Original Article

Molecular Survey of *Leishmania* Infection of Sand Flies in Karun County, Southwestern Iran

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(Received 03 Sep 2023; accepted 13 Mar 2024)

Abstract

Background: Zoonotic cutaneous leishmaniasis (ZCL) is widely distributed in Iran and around the world. Also, Khuzestan Province is an endemic focus of ZCL. This study aims to investigate the natural infection of sand flies with the *Leishmania* parasite in Karun County.

Methods: Sand flies were collected from Jangiyeh, Qaleh Chanan, Kut-e-Navaser, and Ghazavieh in the spring and summer in the year of 2019, by installing 60 sticky paper traps each time (30 traps outdoors and 30 traps indoors). Two hundred female sand flies with different abdominal conditions (empty, blood-fed, semi-gravid, and gravid) were examined for infection rate using the Nested-PCR method.

Results: In this study, seven species of sand flies including *Phlebotomus papatasi*, *Ph. alexandri*, *Ph. sergenti*, *Ph. caucasicus*, *Sergentomyia tiberiadis*, *Se. sintoni*, and *Se. antennata* were reported from Karun County, with a frequency of 79.64%, 16.96%, 1.07%, 0.18%, 0.36%, 1.61%, and 0.18%, respectively. Only eleven specimens of *Ph. papatasi* were found to be positive for *Leishmania major*, with an overall infection rate of 7.8%. The infection of *Ph. papatasi* was specifically reported in blood-fed, gravid, and semi-gravid specimens, with infection rates of 17.02%, 4.35%, and 14.29%, respectively.

Conclusion: In this study, the infection of *L. major* from *Ph. papatasi* was reported. The results can be used in planning the control of ZCL in the study area.

Keywords: *Leishmania*; Sand fly; Karun; Khuzestan

Introduction

Leishmaniasis is still one of the most neglected diseases and has different clinical manifestations in Iran and the world (1). The prevalence of leishmaniasis in the Old World is mostly in the Middle East. Furthermore, cutaneous leishmaniasis has a high prevalence in 12 countries, Algeria, Colombia, Brazil, Iran, Syria, Peru, Morocco, Tunisia, Afghanistan, Pakistan, Turkey, and Saudi Arabia (2). Cutaneous leishmaniasis cases are currently common in many rural areas of 19 out of 31 provinces of Iran (3, 4).

The reality is that only about 20% of all leishmaniasis cases in Iran have been reported through government health centers. According to the reports of the Center for Disease Management, the average number of people who suffer from different types of leishmaniasis is 20000 people per year in Iran, but undoubtedly, the prevalence of the disease is 4–5 times higher than the official reports and about 80% of these cases are related to zoonotic cutaneous leishmaniasis (ZCL) (5).

One of the endemic regions of the disease

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is Khuzestan Province where, at least three species of *Leishmania* (*L. major*, *L. tropica*, and *L. infantum*) have been confirmed (6). Also, *Nesokia indica* and *Tatera indica* play an important role as the reservoir of ZCL in this province (7). Karimi et al. (2020) detected *L. major* infection rate in *T. indica* and *N. indica* at 6.7% and 5.5% in Shush County, respectively (8).

After ending the Iran-Iraq War (1980–1988), reconstruction and making towns in the new regions caused changes in the ecology of the reservoirs and vectors in the Khuzestan Province. Therefore, control measures to reduce the incidence of the disease, identification of vectors and their infection rate, and the peak of infection during the year are very important and crucial issues. Finding infected sand flies with *L. major* is an essential step in identifying the vector and also, the potential of disease transmission in endemic areas (9). Microscopic examination and culture of parasites are common methods to estimate the natural infection rate of promastigote in vectors and the *Leishmania* infection rate of reservoir host. Being a few numbers of parasites in the digestive tract of the sand fly most likely gives us false negative results, because they may be eliminated or destroyed before the parasite arrive to the mouthparts. Also, in these methods, species detection of parasites is not possible (10).

In contrast to the microscopic method, using the DNA-based methods will give us more accurate results regarding identifying the parasite species. The advantages of using the molecular methods include the need for a less amount of DNA, the lack of influence of confounding conditions, the environment, and the host, the ability to examine many samples in a short time, and the high sensitivity of the test (11, 12).

Molecular methods such as Nested-PCR and Semi-Nested PCR are used to identify *Leishmania* parasites in sand flies, and the results have shown that these methods have high sensitivity (13).

