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ORIGINAL ARTICLE

A Field Study of Plague and Tularemia in Rodents, Western Iran

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Abstract

Introduction: Kurdistan Province in Iran is a historical focus for plague and tularemia. This study aimed at assessing the current status of these two foci by studying their rodent reservoirs.
Materials and Methods: Rodents were trapped and their ectoparasites were collected. The genus and species of both rodents and ectoparasites were determined. Serological analyses of rodent blood samples were done by enzyme-linked immunosorbent assay for plague and by standard tube agglutination assay for tularemia. Rodent spleen samples were subjected to bacterial culture, microscopic examination, and real-time PCR to search for active plague or tularemia infection.
Results: During this study, 245 rodents were trapped, of which the most abundant genera were *Apodemus* (40%), *Mus* (24.49%), and *Meriones* (12.65%). One hundred fifty-three fleas, 37 mites, and 54 ticks were collected on these rodents. The results of all direct and indirect tests were negative for plague. Serological tests were positive for tularemia in 4.8% of trapped rodents.
Discussion: This study is the first report on the presence of tularemia infection in rodents in Western Iran. Since *Meriones persicus* is a known reservoir for plague and tularemia, and this rodent carried plague and tularemia vectors in Marivan and Sanandaj districts, there is a real potential for the occurrence of these two diseases in this region.

Keywords: *Francisella tularensis*, Iran, plague, rodent, tularemia, *Yersinia pestis*

Introduction

Approximately 60% of infectious diseases and 75% of emerging infectious diseases are zoonoses (Taylor et al. 2001). Currently, the possible resurgence of some infectious diseases, which were thought to be almost eradicated, represents a major challenge in the field of infectious disease control (Morens and Fauci 2013, O'Connor 2013). Plague and tularemia are two important zoonotic infectious diseases, which are endemic in various regions around the world, including Western Iran (Esmaeili et al. 2013, 2014b).

The causative agent of plague is *Yersinia pestis.* The disease is highly lethal as without prompt and efficient treatment the case fatality rate is ≈100% for pneumonic and septicemic plague, and 50–80% for bubonic plague (Chin 2000). The plague reservoirs are wild rodents (Slack 1989) and the vectors are infected fleas (Chin 2000). Plague continues to be endemic in several countries (Perry and Fetherston 1997, Titball and Williamson 2001). In the last 20 years, plague outbreaks have been recorded in countries neighboring Iran such as Afghanistan (2007), Jordan (1997), Saudi Arabia (1994), Kazakhstan (2002), and India (1994, 2002, and 2004)

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(Bakanidze et al. 2010, Gurbanov and Akhmedova 2010, Dikid et al. 2013).

In Iran, plague epidemics have been responsible for numerous human deaths (Azizi and Azizi 2010). An epidemic of bubonic plague in 1772 and 1773 killed ~2 million people (Seyf 1989, Azizi and Azizi 2010). Hundreds of people died of plague in Western Iran during further epidemics in 1829, 1835, 1870, and 1946–1965 (Schlimmer 1874, Karimi 1976, Theodorides 1998). From 1947 to 1966, Baltazard et al. (1951) studied 14,102 rodents in the Kurdistan Province of Iran and identified 66 (0.46%) plague-infected rodents (Karimi 1976). In 1952, a research center was established in Akanlu village (Hamadan Province, Iran) to investigate and monitor natural hotspots of plague in Western and Northwestern Iran (Karimi 1963). In 1979, 14 strains of *Y. pestis* were isolated from rodents and fleas in Sarab County, Western Iran (Karimi 1980). Several studies in Western Iran showed that four types of *Meriones* were of great importance for the plague cycle, two of which (*Meriones vinogradovi* and *Meriones tristrami*) were sensitive to plague, while the other two (*Meriones persicus* and *Meriones libycus*) were resistant. The main flea vector of plague was *Xenopsylla buxtoni* in Western Iran (Karimi 1976). A recent study performed in 2011 and 2012 in Kurdistan and Hamadan Provinces showed that plague might still be present and active among rodents in this region (Esmaeili et al. 2013).

