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REVIEW ARTICLE

FACTORS OF *Yersinia pestis* PROVIDING FOR CIRCULATION AND PERSISTENCE OF THE PLAGUE PATHOGEN IN ECOSYSTEMS OF NATURAL FOCI. COMMUNICATION 2

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For constant circulation in natural foci, the plague pathogen must penetrate into the host organism, withstand the protective bactericidal systems of the rodent, and reproduce to ensure the bacteriemia necessary for further transmission by fleas to a new host. Each of these stages in the cyclic existence of *Y. pestis* is supported by numerous factors of the plague pathogen, which can act jointly or individually. Each of these factors in turn may be involved in various stages of the infection process or transmission. But only all together do these factors provide for persistence of the plague pathogen in natural foci, no matter how significant or negligible their individual effect may be. The second communication discusses factors of the plague pathogen providing for its transmission from one host to another, and also the relationship of individual pathogenicity factors and the expression of various "housekeeping" genes to the virulence of the plague pathogen. In compiling the review, not only the widely known publications but also studies published in relatively inaccessible sources, especially for English-speaking specialists, have been used.

RETURNING TO WHAT WAS PUBLISHED

In concluding the first communication [4], we directed the reader to "the most interesting, in our opinion, attempts to reproduce possible scenarios of the pathogenic process in plague." After the manuscript was given to the editors of the journal, a whole series of original and analytical articles were published, which I should have liked to cite in this review. Understanding that "you can't encompass the infinite" [45], nonetheless, let us direct the reader to the studies of V. A. Fedorova and Z. L. Devdariani [74, 75]. Without going into a polemic on the possibility of the formation of full-sized LPS with O-polysaccharide chains in the cells of the plague pathogen, I should like to call attention to the fact that the authors of the work used original methodological approaches, which permitted them to establish the ability of *Y. pestis* to penetrate into mammalian erythrocytes and to provide for its own nutrient requirements by utilizing the compounds present in them. In the course of the investigations, they established a whole series of peculiarities of the production of bacterial proteins, utilization of growth factors, and reproduction of *Y. pestis* on media simulating the contents of erythrocytes and phagolysosomes, thereby suggesting a nontraditional concept of the pathogenetic process in plague, considering the erythrocytes as the main target for *Y. pestis* in the bodies of mammals sensitive to the disease. It has been suggested that the destruction of erythrocytes by the plague pathogen is the cause of progressive tissue hypoxia, which ultimately leads to death of the macroorganism.

Also noteworthy is the review article by L. M. Kukleva *et al.* [28], in which "it is suggested that at this stage of evolution, non-main subspecies of the plague microbe (*caucasica*, *altaica*, *hissarica*, and *ulegeica*) occupy an intermediate position between the plague pathogen of the main subspecies *pestis* and *Y. pseudotuberculosis*."

Table 1

Factors Providing for Flea-Borne Transfer

5.1.Factors providing for intensive bacteriemia
5.2.Factors providing for death of infected animal and search for new feeders
5.3.Factors providing for colonization of midgut of flea
5.4.Factors providing for blockage
5.5.Factors providing for vector-borne transfer by unblocked fleas

FACTORS PROVIDING FOR TRANSMISSION OF *Y. pestis*

Questions of the epizootology and epidemiology of plague, namely, the sources of the pathogen of the infection, the mechanisms of transmission, susceptibility of a population or group, patterns of spread of the disease depending on environmental factors, are discussed in detail in a whole series of publications [6, 16-18, 22, 24, 33, 34, 35, 50, 54, 69, 87]. We shall examine only the factors of *Y. pestis* that provide for various mechanisms of transmission of the pathogen of the infection.

5. Factors Providing for Block Formation and Vector-Borne Transfer

The classic way that *Y. pestis* is transferred to a new host is transmission of the plague pathogen by blocked fleas [58]. High lethality of the plague infection is customarily considered as one of the main conditions promoting effective transmission. Blocked fleas that have fed on rodent blood during preagonal bacteriemia seek out new feeders after death of the rodent, and in the course of bloodsucking, part of the swallowed blood washing the clump of microbes blocking the proventriculus is regurgitated by the insect into the blood vessel, infecting the new host. The higher the virulence of the circulating *Y. pestis* population, the faster the animals die, the more often there is a change of hosts, and the more intensively the epizootic progresses [54, 61]. The subdivision of *Y. pestis* factors that provide for flea-borne transfer into four categories is presented (Table 1).

5.1. Factors Providing for Intensive Bacteriemia

It has been established that fleas are capable of being infected at bacteriemia indices beginning with a concentration of 10^3 CFU/ml. However, in these cases the frequency of block formation is significantly lower than in the case of intensive septicemia (10^5-10^9 CFU/ml). Bites of unblocked fleas, on the contrary, possess low infectiveness and, as a rule, lead not to the penetration of *Y. pestis* under the skin but to an insemination of the integument. It has been suggested that the infection of rodents occurs in this case when the skin is scratched at the sites of ectoparasite bites. It has been shown that loss of the plasmid pPst leads to a decrease in the intensity of bacteriemia in mice, midday gerbils, and little susliks in the case of subcutaneous [24, 92], oral, and flea-borne [24] infection.

