



The Resistance to Plague Infection among *Meriones persicus* from Endemic and Non-endemic Regions in Iran: The Role of Gut Microbiota

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Abstract

Background: The present study was conducted approximately 40 years ago, but its results have not been released. At the time of this study, the importance of the gut microbiota was not fully understood.

Methods: *Meriones persicus* rodents, as one of the major reservoirs of *Yersinia pestis* bacterium in Iran, were compared in a disease endemic area (Akanlu, Hamadan, western Iran) and a non-endemic zone (Telo, Tehran, Iran) from 1977 to 1981.

Results: This study was able to transmit the resistance to *Y. pestis* to other rodents creatively by using and transferring gut microbiota.

Conclusion: The study indicated for the first time that the gut microbiota could affect the sensitivity to plague in *Meriones* in Telo.

Keywords: Gut microbiota, Infectious disease, *Meriones persicus*, Plague, Iran

Introduction

Forty years ago, the role of intestinal bacteria, nowadays known as the gut microbiota, in sensitivity of the rodents to plague was studied. In recent years, many studies have evaluated the role of gut microbiota on the pathogens transfer through vectors (1), their survival in carriers (2) and their character in human infection (3-5), which could increase our knowledge about this functional organ.

Generally, plague is a zoonotic disease and transmitted by fleas among wild rodents. Major epidemics of plague have occurred causing the death of many people. Plague is still an endemic disease in a number of countries in Asia, Africa, North and South America. Kurdistan Province in Iran is one of the endemic regions of plague. *Meriones persicus*, *M. libycus*, *M. tristrami*, and *M. vinogradovi* are the main plague-transmitting ro-

dents in Iran. These rodents led to the epidemics occurred in 1964-1965 in the western regions of Iran mainly, Kurdistan and western Azerbaijan (6). Among these reservoir rodents, *M. persicus* and *M. libycus* are relatively resistant to plague, while *M. tristrami* and *M. vinogradovi* are sensitive to *Yersinia* infection (7). The relative stability of plague in resistant *Meriones* plays an important role during the episodes of plague bacillus maintaining (6).

Although *M. persicus* is the main reservoir of the disease in Kurdistan (Akanlu) and eastern Azerbaijan (Sarab) and has been also observed in Tehran, the plague infection has not been reported in non-endemic regions such as Tehran. This hypothesis was raised in 1953 that this could be due to the relative plague-resistance of *M. persicus* in plague endemic regions of Iran (8).

Therefore, the aim of this study was to find the factors making *M. persicus* in the Kurdistan region resistant to *Yersinia pestis* (8). This study conducted whether the resistance of *M. persicus* in Kurdistan is related to the intestinal bacteria flora of the wild rodents in this region, or to other factors such as genetic diversities and morphological characteristic.

Materials and Methods

Study Area

This cross-sectional study was performed on the *M. persicus* of two areas: Kurdistan, as an endemic region of plague, and Tehran, as a non-endemic region, from 1977 to 1981.

The sampling in Tehran was done in the Telo area located in the northeastern part of Tehran, capital of Iran. The sampling in Kurdistan was done in areas near Akanlu village that located in 365km distance from Tehran in the western part of the country. The rodents were trapped by wooden mousetrap for two months.

Rodent identification

The morphological identification of the rodents was conducted by measuring the length of their head and body, the size of their ear, tail, and rear

legs, and the color of their hair and incisors (9, 10). To compare the physiological characteristics of the rodents, their body temperature, heart rate, respiratory frequency, and feed rate were assessed for 24 h. To compare the chromosomal differences, 1 ml (per 100 gr of body weight of the *Meriones*) of 25% diluted solution of colchicine was injected intraperitoneally; the rodents were killed after one hour, and the femur was removed to study the chromosomes (11-13). The number of chromosomes and their connection position, i.e. metacentric, sub-metacentric, and acrocentric manners were then recorded.

