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Molecular identification of *Leishmania* in rodents and their hard ticks in Golestan province, Northern Iran

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Abstract

Background: Leishmaniasis is a zoonotic disease transmitted between humans and animals and is caused by the *Leishmania* parasite. This parasite is transmitted through the bite of the female sandfly. Rodents and canids serve as reservoir hosts, while humans act as incidental hosts for this parasitic disease. Given the crucial role of rodents as reservoirs for zoonotic cutaneous leishmaniasis in the endemic regions of Golestan province, we aimed to investigate the presence of *Leishmania* in rodents and their hard ticks in the Agh Qala and Inche Broun areas of Golestan province.

Methods: This study is a descriptive cross-sectional study. It involved the analysis of 28 liver and 28 skin samples from 28 rodents and their isolated hard ticks for the presence of *Leishmania* parasites using the ITS1-PCR method.

Results: In this study, 6 species were identified among the 28 rodents captured in the Agh Qala and Inche Broun areas of Golestan province, with the dominant species being *Rhombomys opimus*, accounting for 75% of the rodents. Through PCR analysis, 13 rodents (46.4%) and 15 hard ticks (10.7%) were positive for *Leishmania major* parasites. Interestingly, it was observed that 69% of the rodents infected with *Leishmania* parasites were female. Most rodents infected with *Leishmania* were found to inhabit the Inche Broun area. The majority of ticks belonged to the genera *Rhipicephalus* spp., *Ornithonyssus bacoti*, and *Ixodes ricinus*.

Conclusion: Given the positivity of *Leishmania* parasites in some ticks isolated from infected animals, it is important to consider the potential epidemiological role of hard ticks in the transmission of rodent leishmaniasis.

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Introduction

Leishmania is a eukaryotic parasite that belongs to the Kinetoplastida order and the Trypanosomatidae family. At least 20 species of this parasite cause leishmaniasis. Leishmaniasis is a tropical parasitic disease with a wide range of clinical manifestations and a diverse geographical distribution. It is a common disease between humans and animals, transmitted via the bite of the female sandfly. Rodents and canids serve as reservoir hosts, while humans are accidental hosts (1-3). This disease is observed mainly in several forms: cutaneous, visceral (Kala-azar), and mucocutaneous (Spondia) forms (4). Cutaneous leishmaniasis is more common than the other types of the disease; thus, nearly 70% of all new cases reported each year (About 1-1.5 million people) are related to this form (4,5). Leishmaniasis is endemic in 98 countries and is found in tropical and subtropical regions. According to the World Health Organization (WHO) report, more than 1.5 million people are infected with cutaneous leishmaniasis every year, and around 350 million people are at risk of contracting the disease. Phlebotomine sandflies have been proven to be biological vectors for *Leishmania* parasites. Hard ticks have been extensively studied in laboratories for their potential to transmit *Leishmania* (4). Ticks are considered one of the most significant carriers of pathogenic agents, including bacteria, viruses, and some protozoa. While feeding, blood-sucking vectors ingest various microorganisms; however, not all organisms can be transmitted by them (5,6). The hard tick *Rhipicephalus sanguineus* is an important pathogenic vector with a significant zoonotic role (7,8). This tick has the widest distribution in the world (6). The hypothesis of transmission by hard or Ixodidae ticks has long been debated and has recently regained attention from the scientific community (9). *Rhipicephalus sanguineus* often ingests this parasite. However, the potential transmission of *Leishmania infantum* to susceptible dogs by *Rhipicephalus sanguineus* is poorly understood (10). Ticks are abundant in rodent populations, reproduce at high rates, feed on blood, and may survive for long periods without feeding. An adult tick can survive for 160 to 170 days after feeding on blood. Under laboratory conditions, they can survive without food for 12 months or more. The long lifespan and feeding behavior of ticks may contribute to the spread of leishmaniasis by activating vector species in endemic areas (11,12).