5.2. Factors Providing for Death of the Infected Animal and Search for New Feeders

R. Brubaker [61] believes that it is precisely "murine" toxin that, causing the death of *Muridoe* (Old World rats and mice), promotes flea-borne spread of the infection. In our opinion, this consideration is also correct for any of the factors that provide for the development of infectious toxic shock (see section 1.9 [4]). It has recently been suggested that the death of animals is due in turn to hypoxia that arises as a result of intensive destruction of erythrocytes [75].

5.3. Factors Providing for Colonization of the Midgut

It has been established that effective blocking occurs only in cases of preliminary colonization of the flea's midgut by *Y. pestis.* According to the data of B. Hinnebusch *et al.* [77], the Ymt protein is responsible for this process. Morphological manifestations typical of unstable L-forms of bacteria have been detected in plague pathogens

Table 2

Virulence of "Wild Type" *Y. pestis* Strains Grown at 28°C in the Case of Various Modes of Experimental Infection

Mode of infection	Animal species	*Y. pestis* strain	Virulence		Reference
			LD_{50}, CFU	Average life duration, days	
Subcutaneous	Mice	231	**3** (1-18)	**7,3** (4-6)	[71]
		358	**7** (1-27)	**4,6** (3-5)	
		CO92	**1,9**	ND*	[76]
	Guinea pigs	231	**4** (1-22)	**8,1** (5-9)	[71]
		358	**13** (3-63)	**8,6** (5-9)	
Aerosol	Mice	358	$\mathbf{6{,}7 \times 10^2}$	ND	[24]
		CO92	$\mathbf{2{,}3 \times 10^4}$	ND	[76]
	Guinea pigs	358	$\mathbf{2{,}1 \times 10^3}$ (618-10120)	**8,3** (7-10)	[91]
		CO92	$\mathbf{4 \times 10^4}$	ND	[76]
Oral	Mice	358	$\mathbf{1{,}9 \times 10^7}$	ND	[24]
	Meriones meridianus		$\mathbf{1{,}9 \times 10^7}$	ND	
	Citellus pygmaeus		$\mathbf{2{,}2 \times 10^7}$	ND	
	CF1 mice	V?**	$\mathbf{2{,}1 \times 10^6}$	ND	[64]
	Zygodontomys pixuna	B?***	$\mathbf{2{,}9 \times 10^5}$	ND	
	Rattus rattus		$\mathbf{4{,}2 \times 10^9}$	ND	

Note. * ND, no data; **, strain was isolated in Vietnam from a human patient in 1974; *** strain was isolated in Brazil from a human patient in 1977. The confidence interval for the 95% probability level is indicated in parentheses.

surviving in fleas [26].

An analysis of the genome of the strain *Y. pestis* CO92 [86] revealed sequences similar to the genes of insecticidal toxins produced by *Photorhabdus luminescens, Serratia entomophila,* and *Xenorhablus nematophilus.* Probably the products of these genes are also important for the colonization of fleas.

5.4. Factors Providing for Blockage

The factors providing for the block-forming activity of *Y. pestis* were discussed in our previous review publication [2].

5.5. Factors Providing for Vector-Borne Transfer by Unblocked Fleas

Unestablished factors of *Y. pestis* that provide for transfer of the plague microbe by unblocked fleas [24] are also present in *Y. pseudotuberculosis*, infection of *Xenopsylla cheopis* fleas by which leads to a disruption of the valve function of the fleas' proventriculus, which makes it possible for "rodents to be infected after belching of unblocked fleas, the stomach of which contains bacteria of the virulent strain" [13].

6. FACTORS PROVIDING FOR NON-VECTOR-BORNE MECHANISMS OF TRANSFER

Clinical and epidemiological observations and the data of experimental investigations provide evidence that people, experimental and wild animals can be infected by plague not only by flea bites but also by aerosol, contact (through damaged skin), and oral routes [24, 48, 64, 68, 87]. The effectiveness of infection in the routes of penetration of *Y. pestis* into the host organism enumerated above is rather high. A. M. Kokushkin [24] notes: "On the average, considering the impossibility of determining the LD_{50} index during

Table 3. Factors Providing for Non-Vector-Borne Mechanisms of Infection

6.1. Factors providing for penetration into the host organism during aerosol and oral infection
6.2. Factors providing for persistence of *Y. pestis* in the "environment"
6.2.1. Intracellular parasitism in soil protozoa
6.2.2. Putative ability to exist in the phytophase — in xerophyte plants
6.2.3. Formation of unculturable forms
6.2.4. Formation of bacterial nanocells
6.2.5. Transition into a prototrophic state
6.2.6. The plasmid pYC

flea-bite transmission, we can say that when the plague pathogen possessing a full set of virulence determinants is introduced into outbred mice, up to 100% die of the generalized form of the disease in the case of subcutaneous infection, about 50% in the case of aerosol infection, up to 89.3% in oral infection, and about 30% of the animals in flea-borne infection." The values of LD_{50}, for various modes of infection of the animals are presented in Table 2.