Blood biochemistry

The blood groups were evaluated by comparing the methods of non-insiders (heteroagglutination) and insiders (isoagglutination) (14). The immunity acquired was evaluated by passive micro-haemagglutination assay (15-17). A total number of 75 *Meriones* serums were compared in both regions. The serum proteins of *Meriones*, including albumin, alpha-2-globulin, and beta and gamma globulin obtained from Akanlu and Telo regions were evaluated by the electrophoresis method (18-20); furthermore, the slides of the serum proteins and their curves were compared with the human serum proteins. The alpha-1-antitrypsin in *M. persicus* in the Akanlu region was compared with that of the Telo region via isoelectrofocusing on the polyacrylamide gel (21), using human serum samples as a control. The haptoglobin variety was also evaluated by starch gel electrophoresis method at the two regions (22). The transferrin variety was also studied, using high-voltage thin-layer agarose gel electrophoresis (23). Human serum was employed as a standard control. Moreover, ceruloplasmin heterogeneous and immunoglobulin A and G were evaluated by quantitative method, i.e. gel diffusion and tri-partigen plates (24-26). The base of these methods is antigen-antibody reactions. Ceruloplasmin and immunoglobulin were separately injected into adult rabbits to prepare anti-ceruloplasmins and anti-immunoglobulin. Then the serum samples of *Meriones* at the two regions were evaluated by anti-ceruloplasmins and anti-

immunoglobulin. Isolation and purification of ceruloplasmin and immunoglobulin were performed and measured via ion exchange chromatography and biuret method, respectively.

The electrophoretic patterns of acid phosphatase (AP) (27), esterase D (ESD) (28), 6-phosphogluconate dehydrogenase (6-PGD) (29), phosphoglucomutase-1 (PGM-1) (30), and adenylate kinase (AK) (31, 32) enzymes in red blood cells in the two groups of *Meriones* were compared by thin-layer starch gel electrophoresis method and these enzymes in red blood cells were investigated for genetic polymorphisms.

Parasitology

Ectoparasite and intestinal parasites were also evaluated in both areas. Ticks and fleas were removed from the body of the *Meriones* by forceps and observed by microscope (33, 34). The morphology of intestinal parasites was examined after opening a part of the intestine of *Meriones* and its staining with hemalum and Carmine-Alum (35, 36).

The resistance of *Meriones* to plague infection (with and without gut microbiota exchange)

The sensitivity of *Meriones* to *Yersinia pestis* was investigated by *Y. pestis* PKR 775 strains obtained from Pasteur Institute (37). After preparing the microbial solution in different dilutions (the initial concentration was equal to 10^8 CFU/ml), the *Meriones* of each region were divided into six groups, each including 10 *Meriones*. Then, 2 ml of each diluted microbial solution was injected subcutaneously; the *Meriones* was transferred to a laboratory and were isolated in plastic cages for 30 d. During this time, the dead *Meriones* were autopsied, and the blood smear was stained with Toluidine blue and studied by microscope. *Y. pestis* infection was also studied by culturing the spleen sample on blood agar plates.

The intestinal microbial flora of the *Meriones* was also investigated (38). For this purpose, the *Meriones* were killed. Afterwards, a part of their intestine was removed under sterile conditions, and their intestinal contents were cultured at 37 °C for 24 h on agar mediums. Each of the grown

colonies was cultured on blood agar and MacConkey agar. The bacteria were identified by standard biochemical techniques and confirmed by serological tests, using specific microbial antibodies. Furthermore, suspensions equal to 3×10^8 CFU/ml were prepared from the intestinal bacteria of the *Meriones* in the Akanlu region that did not exist in the *Meriones* in the Telo region; additionally, they were fed by syringe to the Telo group that had 12 *Meriones*. In addition, a group of 12 *M. persicus* from Tehran (Telo) and a group of 10 *Meriones* from Kurdistan (Akanlu) were selected as the control group that did not receive the bacteria. Each of the *M. persicus* was placed in a separate glass cylinder. After 15 d, sterile swab samples were taken from their stool of the rectum, and their bacteria were examined. Following that, 200 *Xenopsylla* fleas infected with *Yersinia pestis* strains of PKR775 were released near each of the *Meriones* studied, and their mortality from plague was assessed.

Ecology

The ecological conditions of 100 nests, 50 from Telo and 50 from Akanlu, were also investigated (39). The channel diameter of the nests of *Meriones* and the temperature and humidity inside and outside the nests were measured. To study the plant ecosystem in Telo and Akanlu areas, all parts (root, stems, leaves, and flowers) of the plants existing in the regions were collected and transferred to the laboratory for the identification of the species.