Tularemia, a zoonotic disease caused by *Francisella tularensis* (Sjöstedt 2007), is transmitted to humans either by arthropods (ticks, flies, and mosquitoes), ingestion of contaminated water or food, or aerosols (Hestvik et al. 2015). *F. tularensis* is tightly associated with aqueous environments. Tularemia affects more than 200 species of mammals, birds, reptiles, and fish (Hestvik et al. 2015). Farmers, veterinarians, and hunters are the populations that are at most risk of infection (Hestvik et al. 2015). This disease is common in the Northern Hemisphere, with hotspots in the United States, Russia, Kazakhstan, and Turkmenistan. Tularemia is also reported annually in many countries in Eastern Europe (Tarnvik et al. 2004, Zargar et al. 2015), and in Turkey, a country neighboring Iran (Akalın et al. 2009, Erdem et al. 2014).

The first reports of tularemia in Iran were in 1969 and 1970, in domestic and wild animals from Northwest (Azerbaijan province) and Southeast (Zabol County) of Iran (Arata et al. 1973). The first human case of tularemia in Iran was reported in Kurdistan Province in 1980 (Karimi et al. 1981). A recent study conducted in 2011 and 2012 in this province showed that 14.4% of 250 human serum samples analyzed had anti-*F. tularensis* antibodies (Esmaeili et al. 2014b). In another study performed in 2011 on blood samples from slaughterhouse workers and butchers in Sistan and Baluchestan province (Eastern Iran), 6.52% of 184 analyzed sera had anti-*F. tularensis* antibodies (Esmaeili et al. 2014a). Another study performed in 2013 in these two provinces, showed that one trapped rodent had a positive serology for tularemia by the serum agglutination test (Pourhossein et al. 2015).

As there have been recent reports of positive serology for plague in rodents and dogs (Esmaeili et al. 2013) and for tularemia in humans in Kurdistan Province (Esmaeili et al. 2014b), and as periods of silence and re-emergence are an intrinsic feature of both diseases, our study aimed to further investigate the presence of these two diseases in rodents in hotspots located in the Western and Southern regions of Kurdistan Province.

Materials and Methods

Rodent trapping

Trapping was conducted using traditional live catch traps with baits (dates, cucumber, and cheese puffs). Spatial information on rodent nest locations was recorded using the Global Positioning System. Simultaneously, sampling location, topography, and vegetation of these places were recorded.

Isolation and identification of ectoparasites

Rodents were handled with long forceps and placed in a basin filled with water. Ectoparasites were released into the water by slowly brushing and blowing in the rodent fur. They were collected from the water with forceps and placed into individual microtubes filled with 70% ethanol. The samples were sent to the Entomology Department, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, for identification.

Rodent analyses

After removal of ectoparasites and insecticide spraying, the trapped rodents were transferred to the local research laboratories for identification by morphological features according to identification keys (Corbet 1978, Kryštufek et al. 2005). These rodents were then anesthetized with chloroform, and their blood was collected by heart puncture. For each sample, a blood smear was prepared and Gram stained. The remaining blood samples were left to coagulate at room temperature and centrifuged at 3000 rpm for 10 min. The supernatant was stored at −20°C for further investigations. For microbiological studies, a piece of spleen was aseptically removed and kept at −4°C. Frozen sera and spleen samples were sent to the Research Center for Emerging and Reemerging Diseases, Pasteur Institute of Iran.

Culture of spleen samples

After addition of 0.5 mL normal saline, each rodent spleen sample was homogenized using a manual grinder under sterile conditions, and 50 μL of the suspension was streaked onto sheep blood agar (SBA), brain heart infusion agar, and MacConkey agar plates for *Y. pestis,* and on SBA and cysteine heart agar media enriched with polymyxin B, amphotericin B, cefepime, cycloheximide, and vancomycin antibiotics. The plates were incubated at 28°C for *Y. pestis* and at 37°C for *F. tularensis*, and checked daily for 1 week. Each suspected colony was Gram stained and gram-negative coccobacilli resembling *Y. pestis* or *F. tularensis* were subjected to biochemical tests, including catalase, oxidase, lactose fermentation, citrate, urease activity, and indole. RT-PCR was performed on suspected colonies.