According to the report of the Centers for Disease Control and Prevention, there were 67 cases of ZCL in Karun County, which is located in the west of Ahvaz and the north of Khorramshahr and Shadegan, between 2014 and 2017. Considering the semi-urban lifestyle of the people of this county, its proximity to Ahvaz County as the focus of cutaneous leishmaniasis diseases, Annual cases of cutaneous leishmaniasis, and the lack of a study on the status of sand fly infection, the present study was designed to determine the natural infection of sand fly with *Leishmania* parasite in Karun County.

Materials and Methods

Study area

Karun (31.2564° N, 48.6586° E) is one of the counties in Khuzestan Province, its area is 1197 Km^2 and the population of this county is 105,872 people. This county leads to Ahvaz County to the north, Shadegan County to the south, Karun River to the west, and Ahvaz County to the east. It leads to Ahvaz City from the north, Shadgan from the south, Karun River from the west, and Ahvaz from the east. This county has a hot and humid climate, and in the summer, the temperature reaches above 50°. This county includes two parts, named: Markazi and Suyseh, and four rural districts named Kut Abdullah, Qaleh Chanan, Suyseh, and Morran. Kut Abdullah, Kanan, Rabie, and Shirin Shahr cities are located in Karun County (14).

Sand fly collecting

Based on the report of cutaneous leishmaniasis in recent years, the rural districts of Jangiyeh, Qaleh Chanan, Kut-e-Navaser, and Ghazavieh of Karun County were selected for sand fly collection (Fig. 1). The sand flies were collected from study areas twice during the spring and summer in 2019. The specimens were transferred to the Medical Entomology Laboratory of Ahvaz Jundishapur University of Medical Sciences. Firstly, the abdominal

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condition (empty, blood-fed, semi-gravid, and gravid) of female sand flies was detected. Then, the head and three terminal segments of the abdomen of female specimens were separated, and microscopic slides were prepared using a drop of Puri's medium and the other parts of the body were placed in 96% alcohol and stored in a -20° freezer for molecular examinations. The characteristics of the samples, including the sample code, the collector's name, the date of collection, and the name of the location were recorded on separate forms. The samples were identified using the reliable key (15).

Molecular analysis

DNA extraction

DNA was extracted from the thorax and abdomen of sand flies using DNA (AccuPrep® Genomic DNA Extraction Kit; Cat No. K-3032) according to the manufacturer's protocol. The extracted solution was stored at 4 °C for the next steps of the experiment.

Selected primers

To detect *Leishmania* DNA in sand flies, we used a Nested-PCR method using two primers for the first step including CSB2XF (5'-C/GA/GTA/GCAGAAAC/TCCCGTTCA-3') and CSB1XR (5'-ATTTTTCG/CGA/TTTT/CGCAGAACG-3') and for the second step reported 13Z (5'-ACTGGGGGTTGGTGTAAAA TAG-3') and LiR (5'-TCGCAGAACGCCCCT-3') (14). These primers are designed based on the conserved region of the small kDNA (mini-circle) loops which produce PCR products with a length of 680 bp, 750 bp, and 560 bp for *L. donovani*/*L. infantum*, *L. tropica*, and *L. major*, respectively.

Semi-Nested PCR

First-round PCR mixtures contained 2.0 mM MgCl2, 200 μM deoxynucleoside triphosphates, 20 mM (NH4)2SO4, 75 mM Tris-HCl (pH 9.0), 0.01% Tween, 0.4 U of Red Hot *Taq* polymerase (Advanced Biotechnologies, Leatherhead, United Kingdom), and 40 ng each of primers CSB2XF and CSB1XR in a final volume of 20 μl. The first round of PCR was conducted using the following conditions: firstly, denaturation 1 cycle at 94 °C for 5 min, followed by 30 cycles including denaturation at 94 °C for 45 s, annealing at 51 °C for 45s, extension at 72 °C for 45 s, and a final extension at 72 °C for 45 s. The total volume of the reaction was increased to 20 μl by adding the appropriate amount of double distilled water (ddH2O), and the above thermal program was used in the thermocycler to amplify the DNA fragment.

The second round of Nested-PCR was done in the same condition as the first step and with 1μl of the product of the first round as the template for the second round. Double distilled water was used as negative control and the reference strain of *L. major* (MHOM/IR/54/LV39) was used as the positive control. At the last stage, 10 μl of the PCR results were electrophoresed and visualized on gel agarose 1% including ethidium bromide.