In Iran, cutaneous leishmaniasis and kala-azar are observed. Golestan province is located between 54 degrees and 56 degrees east longitude and 36.30 to 38.15 degrees north latitude. This province is one of the northern provinces of Iran, characterized by vast plains. Different species of rodents can be found in these plains. Ag-Qala and Inche Broun cities are located in the eastern part of this province. In eastern Golestan province, zoonotic cutaneous leishmaniasis is endemic. The rodent *Rhombomys opimus* is considered a nuisance species, and *Leishmania major* is the dominant species in this province. According to a study conducted by Sofizadeh (13), both *Rhombomys opimus* and *Leishmania major* can be present in the north, based on the MaxEnt model. The eastern region of Golestan province is distinct from other areas. It is important to consider various factors, such as temperature and altitude, when predicting the environmental suitability of reservoirs of zoonotic cutaneous leishmaniasis. It has been observed that plain areas located in the north and northeast of Golestan province have more favorable conditions for sustaining rodents such as *Rhombomys opimus*, making these areas potential foci of zoonotic cutaneous leishmaniasis (14). The purpose of this study was to examine the relationship between rodents and their hard ticks in areas of Golestan province that are prone to leishmaniasis. The study aimed to identify the presence of *Leishmania* parasites in these animals, which could provide valuable information for preventing and controlling the spread of this disease in the province's endemic areas.

Methods

This study is a descriptive cross-sectional study and was performed in the Inche Broun and Ag-Qala areas of Golestan province in northern Iran. Eighty-one rodents were collected during the months of March to September 2021. Rodents were captured using Sherman metal traps, which were set in the morning. Cucumbers, tomatoes, and roasted walnuts were used as bait. Of the 81 samples, only 28 rodents had ticks on their bodies. Therefore, skin and liver samples from these rodents, as well as their hard ticks, were examined. Five hard ticks were collected from each rodent. Hard ticks at all developmental stages (Larvae, nymph, and adult) were collected after anesthetizing the rodents, using forceps and swabs, and each was transferred to microtubes maintained at 90-95% relative humidity and stored in a freezer at -70°C.

To identify ectoparasites, the samples stored at -70°C were examined under a stereomicroscope. Accurate identification of external parasites requires consideration of all available information, including sampling location, season, and sex. Therefore, a complete set of characteristics was evaluated for each sample, and identification was performed using valid taxonomic keys at the genus and species levels (Figure 1). Five hard ticks were separated from each rodent. Subsequently, DNA was extracted from the hard ticks, liver, and skin samples of these rodents using a DNA extraction kit from Dena Zist Company (Mashhad, Iran), according to the manufacturer's instructions.

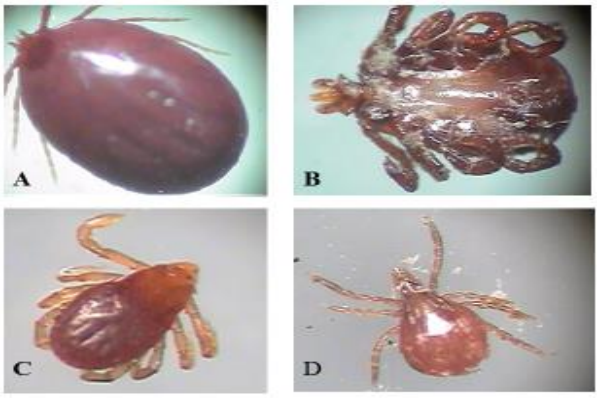

Figure 1. Ectoparasites collected from rodents: A, Dorsal view of *Rhipicephalus* spp. nymph from *Rattus norvegicus*; B, Ventral view of *Rhipicephalus* spp. adult from *Rhombomys opimus*; C, Dorsal view of *Rhipicephalus* spp. adult from *Rattus norvegicus*; D, Dorsal view of *Ixodes ricinus* adult from *Microtus arvalis*.

A PCR assay was performed using primers LINR4 (Forward): 5′-GGG GTT GGT GTA AAA TAG GG-3′ and LIN17 (Reverse): 5′-TTT GAA CGG GAT TTC TG-3′ to amplify the ITS1 gene of *Leishmania*. The PCR program consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 8 min. This protocol amplified fragments of 650 bp for *L. major* and 760 bp for *L. tropica*. Electrophoresis was performed on a 1.5% agarose gel.

Statistical analysis

The obtained data were analyzed using IBM SPSS version 18 software, employing descriptive statistical indicators such as ratios and percentages.

Results

In the present study, a total of 28 rodents were captured and examined. The species distribution revealed that *Rhombomys opimus* was the most prevalent, accounting for 21 individuals, which constitutes approximately 75% of the total rodent population. The second most common species was *Mus musculus*, representing 10.7% of the sample, with 3 individuals identified. Two rodents, representing 7.1%, belonged to the species *Rattus norvegicus*. Additionally, one rodent each of *Meriones libycus* and *Microtus arvalis* was recorded, each contributing 3.6% to the overall rodent composition (Table 1).

