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A serological and molecular study on *Francisella tularensis* in rodents from Hamadan province, Western Iran

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ABSTRACT

Introduction and purpose: Tularemia is a zoonotic disease, the most important hosts of which are rodents. Endemic regions and reservoirs of *F. tularensis* are not well-researched areas in Iran. The present study aimed to study *F. tularensis* infection in the rodent populations of western Iran.

Materials and methods: Samples were collected in different areas of Kabudar Ahang County in Hamadan province (west of Iran) from 2014 to 2017. Tularemia serological and molecular tests were conducted using the tube agglutination test and Real-time PCR method tracking the ISFtu2 gene. Positive serum samples were evaluated for cross-reactivity with brucellosis.

Results: A total of 433 rodents, collected from 33 localities, were included in the study. The most abundant species belonged to the Persian jird (*Meriones persicus*; 75.5%), and Libyan jird (*Meriones libycus*; 10.1%). Among the studied samples, three (0.74 %) were seropositive and five (1.15%) were PCR positive. Seropositive samples were two *M. persicus* and one *M. libycus*, and PCR positive rodents were four *M. persicus* and one *M. vinogradovi*. Tularemia seropositive samples showed no cross-reactivity with brucellosis.

Conclusion: Given the presence of infection in rodents with tularemia agent in the studied area, it is crucial to elucidate the risks of rodent exposure to tularemia for physicians, health personnel and the general population.

1. Introduction

In Iran, tularemia is an emerging zoonosis, which causative agent is a gram-negative bacterium called *Francisella tularensis*, a pathogen reported in more than 250 animal species, including Lagomorphs, squirrels, a variety of rodents, birds and amphibians [1]. In addition to countries formerly belonging to the Soviet Union, tularemia has been reported in many regions of the USA, Eurasia, China, and Japan [2–4]. Four subspecies of *F. tularensis* have been diagnosed with different geographic distribution and severity. Currently, only two subspecies of *F. tularensis* are recognized as etiological agents of tularemia. The most important subspecies of tularemia are *F. t. tularensis* (type A) and *F. t. holarctica* (type B), the former having a higher severity compared with the latter; however, these two types differ in terms of hosting, cycle and geographic dispersion. In general, aquatic and terrestrial cycles have been described for this bacteria. In the terrestrial cycle, associated with type A of the disease, Lagomorpha and Rodentia are reservoirs of the disease, with ticks being a main vector. In the aquatic cycle, related to type B, mosquitoes, semi-aquatic rodents, or small mammals living in close proximity to the water, dispose the living organism into the aquatic environment, and the bacteria are further transmitted through eating or contact with contaminated water [5]. *F. tularensis* are also transmitted through direct contact with liquids and tissues of infected animals, and inhalation of infected aerosol [6].

Depending on the transmission of the infection, the disease manifestations are different in humans. The common form of tularemia in

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humans is the glandular type that is associated with erythematosus papule, necrosis, fever, and lymphadenopathy in the affected area [7]. In rodents, tularemia induces chronic lesions such as necrosis in lymph nodes, liver, and spleen, usually ensuing mortality [8]. Tularemia diagnosis methods include cultivation, and molecular and serological tests. Since the cultivation of organism requires at least 5 days and a high biosafety level [9], the serological and molecular diagnosis is more common in many regions of the world [10].

There is scarcity of information on the prevalence of tularemia in rodents in the Middle East, with the conducted studies mainly limited to Iran; however, no research has been performed on the detection of *F. tularensis* in rodents in Hamadan province, which is an important region due to the presence of a human plague focus on the wildlife [11]. Given the reports of tularemia outbreaks in Turkey [12], the northwestern neighbor of Iran, and the detection of tularemia in rodents and human in Kurdistan province [3,13,14], adjacent to Hamadan province, this region is a potential site for tularemia infection, hence the need for further research on the disease in rodents as potential reservoirs and information regarding their dispersal patterns. The present study aimed to investigate the contamination of rodents in western Iran as a potential reservoir of tularemia.