DNA extraction and RT-PCR

DNA was extracted from each spleen sample and suspected colony using the Qiagen DNA Extraction Kit and subsequently stored at −70°C until use. RT-PCR was performed using the Rotor-Gene 6600 (6-Plex) RT-PCR System (Corbett Life

TABLE 1. PRIMERS AND PROBES USED TO DETECT *YERSINIA PESTIS* AND *FRANCISELLA TULARENSIS*

Target	*Primer/probe*	*Sequence (5′ to 3′)*	*Product (nt)*	*Reference*
yihN (chromosome)	Forward	CGCTTTACCTTCACCAAACTGAAC	128	Stewart et al. (2008)
	Reverse	GGTTGCTGGGAACCAAAGAAGA		
	Probe	FAM-TAAGTACATCAATCACACCGCGA CCCGCTT-BHQ1		
caf1 (pMT1)	Forward	CCGTTATCGCCATTGCATTATTTGG	194	Stewart et al. (2008)
	Reverse	GCCAAGAGTAAGCGTACCAACAAG		
	Probe	TxR-AAGCACCACTGCAACGGCAACTCTT-BHQ2		
tul4 (chromosome)	Forward	ATTACAATGGCAGGCTCCAGA	89	Versage et al. (2003)
	Reverse	GCCCAAGTTTTATCGTTCTT CTCA		
	Probe	FAM-TTCTAAGTGCCATGATACAAGCTT CCCTTACTAAGTA-TAMRA		
fopA (chromosome)	Forward	AACAATGGCACCTAGTAATATTTCTGG	86	Bushon et al. (2010)
	Reverse	CCA CCAAAGAACCATGTTAAACC		
	Probe	FAM-TGGCAGAGCGGGTACTAACAT GATTGGT-TAMRA		

Science). *Y. pestis*-positive controls were the chromosomal *yihN* gene and the pMT1-borne *caf1* gene cloned into plasmid pUC57 (provided by the Pasteur Institute of Iran). The *F. tularensis*-positive controls were the *tul4* and *fopA* genes cloned into plasmid pUC57 (Table 1) (Emanuel et al. 2003, Bushon et al. 2010). The beta-actin gene (Qiagen Company) was used as an internal control or as a housekeeping gene to normalize the expression of the target gene levels between different samples.

Plague and tularemia serology

An enzyme-linked immunosorbent assay (ELISA) test was used to detect antibodies against the capsular F1 antigen of *Y. pestis*, following the protocol described in Chanteau et al. (2003). F1 antigen, positive and negative control sera, anti-rodent-conjugated antibodies, and solutions necessary for the ELISA test were provided by the Institut Pasteur in Madagascar. For tularemia, serum agglutination was done according to the protocol provided with the kit (Bioveta, Inc., Ivanovice, Czech Republic). Dilution titers higher than 1:80 were considered as positive, while a titer of 1:40 was recorded as suspect (Gyuranecz et al. 2011). Positive sera for tularemia were tested for brucellosis by the tube agglutination method (Bioveta, Inc.). Dilution titers higher than 1:80 were considered as positive for brucellosis.

Results

The Kurdistan Province where the study was performed is shown in Figure 1. The 29 villages investigated covered 5