Subsequently, these rodents were analyzed for infection with *Leishmania major*, the causative agent of zoonotic cutaneous leishmaniasis. Out of the 21 *Rhombomys opimus* specimens examined, 11 (52.3%) were found to be infected with *Leishmania major*. These findings are summarized in Table 2 and visually represented in Figure 2.

Table 1. Frequency of caught rodents by species and sex

Species of rodent	Sex		Number (%)
	Female (%)	Male (%)	
Rhombomys opimus	11 (52.3)	10 (47.6)	21 (75)
Rattus norvegicus	1 (50)	1 (50)	2 (7.1)
Meriones libycus	0 (0)	1 (100)	1 (3.6)
Mus musculus	1 (33.3)	2 (66.7)	3 (10.7)
Microtus arvalis	1 (100)	0 (0)	1 (3.6)
Total	14	14	28

Table 2. Frequency of infected rodents by species and sex

Infected rodents with *Leishmania*	Sex of infected rodents		Infected rodents Number (%)	Total %
	Female %	Male %		
Rhombomys opimus	8 (70)	3 (30)	11 (52.3)	82.4
Ratus nergicus	1 (50)	1 (50)	2 (100)	17.6
Meriones libycus	0 (0)	0 (0)	0 (0)	0 (0)
Mus musculus	0 (0)	0 (0)	0 (0)	0 (0)
Microtus arvalis	0 (0)	0 (0)	0 (0)	0 (0)
Total	9	4	13 (46.4)	100

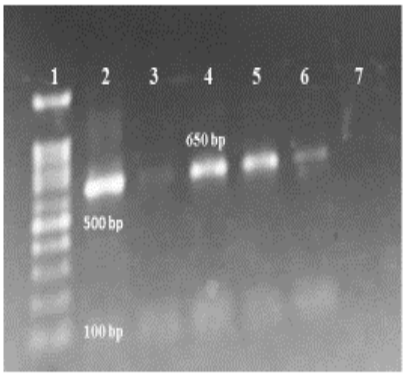

Figure 2. PCR of rodent samples: Lane 1, DNA ladder marker (100 bp); Lane 2, Positive control of *Leishmania major*; Lanes 3 - 6, Samples; Lane 7, Negative control of *Leishmania major*.

In parallel, a total of 140 hard ticks were collected from the captured rodents and tested for the presence of *Leishmania* parasites. Of these, 15 ticks (10.7%) were confirmed to be infected with *Leishmania major*. The majority of the collected tick species included members of the genus *Rhipicephalus*, along with *Ornithonyssus bacoti* and *Ixodes ricinus*. Notably, all infected ticks carried *Leishmania major* as the causative parasite. These results are illustrated in Figure 3 and further support the potential role of ticks as possible vectors or carriers in the transmission cycle of this disease.

These data contribute to a better understanding of the host-parasite dynamics within the studied region and highlight the importance of monitoring rodent and tick populations in endemic areas for effective disease control and prevention strategies.

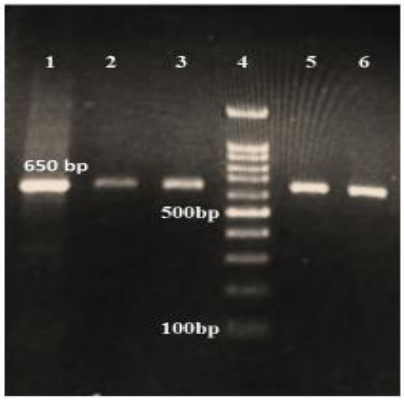

Figure 3. PCR of tick samples: Lane 1, Positive control of *Leishmania major*; Lanes 2, 3, 5, and 6, Positive samples of *Leishmania major*; Lane 4: DNA ladder marker (100 bp).

