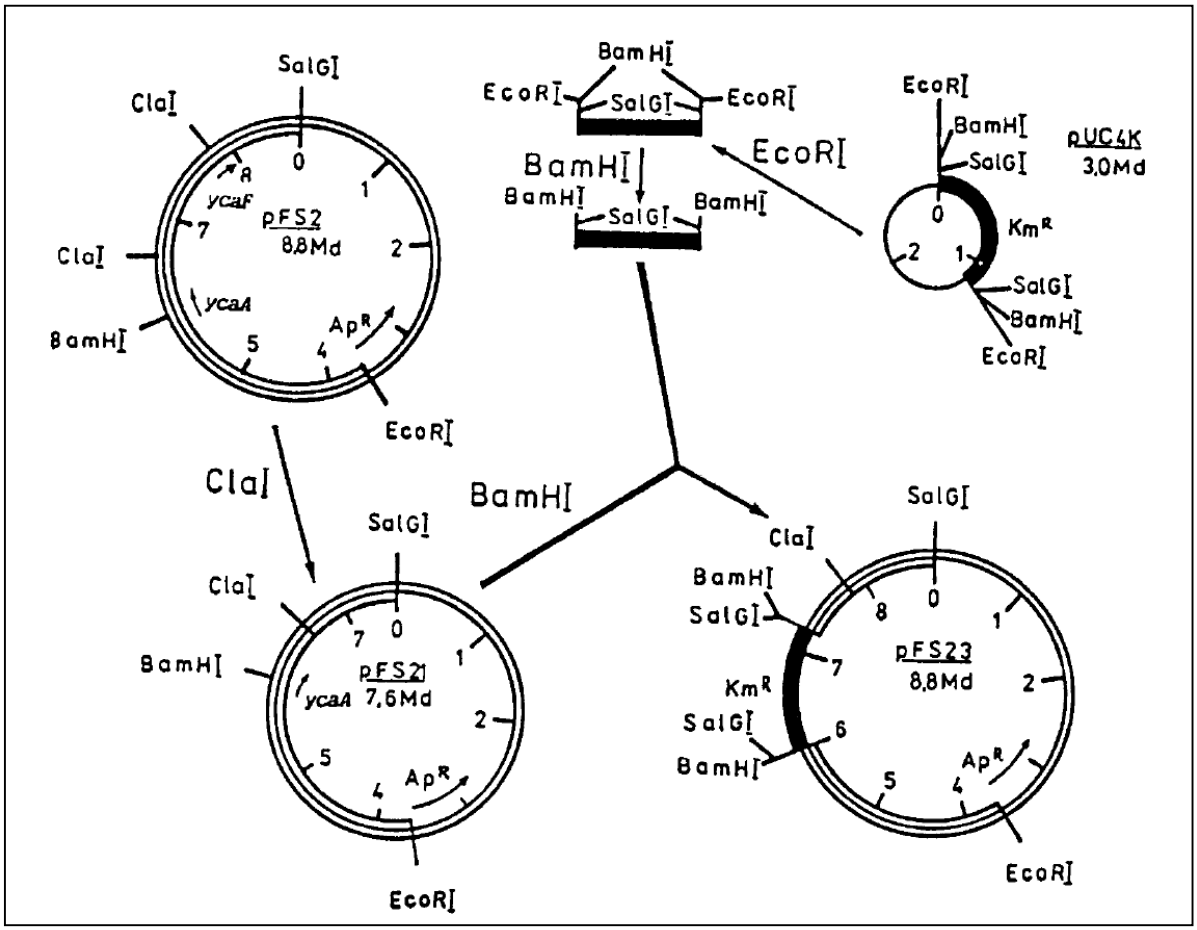

Fig. 1. Construction of pFS23. Isolation of plasmid DNA by alkali-lysis method and genetic engineering methods was performed essentially as described by Maniatis *et al*.[14] Plasmid pFS21 was constructed by deletion of the *Cla*I-*Cla*I fragment from previously constructed plasmid pFS2.[10] The *Bam*HI-*Bam*HI fragment with *kan* (Km^R) locus from pUC4K[16] was isolated and cloned into *Bam*HI site of pFS21. The plasmid generated was named pFS23. The vector fragment of the plasmid pFS23 carried the *bla* (Ap^R) gene and the replication region of the plasmid ColE1. This Co1E 1 *ori* could not provide stable preservation of recombinant plasmids constructed on its basis in *Y. pestis* recipient cells under the non-selective conditions.[17]

Main phenotypic determinants

The main phenotypic determinants encoded by *Y. pestis* plasmids and chromosome were analysed as described previously[15] (FI antigen production was tested by haemagglutination tests for FI antigen detection).