2. Materials and methods

The sampling was done from April 2014 to September 2017 in different regions of Kabudar Ahang County of Hamadan province on the border between Kurdistan and Zanjan provinces. To trap rodents, the locations of presence and active nests of rodents were identified, and live traps with dates, cheese snack and cucumber were then employed. Geographical coordinates of the sampling sites were recorded using the GPS (Fig. 1). Trapped rodents were transferred to the Research Centre for Emerging and Reemerging Infectious Diseases, the branch of Pasteur Institute of Iran, located in Akanlu village (Kabudar Ahang County-Hamadan). Rodents were identified mainly based on morphological characteristics, such as pelage coloration and external and craniodental features, using available identification keys [15]. All steps were conducted in accordance with international ethical standards.

In the laboratory, rodents were initially anaesthetized with chloroform, and blood samples were then taken. The serum was obtained via centrifugation at 3500 rpm for 10 min, and stored at −20 °C.

Serological test: Detection of antibodies against *F. tularensis* in rodent's sera samples was done by tube agglutination test (TAT) according to the protocol provided with the kit (Bioveta, Inc., Ivanovice, Czech Republic). TAT was performed with 0.5 mL aliquots of serial dilutions (from 1:10 to 1:160) of sera mixed with 0.5 mL of diluted (1:4) antigen. The test was considered positive if visible agglutination was observed with clear supernatant fluid occurring after 20 h of incubation at 37 °C and 1 h at room temperature. According to the manufacturer's directions, agglutination at dilutions of 1:80 or higher was considered as positive, whereas 1:40 was still ambiguous [16]. The positive control sera were provided by the manufacturer, and studied in terms of cross-reactivity with brucellosis by the wright method; the results were further examined following 48 h based on the agglutination observation and its severity.

Molecular test: The target ISFtu2 gene was traced for the molecular test of *F. tularensis* in the spleen sample of trapped rodents (Table 1). Spleen DNA was extracted using DNA extraction kit from tissue (Favrogene Company), and was then examined using Real-time PCR by the Rotor-Gene 6600 (Corbett Life Science). DNA amplification was done at the volume of 20 µl in 40 cycles' initial denaturation at 95 °C for 10 min. The cycling was at 95 ∘C for 15 s and 60 ∘C for 60 s. DNA of *F. tularensis* subsp. holarctica NCTC 10857 was used as the positive control.