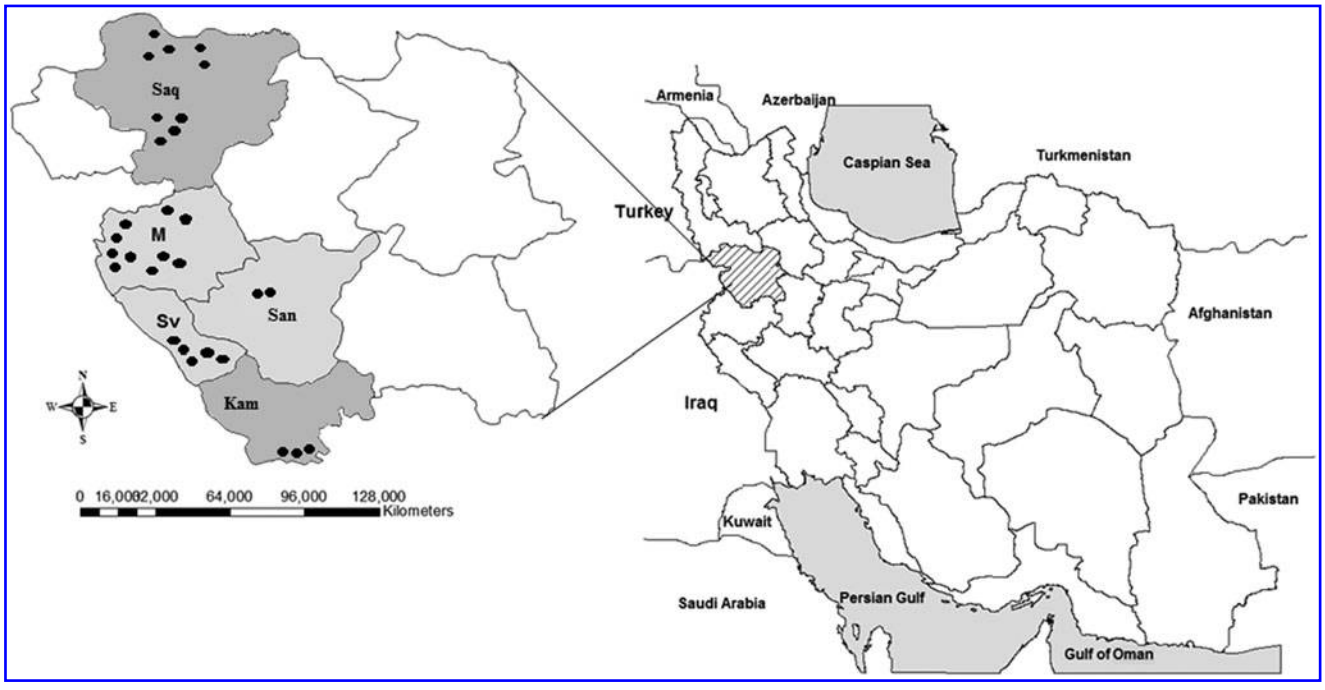

FIG. 1. Sampling location in Kurdistan Province, west of Iran. The *dots* represent the location of the villages sampled. Kam, Kamyaran–Sonqor; M, Marivan; San, Sanandaj; Saq, Saqqez; SV, Sarvabad.

counties and comprised 12 villages, which were previous hotspots of plague and 17 villages that had no previous reports of infection but were neighboring the ancient plague foci (Fig. 1).

A total of 245 rodents were trapped. The most frequently were *Apodemus* (40%, mostly *Apodemus witherbyi*), *Mus* (24.5%, predominantly *Mus macedonicus*), and *Meriones* (12.6%, mostly *M. persicus*). Most catches were from Saqqez (84 rodents), followed by Marivan (65 rodents), Kamyaran–Sonqor (35 rodents), Sarvabad (34 rodents), and Sanandaj (26 rodents) (Table 2).

In total, 153 fleas, 37 mites, and 54 ticks were collected. Of these, 109 fleas (71.2%), 4 mites (10.8%), and 12 ticks (22.2%) were collected on *M. persicus*.

The 153 fleas were collected in all localities. They were identified as *X. buxtoni* (123), *Paraceras melis melis* (11), *Ctenophthalmus iranus persicus* (11), *Leptopsylla segnis* (6), *Parodoxopsyllus microphtalmus* (1) and *Ctenophthalmus rettigi smiti* (1). The 37 collected mites were *Eulaelaps stabularis* (15), *Haemolaeps glasgowi* (13), *Laelaps nuttalli* (6), *Echinolaelaps echidninus* (2), and *Dermanyssus sanguineous* (1). The 54 ticks were identified as *Haemaphysalis* spp. (35), and *Hyalomma* spp. (19).

Microscopic analyses, bacterial cultures, and RT-PCR were negative for the presence of *Y. pestis* or *F. tularensis* in the spleen of the rodents trapped. The ELISA assay did not detect the presence of anti-*Y. pestis* F1 antibodies. In contrast, the tube agglutination test was positive (titers $\geq$ 1:80) for 8 of 164 rodents (4.8%), and suspected (titers of 1:40) for 9 rodents. Positive rodents for tularemia were collected from four regions of Kurdistan (Table 3). Only one of the positive sera of tularemia was also positive for brucellosis at 1/160 titer.

Discussion

This study is the first report on the presence of tularemia infection in rodents in Western Iran. The study did not detect signs of plague in the studied rodents. However, most of the collected rodents, fleas, and ticks had the potential to transmit plague and tularemia.

Most trapped rodents in this study were *A. witherbyi*, *M. macedonicus*, and *M. persicus*. Several studies on plague and tularemia in various regions around the world showed that rodent species (reservoirs), which take part in the epidemiological cycles of each of these two diseases, are exclusive (Bengis et al. 2004). However, the three aforementioned