From Table 2 it is evident that the LD_{50} values in the case of oral infection significantly exceed those in the case of subcutaneous infection of animals. However, as is correctly noted by A. M. Kokushkin [24], they correspond to or are even lower than the LD_{50} indices characteristic of the pathogens of typical "enteric infections of rodents — salmonellosis and yersinosis" in the case of an analogous mode of infection.

The factors providing for non-flea-bite mechanisms of transmission, in our opinion, can be subdivided as follows (Table 3).

6.1. Factors Providing for Penetration into the Host Organism During Respiratory and Oral Infection

These factors were discussed in section 1.5.2.

6.2. Factors Providing for Persistence of *Y. pestis* in the "Environment"

To explain the mechanism of the persistence of *Y. pestis* during the interepizootic period, M. Baltazard [59] advanced the hypothesis of "telluric plague," postulating two ecological phases of plague: brief (parasitic) and providing for persistence of the plague pathogen (soil). Actually, according to the data of different authors, culturable forms of *Y. pestis* are capable of persisting for 7-16 months and even of reproducing for some time in various types both of sterile and of non-sterile soils [18, 40, 50].

In recent years this hypothesis has received further development in the new concept of the natural focality of infectious diseases, developed under the guidance of V. Yu. Litvin [34, 35]. According to this concept, natural foci of infection represent a whole complex of surface, soil, and/or aquatic ecosystems, including a population of the pathogenic microorganism. In contrast to surface ecosystems, in soil and aquatic ecosystems the hosts of the pathogens of "sapronoses" are soil invertebrates and hydrobionts, among which microbes can circulate along various food chains of the biocoenosis from lower trophic levels to higher ones. The pathogens of sapronoses in soil and aquatic ecosystems can lead a truly saprophytic existence or can be parasites or enter into other symbiotic relationships with fauna and flora, while retaining their potential pathogenicity for terrestrial warm-blooded hosts. According to the ecological and epidemiological classification of infectious diseases of humans, plague has been assigned to the zoophilic sapronoses (saprozoonoses) [33].

We should mention that the concept of the natural focality of infectious diseases proposed by V. Yu. Litvin [34, 35] rests to a significant degree on L. Rahme's idea of the polyhostal nature of pathogenic bacteria, brilliantly confirmed in a whole cycle of investigations studying the polypathogenicity of *Pseudomonas aeruginosa* with respect to mammals, insects, nematodes, protozoa, and plants [65, 85, 88-90]. Recently the study of the interaction of pathogens with nontraditional hosts has been attracting increasing numbers of adherents [93].

6.2.1. Intracellular Parasitism in Soil Protozoa

S. V. Nikul'shin *et al*. [42] have shown the possibility of long-term persistence of the plague pathogen in a viable state in cysts of amebas.

According to the data of Yu. G. Suchkov *et al*. [52] *Euglena gracilis* is capable of supporting of the existence of *Y. pestis.* The numbers of bacteria were sharply reduced over a period of three days, down to 0.001% of the initial concentration; however, in this concentration they continued to be isolated up to the end of the observations – 30 days.

6.2.2. Putative Ability to Exist in the Phytophase — in Xerophyte Plants

Yu. Z. Rivkus and V. M. Bochkar [47] have experimentally demonstrated the possibility of penetration of the bacterial strain *Y. pestis* EV into the stem of *Impatiens walleriana* through roots immersed in a microbial suspension. In their opinion, this is evidence of the ability of *Y. pestis* to colonize the root system and vegetative organs of higher plants, which in turn confirms their earlier hypothesis [46] of the possibility of persistence of the plague pathogen in plants during epizootic periods. "In a period of 1-2 days the bacteria rose 10-20 cm along the stem, overcoming the natural barriers (structures of the conducting system, chemical and immunological resistance of the plant)" [47]. The authors of the publication note that "the object of an infection belongs to the genus *Impatiens* − a component of the grassy floor of forests of Central Asia, in particular, the Fergan ridge. However, this species, used as a decorative plant, does not grow in natural foci. It was selected ... only as a representative of vascular plants, the group of which also includes the *Chenopodiaceae* (saxaul, saltworts) − background species of the flora of desert plague foci."

In connection with the development of the hypothesis of the "phytophase" ... and the high degree of "analogy in the biological structure and physiological function of hemoglobin of the blood and chlorophyll," an attempt was undertaken to evaluate the possibility of satisfying the nutrient needs of fleas using plants [56]. An ability of flea imagos to assimilate plant food was established. Although the fleas *Ciltellophyllus tesquorum sungaris* and *Xenopsylla cheopis* fed less willingly on a suspension of chlorophyll in physiological saline solution or a mixture of it with guinea pig blood than on the blood of an animal without admixtures, the survival of the insects was approximately the same in all three groups. We should mention that, according to the data of a number of researchers, block formation was noted when the fleas were fed with blood preparations or blood substitutes not containing formed elements. When hemoglobin was present in them, it promoted the formation of conglomerates of *Y. pestis* cells in liquid nutrient media as well [5]. Considering the publications cited above, it would be interesting to evaluate the possibility of inducing blockage when fleas are fed with preparations of chlorophyll and the plague microbe is transmitted along the chain r o d e n t—f l e a—r o d e n t—s o i l—p l a n t—f l e a—r o d e n t.