3. Results

From 2014–2017, 33 localities in different districts of Kabudar

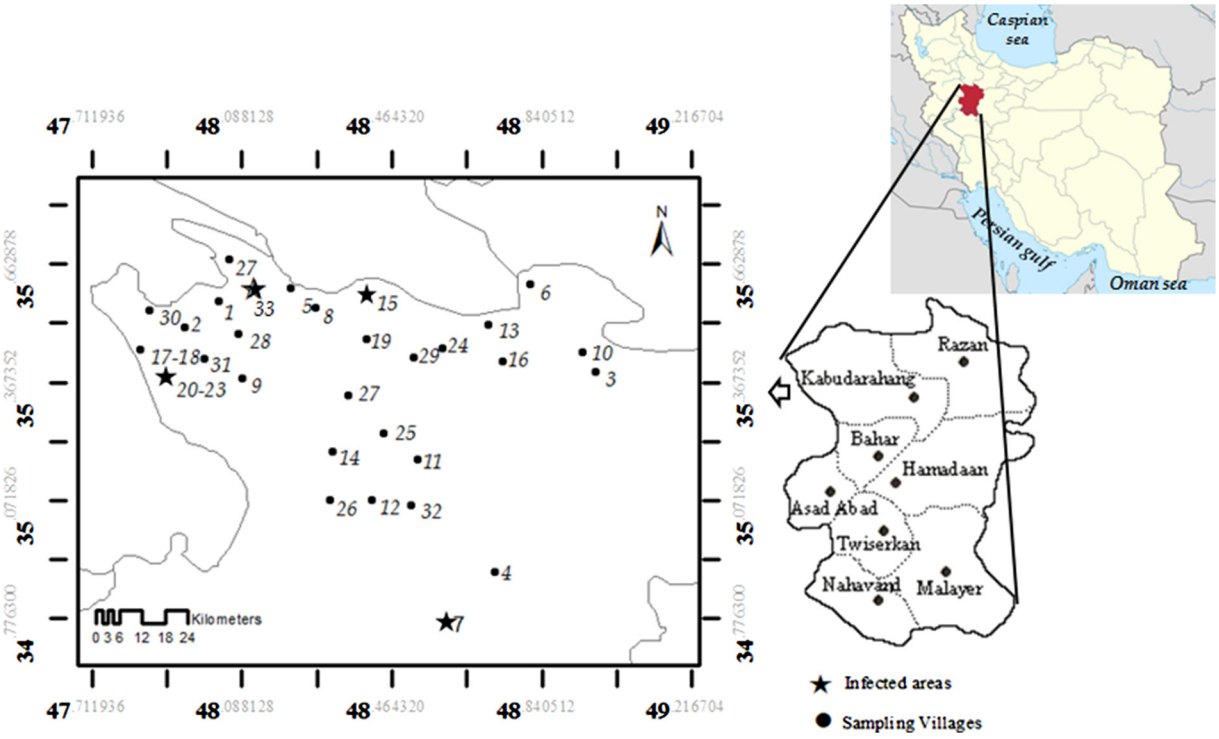

Fig. 1. Map of Hamadan Province and Trapped Areas in Kabudar Ahang County; 1- Kohneh Hesar, 2- Dali Chu, 3- Tazeh Kand, 4- Aq Dash, 5- Ab Meshkin, 6- Chebikhli Darreh si, 7- Sorkhabad, 8- Ghazanghare, 9- Reza Darreh Si, 10- Haji Abad Darreh Si, 11- Pir Badam, 12- Ban Quei Darreh Si, 13-Dareh Jeyran Bolaqi, 14- Yekeh Chalab Kalik, 15- Shahgodar, 16- Chal Tappeh, 17- Qeytar Mezruk, 18- Bikaseh, 19- Bashghurtaran, 20- Yekeh Guni, 21- Sarin Bolagh, 22- Qamish Dare, 23- Uch Darreh, 24- Daq Dali, 25-Yekeh Chal Ab Kalik, 26- Kalik, 27- Darreh Sinaf, 28- Choqondar Goli, 29- Darreh Dash Quei, 30- Akhar Picheh, 31- Polo khoreh, 32- Payin Ghaleh, 33-Akanlu.

Table 1
Primers and probes employed in detecting *F. tularensis*.

Gene target	Primer/probe	Sequence (5′ to 3′)	Amplicon size (bp)	Reference
ISFtu2	*ISFtu2F*	TTGGTAGATCAGTTGGTAGGATAACC	97	[17]
	ISFtu2R	TGAGTTTTATCCTCTGACAACAATATTTC		
	Probe	FAM-AAAATCCATGCTATGACTGATGCTTTAGGTAATCCA-TAMRA		

Table 2
Number and species of the studied rodents.

No	Rodent species	Number of live rodents (%)	Total number (%)
1	*Meriones persicus*	304 (74.7)	327 (75.52)
2	*Meriones libycus*	42 (10.32)	44 (10.16)
3	*Meriones vinogradovi*	23 (5.65)	24 (5.54)
4	*Ellobius lutescens*	13 (3.19)	13 (3.0)
5	*Microtus qazvinensis*	8 (1.97)	8 (1.85)
6	*Spermophilus fulvus*	7 (1.72)	7 (1.62)
7	*Meriones tristrami*	5 (1.23)	5 (1.16)
8	*Arvicola persicus*	2 (0.49)	2 (0.46)
9	*Calomyscus elburzensis isatissus*	2 (0.49)	2 (0.46)
10	*Mus musculus domesticus*	1(0.24)	1(0.23)
		407(100)	433(100)

Ahang County were investigated. From a total of 3902 trapping efforts, 433 rodents were collected and classified into 10 distinct species. The dominant species of the studied region was *M. persicus* (75.5%) (Table 2). Twenty-six (6%) rodents were found dead while the checking traps, rendering the serological study impossible. Accordingly, 407 and 433 samples were tested by TAT and PCR, respectively. Trapping was carried out in different regions and their details are presented in Table 3.