Results

Sand fly species

In total, 560 sand flies were collected from Karun County. Furthermore, seven species were identified, including *Phlebotomus papatasi*, *Ph. alexandri*, *Ph. sergenti*, *Ph. caucasicus*, *Sergentomyia tiberiadis*, *Se. sintoni*, *Se. antennata*. The most abundant species were reported as *Ph. papatasi* (79.64%), and the lowest frequency belonged to *Ph. caucasicus* and *Se. antennata* (0.18%). *Phlebotomus papatasi* was the most abundant species captured from the outdoors with a frequency of 68.98% in Karun County. *Phlebotomus sergenti*, *Ph. caucasicus* and *Se. antennata* were reported to be 0.53% from outdoors.

Fig. 2 shows the frequency percent of sand flies based on gender. In all study areas, the abundance of females was more than males.

In this study, out of a total of 323 female sand flies captured, 200 of them were selected for PCR testing to determine *Leishmania* in-

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fection, which included 141 *Ph. papatasi*, 46 *Ph. alexandri*, 6 *Ph. sergenti*, and 7 *Se. sintoni*. Out of 200 samples, only 11 (8.7%) *Ph. papatasi* were positive for *L. major*. The infection rate of *Ph. papatasi* was 11.67% in Jangiyeh, but the examined sand flies in Kut-e-Navaser and Ghazavieh were negative (Table 1).

The infected *Ph. papatasi* rate was reported at 8.79% and 6.0% in the summer and spring, respectively. Furthermore, the infected sand flies were not reported from Ghazavieh and Kut-e-Navaser. Approximately 17.02% of blood-fed sand flies were infected with *L. major*. However, no *Leishmania* infection was observed in the sand flies that were empty in terms of their abdominal status. In outdoor sampling, 41 sand flies with different abdominal conditions (blood-fed, gravid, semi-gravid, and empty) were also tested for *Leishmania* infection using Nested PCR, but no infection was observed (Table 2). *Phlebotomus papatasi* infection was reported in the summer with 8 out of 91 (8.79%) samples.

In general, the highest *Leishmania* infection was reported in blood-fed *Ph. papatasi* at 14.8%, and no positive results were observed in empty abdominal condition (Fig. 3). The result of electrophoresis tests showed that eleven sand flies were infected with *L. major* and the length size of the bands were 560 pb (Fig. 4).

Table 1. Frequency of *Leishmania* infection of *Phlebotomus papatasi* determined by Nested PCR in Karun County, Iran, 2019

Study area	Number of examined specimens	Infection	
		N	%
Jangiyeh	60	7	11.67
Qaleh Chanan	46	4	8.7
Kut-e-Navaser	21	0	0
Ghazavieh	14	0	0