6.2.3. Formation of Unculturable Forms

The possibility of transition of the plague microbe into an unculturable state both in a sterile soil extract and in association with infusoria *Tetrahymena pyriformis* and the green algae *Scenedesmus quadricauda* was demonstrated on a model of the vaccine *Y. pestis* strain EV. In the absence of growth on nutrient media, high concentrations of the plague microbe − up to 10^4 microbial cells per ml on the 30^{th} day of the experiment − were detected in the polymerase chain reaction (PCR). Transition to the vegetative state could be achieved after enrichment of the nutrient medium with fetal serum [52].

The highly virulent strain *Y. pestis* 231 (Dcl = 10 microbial cells) also passed readily into an unculturable state when incubated in deionized water at temperatures of 28 and 37°C. The bacteria lost the ability to grow on LB agar with 2% hemolyzed blood: "the 37°C cultures by the 7^{th} day, and those incubated at 28°C by the 17^{th} day." Intraperitoneal injection into intact mice or animals preliminarily treated with hydrocortisone (5 mg/mouse) of approximately 10^3 microbial cells (according to the PCR data) did not cause their death. In an investigation of the spleens of mice sacrificed on the 30^{th} day after infection, in a number of cases PCR with primers for the *caf1* gene was positive [39].

An investigation of soil samples from the burrows of great gerbils during an interepizootic period showed that, despite the absence of positive results when a bacteriological method was used, in more than 5% of the samples the presence of the plague microbe was confirmed by PCR [52].

Table 4

Dependence of the Virulence of Δ*pgm* Mutants of *Y. pestis* on the Mode of Infection of Mice

Y. pestis strains	LD_{50} in infection, CFU			Reference
	Subcutaneous	Iniracerebral	Intravenous	
KIM5+ ("wild type")	< 10	ND	< 10	[94]
KIM5 (Δ*pgm*)	$> 10^7$	ND	15	
EV line NIIEG (Δ*pgm*)	5×10^8	$4{,}4 \times 10^3$	ND	[12]

Note. ND, no data.

6.2.4. Formation of Bacterial Nanocells

Nanocells of bacteria with linear dimensions 0.2-0.3 μm at the volume of hundredths of a cubic micrometer have been detected in natural samples and produced under experimental conditions. In geological samples they constitute more than 95% of the total number of bacteria detected, and in soil — 72%. Taxonomic analysis of natural nanobacteria revealed that they belong to the known existing divisions of the kingdom of eubacteria. Cytological investigations of artificially formed nanocells suggested that their formation is a universal response of the microorganism to unfavorable conditions and stress factors [7]. The formation of nanocells by the plague pathogen also cannot be ruled out.

6.2.5. Transition into the Prototrophic State

As it was noted above, the accessibility of a whole series of organic compounds to pathogenic microorganisms leads to a loss of the enzyme systems necessary for their synthesis and to the formation of systems providing for effective uptake of ready-made organic substances from the organism. The plague pathogen is auxotrophic for phenylalanine and methionine; in many cases cysteine, threonine, and isoleucine are also required for its growth on artificial nutrient media; the requirements for other amino acids are determined by strain differences [36]. The generally recognized auxotrophy of *Y. pestis* is a serious obstacle to the possibility of saprophytic existence; however, a whole series of strains of the plague pathogen capable of growing on an agar medium with mineral salts and 0.1% glucose have been described [3, 36, 57].

Thus, the prototrophic strain 296 was isolated from a red suslik in 1961 in the Alai valley of Kirghizia [57].

A prototrophic mutant possessing high virulence has been produced experimentally — guinea pigs and mice died at periods from 4 to 7 days; Dcl of the prototroph was 25 CFU. It is interesting that guinea pigs infected with the original strain sometimes died on the 15^{th} to 18^{th} days, and its Dcl varied from 50 to 500 CFU. The prototrophic mutant retained high viability during 11-day storage [57].

6.2.6. The Plasmid pYC

The detection of sequences coding for proteins (homologs of DinJ1 and DinJ2 of *E. coli),* which may be involved in the repair of DNA damages, in the structure of the cryptic plasmid pYC (5919 bp), characteristic of strains of *Y. pestis* isolated in Yunnan province in China, provided the basis for suggesting that the acquisition for this plasmid "may promote survival of *Y. pestis* in the host organism or possibly in the environment" [70].

INFLUENCE OF FACTORS OF *Y. pestis* PROVIDING FOR SURVIVAL IN THE HOST ORGANISM ON VIRULENCE

As it was noted in section 1, the virulence of microorganisms depends on the mode of infection. Thus, Δ*pgm* mutants of *Y. pestis,* defective for the production of the siderophore and the ability to adsorb hemin [87] and used as vaccine strains for cutaneous, intracutaneous, subcutaneous, respiratory, and oral immunization [41], when injected

Table 5

Virulence of *Y. pestis* Strains 231 and 358, As Well As Their Experimental Derivatives, in the Subcutaneous Mode of Infection of Mice and Guinea Pigs (Comparison on the Basis of the Data of [3, 43, 55, 71].