The tube agglutination test was performed on rodent serum, and three out of 407 serum samples became positive (0.74%) with 3+ concerning the agglutination intensity in all positive samples. Seropositive samples belonged to two *M. persicus* (once in Akanlu and another in Ab Meshkin) and one *M. libycus* (in Shahgodar) (Table 3). Serum samples positive for *F. tularensis* did not cross react with *Brucella sp.* antigen.

In PCR test, five out of the 433 extracted DNA samples were positive (1.15%) for tularemia. Among these samples, four (1.85%) belonged to *M. persicus* (one sample of Ouch Dare, one sample in Shahgodar, one in Akanlu and one in Sorkhab) and one specimen (0.23% of the total number of samples) belonged to *M. vinogradovi* (in Sarin Bolagh) (Table 3). None of the seropositive samples were positive in PCR testing.

4. Discussion

The present survey studied the prevalence of *F. tularensis* infection in rodents in Kabudar Ahang County in Hamadan province (Western Iran) from 2014-2017. *F. tularensis* positive samples were detected in eight rodents, including three seropositive (two *M. persicus* and one *M. libycus*) and five PCR-positive animals (four *M. persicus* and one *M. vinogradovi*), corroborating the circulation of this bacteria in this region. The first detection of tularemia in Iran dates back to 1973 [2], where one seropositive hedgehog sample was detected in southeastern Iran (Sistan and Baluchistan province). Following a 40-year interruption, a seropositive Indian gerbil (*Tatera indica*) was reported in this province in 2013 [18]. Three positive rodents were reported in a recent study (2017) on rodents from Kurdistan province [3]. Nonetheless, a broader study (2018) on 17 different provinces in Iran reported *F. tularensis* in eight rodents and lagomorphs, underscoring the circulation of the bacterium in other regions as well [19].

Approximately 44% of all mammal species are rodents distributed in different habitats, ranging from mountainous regions to agricultural areas, from arid desert and temperate habitats to highly mesic ecosystems. This group is considered as a major reservoir of zoonotic pathogens inducing the transmission and spread of many infectious diseases including tularemia [20]. In addition to the difference regarding the prevalence of *F. tularensis* infection in rodents between the Iranian studied areas [2,3,19,21] and the results of the present study, the reservoir species were also different in the present study compared with the previous ones. More than half of the rodent-borne diseases in Iran are caused by domestic or pre-domestic rodents such as *M. musculus*, *Rattus rattus* and *R. norvegicus* [22]. In previous studies, *M. persicus* was identified to be infected by *F. tularensis* [3], yet the present is the first report on the infection of *M. vinogradovi* in Iran. Because all the seropositive rodents belonged to jirds, which are widespread in the Iranian Plateau, the risk of human infections acquired from these animals is high in the studied region.

In this study, 0.74 % and 1.15% of rodents were seropositive and PCR positive for *F. tularensis*, respectively. Comparing the present study with studies in other regions of the world indicates a relatively large diversity of reservoir species and the difference in the prevalence of tularemia in rodents; among the neighboring countries of Iran, tularemia is prevalent in Turkey, but less information is available regarding other countries. Most studies on tularemia in Turkey have been conducted in human populations; however, in a study on rodents in 2012, no seropositive sample was detected, while two positive samples were detected from the 19 samples in the molecular analysis. In another serological study on 163 rodents in Hungary (2010), no seropositive sample was detected [23]. Despite the fact that the infection rate in the rodent population was 6.5% in an endemic region in Ukraine [24], 4.76% in certain regions of China [4], and 4.92% in several endemic regions of Germany [25], the average prevalence of tularemia was estimated at 20.16% in voles of the Northwest Spain in a study conducted in 2017 [26]. A possible reason for the higher rate of infection in the foregoing studies is they were done in the endemic region of tularemia with reported human cases. Seemingly, changing the process of rodent sampling from live trapping to finding dead rodents has played a crucial role in increasing the infection rate in this region.

