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Report on Leishmania Infection of *Gerbillus nanus* (Rodentia: Muridae) as the Reservoir Host of Leishmania Major in Hormozgan Province

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Abstract

***Background*:** Cutaneous leishmaniasis or oriental sore has continuously expanded during the recent years in the country. Jask County in the easternmost point of Hormozgan province with 245 cases in 2007 has been the main focus of the disease. The present study was conducted to investigate the role of *Gerbillus nanus* as the reservoir of cutaneous leishmaniasis in this center.

***Materials and Methods*:** In a cross-sectional study during 2007-2008, rodents were caught from infected villages and after anesthesia, two slides were prepared from each ear of rodents using abrasive grinding and after recording morphometric specifications, their liver and spleen were kept in the 70% ethanol and the rest of the body was kept in 10% formalin for identification. Studies to determine leishmania infection were conducted through microscopic and molecular techniques. DNA was extracted through phenol/chloroform/lsoamyl alcohol method and it was proliferated through Nested-PCR method with primers LINR4, LIN17 and LIN19.

***Results*:** A total of 106 rodents were caught. Species *Gerbillus nanus* (Muridae: Gerbillinae), with 17 heads, included 16.03% of haunting. Leishmania infection was found in a male sample of this species of rodent through microscopic method and two male and female samples (11.76%) through molecular method and specific PCR specified the parasite *Leishmania major*.

***Conclusion*:** Cutaneous leishmaniasis in this center is of zoonotic or damp type with *leishmania major* agent and the rodent *Gerbillus nanus* will be introduced in Hormozgan province for the first time as a possible reservoir host of the disease in this center. Infection of this species with *Leishmania major* is reported for the first time in the world.

Introduction

World Health Organization (WHO) has introduced leishmaniasis as one of eight important tropical diseases in the world [1]. Among the four major forms of the diseases caused by different flagellated protozoan species of Leishmania type (Kinetoplastida: Trypanosomatidae), [2] two forms of skin (cutaneous leishmaniasis) and visceral in Iran are endemic [3]. Despite all efforts by health authorities in controlling cutaneous leishmaniasis, unfortunately, we have always witnessed the spread of the disease in different areas of the country in recent years. Vectors of leishmaniasis are sand flies from subfamily of Phlebotomine (Diptera: Psychodidae) and among about seven hundred species known, a number of members of the genus Phlebotomus Spp. have been reported as proven or probable vector [4] of the diseases in Iran and a vector *Phlebotomus papatasi* has been introduced as proven and primary vector in most endemic disease centers [5]. In the wet or zoonotic form of cutaneous leishmaniasis (Zoonotic Cutaneous Leishmaniasis= ZCL), tailed rodents subfamily of Gerbillinae (Rodentia: Muridae) have been introduced as the main reservoirs in Iran and the world [6, 7]. In the central and northeastern centers of the country and the South Tehran, the species *rhombomys opimus* has been reported as proven and primary reservoir of the original and *meriones libycus erythrorus* species has been reported as the secondary reservoir of the disease [3-10]. Yaghoubi Ershadi et al. in the Kurdistan region (central Iran), has reported rodent *meriones libycus* with 7.1% of infection as the primary reservoir and species *nesokia indica* as the secondary reservoir [11]. Recently Rassi et al. reported 37.5% infection in the *R. opimus* species in Kalale, Golestan province [12]. In West and Southwestern regions of the country, *Tatera indica* and in the southeast of the country *Meriones hurrianae* species have been reported as proven and primary reservoirs [13, 14]. In the nearest ZCL endemic center to Hormozgan province, i.e. Fars province, two species have been mainly reported as reservoir hosts. In rural areas of the cities Arsanjan and Neiriz, *M. libycus* species plays the role of primary and proven reservoir of the disease [15]. The same species with 8.4% infection has been reported in Marvdasht [16].

T. indica species infected with *Leishmania major* has been reported in Kharameh [17]. Mehrabani et al. reported leishmania infection in Larestan region of Fars and in the closest center to the studied area in four cases of rodent *T. indica* and two cases of infection in two rodent samples of Gerbillus Spp. genus [18].