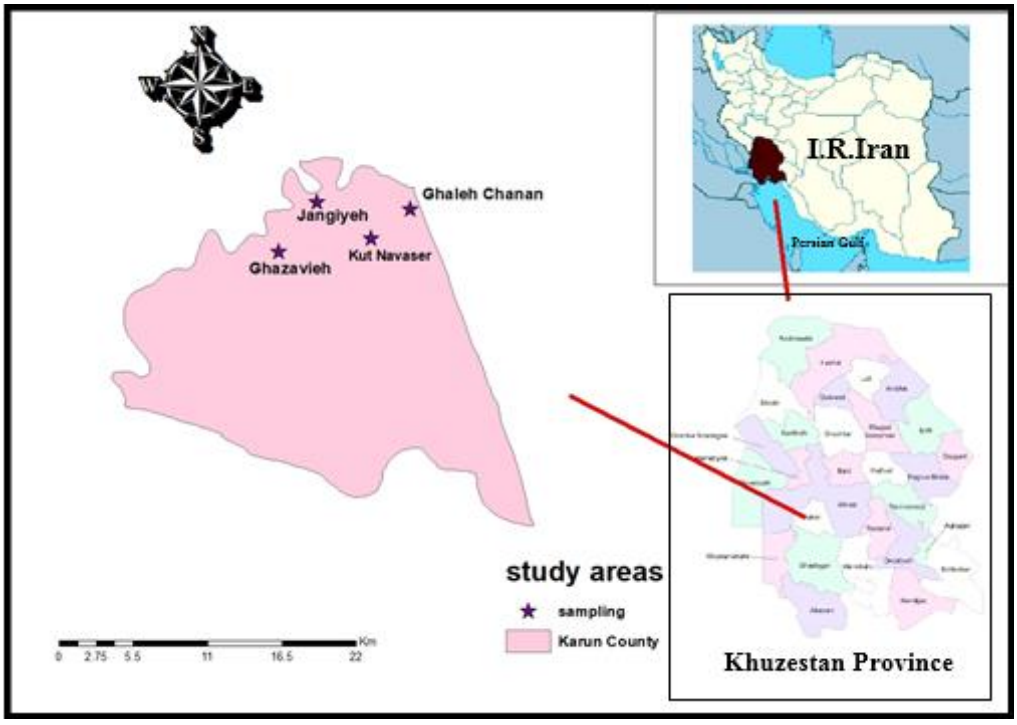

Fig. 1. Study areas in Karun County, Khuzestan Province, 2019

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Table 2. Frequency of *Leishmania major* infection in *Phlebotomus papatasi*, Karun County, Iran, 2019

Areas	Abdominal conditions	Number of examined specimens	*Leishmania* infection	
			N	%
Indoor	blood-fed	47	8	17.02
	gravid	23	1	4.35
	semi-gravid	14	2	14.29
	empty	16	0	0
	Total	100	11	11.0
Outdoor	blood-fed	7	0	0
	gravid	26	0	0
	semi-gravid	3	0	0
	empty	5	0	0
	Total	41	0	0

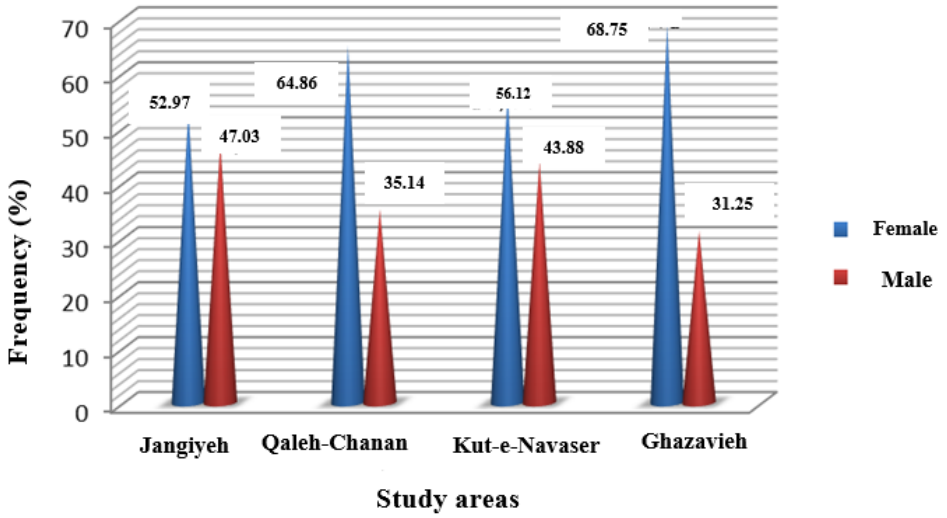

Fig. 2. Frequency of collected sand flies based on location and gender in Karun County, Iran, 2019

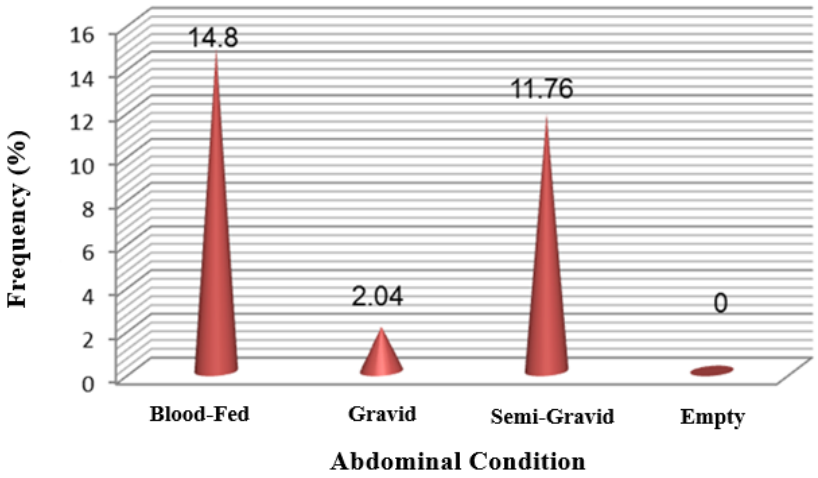

Fig. 3. Frequency of *Leishmania major* infection based on *Phlebotomus papatasi* abdominal status, Karun County, Iran, 2019