Statistical analysis

The data were analyzed using SPSS software ver. 16 (SPSS, Inc., Chicago, IL). The descriptive (mean and frequency) and analytical analyses (Chi-squared and two independent sample proportion test) were done on the data. P-value less than 0.05 were considered as statistically significant.

Results

The total number of *M. persicus* was 580 (380 from Telo and 200 from Akanlu). There was no

significant difference between the *Meriones* of the two regions studied regarding their physiological characteristics, i.e. body temperature, heart rate, respiratory rate, and feed rate during 24 h, and morphological characteristics, i.e. head and body length, the size of ear, tail, and rear legs, and the color of hair and incisors.

No differences were observed in the blood characteristics of the groups: serum, and alpha-1-antitrypsin pattern.

The cytogenetic and chromosomal analyses indicated that none of the studied *M. persicus* had chromosomal abnormalities. The number of chromosomes in all of the samples studied was forty-two. Non-sex chromosomes were 24 metacentric, 10 sub-metacentric, and 6 acrocentric manners. Sex chromosomes were X and Y chromosomes. Female *Meriones* had two metacentric X chromosomes, while the male *Meriones* had X and Y chromosomes.

According to the results of passive microhemagglutination, none of the studied *M. persicus* showed any plague antibodies.

The serum albumin and alpha-2 globulin levels in the *Meriones* of Telo and Akanlu were not significantly different. There was not a significant difference between the levels of IgA and IgG in the serum of *M. persicus* in Akanlu and Telo regions. However, a significant difference was observed between the two groups in terms of the immune diffusion rate of IgA and IgG ($P < 0.0001$). Moreover, there was a significant difference between the two regions in terms of the immune diffusion rate ceruloplasmin ($P < 0.0001$).

The results of complement variety showed three phenotypes, namely S-S, F-F, and S-F, in the *Meriones* in both regions. There was no difference regarding F-F and S-S phenotypes in Akanlu and Telo; however, S-F phenotype was different in both regions.

M. persicus in both areas did not differ in terms of intestinal parasite species, but the percentage of contamination was statistically different ($P < 0.0001$), Contamination percentage was higher in *Meriones* of Akanlu. *Moniliformis moniliformis*, *Hymenolepis nana*, and *Trichuris leparis* were observed as intestinal parasites in both regions (Ta-

ble 1). Nine species of fleas were collected from the bodies of *M. persicus* in both areas. Four species were common in both groups, including *Amphipsylla schelkownikovi*, *Echidnophaga oschanini*, *Nosopsyllus iranensis*, and *Xenopsylla buxtoni*. Four species were observed only on the skins of the *Meriones* in Akanlu, including *Ctenophthalmus dolichus kurdensis*, *Ophthalmopsylla volgensis*, *Paradoxopsyllus grenieri*, and *Rhadinopsylla ucrainica*. *Xenopsylla nuttalli* was only observed on the skins of the *Meriones* in Telo. Haptoglobin was monomorphic in both groups of *M. persicus*. There was not any difference between the *M. persicus* of both areas; the *M. persicus* of both areas were monomorphic and of the C-C type.

The electrophoresis pattern of the enzymes in red blood cells, including AP, ESD, 6-PGD, PGM1, and AK was not different among the *Meriones* of both regions.

In Kurdistan, the plant diversity was higher, and the majority of these plants had not been observed in Telo. Comparison of the ecological status of *M. persicus* in Telo and Akanlu demonstrated that heat and moisture were different in the two regions. In addition, the average nest depth of *M. persicus* in Akanlu and Telo was 4.5 and 1.5 meters, respectively. The results of *Meriones* sensitivity to *Yersinia pestis* revealed that 10^{-1} dilution of 10^8 CFU/ml in the *Meriones* in the Telo region was associated with 100% mortality, but this dilution in the *Meriones* in the Akanlu region resulted in 20% mortality. Moreover, the 10^{-4} , 10^{-5} , and 10^{-6} dilutions of microbial solution were equal to 70%, 60%, and 20% mortality in the *Meriones* in the Telo region, respectively. These dilutions did not make any mortality in the *Meriones* in the Akanlu region (Table 2).