Table 2. Sampling Locations and Type of Rodents Trapped in Kurdistan Province

Studied region		
County	*Village*	*Species of trapped rodent (No.)*
Saqqez	Kani Niaz[a]	*Mus macedonicus* (16), *Meriones persicus* (4), *Apodemus* sp. (2), *Microtus* sp. (1)
	Qahrabad	*M. persicus* (2), *Apodemus witherbyi* (3), *Apodemus* sp. (1)
	Seyedabad[a]	*A. witherbyi* (6), *M. macedonicus* (3)
	Bughda Kandi	*Calomyscus* sp. (4), *M. persicus* (2), *M. macedonicus* (1)
	Ahmadabad	*A. witherbyi* (4), *M. macedonicus* (1), *M. persicus* (1)
	Quchaq	*M. persicus* (3), *M. macedonicus* (2), *Calomyscus* sp. (2), *A. witherbyi* (2), *Dryomys nitedula* (2)
	Jameiyan	*M. macedonicus* (5), *A. witherbyi* (1)
	Koose	*M. macedonicus* (3), *Cricetulus migratorius* (1), *A. witherbyi* (2), *M. persicus* (2)
	Sara	*A. witherbyi* (5), *M. macedonicus* (3)
Marivan	Bayveh	*A. witherbyi* (2), *A. flavicollis* (1)
	Anjiran	*Apodemus* sp.
	Do Palureh[a]	*Apodemus* sp. (11), *A. witherbyi* (4)
	Border checkpoint	*M. persicus* (4)
	Esmaeilieh	*Apodemus* sp. (2)
	Bardeh Rasheh[a]	*Apodemus* sp. (2) *M. musculus* (1), *A. witherbyi* (3), *A. flavicollis* (1)
	Dega Shaykhan	*Cricetulus migratorius* (1), *A. witherbyi* (2), *Apodemus* sp. (2), *A. flavicollis* (1)
	Kheyrabad	*D. nitedula* (8), *A. flavicollis* (1), *M. macedonicus* (1)
	Qamishleh[a]	*M. macedonicus* (6), *Microtus* sp. (6), *A. witherbyi* (4), *M. persicus* (1)
	Marlang	*D. nitedula* (1)
Sarvabad	Sarumal[a]	*Microtus* sp. (3), *A. flavicollis* (2), *Meriones* sp. (1), *A. witherbyi* (1)
	Tefin	*M. macedonicus* (3)
	Sorkheh Tut	*Microtus* sp. (1)
	Palangan	*Apodemus* sp. (1)
	Bisaran[a]	*A. witherbyi* (11), *A. flavicollis* (2), *Microtus* sp. (4), *M. macedonicus* (1), *D. nitedula* (3), *M. persicus* (1)
Kamyaran–Sonqor	Jamishan[a]	*M. macedonicus* (9), *Calomyscus* sp. (4), *Microtus* sp. (5), *M. persicus* (2), *A. witherbyi* (2)
	Sarbaghleh[a]	*D. nitedula* (1), *A. witherbyi* (6)
	Sameleh[a]	*M. macedonicus* (4), *A. witherbyi* (1), *M. persicus* (1)
Sanandaj	Qaderabad[a]	*M. persicus* (7), *A. witherbyi* (7), *Microtus* sp. (3), *M. macedonicus* (1)
	Gozar-darreh[a]	*Microtus* sp. (5), *A. witherbyi* (3)

[a]Villages where plague outbreaks were reported over the past 100 years.

Table 3. Features of Trapped Rodents with a Positive Serology for Tularemia

Geographical location of trapping	*County, village*	*Antibody titer*	*Type of trapped rodent*
Central Kurdistan Province	Sanandaj, Ghader Abad	1:80	*Microtus* spp.
Southwestern Kurdistan Province	Sarvabad, Sarumal	1:80	*Meriones* spp.
Western Kurdistan Province	Marivan, Qamishleh	1:80	*M. macedonicus*
	Marivan, Kheyrabad	1:80	*D. nitedula*
Southern Kurdistan Province	Kamyaran–Sonqor, Sameleh	1:80	*M. macedonicus*[a]
	Kamyaran–Sonqor, Jamishan	1:80	*Calomyscus* spp.
		1:80	*M. macedonicus*
		1:160	*Calomyscus* spp.

[a]Positive for brucellosis.

rodent species in this study can be the reservoir of plague and tularemia. *Apodemus* spp. (Xing Yuan et al. 2007, Akalın et al. 2009), *Mus* spp. (Saunders and Giles 1977, Sjöstedt 2007), and *Meriones* spp. (Zhang et al. 2006) are reported to be the reservoir of both plague and tularemia in different regions of the world.