Rodents are potentially able to carry live bacteria over a period of time and maintain a detectable antibody level for a long time [23], hence reflecting the usefulness of serological tests for the diagnosis and surveillance of tularemia in different regions. However, false positive and false negative results are always considered as limitations of this method [10]. Although no cross-reactivity was observed between the positive tularemia samples and brucellosis in the present study, false-positive results are to be always taken into account due to the similarity of the O-polysaccharide epitope of *Francisella* to other bacteria such as *Yersinia* [10]. As mentioned, confronting dead rodents in field expedition possibly increases the chances of detecting tularemia infection by PCR in such corpses, yet serologic test is not be a possible practice.

Live trapping is another challenge for the real estimation of infection rate in rodents by serological tests. Certain species of rodents such as *Mus musculus* and *Apodemus* spp. are highly susceptible to tularemia, dying shortly after infection [23]. As *Mus musculus* and *Apodemus* spp. were infrequent among the captured rodents in the present study, our estimation is possibly lower than the actual rate of infection in wildlife. Seropositive samples are sometimes reported despite their negative results in PCR tests [3,27]. It seems that the removal of bacteria from

Table 3
Sampling locations, date and species collected from the studied region in Hamadan province; *: location and time of captured seropositive species; **: location and time of capture PCR positive species.

No	Location	No. of trap efforts	Sampling time	Captured rodent species (No.)
1	Kohneh Hesar	298	Jun.2014 & Agu.2015 & Agu.2017	*M. persicus (12), M. libycus(3), M. vinogradovi (1), Ellobius lutescens (1), M. tristrami (1)*
2	Gheytar Mezruk	66	Jun.2014	*M. persicus (6), M. libycus(1)*
3	Bikaseh	309	Jun.2014 & Sep.2017	*M. persicus (10), M. vinogradovi (1), Microtus qazvinensis (1), Ellobius lutescens (1), Calomyscus elburzensis isatissus (1)*
4	Gazan Qarah	1119	Jun.2014 & Sep.2015 & Agu.2017	*M. persicus (57), M. libycus(7), M. vinogradovi (4), Spermophilus fulvus (1), M. tristrami (1), Ellobius lutescens (1)*
5	Bashqurtaran	395	Jun.2014 & Sep.2014 & Sep.2017	*M. persicus (23), M. libycus (3), Arvicola persicus (1), M. tristrami (1), E. lutescens (1), S. fulvus (1)*
6	Sarin Bolagh	116	Jun.2014** & Sep.2017	*M. persicus (6), M. vinogradovi** (1)*
7	Ghomish Darreh	135	Jun.2014 & Sep.2014	*M. persicus (7), M. libycus (1), E. lutescens (1)*
8	Yekkeguney	56	Sep.2014	*M. persicus (2)*
9	Ouch Darreh	245	Sep.2014 & Agu.2015** & Feb.2016	*M. persicus** (17), M. libycus (2), M. qazvinensis (2), M. tristrami (1), E. lutescens (1), S. fulvus (1)*
10	Hajiabad Darreh Si	59	Sep.2014	*M. persicus (6), M. vinogradovi (1),*
11	Sorkhab	196	Sep.2014 & May.2016 & Sep.2017**	*M. persicus ** (12), M. libycus (2), M. vinogradovi (1), S. fulvus (1)*
12	Tazeh Kand	244	Sep.2014 & Oct.2014 & Feb.2017	*M. persicus (15), M. libycus (1), M. vinogradovi (1), E. lutescens (1)*
13	Pir Badam	340	Sep.2014 & Agu.2015 & Agu.2016 & Agu.2017	*M. persicus (20), M. vinogradovi (2), M. libycus (2), M. qazvinensis (1), S. fulvus (1), Mus musculus domesticus (1)*
14	Yekeh Chal Abkelik	58	Sep.2014 & Agu.2016	*M. persicus (3), M. libycus(1)*
15	Bankelik	98	Sep.2014 & Agu.2015 & Agu.2016	*M. persicus (5), M. libycus(2), M. tristrami (1)*
16	Ban Ghoei Darreh Si	72	Jun.2014	*M. persicus (4)*
17	Chopoqly	80	Jun.2014	*M. persicus (4)*
18	Aqdash	543	Jun.2014 &Sep.2014 & May.2016	*M. persicus (23), M. libycus (4), M. qazvinensis (1), M. vinogradovi (1), E. lutescens (1), S. fulvus (1)*
19	Ab Meshkin	687	Oct.2014 & Nov.2016*& Feb.2017	*M. persicus* (28), M. vinogradovi (4), M. libycus (2), M. qazvinensis (1), E. lutescens (1), M. tristrami (1)*
20	Akanlu	447	Jun., Sep., and Oct. 2014*& Feb., and May. 2016 & Agu.2017**	*M. persicus* ** (13), M. libycus (2), M. vinogradovi (2), M. qazvinensis (1), E. lutescens (1), A. a. persicus (1)*
21	Darreh Sinaf	88	Sep.2014 & Agu.2015	*M. persicus (3), M. libycus (1), E. lutescens (1)*
22	Akharpiche	150	Sep.2014 & Agu.2015 & Sep.2017	*M. persicus (6), M. libycus (2), M. vinogradovi (1), E. lutescens (1), C. e. isatissus (1)*
23	Reza Darreh si	284	Agu.2015 & Feb., and Nov.2016	*M. persicus (11), M. libycus (2), M. vinogradovi (1), M. tristrami (1)*
24	Paein Ghaleh	58	Agu.2015	*M. persicus (3),*
25	Yekeh Kalab Kalik	111	Agu.2015 & Agu.2017	*M. persicus (6), M. libycus(1), M. vinogradovi (1)*
26	Polokhore	45	Agu.2015	*M. persicus (2), M. qazvinensis (1), E. lutescens (1)*
27	Choghondar Goli	60	Feb.2016	–
28	Shahgodar	129	May.2016* **, Nov.2016	*M. persicus** (7), M. libycus*(2)*
29	Darreh Jeyran Bolaghi	45	May.2016	*M. persicus (3)*
30	Darreh Dash Ghoei	39	May.2016	*M. persicus (2), M. libycus (1)*
31	Chaltappe	75	Feb.2017	*M. persicus (4)*
32	Dagh Dali	160	Feb.2017 & Agu.2017	*M. persicus (7), M. libycus (2), M. vinogradovi (2), S. fulvus (1)*
33	Baldali Cho	80	Agu.2015	–
	Total	3902		433