The isolation of intestinal microbial flora detected 13 bacteria in the intestines of the *Meriones* in Akanlu. These bacteria did not exist in the intestines of the *Meriones* in Telo. A suspension equal to 3×10^8 CFU/ml was prepared from each of the aforementioned intestinal bacteria and was fed to a group of 12 *Meriones* in Telo. After 15 d, the bacteria that were fed to these *Meriones* were colonized in their intestine and identified by standard biochemical techniques.

Table 1: Comparison of serum proteins, immunoglobulins, ceruloplasmin, complement variety, and intestinal parasites between the Meriones of the two regions

Tested variable	Average (range) or Number (%)		P-value
	Akanlu	Telo	
	n=34	n=48	
Albumin	46(36-65)	45(22-56)	-
Alpha-2 globulin	25(12-37)	23(12-52)	-
Beta and gamma globulin†	43(7-39)	31(20-44)	-
Igg	180.4(37-306)	186.6(18-306)	-
Iga	37.3(9-69.2)	41.1(12-69.2)	-
CP	20.88(8-26)	19.39(5.2-26)	-
Migration rate	N=34	N=48	
Albumin	40%	45%	0.65
Alpha-2 globulin	22%	24%	0.83
Beta and gamma globulin†	38%	31%	0.50
IgG†	100 (75%)	100 (48%)	P<0.001
IgA†	100(75%)	100 (49%)	P<0.001
CP†	100 (75%)	100 (46%)	P<0.001
Phenotype(n=77)	N=51	N=26	
S-S	41(80%)	23(88%)	0.38
S-F	8(16%)	2(8%)	0.32
F-F	2(4%)	1 (4%)	~1
Intestinal parasite	N=72	N=129	
Contamination†	40(55.5%)	15(11.6%)	P<0.001

†Beta and gamma globulin showed a single band and were not separable from each other.

Table 2: The sensitivity of *Meriones* toward microbial solution of *Yersinia pestis* in the two areas studied

Bacterial Concentration	Telo Dead(%), n=10	Akanlu Dead(%),n=10	P-value
10 ⁻¹	100	20	<0.001
10 ⁻²	90	10	<0.001
10 ⁻³	60	10	<0.001
10 ⁻⁴	70	0	<0.001
10 ⁻⁵	60	0	<0.001
10 ⁻⁶	20	0	< 0.001

The *Meriones* and their mortality were assessed by means of 200 *Xenopsylla* fleas infected with *Yersinia pestis* strain of PKR775. The dead *Meriones* were autopsied. In addition, the plague bacillus was isolated from their spleen and confirmed by bacteriophage cultures. The resistant *Meriones* were killed and their spleens were examined for

the plague infection. In none of them, the plague infection was isolated. Among the 13 types of bacteria isolated from the intestines of the *Meriones* in Kurdistan, seven types induced more than 80% resistance to plague bacillus in the *Meriones* of Tehran (Table 3).

Table 3: The effects of inducing cross-resistance to plague in bacteria isolated from the intestines of the *Meriones* in Akanlu fed to the *Meriones* in Telo

Bacterium	No. of Meriones	Mortality	Resistance
<i>Streptococcus faecalis</i>	12	8	33.4
<i>Klebsiella Spp</i>	12	8	33.4
<i>Streptococcus mitis</i>	12	6	50
<i>Staphylococcus albus</i>	12	6	50
<i>Hafnia Spp</i>	12	5	58.4
<i>Citrobacter Spp</i>	12	4	66.7
<i>Streptococcus sanguinis</i>	12	2	83.4
<i>Serratia marcescens</i>	12	2	83.4
<i>Bacillus pasterii</i>	12	2	83.4
<i>Bacillus mycoides</i>	12	2	83.4
<i>Enterobacter agglomerans</i>	12	0	100
<i>Corynebacterium xerosis</i>	12	0	100
<i>Bacillus alvei</i>	12	0	100
Control			
Telo	12	12	0
Akanlu	10	0	100

Discussion

There was no significant difference between the *Meriones* of the two studied regions in terms of physiological characteristics, i.e. body temperature, heart rate, respiratory rate, and feed rate during 24 h, and morphological characteristics such as head and body length, the size of ear, tail, and rear legs, and the color of hair and incisors. No differences were observed in the characteristics of the blood groups, serum, and alpha-1-antitrypsin pattern.