Construction of the pFS23 plasmid

The scheme of the pFS23 plasmid construction is shown in fig. 1. Plasmids were isolated from *E. coli* and *Y. pestis* by the alkali-lysis method of Birnboim and Doly.[14] Protocols for restriction enzyme digestions, and the use of T4 DNA ligase, were essentially as

described by Maniatis *et al.*[14] All enzymes were used as recommended by the manufacturers.

Plasmid pFS21 was constructed from previously constructed plasmid pFS2[10] by deleting the 1986-bp *Cla*I-*Cla*I fragment and ligating the two ends. This deletion includes the 173-bp region from the beginning of the FI structural gene *ycaF*. The *Bam*HI-*Bam*HI fragment with *kan* (Km^R) locus from pUC4K[16] was isolated and cloned into the *Bam*HI site of pFS21. The plasmid generated was named pFS23. The vector fragment of the plasmid pFS23 carried the *bla* (Ap^R) gene and the replication region of the plasmid ColE1. This ColE1 *ori* could not provide stable preservation of recombinant plasmids constructed on its basis in *Y. pestis* recipient cells in unselective conditions.[17]

Transformation of Y. pestis

Y. pestis was transformed with plasmid DNA by electroporation as described by Yezhov *et al.*[18]

Construction of the ycaF Y. pestis mutants

Recipient *Y. pestis* cells were transformed by the specially constructed target plasmid pFS23. The plasmid pFra *Sal*GI-*Eco*RI fragment cloned in pFS23 had a deletion of the *Cla*I-*Cla*I region consisting of a part of *ycaF* gene and insertion of *kan* gene into the *Bam*HI site of *fra* operon. The Km^R marker flanked by the regions homologous to the pFra *fra* operon was able to enter into homologous recombination with the latter. This allowed generation of homologous recombinants with the intact DNA sequence substituted by a *ycaF*-defective DNA fragment of the plasmid pFS23 harbouring the Km^R marker. Cultivation of recombinant cells on ampicillin-free medium did not prevent elimination of the pFS23 vector region and allowed selection of *Y. pestis* $Km^RAp^SFra^-Tox^+$ variants. The advantages of this method were the absence of translocations and the stability of the recombinants.

Selection was performed in the following way. Km^R clones of the *Y. pestis* transformants were examined for expression of the Ap^R phenotype. Km^RAp^S variants were found at a frequency of 0·1-15 % of the whole recombinant cell population.

Preliminary passage of the Km^R transformant mixture in previously immunised mice (10^8 cfu/animal) increased the proportion of the tested Km^RAp^S clones in the *Y. pestis* cultures from dead animals up to 50-100 %. These mice were immunised by FI antigen (20 μg/animal) 21 days before injection. The Km^RAp^S clones were tested for FI antigen production by haemagglutination. Km^RAp^S *Y. pestis* cultures were shown to be Fra^- in all clones analysed.

Selection of $pFra^-$ ($pFra/pFS23^-$) clones

The main difficulty in the construction of *Y. pestis* $pFra^-$ variant is the absence of a convenient marker for negative selection. In the selective method described below, the use of *Y. pestis* strains carrying pFra/pFS23 plasmids with the Km^R marker provided a solution to this problem.

The *Y. pestis* 231pFra/pFS23 and 358pFra/pFS23 cultures were grown on LB agar plates containing kanamycin at subinhibitory concentrations. These concentrations were defined experimentally for every strain. About 300 cells were sown on agar plates containing kanamycin (1-10 μg/ml) and grown at 28°C. The loss of Km^R was manifest by inhibition of cell growth. Two variants of bacterial colonies were seen on the plates containing kanamycin (1-2 μg/ml) after incubation for 3-5 days; these were 0·5-1·5 mm and 2·0-3·5 mm in diameter respectively. The clones from the first group were analysed on agar plates containing a completely inhibitory concentration of kanamycin (50 μg/ml). Km^S mutations of these strains arose at the frequency of ($2{\cdot}5 \times 10^{-5}$-$5{\cdot}0 \times 10^{-5}$). Km^S clones examined by plasmid screening[14] were shown to be $pFra^-$ in all clones analysed.