***Y. pestis* strains (phenotype)**	**Mice**		**Guinea pigs**	
	LD_{50}, CFU	**Average life duration, days**	**LD_{50}, CFU**	**Average life duration, days**
231 ($Caf1^+ Ymt^+ Lcr^+ Pst^+ Pla^+ Pgm^+ pH6^+$)	**3** (1-18)	**7,3** (4-16)	**4** (1-22)	**8,1** (5-9)
231pCad⁻($Caf1^+ Ymt^+ Lcr^- Pst^+ Pla^+ Pgm^+ pH6^+$)	> **10^8**	–	> **$1{,}5\times10^{10}$**	–
231/830 ($Caf1^+ Ymt^+ Lcr^+ Pst^+ Pla^+ Pgm^+ pH6^-$)	> **10^8**	–	> **$1{,}5\times10^{10}$**	–
231Pgm⁻ ($Caf1^+ Ymt^+ Lcr^+ Pst^+ Pla^+ Hms^- Pst^R pH6^+$)	> **10^8**	–	> **$1{,}5\times10^{10}$**	–
231Psb⁻ ($Caf1^+ Ymt^+ Lcr^+ Pst^+ Pla^+ Hms^- Pst^S pH6^+$)	**4** (1-21)	**7,8** (5-8)	**10** (2-24)	**8,9** (6-10)
231pFra/pFS23 ($Caf1^- Ymt^+ Lcr^+ Pst^+ Pla^+ Pgm^+ pH6^+$)	**5** (1-42)	**8,2** (7-10)	**27** (5-210)	**10,2** (8-13)
231pFra⁻ ($Caf1^- Ymt^- Lcr^+ Pst^+ Pla^+ Pgm^+ pH6^+$)	**8** (2-41)	**7,9** (7-10)	**2** (1-5)	**9,7** (8-10)
231Psb⁻pFra/pFS23 ($Caf1^- Ymt^+ Lcr^+ Pst^+ Pla^+ Hms^- Pst^S pH6^+$)	**13** (3-63)	**8,6** (6-12)	**267** (67-966)	**24,5** (9-26)
231pPst⁻ ($Caf1^+ Ymt^+ Lcr^+ Pst^- Pla^- Pgm^+ pH6^+$)	**1** (1-4)	**6,9** (4-7)	**4** (1-21)	**8,6** (5-9)
231pPst⁻pFra/pFS23 ($Caf1^- Ymt^+ Lcr^+ Pst^- Pla^- Pgm^+ pH6^+$)	**1** (1-5)	**6,3** (6-10)	**9** (2-38)	**12,7** (9-16)
231pFra⁻pPst⁻ ($Caf1^- Ymt^- Lcr^+ Pst^- Pla^- Pgm^+ pH6^+$)	**1** (1-4)	**6,5** (6-11)	**9** (1-45)	**12,4** (9-15)
358 ($Caf1^+ Ymt^+ Lcr^+ Pst^+ Pla^+ Pgm^+ pH6^+$)	**7** (1-27)	**4,6** (3-5)	**13** (3-63)	**8,6** (5-9)
358pCad⁻($Caf1^+ Ymt^+ Lcr^- Pst^+ Pla^+ Pgm^+ pH6^+$)	> **10^8**	–	> **$1{,}5\times10^{10}$**	–
358Pgm⁻ ($Caf1^+ Ymt^+ Lcr^+ Pst^+ Pla^+ Hms^- Pst^R pH6^+$)	> **10^8**	–	> **$1{,}5\times10^{10}$**	–
358pFra/pFS23 ($Caf1^- Ymt^+ Lcr^+ Pst^+ Pla^+ Pgm^+ pH6^+$)	**5** (1-42)	**7,8** (6-10)	**13** (3-63)	**11,2** (9-15)
358pFra⁻ ($Caf1^- Ymt^- Lcr^+ Pst^+ Pla^+ Pgm^+ pH6^+$)	**3** (1-18)	**6,2** (6-8)	**10** (2-15)	**10,6** (8-13)
358pPst⁻ ($Caf1^+ Ymt^+ Lcr^+ Pst^- Pla^- Pgm^+ pH6^+$)	**1** (1-2)	**5,5** (4-6)	**11** (2-68)	**6,3** (5-9)
358pPst⁻pFra/pFS23 ($Caf1^- Ymt^+ Lcr^+ Pst^- Pla^- Pgm^+ pH6^+$)	**2** (1-16)	**9,2** (7-12)	**26** (6-22)	**13,8** (10-16)