The present study was conducted about 40 years ago, and at that time, the effect of cytokines on the immune system was not well comprehended. *Meriones* microflora in the Akanlu region may also lead to inducing cytokines such as IL-22 and IL-17, which could cause the resistance to plague by provoking the host immune responses. The same kind of resistance is created by transferring the *Meriones* microflora in Akanlu to the *Meriones* in Telo. Another important immune response is neutrophil cytotoxicity that is increased by peptidoglycan (gram-positive bacteria) microbiota in systemic infections (40). Eight out of 13 bacteria

isolated from the intestinal microflora of the *Meriones* in Akanlu were gram-positive bacteria.

For the first time, the serum proteins in both regions were studied. The levels of IgA and IgG were significantly higher in the *Meriones* of Akanlu in comparison with those of the *Meriones* in Telo. By considering the vital role of each of these factors, a higher level of *M. persicus* in Akanlu could enhance the resistance to plague (22, 41).

The blood ceruloplasmin level in the *Meriones* of Akanlu is significantly higher than that of the *Meriones* in Telo. By considering the effect of ceruloplasmin-transmission of copper to tissues, its important role in the metabolism of iron, and the crucial role of iron in the pathogenicity of *Yersinia pestis*, the higher resistance to plague observed amongst the *Meriones* of Akanlu to plague could be due to the high levels of ceruloplasmin in their blood (42). The importance and the biologic role of complements in cell lysis and microbial destruction were also noted. The phenotype of S-F in Akanlu could be another reason for the high resistance of *Meriones* to plague.

In addition, four types of fleas were isolated from *M. persicus* in Akanlu, while they were not found

in the Telo region. This is a significant observation due to the relationship between the vector arthropods and their reservoirs. With regard to various lifespans and other biologic characteristics amongst different types of flea, these fleas could play a crucial role in the maintenance of plague in Akanlu.

Based on the ecosystem and the list of the known plants (43), there was a significant difference between Akanlu and Telo regions. Since there were differences between the *Meriones* of these two regions regarding blood and serum factors, a number of plants in Akanlu, which are a part of the diet of these animals, could be attributed to the differences in increasing or decreasing the blood factors. Future studies investigate the role of these plants.

Furthermore, the nest of *M. persicus* in Akanlu was deeper in comparison with that of *M. persicus* in Telo. This difference is of utmost importance since changes in temperature in winter in Akanlu would not affect plague microbes very much in terms of coldness or dryness; therefore, these microorganisms can live longer. This finding was also reported in another study that reported the soil in these nests could keep the plague bacteria alive for more than a year (44). As this study was conducted about 40 years ago, some of the materials and methods used in this investigation are old-fashioned; therefore, conducting similar studies with updated tools could result in more findings that are precise.

The bacteria of the intestinal micro flora of *M. persicus* in Akanlu could play a role in relative resistance to plague amongst the *Meriones* in Kurdistan, the transmission of certain bacteria in the intestines of *M. persicus* in Akanlu can lead to the resistance against plague amongst the *Meriones* of the Telo region. This transmission could significantly decrease the rate of death amongst *M.* after exposure to *Yersinia pestis*-infected fleas.

The beneficial effects of intestinal microflora on mammals and human health have been the subject of several studies (45-47). Intestinal microflora contributes to supplying the essential nutrition and improving the metabolism of non-digestible substances in the host body. However, one of the

most important roles of intestinal microflora is modulating the host immune system to fight against infectious diseases (48). The microbiota composition plays a key role in the development of resistance or susceptibility to infections. In addition, oral transmission of microbiota from susceptible mice to a kind of infection can cause the same kind of sensibility in animals that were not previously susceptible to infection. Moreover, microbiota transmission from a resistant animal to a sensitive one can create the same type of resistance in the sensitive animal, thus reducing the colonization of the pathogens and the mortality rate. This resistance to infectious diseases is associated with the overexpression of IL-22. This cytokine can lead to the proliferation of IL-17 by producing T helper cells as well as anti-microbial peptides, including Reg3 γ and Reg3 β , which enhances the activities of the host immune system against pathogens (49). In animal models, the absence of these cytokines (IL-22) may lead to sensitivity to infection (50).

Conclusion

Various factors could affect the sensitivity to plague among the *M.* Various blood factors could influence the sensitivity toward plague, and resistance to plague could be transmitted from the resistant *M.* to the sensitive ones by fecal or blood transplants.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of Interests

The authors declare that there is no conflict of interests.

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