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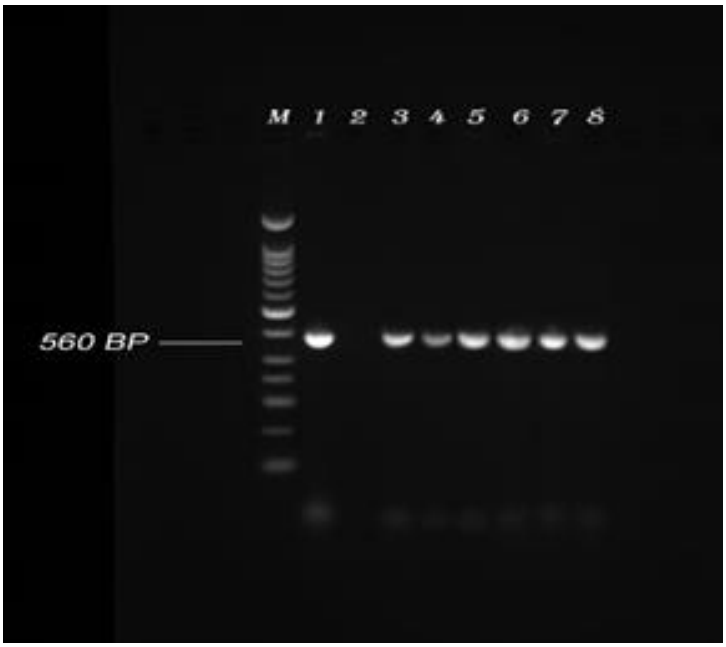

Fig. 4. Electrophoresis of the Nested-PCR products for *Leishmania* kDNA amplification in *Phlebotomus papatasi* sand flies, Karun County, South of Iran, 2019. M: 100 bp molecular weight marker, Sinagen, 1: *L. major* standard (MHOM/IR/54/LV39), 2: negative control ddH2O, 3-8: *L. major* parasites isolated from *Ph. papatasi.*

Discussion

Zoonotic cutaneous leishmaniasis is an endemic disease in Khuzestan Province. This disease has also been reported in rural areas of neighboring countries such as Iraq, Turkey, Saudi Arabia, Afghanistan, and Pakistan (12). To find vector species and the potential for ZCL transmission in endemic areas, finding sand flies with parasite infections is a crucial first step. To complete the ecological knowledge on sand flies for their control during epidemics, this study set out to ascertain the natural infection of sand flies with *L. major* in Karun County.

Jahanifard et al. (2014) reported *Ph. papatasi*, *Ph. alexandri*, and *Se. sintoni* in Khorramshahr, as well as *Ph. papatasi*, *Ph. alexandri*, *Se. sintoni* and *Se. tiberiadis* in Shush (16). However, they also showed 72 other sand fly species in these two counties (17) that were not observed in our study. *Phlebotomous papatasi* was observed with high frequency (n=1425) at 57.344% in Khuzestan Province (18).

The reason for changes in the species composition of sand flies may be due to human intervention in their natural environment, changes in the region's climate, differences in sampling intervals in each study, variety of sampling methods, and the period and time of sampling.

Sergentomyia sintoni was a species that was exclusively found in rodent burrows in the current investigation. This species was found in great numbers in the gerbil burrows in Abarkouh, Yazd Province (19).

Sergentomyia tiberiadis and *Se. sintoni* were collected from the burrows of rodents in Rafsanjan County (20). The Jangiyeh rural district had the most sand fly species (51.25%), whereas Ghazavieh had the lowest abundance of sand flies (11.42%). The reason for the difference in sand fly abundance among the study areas may be due to the existence of suitable breeding places for sand flies, agricultural and livestock activities in some areas, and differences in weather conditions during the sam-

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pling time. Our findings indicate that *Ph. papatasi* was the dominant species indoors and outdoors. It seems that this species not only plays a role in transmitting ZCL to humans but also, it is a vector of *Leishmania* transmission among rodents. This species has adapted to live in human settlements and animal shelters (21).