Discussion

This study examined the liver and skin of 28 rodents and their ticks in the Aq-Qala and Inche Borun regions of Golestan province for *Leishmania* contamination using the ITS1-PCR method. Among the captured rodents, six species were identified, including *Rhombomys opimus*, *Mus musculus*, *Microtus arvalis*, *Meriones libycus*, and *Rattus norvegicus*. The dominant species (75%) among these rodents was *Rhombomys opimus*. Rafizadeh et al.'s study in Esfaraien and North Khorasan on cutaneous leishmaniasis reservoirs using the PCR-RFLP method also showed that most of the captured rodents (72.7%) were infected with *Leishmania* parasites (15), indicating a higher percentage of infected rodents in Esfaraien and North Khorasan compared with Golestan province. In a study conducted by Mehrabani in the Larestan region, rodents captured and examined using microscopic examination of stained tissue smears and tissue sample cultures showed that 18.7% of the rodents were infected with *Leishmania* parasites (16). Fallah et al.'s study on rodents to investigate visceral leishmaniasis in the Sarab region using parasitological, serological, and molecular methods showed that out of 100 rodents from four different species and both sexes examined, one rodent had a positive serum titer, six rodents had serum titers lower than the positive threshold, and 93 rodents were seronegative; moreover, *Leishmania* bodies were not observed in any of the liver and spleen tissue smears of the rodents (17). In Darvishi's study on rodents in Tangistan city, Bushehr province, no contamination with *Leishmania* parasites was observed in any of the slides prepared from the ear lobes of the captured rodents (18). In the study by Azizi et al. in Jask city, out of 106 examined rodents, leishmaniasis infection was observed microscopically in one female *Tatera indica*, one female *Meriones hurianeh*, and one male *Gerbilus nanus*. Using molecular methods, contamination was observed in one case of *Tatera indica*, two cases of female *Meriones hurianeh*, and two cases involving male and female samples of *Gerbilus nanus*, and the dominant species of *Leishmania major* parasite was identified (19).

In the present study, the parasite species identified in all infected rodents was *Leishmania major*, which is consistent with the studies of Mehrabani in Larestan (16), Rafizadeh in North-East Iran (15), and Azizi in Jask (19). All of the above studies are in agreement with our study, which examined rodents for *Leishmania*, because in all of the mentioned areas, rural cutaneous leishmaniasis is endemically present; however, the predominant rodent species infected with *Leishmania* differs from our study, as the dominant rodent species varies across regions.

The results of this study showed that 10.7% of hard ticks collected from rodents were positive for the *Leishmania* parasite. Dantas-Torres reported that in Brazil and Italy, PCR results showed that 12% of dog ticks were positive for the *Leishmania* parasite (10). In Salvatore's study in Italy, 7.5% of ticks were positive for *Leishmania* parasites (20). Solano-Gallego's study in England showed that 13% of hard ticks isolated from dogs infected with visceral leishmaniasis were positive for *Leishmania* parasites (21). In the study by Rakhshanpur in Tehran and Alborz provinces (Iran) on ticks collected from dogs infected with visceral leishmaniasis, 67% of the ticks were infected with *Leishmania infantum* (22).

Regarding the transmission of *Leishmania* parasites through blood feeding by hard ticks, there is also supporting evidence. For example, in the study by Dabaghmanesh in Shiraz (Iran) on the transovarial and transstadial transmission of *Leishmania infantum* by *Rhipicephalus sanguineus* ticks feeding on an infected dog, the presence of *Leishmania infantum* kDNA was demonstrated in nymphs and adult ticks after repeated blood feeding at time intervals, as well as across three subsequent generations. Both transmission routes of *Leishmania infantum* were observed and confirmed by PCR (23). In a study conducted by Furtado in Brazil, hard ticks infected with *Leishmania* parasites were placed on healthy dogs, and PCR results showed that 22.9% of the dogs exposed to feeding by infected ticks tested positive for *Leishmania* parasites (24).

Conclusion

Considering the detection of the *Leishmania* parasite in some ticks isolated from infected animals, the epidemiological role of hard ticks in the transmission of rodent leishmaniasis should be considered, and further investigations should be conducted in this regard.

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Ethical statement

In this research, all ethical and professional principles for working with laboratory animals were followed, in accordance with the recommendations and guidelines of the International Animal Institute. Samples were collected accordingly, and the animals received the ethics code IR.GOUMS.REC.1401.130 from the Ethics Committee of the Research and Technology Vice-Chancellor.

Conflicts of interest

The authors declare that there is no conflict of interest.

Author contributions

Farideh Tohidi: Conceptualization, writing, review and editing, methodology, and analysis; Zeinolabedin Mohammadi: Collection of samples and identification of rodents and ticks; Mohammad R. Ghanbari: Statistical consultant; Abazar Nejati and Ayeneh Hagieh Pangh: Conducting molecular experiments.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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