Definition of bacterial virulence for mice and guinea-pigs

Outbred mice weighing *c.* 20 g or outbred guinea-pigs weighing *c.* 250 g were given suspensions of 48-h agar cultures in NaC1 0·89 % solution in a total volume of 0·2 ml (with 10-fold dilutions) by subcutaneous injection. The animals were observed for 21 days. LD50 values and confidence intervals were measured according to the method of Kärber modified by Ashmarin and Vorob'ov.[19] The confidence interval was determined for a probability of 95 %.

Results

Comparative analyses of Y. pestis Fra^+ and Fra^- strains

The main biological properties of the strains were compared. There were no differences between wild strains and their Fra^- variants in: (i) cultural properties; (ii) nutritional requirements; (iii) fermentation of glycerol, rhamnose and urea; (iv) phage sensitivity. The parent strains and their Fra^- derivatives did not differ in other virulence-related properties such as low calcium response, synthesis of V-antigen, synthesis of pesticin, fibrinolysin activity, coagulase activity or storage of exogenous haemin. Synthesis of murine exotoxin was retained by the Fra^- strains generated with the help of the insertion mutagenesis but not in the $pFra^-$ variants.

No consistent differences were found in the LD50 values for mice and guinea-pigs of parent Fra^+ strains and their Fra^- derivatives. The only exception was a pair of strains, $231Psb^-$ and $231Psb^-$pFra/pFS23, whose LD50 values for guinea-pigs were 10 (2-24) cfu and 267 (67-966) cfu, respectively (fig. 2). Mice infected with strain 231 or its derivatives died in approximately the same periods of time (fig. 3).

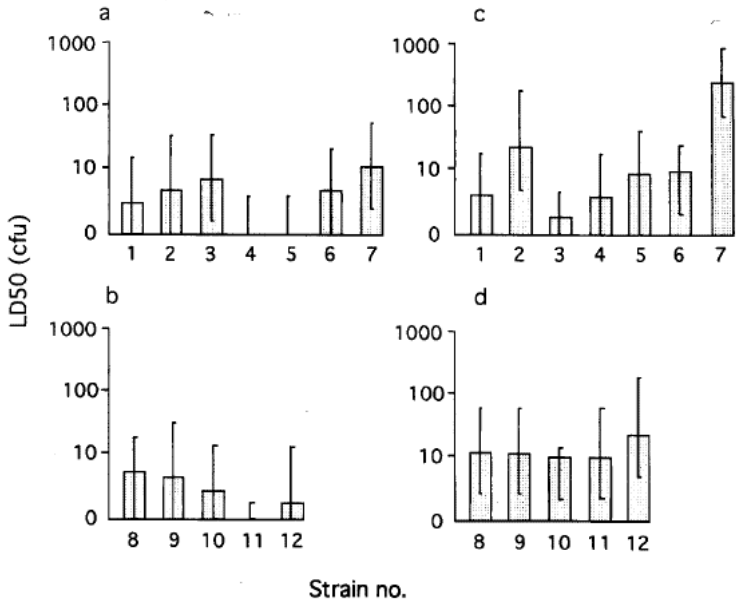

Fig. 2. LD50 of Fra⁻ strain 231; 2, 231pFra/pFS23; 3, 231pFra⁻; 4, 231pPst⁻; 5, 2 pFS23; 10, 358pFra⁻; 11, 358pPst⁻; 12, 358pPst⁻pFra