The abundance of collected vectors from indoor, outdoor, and rodent burrows in Khuzestan Province were 312 (61.7%), 136 (26.9%), and 58 (11.5%), respectively (22). This species was reported with 69.68% abundance from Roffayeh City (23). In Shush and Khorramshahr Counties, *Ph. papatasi* was introduced as the species that was most common in animal shelters and human settlements (17).

This species is shown to be an endophil species by the high frequency of its occurrence in the counties of Shush, Khorramshahr, and Karun. Leishmaniasis ecology and epidemiology must be taken into consideration if the disease is to be managed in endemic areas. This is the major challenge that epidemiologists face in identifying the reservoir and vector of the disease. It should be noted that identifying infected sand flies as the vector of leishmaniasis and determining the infection rate in endemic areas is necessary (24, 25).

In this study, infected *Ph. papatasi* with *Leishmania* parasites were identified by molecular methods indoors in Karun County. *Leishmania* infection of this species was reported for the first time in this county, and its infection rate indoors was 11%.

Leishmania major infection in *Ph. papatasi* was also reported by molecular methods in Golestan Province (26, 27), Fars Province (28), Kerman Province (13), Bushehr Province (29) and Khuzestan Province (30). In the present study, the *Leishmania* infection rate of *Ph. papatasi* was calculated as 7.8%. In the previous studies, the *Leishmania* infection rate of the main vector of ZCL in Orzouyeh County was 6.5% (29), 7.4% in Roffayeh County (30), 4.35% in Shush County (8), 10.41% in Bayza County (28), and 10.1 % in Gonbad-e Kavus County (26). Also, in 1974 Javadian and Mesghali (31) reported leptomonad infection in three *Ph. papatasi* specimens in Khuzestan Province. It should be noted that the highest infection rate of *Ph. papatasi*, which was 37.8%, was observed during an epidemic in Badrood, Isfahan Province (32). The prevalence of *Leishmania* parasites in sand flies can be significantly changed during the transmission season in the ZCL foci (33). Moreover, Khuzestan Province is one of the ZCL foci.

Most of the previous studies in this province are related to the sand fly fauna and a few studies have been carried out to identify the *Leishmania* species in the vector and reservoirs of the disease by molecular methods (30, 8). Molecular methods are useful for identifying parasite infection and the Nested-PCR is one of the sensitive methods for detecting *Leishmania* compared to microscopic methods (34). However, one of the problems of using molecular methods to determine *Leishmania* in vectors is the inability to differentiate between amastigotes and promastigotes forms in infected sand flies that have fed on blood (35). Therefore, finding the genome of *Leishmania* in the sand fly cannot be a definitive criterion for determining that sand fly is a definite vector of the parasite. In this study, the highest level of infection (17.02%) was related to blood-fed *Ph. papatasi*. However, the infection rate was reported to be 4.35% and 14.29% in the gravid and semi-gravid species, respectively.

Therefore, in gravid and semi-gravid sand flies, it seems that the parasite had the opportunity to complete a part of its life cycle inside the body of the sand fly, which could confirm its vector-borne transmission. In this study, Nested PCR using four primers, CSB2XF and CSB1XR, 13Z, and LiR, was used to amplify 560 bp for *L. major*. Arjmand et al. (2014) reported *L. major* using these primers from human samples in Varzaneh City, Isfahan Province (16).

Maraghi et al. (2007) reported fragments of 750 bp and 560 bp for *L. tropica* and *L.*

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major using Nested-PCR on human samples, respectively (36). Also, 2.2% of collected sand flies were infected with *L. major* in Damghan City (37). Vazirianzadeh et al. (2013) reported *L. major* in *Ph. papatasi* and *Tatera indica* in Roffayeh City (30). It should be noted that all the studies mentioned are consistent with our study in terms of producing the fragment of 560 bp for *L. major*.

Conclusion

Considering the isolation of *L. major* from *Ph. papatasi* in this county, it is suggested that other counties of Khuzestan Province should be monitored to determine the circulation of *Leishmania* spp. and outbreak of leishmaniasis in the province. Also, an entomological survey is necessary for the annual monitoring of leishmaniasis vectors such as *Ph. papatasi, Ph. sergenti,* and *Ph. alexandri* in this area.

Acknowledgements

This article is a part of the MSc thesis Mrs. Shahrzad Taheri in Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences No. OG−9814.

Ethical considerations

The ethical code IR.JUMS.REC.1398.328 has been registered for this study.

Conflict of interest statement

The authors declare there is no conflict of interest.

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Published Online: March 31, 2024