Note. The confidence interval for the 95% probability level is indicated in parentheses. Lcr (low-calcium response) denotes a requirement for calcium ions for growth *in vitro* at 37°C in conjunction with the ability to produce V antigen and outer membrane proteins Yops at the same temperature. Pst^S indicates sensitivity of $pPst^-$ variants of the indicated strain to pesticin, evidencing the functional adequacy of the receptor for pesticin/yersiniabactin. Pst^R indicates insensitivity of $pPst^-$ variants of the indicated strain to pesticin. Pgm^+ denotes the simultaneous presence of Hms^+ and Pst^S traits. Pgm^- indicates a combination of the Hms^- and Pst^R traits (Δ*pgm* mutant).

intravenously or intracerebrally, lead to death of animals from a generalized infection process (Table 4). Intravenous or an analogous retroorbital route of infection with Δ*pgm* mutants of *Y. pestis* is widely used as a model for evaluating the influence of supplemental mutations on the virulence of the plague pathogen. On the one hand, the modeling of such an especially dangerous infectious disease as plague (virulent strains belong to pathogenicity group I according to the Russian classification or risk group IV according to the WHO classification), by intravenous infection of animals with attenuated strains (pathogenicity group III or risk group I) completely eliminates the possibility of infection of the experimenter. In this case investigations can be conducted not under conditions of maximal isolation but in microbiological base laboratories, designated for work with pathogens presenting a "low or moderately low hazard for workers and low or limited hazard for society" [49]. On the other hand, this is an extreme degree of simplification of such a complex phenomenon as an infectious disease. Therefore, the correctness of the results obtained in experiments with Pgm^+ strains in the case of peripheral and Pgm^- variants in the case of intravenous routes of infection will need serious critical evaluation.

Along this line, it is interesting that the Δ*pgm* mutant of the strain *Y. pestis* KIM5-3001 (Sm^R) - KIM5-3001.1 (Sm^R, *psa3::m*-Tn*3*), which had lost the ability to produce the pH6 antigen, possess rather high virulence in the retroorbital mode of infection – $LD_{50} = 9 \times 10^3$ CFU, only 214.3 times lower than for the original strain in the case of an analogous mode of infection (42 CFU) [82]. However, the pH6 variant of the "wild-type" strain *Y. pestis* 231 - 231/830 - entirely lost virulence for white mice and guinea pigs in the subcutaneous mode of infection (Table 5) [3, 43, 55]. Moreover, we do not know of any publication on the isolation of $pH6^-$ mutants of *Y. pestis* in nature, although strains defective for other traits are isolated relatively often [9, 20, 25]. Of no less interest is the fact that the pH6 antigen has been detected in all the investigated virulent strains of *Y. pseudotuberculosis,* whereas $pH6^-$ strains proved to be avirulent [14]. In subcutaneous infection of mice with *Y. pestis* cultures grown under conditions optimum for synthesis of the pH6 antigen (37°C, pH 6.0), death of the animals occurred earlier than in the group of mice infected with bacteria cultured also at 37°C, but with pH 7.0. The total number of animals that died was the same in both cases [60]. It is noteworthy that the strain EV of the NIIEG line entirely lost its protective properties

Table 6

Virulence of Isogeneic pPst$^-$ Variants of Strains of Five "Subspecies" of *Y. pestis* (Compiled on the Basis of the Data of [21])

Subspecies	Strain, clone No.	Phenotype	Virulence for mice	
			10^3 CFU	10^7 CFU
pestis	231 # 1	$Hms^+ Pst^S$	3/3	3/3
	231 # 2	$Hms^+ Pst^R$	0/3	0/3
	231 # 3	$Hms^- Pst^S$	3/3	3/3
	231 # 4	$Hms^- Pst^R$	0/3	0/3
hissarica	A-1249 # 1	$Hms^+ Pst^S$	2/3	3/3
	A-1249 # 2	$Hms^+ Pst^R$	0/3	0/3
	A-1249 # 3	$Hms^- Pst^S$	3/3	3/3
	A-1249 # 4	$Hms^- Pst^R$	0/3	0/3
altaica	I-2359 # 1	$Hms^+ Pst^S$	3/3	2/3
	I-2359 # 2	$Hms^+ Pst^R$	0/3	0/3
	I-2359 # 3	$Hms^- Pst^S$	3/3	3/3
	I-2359 # 4	$Hms^- Pst^R$	0/3	0/3
ulegeica	I-3069 # 1	$Hms^+ Pst^S$	2/3	3/3
	I-3069 # 2	$Hms^+ Pst^R$	0/3	0/3
	I-3069 # 3	$Hms^- Pst^S$	2/3	3/3
	I-3069 # 4	$Hms^- Pst^R$	0/3	0/3
caucasica	6499 # 1	$Hms^+ Pst^S$	3/3	3/3
	6499 # 2	$Hms^+ Pst^R$	1/3	3/3
	6499 # 3	$Hms^- Pst^S$	3/3	3/3
	6499 # 4	$Hms^- Pst^R$	0/3	0/3

Note. In the numerator: number of animals that died; in the denominator: number of infected animals.

for guinea pigs and white mice in the case of subcutaneous immunization [43, 55]. We are inclined to attribute this to its loss of "latent" virulence, i.e., the ability to reproduce for a limited time in the organism to be immunized. The amount of antigenic material in the dose of live bacteria introduced is small and incapable of supporting the development of the vaccinal process.