In Hormozgan province, few studies have been conducted on Leishmaniasis reservoirs. Through microscopic examination, Soleimani-Ahmadi in Kahurestan region reported [9] infection cases in the species *R. opimus* [19]. In the study on rodents of Hajiabad County, Hanafi Bojd et al. also caught [11] rodents of two species *Meriones persicus* and *T. indica* none of which showed any leishmania infection [20].

Jask County in the easternmost point of Hormozgan province in the vicinity of Sistan and Balouchistan province in recent years has witnessed a dramatic increase in the number of cases of cutaneous leishmaniasis, so that in 2007, 245 cases and in 2008, 195 cases of disease have been reported [21]. Since no study had been so far conducted on review of leishmaniasis reservoirs in this region of the country, the present study was designed and implemented for the first time in Hormozgan province using molecular method of PCR to determine the disease reservoir hosts at this center.

Materials and Methods

In a descriptive cross-sectional study from November 2007 till March 2008, rodents of infected areas of Jask County were caught alive using Sherman wire live traps. Jask County is located in the easternmost point of Hormozgan province (25 degrees and 24 minutes to 26 degrees and 58 minutes north latitude and 57 degrees and 11 minutes to 59 degrees and 15 minutes east longitude). This county is located in the northern Oman Sea and it has warm and humid weather.

After anesthesia of the caught rodents with chloroform, two slides were prepared from each ear of them by abrasive grinding techniques and their morphometric characteristics were recorded simultaneously [22]. Then, the rodent stomach was opened using surgical blade and their liver and spleen were transferred in 70% ethanol and the rest of their body was kept in the containers containing 10% formalin for later identification.

Prepared slides were fixed using methanol and were stained in the laboratory using Gimsa and they were examined microscopically to observe leishman bodies of leishmania parasite. The slides were then also tested molecularly to extract DNA and PCR. Rodents' bodies were sent to the Museum of Natural History of Shiraz University to determine the identity using diagnostic keys and some samples of them were sent to the rodentology group of Mashhad University for authentication.

DNA was extracted from the slides containing parasites and liver and spleen samples of rodents through the method of Azizi and Motazedian [23-25]. In short, so that the surface of slides were carved by a scalpel and the substances resulting from slides carving were added into an sterile Eppendorf tube containing 200 µl of lysis Buffer [50 Mm Tris-HCl (pH=7.6), 1m EDTA 1% (V/V) Tween 20] containing 8.5 µl proteinase solution K (19 µl/Ml) in tubes of 1.5 mL. Tube was incubated for two hours at 56ºC and then, 200 µl of mixture of phenol: chloroform: Isoamyl Alcohol (25:24:1) was added to it: After intense shaking, the tube was centrifuged for 10 minutes in round 6000 xg. Then, two to three times the volume of the solution containing DNA, cold ethanol 100% (Absolute) was added and slowly mixed several times and was put in freezer -20ºC for 2 hours. Then, it was centrifuged at 10,000 rpm for 6 minutes. Then, proportional to the amount DNA (In this study 50 Lambda) double-distilled sterile water was added to the tubes and they were kept in the refrigerator of 4ºC until PCR performance extraction of DNA from liver and spleen samples of reservoirs was so that first, a small piece of these organs was cut, was put in sterile 1.5 ml Eppendorf and was well crushed with a sterile pastor pipet to be homogenized. Then, adding lysis Buffer and Proteinase K, other steps were performed like the described method.

DNA was proliferated using Nested-PCR method which was used by the author et al. several times to identify leishmania parasites in the body of cutaneous leishmaniasis vectors and reservoirs and Kala-Azar in the south country [5, 24, 25].

In this method, the primer LINR4 was used in both stages as Forward primer, and primers LIN17 was used as reverse primer of first stage and LIN19 as reverse primers of the second stage. The primer sequences were as follows [26]:

-LINR4: 5'-GGG GTT GGT GTA AAA TAG GG-3 '(Forward).

-LIN17: 5'-TTT GAA CGG GAT TTC TG-3 '(Reverse for 1st round).