the infected rodents or its lower number in the taken samples compared with the detectable level by PCR induces [28] such a condition; similarly, in the present study, three seropositive samples were PCR negative. Given the two weeks of delay in the immune response following the infection with the tularemia agent, PCR was able to reveal certain false-negative results in the serological test during this time. Therefore, both serological and PCR tests are proposed for sample study [29].

It is to be noted that the report of tularemia in humans often occurs after the observation and reports of the disease in animals [30]. Due to the adaptation of the jirds of the genus *Meriones* to its diverse habitats, its high density in the rodent population of the studied regions, and the high infection rate of this species by *F. tularensis* in Hamadan province, the risks of tularemia infection must be explained for people and local and national organizations associated with the public health and treatment. So as to fathom the complexity of this disease, it is necessary to study the characteristics of the rodent ecosystem and the zoonotic transmission pathways of the disease.

Authors' contributions

Idea of the project: Majid Hemati, Mohammad Khalili, Ehsan Mostafavi.

Interpretation of data: Majid Hemati, Mohammad Khalili, Saber Esmaeili, Ahmad Ghasemi, Ahmad Mahmoudi, Ehsan Mostafavi, Balal Sadeghi and Miklós Gyuranecz.

Preparing the manuscript: Majid Hemati, Saber Esmaeili, Ahmad Ghasemi, Mostafavi, Khalili, Ahmad Mahmoudi, Balal Sadeghi and Miklós Gyuranecz.

Critical revision of the manuscript for the development of the protocol and abstracting the data: Majid Hemati, Ehsan Mostafavi, Mahdi Rohani, Balal Sadeghi and Miklós Gyuranecz.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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