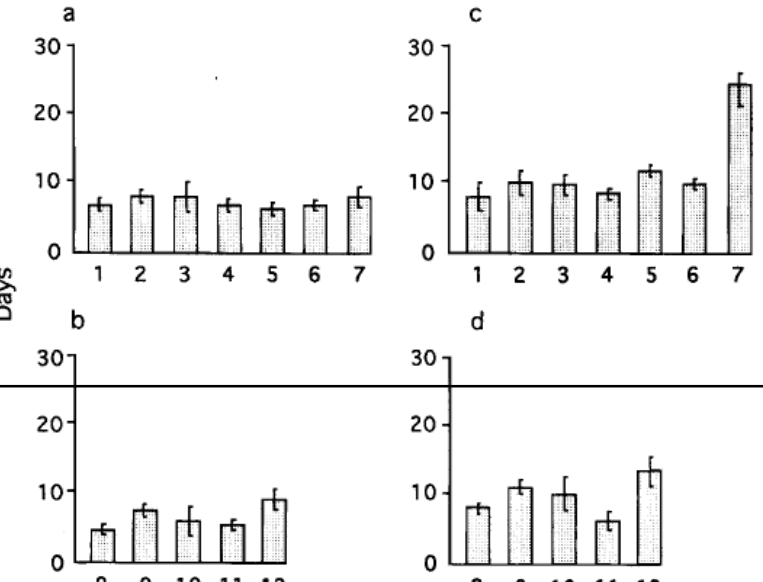

Fig. 3. Average life dura ariants. 1, strain no. 231; 2, 231pFra/pFS23; 3, ra/pFS23; 8, 358; 9, 358pFra/pFS23; 10, 35 for probability of 95 %.

However, infection isms that allow derivatives of strain ns to resist the significantly longer species is one of of the strains and biological 231pFra/pFS23, ca s problem may be after substantially lo parent strains.

achieved by identification of the genetic determinants encoding immunogenic proteins, clarification of mechanisms of regulation of their expression, and modelling their antigenic and immunogenic activity in experiments. *Y. pestis* Fra$^-$ strains, in comparison with the wild strains, had a selective survival advantage in mice immunised with the live plague vaccine.[20] Such strains might be produced by insertion of *Y. pestis* insertion elements[6,21] into the region of the *fra* operon or by spontaneous elimination of the plasmid pFra. The results of creating pFra$^-$ strains under non-selective conditions suggested that such *Y. pestis* variants could be generated in natural plague foci at fairly high frequency and may participate in supporting the epizootic process.

It was reported previously that *Y. pestis* mutations to Fra$^-$ phenotype caused a significant reduction of virulence.[3,4] Present data suggest that this may have been due to the fact that the majority of investigators used *Y. pestis* variants carrying additional unidentified mutations. The present study, with isogenic strain sets, showed that the loss of FI production ability alone or in combination with the absence of murine toxin production did not lead to an increase in LD50 absolute values. Simultaneous loss of these two virulence determinants did not influence the duration of survival of the infected animals. However, in the case of absence of FI antigen production alone in the infecting strain, the duration of survival in the infected animals increased. Conversion of plague infection into its chronic form depended on the animal species and the *Y. pestis* parent strain subjected to mutagenesis.

These data allow us to speculate that loss of the ability to produce antigen FI may lead to a decrease of the propagation rate of *Y. pestis* in the host organism. This would be manifest as an increased duration of survival of the infected animals. The loss of both virulence determinants encoded by the plasmid pFra could lead to a further increase of duration of infection and even to an increase in LD50. However, it is compensated by conversion of energy sources of the bacterial cell from synthesis of the plasmid pFra products to synthesis of other pathogenicity factors and to intensification of cell division. This, respectively, leads to the reduction of duration of survival in the infected animals corresponding to that in animals infected with the wild strains of *Y. pestis.*

These findings are in agreement with the data of Shanina[20] who described the highly virulent pFra$^-$ strain 358/12.