I. V. Domaradskii [17] correctly notes "that the main difference of avirulent strains of the plague microbe from virulent strains lies in the ability of the latter to spread and reproduce without restraint in the organism." Thus, considering the data of the publications cited above, we can conclude that the pH6 antigen is essential primarily for overcoming the protective barriers of the host organism and dissemination of bacteria at the initial stages of development of the infection process. Taking into consideration the pH dependence of the production of this pathogenicity factor, we can also suggest that synthesis of the pH6 antigen, induced in phagolysosomes of macrophages [11, 82, 83], also determines the ability of the bacteria to survive and reproduce in these phagocytic cells on account of suppression of their antibacterial functions. The intracellular localization of the pathogen at this stage of the infection in turn promotes a further spread of *Y. pestis* with the flow of lymph and blood and a generalization of infection.

To study the role of individual factors in the pathogenicity of *Y. pestis,* and to reveal their possible involvement in the mechanisms that provide for intracellular dissemination and reproduction of the plague pathogen in plasma, interstitial fluid, and within phagolysosomes, the presence of genetically determined isogeneic variants of the virulent strain is necessary. It is evident that the value and convincingness of the results obtained increase if they are reproduced in different experiments conducted on different models. Therefore, the most reliable information can be obtained when several sets of genetically characterized isogeneic mutants, constructed on the basis of different parental strains, are used. Researchers from the Russian Research Anti-Plague Institute "Microbe" (Saratov) and the State Research Center for Applied Microbiology (Obolensk) [3, 10, 24, 29, 30, 38, 43, 55, 71, 80, 91] have created such isogeneic sets based on two strains of the "major subspecies" of *Y. pestis* - 231 and 358. The strain 231 was isolated in 1947 from a dead marmot *(Marmota bobac)* in the Central Asian mountain focus, and strain 358 was isolated in 1955 from a person who died of the bubonic-septic form of plague in the Central Asian desert focus.

The virulence of the original strains 231 and 358 and their derivatives with respect to two species of laboratory animals in the case of subcutaneous infection is presented in Table 5.

The results of our experiments confirm the generally recognized opinion of unconditional necessity of the Yop virulon encoded by the plasmid pCad for the manifestation of virulence by *Y. pestis*. The influence of individual products of pCad on the virulence of the plague pathogen is discussed in detail in the review publications [61, 66, 67, 87].

The necessity of the pH6 antigen for the development of the infectious process in the case of peripheral infection was discussed above.

The necessity of expression of "housekeeping" genes responsible for satisfaction of the nutrient requirements of *Y. pestis* for iron with the aid of a siderophore − yersiniabactin (Pgm^+ or Hms^-Pst^S phenotype) for the manifestation of virulence in the case of peripheral routes of infection [61, 79, 87] was also confirmed in our experiments. However, we should mention the experiments of I. V. Zudina [21], in which it was shown that Hms^+Pst^R mutants of *Y. pestis* of the subspecies *caucasica* retain virulence in the case of subcutaneous infection at a rather high level (Table 6).

Our data [3, 71, 91] and the results of other researchers [37, 72, 73] are evidence that the loss of ability to synthesize Ymt does not lead to a decrease in virulence of mutants with respect to mice and guinea pigs.

The data presented in Table 5 on the necessity of the presence of the capsule antigen Caf1 in cells of the plague microbe for "complete" virulence of *Y. pestis* for mice and guinea pigs agree with the data of V. V. Akimovich and L. N. Shanina [1] and the results of the investigations of V. V. Kutyrev [29, 30]. However, ignoring the data of his own investigations of the experimental strain 358/12 and its isogeneic derivatives and the "wild-type" strain I-2422 $Fra1^-$", solely on the basis of data on the study of the virulence of isogeneic derivatives of the wild-type strain I-1843, V. V. Kutyrev draws the conclusion that the low virulence of natural $F1^-$ "strains for guinea pigs is due to the absence of expression of the capsule antigen" [30]. At the same time, the results of our experiments contradict the data of the overwhelming majority of researchers who have studied the virulence of $Caf1^-$ variants of the plague pathogen. This fact permits us to suggest that the significant decrease in the virulence of $Caf1^-$ strains with respect to various species of "wild" rodents and laboratory animals [8, 27, 29-32, 37, 51, 53, 62, 63, 95, 96] may be associated with the presence of unidentified supplemental mutations in the strains studied. Unfortunately, in the publications of the researchers from USAMRIID (Fort Detrick, USA) [68, 76, 97], who used a method of producing $Caf1^-$ variants of *Y. pestis* by means of localized mutagenesis similar to ours 171], there are only data on the virulence of $Caf1^-$ mutants for mice and African green monkeys (*Cercopithecus aethiops*), but no data on virulence for guinea pigs.