Fleas are the main vectors of plague. In our study, the most abundant fleas were *X. buxtoni,* which were previously considered as a major carrier of plague in Iran (Baltazard et al. 1960, Karimi 1980). Transmission of tularemia by *Xenopsylla cheopis* has also been reported (Prince and McMahon 1946), but ticks are the main vectors, especially those of the species *Hyalomma* (Amirova et al. 1988). In this study, the density of fleas on the body of *M. persicus* was much higher than on other rodents, which is in accordance with previous studies (Karimi 1980). Since this rodent species is a known reservoir for both plague and tularemia, and since it carries vectors for both diseases, our data suggest that there is a real potential for the occurrence of plague and/or tularemia cases in Marivan and Sanandaj Counties.

Plague has a long history in Iran (Hashemi Shahraki et al. 2016). Various cities in Iran suffered epidemics and there are recent indirect evidences that plague is still circulating in the animal reservoir in Kurdistan Province: 3.5% of dogs and 1% of the rodents analyzed in 2011 and 2012 had a positive serology (Esmaeili et al. 2013). Although outbreaks had been reported in locations in which the rodents were collected in this study (Baltazard et al. 1951, 1960, Esmaeili et al. 2013), no sign of *Y. pestis* activity was detected. There are numerous recent examples of the disease entering a long period of silence before reappearing. During these silent periods, the plague causative agent continues its life cycle in rodents and fleas. Changes of climate can also play a significant role in the decline and rise of plague in a region (Duplantier et al. 2005). Lack of evidence of *Y. pestis* carriage in our analysis can also be due to the relatively low number of samples collected. Continued surveillance of the potential reservoirs and vectors is necessary to get a better overview of the disease in this region.

Specific *Y. pestis* and *F. tularensis* antibodies reach detectable levels 10 to 20 days after initiation of the infection, but sensitive species die before detectable levels of specific antibodies can be obtained. One possible solution to increase chances of detecting plague or tularemia-infected rodents would be to put in place a monitoring system in the villages to report dead rodents to public health teams that will collect and study them by culture and RT-PCR.

The first report of human tularemia in Kurdistan Province was recorded in Marivan County in 1980 (Karimi et al. 1981). A study performed in Kurdistan Province in 2011 showed that 14.5% of people from various population groups had anti-tularemia IgG antibodies. This study also indicated that being in contact with wild animals is the major risk factor for tularemia infection (Esmaeili et al. 2014b). In a more recent study performed in 2013 in Sistan and Baluchestan Province (Eastern Iran), serum agglutination tests for tularemia were positive in one of nine trapped rodents (Pourhossein et al. 2015). However, in the current study, the results of standard tube agglutination test showed seropositive cases among 4.8% of the rodents, including *Microtus* spp., *M. macedonicus*, *Dryomys nitedula*, and *Calomyscus* spp.

Agglutination is the standard serological test used for determining the presence of antibodies against *F. tularensis*. The slide agglutination test we used in this study exhibits 100% sensitivity and 83% specificity (WHO guidelines on tularemia 2007). The main agent that may cause cross-reactions with *F. tularensis* is *Brucella*, but we found that only one out of the nine sera that were tularemia positive was positive for *Brucella*. A coinfection with the two pathogens may have occurred, but even if we eliminate the *Brucella*-positive animals, this indicates that the eight other rodents had most likely been infected with *F. tularensis.* Performing western blot analyses would be useful to further eliminate cross-reactions. However, this test cannot be commercially purchased and is not available in our laboratory.

There are various reports on human tularemia in countries near Iran, such as Armenia (Melikjanyan et al. 2014), Azerbaijan (Clark et al. 2012), and Turkey (Dedeoğlu et al. 2007), all of which indicate the importance from a public health point of view of this disease in this geographical area. However, there is no study of tularemia in rodents and wild animals in Iran's neighboring countries, except in Turkey. Therefore, further studies on possible reservoirs and vectors in Iran and nearby countries can provide insights into the epidemiology of tularemia in this region of the world.

Conclusion

This study is the first report on the presence of tularemia among rodents in Western Iran. Continuation of such studies on a larger scale and on the potential vectors of plague and tularemia both in traditional hotspots such as Kurdistan Province and in other Western regions of Iran can improve our understanding of the current situation of these two highly dangerous diseases. The isolation of *Y. pestis* or *F. tularensis* would be a milestone in the research on plague and tularemia in Iran.

Following the 3–4-year cycles of plague in rodents in the region in recent years, regular monitoring of the disease within a 4-year period as well as in longer periods, along with blood sampling of dogs and cats, could improve our understanding of the status of this disease in the region. Rapid and exact diagnosis of these infections in humans is also important to decrease mortality and to control potential epidemics.

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Author Disclosure Statement

No competing financial interests exist.

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