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References

1. Williams JE, Cavanaugh DC. Chronic infections in laboratory rodents from inoculation of nonencapsulated plague bacilli *(Yersinia pestis). Experientia* 1983; **39:** 408-409.
2. Punski AA, Adamenko NI. Epizootological meaning of *Yersinia pestis* strains atypical in connection with their different ability of fraction I production. In: Bagirov BG (ed) Medicogeographical problems of arid zone. Ashgabad, Ilim Press. 1982:36-38.
3. Brubaker RR. The genus *Yersinia:* biochemistry and genetics of virulence. *Curr Top Microbiol Immunol* 1972; **57:** 111-158.
4. Akimovich VV, Shanina LN. *Yersinia pestis* variants unable to produce capsular antigen. In: Nikolaev NI (ed) Microbiological and laboratory questions of diagnostics of particularly dangerous infections. Saratov, Privolzhskoe Knizhnoe Press. 1965: 54-58.
5. Zarenkov MI, Goncharova NA. The method of creating *Yersinia pestis* Fra$^-$ mutants. Authors' Certificate No 1439122, USSR, Int. Classif. Index C12 N15/00. *Byulleten Izobreteniy i 0tkrytiy* (Moscow) 1988: **43.**
6. Portnoy DA, Falkow S. Virulence-associated plasmids from *Yersinia enterocolitica* and *Yersinia pestis. J Bacreriol* 1981; **148:** 877-883.
7. Tsukano H, Wake A, Sakakibara Y. Plasmid-like properties of the four virulence-associated factors of *Yersinia pestis. Microbiol Immunol* 1986; **30:** 837-848.
8. Cherepanov PA, Mihaylova TG, Karimova GA, Zaharova NM. The *fra* operon: the complete nucleotide sequence. In: Borovik RV (ed) New technologies and biosystems. Achievements and perspectives. Obolensk, Medbioekonomika Press 1991: 17-20.
9. Galyov AA, Karlishev AV, Chernovskaya TV *et al.* Expression of the envelope antigen F1 of *Yersinia pestis* is mediated by the product of *caf1M* gene having homology with the chaperone protein PapD of *Escherichia coli. FEBS Lett* 1991; **286:** 79-82.
10. Galyov AA, Smirnov OYu, Karlishev AV *et al.* Nucleotide sequence of the *Yersinia pestis* gene encoding F1 antigen and the primary structure of the protein. *FEBS Lett* 1990; **277:** 230-232.
11. Karlyshev AV, Galyov AA, Abramov VM, Zav'yalov VP. *Caf1R* gene and its role in the regulation of capsule formation of *Y. pestis. FEBS Lett* 1992; **305:** 37-40.
12. Karlyshev AV, Galyov AA, Smirnov OYu, Guzayev AP, Abramov VM, Zav'yalov VP. A new gene of the *f1* operon of *Y. pestis* involved in the capsule biogenesis. *FEBS Lett* 1992; **297:** 77-80.
13. Kutyrev VV, Filippov AA, Oparina OS, Protsenko OA. Analysis of *Yersinia pestis* chromosomal determinants Pgm$^{\mp}$ and Pst^S associated with virulence. *Microb Pathog* 1992; **12:** 177-186.
14. Maniatis T, Fritsch AA, Sambrook J, Molecular cloning: A laboratory manual. Moscow, Mir Press. 1984: 89-244.
15. Klassovskiy LN, Stepanov VM, Martinevskiy IL, Shmuter MF, Pak GU. Guidance for studying new *Yersinia pestis* strains isolated from natural foci. Alma-Ata, Kazakh Agricultural Institute Press. 1972: 1-35.
16. Vieira J, Messing J. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* 1982; **19:** 259-268.
17. Anisimov AP, Karlishev AV, Pavlov VM, Kravchenko VI. Construction of recombinant plasmids with stable maintenance in *Yersinia pestis* recipient cells. In: Anisimov PI (ed) Genetics, microbiology and improvement of laboratory diagnostic methods of dangerous diseases. Saratov, SVVKIU Press. 1991: 18-25.
18. Yezhov IN, Yeremin SA, Tuchkov IV, Nikitin AN, Drozdov IG, Anisimov PI. Transformation of *Yersinia pestis* by electroporation. In: Anisimov PI (ed) Laboratory diagnostics and genetics of virulence of causative agents of dangerous diseases. Saratov, Kommunist Press. 1990: 92-95.
19. Ashmarin IP, Vorob'ov AA. Statistical methods in microbiological research. Leningrad, State Press of Medical Literature. 1962:85-104.
20. Shanina LN. Immunogenic properties of *Pasteurella pestis* nonencapsulated strains established in experiments performed on the model of guinea pigs and mice. In: Domaradskiy IV (ed) Genetics, biochemistry and immunochemistry, vol 1. Rostov on Don, Rostov University Press. 1967: 373-381.
21. Filippov A A, Oleinikov PN, Drozdov AV, Protsenko OA. The role of IS-elements of *Yersinia pestis* in the emergence of calcium-independent mutations. *Genetika* 1990; **26:** 1740-1748.