-LIN19: 5'-CAG AAC GCC CCT ACC CG-3 '(Reverse for 2nd round).

Two µl from first stage product with two primers LINR4 and LIN17, with ratio 4:1 was diluted with DDW and was used as template for the second stage. The required amount of materials and thermal profiles in the second stage was like the first one.

The first stage was repeated for 30 cycles. The second stages were repeated for 33 cycles. For detection of PCR products, 5 µl of the product of the second stage on agarose gel 1.5%, mixed with ethidium bromide, was put in the electrophoresis and the gel was transferred to the device UV Transilluminator after 45 minutes and the resulting bands were examined and photographed and it was interpreted comparing with bands obtained from reference strains and the molecular weight marker.

The bands resulting from these two methods for standard species of Iran were 720, 760 and 560 bp for *leishmania infantum*, *leishmania tropica* and *leishmania major* respectively. Standard strains of Leishmania species in Iran, including *L. infantum*: MCAN/IR/96/Lon 49, *L .tropica*: MHOM/IR/89/ARD 2 and *L. major*: MHOM/IR/54/LV 39 used as positive controls.

Results

In this study, a total of 106 rodents of five species were caught and species *Meriones persicus* with 29% catch was the predominant species and species *Gerbillus nanus* with 16.03% catch was the fourth predominant species. It is worth noting that in this study, rodents catch was simply performed from outside and marginalized rural areas. Thus, no *Mus musculus*, which is usually caught in studies to determine rodents' fauna, was caught. Percentage of species catch is shown in figure 1.

To detect leishmania infection, all samples of rodent *Gerbillus nanus* (17 heads) were reviewed microscopically also to determine DNA of leishmania parasites in their liver and spleen. Leishmania infection in a male sample was observed microscopically, but molecular analysis revealed leishmania major parasite in a male and a female sample (Fig. 1 & 2).

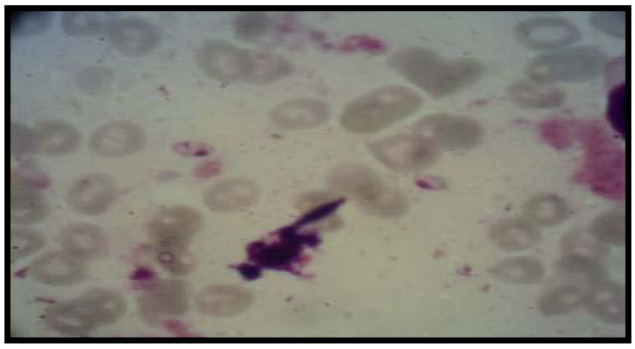

Figure 1. Leishmania major amastigotes in the sample collected from the rodent *Gerbillus nanus*, Jask County, Hormozgan province, 2008

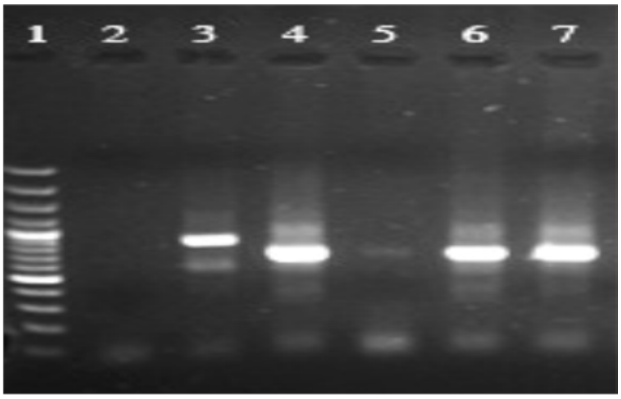

Figure 2. The result of electrophoresis of PCR products of *Gerbillus nanus* rodent's liver and spleen specimens in Jask County on agarose gel 1.5%, stained with ethidium bromide. Size Marker (Lane1), Negative Control (2), Leishmania tropica Standard Strain (3), Leishmania major Standard Strain (4), Gerbillus nanus Liver & Spleen Specimens (5-7)