Still another evidence of the possibility of an influence of unidentified mutations on virulence may be the discrepancy of the results of our investigations from the data of A. M. Kokushkin [24] on the virulence of isogeneic derivatives of the strain 358. In his investigation it was shown that a strain that had lost both "species-specific" plasmids of the plague pathogen had a decrease in virulence of approximately two orders of magnitude in comparison with the original wild-type strain and an analogous variant $358pFra^-pPst^-$, used in our investigations (see Table 5). We are inclined to attribute this contradiction to methodological differences in the generation of such strains. In the studies cited, the virulent strain $358Psb^+pPst^+pCad^+$ was obtained from the avirulent strain $358/12P^+I$ by transfer of the plasmid pCad to it by the method of cryotransformation. A strain lacking the plasmid pPst was produced on the basis of it according to the traditional procedure [81]. Then the plasmid CKΔII, determining resistance to kanamycin, was transferred to both strains as a selective marker by the method of cryotransformation. We should mention that according to our data [3], in the case of cryotransformation by A. M. Kokushkin's method [23], plasmids are transferred chiefly to *Y. pestis* cells "competent" for cryotransformation, possessing defects that we did not detect, manifested in a decrease in the virulence of the population of transformants. On the basis of his analysis of the literature data, I. V. Domaradskii [15] suggested that "transfers of genetic information are more readily removed in the case of atypical forms of certain bacteria. In the case of such as explanation, one should speak not of a decrease in virulence of the cells under the influence of plasmids but of a change in the composition of the population, caused by the accumulation (selection) of recombinants with an initially low virulence or entirely avirulent." According to our data [3], the method of transduction using the phage P1 vir [44] or P1 *cml clr* 100ts [19] lacks the indicated shortcomings. The selection of clones that have retained virulence at the level of the original strains requires an "animalization." The necessity of a stage of animalization in evaluating the virulence of cultures of pathogens of infectious diseases subjected to experimental manipulations or stored for long periods under laboratory conditions is explained by the fact that passages through the organism of a sensitive animal "purify" the microbial population from avirulent segregants (in the case of *Y. pestis* these are bacteria with the phenotypes Lcr^-, Pgm^-, *etc.*) [3].

Thus, the use of two cryotransformations without subsequent selection in the experiments of A. M. Kokushkin (24) might lead to enrichment of the population of transformants with bacteria possessing reduced virulence, and a loss of highly virulent clones.

In a comparison of the results obtained using strains of *Y. pestis* of differing origin, we should also consider the so-called strain differences, which included a different significance of individual pathogenicity factors for the implementation of virulence of various "ecotypes" of the plague pathogen in the organisms of various animal species [31]. These differences probably arose in the course of microevolutionary adaptation of geographically separated populations of *Y. pestis* to definite species of rodents characterized by significant individual metabolic peculiarities.

Thus, the realization of the pathogenic properties of *Y. pestis* in the organism of a susceptible host requires the presence in the plague pathogen of a whole set of pathogenicity factors of different functional direction and systems of regulation providing for their coordinated expression. It is incorrect to consider the role of any of the indicated factors taken separately as absolute. However, a detailed analysis of each of these factors lies at the basis of the systemic approach in studying the pathogenicity and virulence of *Y. pestis*. Since the complex of factors providing for the pathogenicity of *Y. pestis* has been virtually entirely defined by now, the necessity arises of establishing "the minimum of traits that is essential for ensuring expression of virulence" [29] and of subdividing all the pathogenicity factors of the plague pathogen into major (compulsory) and supplemental. This is essential for selecting the optimum "targets" and optimizing the strategy of development of diagnostic, vaccine, and therapeutic preparations. In our opinion, the compulsory pathogenicity factors of the plague pathogen for mice and guinea pigs are:

- the Yop virulon encoded by the plasmid pCad;
- the pH6 antigen.

The following should be classified as supplemental pathogenicity factors:

- capsular antigen FI,
- murine toxin,
- plasminogen activator, *etc*.

After conducting investigations *in vivo*, putative factors detected on the basis of an analysis of the *Y. pestis* genome *in silico* and possessing structural homology to the pathogenicity factors of other pathogenic bacteria [78, 84, 86] perhaps should also be classified as supplemental pathogenicity factors.

Summarizing all that has been stated above, let us emphasize once again that each of the stages of cyclic existence of *Y. pestis* is provided for by numerous factors of the plague pathogen, which can act jointly or individually. Each of these factors in turn may be involved in different stages of the infection process or transmission. But only in aggregate do these factors ensure persistence of the plague pathogen in natural foci, no matter how significant or negligible their individual effect might be.

I should also like to mention that some of the scientific ideas examined above are still speculative; however, their value is nonetheless undoubted, since some of them are already accessible to experimental verification and may give results that are valuable not only theoretically but also in practical terms. On the whole, they reflect the trends in research in this field. Attempts to canonize various concepts on the basis of the fact that they are currently recognized by the majority of scientists or by the most authoritative of them cannot be considered either serious or productive. The process of molecular microbiology leads to a continuous generation of new hypotheses and theories, which attempt to adequately explain the facts accumulated by science. In essence, each of these hypotheses and theories emerges as an attempt of one researcher or another to subjectively reason out definite scientific facts, to approach a knowledge of objective truth.

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