Discussion

In this study, 106 rodents were caught among which *Gerbillus nanus* was the fourth dominant species in which two Leishmania infections were observed. In this descriptive cross-sectional study, broom-tailed rodents' fauna and reservoirs of zoonotic cutaneous leishmaniasis (ZCL) in Jask County were studied using microscopic and molecular techniques. Jask Country in the southeast point of the country in recent years witnessed a gradual increase in the incidence of illness and in the classification of the disease endemicity it is in stratum 1 (API> 1) along with Bastak County [21]. This gradual increase and continuity of case reports raised and stipulated the probability of endemic focus formation. The present study was conducted to scrutinize reservoirs of the disease. Since Gerbillus rodents partly have abundant migration power and good climatic conditions of Hormozgan, especially the continuity of coastal plains bordering North Persian Gulf and Oman Sea increased the possibility of spreading disease towards West Province and the city of Bandar Abbas.

Given that the rodents were caught from border villages, *Mus musculus* which is reported in most studies of determining rodents' fauna was not caught. In this study, among 106 caught rodents, species *Gerbillus nanus* with 17 heads (16.03%) was the fourth caught species in which Leishmania infection was microscopically observed in a male sample and in two male and female samples through PCR molecular method.

According to the extensive research of the authors, the infection of *Gerbillus nanus* with parasite *Leishmania major* has not been mentioned so far in any scientific source and it seems that infection of this species with leishmania parasite in this study is for the first time in the world. Mehrabani et al. for the first time reported two samples of *Gerbillus spp*. rodents in Larestan of Fars to be infected with *Leishmania major* through microscopic and molecular techniques [18]. They did not mention the species of this rodent. However, according to the size of samples' body (two infection cases) which have been big; these two species certainly have not been nanus. However, in Middle Eastern countries including Egypt, Jordan and the occupied Palestinian, some cases of infection of Gerbillus species with *leishmania major* have been reported, but none of these reports have been regarding species nanus. For example, Morsey et al. have reported the infection of *Gerbillus pyramidum* with *leishmania major* in the northern Sinai Peninsula in Egypt [27] or Wesserberg et al. reported the infection of species *Gerbillus dasyurus* with the same parasite in the eastern Negev in occupied Palestine [28].

In this study, infection of the rodent species mentioned above was revealed through both microscopic and PCR methods. Molecular method was more successful in diagnosis of infection, so that the microscopic method identified one case of infection, and molecular method identified two cases of infection. Since DNA of parasites is searched in PCR method, and its specificity and sensitivity are very high, its diagnostic power is expected to be higher than microscopic method, which is highly dependent on the skill and distinction of the microscopist. This has been also emphasized in other studies [12, 25]. So far, few studies have been conducted regarding the examination of cutaneous leishmaniasis reservoirs in Hormozgan province. According to the study of Soleimani et al. only species *Rhombomys opimus* was caught in Kahurestan of Bandar Abbas [19]. It should be noted that in this study, species *R. opimus*, the main reservoir of the disease in most central and northeastern

regions of the country, was not caught. Although fauna definitely may be different in different geographical conditions, great similarity of climatic conditions of regions Jask and Kahurestan determines the necessity of study revisions and repeat in Kahurestan. Hanafi Bojd et al. reported two species *Meriones persicus* and *Tatera indica* in Haji-Abad County, which were also the two dominant species caught in this study, but leishmania infection was not observed in none of the samples caught by the researchers [20].

The present study has been the first study conducted using PCR molecular method to determine reservoirs of cutaneous leishmaniasis in Hormozgan province and *Leishmania major* parasite from rodents Gerbillus has been first identified in this region of the country. Infection of rodent species *G. nanus* with *Leishmania major* is reported in this province for the first time. Infection of this species with *Leishmania major* is reported for the first time in the world.

According to the results of this research, in this emerging center, cutaneous leishmaniasis is of the zoonotic or damp type with *Leishmania major* pathogen and rodent *Gerbillus nanus* will be introduced as the disease reservoir host and it seems that this species acts as the active reservoir host of the disease in the region. The infection of gerbillus nanus with leishmania major was reported for the first time in the world.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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