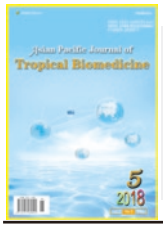

Asian Pacific Journal of Tropical Biomedicine

Journal homepage: www.apjtb.org

doi: 10.4103/2221-1691.233010

Co-detection and isolation of *Leishmania* and *Crithidia* among naturally infected *Tatera indica* (Rodentia: Muridae) in Fars province, southern Iran

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ARTICLE INFO

Article history:
Received 4 February 2018
Revision 18 March 2018
Accepted 12 May 2018
Available online 23 May 2018

Keywords:
Tatera indica
Leishmania
Crithidia
PCR
Iran

ABSTRACT

Objective: To explore the co-detection of natural infection of Trypanosomatidae parasites such as *Leishmania* and *Crithidia* in reservoir hosts of leishmaniasis. **Methods:** Rodent populations were monitored in two endemic foci of cutaneous leishmaniasis of Fars province, southern Iran from March to October 2016. Rodents were trapped alive in several parts of Shiraz and Kharameh cities. Afterwards, their organs were prepared for detection of *Leishmania* and *Crithidia* species by molecular, microscopic, and culture methods. **Results:** Totally, 115 rodents of five species; *Tatera indica* (*T. indica*) (85), *Rattus rattus* (12), *Meriones libycus* (9), *Mus musculus* (7), and *Rattus norvegicus* (2), were trapped alive and their tissue samples were examined using microscopic, cultivation, and molecular assays. Overall, 59 (51.3%) rodents were positive for *Leishmania* or *Crithidia* parasites. The highest rate (61.2%; 52/85) of *Leishmania* infection was related to the *T. indica* population. The cultivation, and molecular observations showed that two (2.4%; 2/85) of *T. indica* (foot-pad, and spleen samples) were positive to *Crithidia*. **Conclusions:** This is the first report of *Crithidia* infection in *T. indica* in Iran. Consequently, more epidemiological and ecological studies are needed to understand the role of *Crithidia* and *Leishmania* in *T. indica*.

1. Introduction

Leishmaniases, protozoan diseases caused by *Leishmania* species, are transmitted by the female sand flies bites. The diseases are broadly reported in tropical and subtropical areas including Iran[1]. It is estimated that more than 1.5 million cases of diseases occur in about 100 countries yearly. Moreover, about 350 million people are at risk of the diseases in the world[1]. Cutaneous leishmaniasis (CL), is more prevalent clinical form of the disease which has been reported from different geographical areas of Iran. The prevalence

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Foundation project: This research was financially supported by the Research Vice-chancellor of Shiraz University of Medical Sciences.

How to cite this article: Kalantari M, Motazedian MH, Asgari Q, Mohammadpour I, Soltani A, Azizi K. Co-detection and isolation of *Leishmania* and *Crithidia* among naturally infected *Tatera indica* (Rodentia: Muridae) in Fars province, southern Iran . Asian Pac J Trop Biomed 2018; 8(5): 279-284.

rates of CL are considerable and more than 30 000 new cases annually occur in different parts of the country[2].

Among nine recognized genus of Trypanosomatidae family (*Leishmania*, *Trypanosoma*, *Crithidia*, *Phytomonas*, *Endotrypanum*, *Rhynchoidomonas*, *Leptomonas*, *Herpetomonas*, and *Blastocrithidia*), two are known to include species pathogenic for humans and animals (*Leishmania* and *Trypanosoma*), and some species of *Phytomonas* are recognized as pathogens of plants[3]. *Crithidia* species exclusively parasitize arthropods, mainly insects. The genus is defined by the presence of the choanomastigote (barley corn-like forms) with free flagellum. Also, their cysts forms are able to move from host to host by fecal-oral rout and naturally, the digestive tracts of insects are the developing parasite sites[4]. Besides, *Crithidia* species make relationship with some parasites of the trypanosomatidae family, and transfer to various hosts along with them[5]. Some molecular investigation on human leishmaniasis revealed that *Crithidia* species were detected from the human cutaneous leishmaniasis cases in Iran[5]. Recently, prevalence and incidence rates of zoonotic cutaneous leishmaniasis (ZCL) have been increased in southern Iran, especially in Fars province[6]. *Crithidia* species are not pathogenic for human, but they have been detected and isolated from human CL lesions and it seems that they are able to survey in these circumstances with *Leishmania* and compete with them[5]. Gerbillinae group is one of the most important reservoir host of several pathogens such as *leishmania* in Iran. Moreover, variant *Leishmania* species have been reported from rodents in different parts of the Country[6,7]. Among 52 species which have been characterized in Iran[8], *Tatera indica* (*T. indica*), commonly known as "The Indian Gerbil" or "Antelope rat", is considered as an important reservoir host of ZCL in southwest regions of the country[9].

Based on taxonomic studies, 12 species of *Tatera* were characterized of which only one (*T. indica*) lives in Asia. This species was first reported from southeastern Turkey by Misonne in 1957[10], and ranges from Indomalayan region throughout the northern Arabia including Iran, and the others (*Tatera afra*, *Tatera bohemi*, *Tatera brantsii*, *Tatera guineae*, *Tatera inclusa*, *Tatera kempi*, *Tatera leucogaster*, *Tatera nigricauda*, *Tatera phillipsi*, *Tatera robusta*, and *Tatera vicina*) live in Africa[11]. *T. indica* is one of the largest species in Gerbillinae group, its color ranges from reddish brown to fawn and has a light brown strip on each side. The soles of their feet are pigmented and bald, and the ears are also nude and elongated. Moreover, ecological studies revealed that *T. indica* which is nocturnal does not move far from their burrows, and choose sandy plains and grasslands in habitats which are close to agricultural fields[12]. Males and females live alone and their mating place is uncertain. Moreover, their numbers increase between March to September and decrease in January[12,13].

Based on molecular categorizations, *Tatera* genus is considered as polyphyletic taxon, so, *T. indica* was divided to *Tatera sensu stricto* and other African spp. located in *Gerbilliscus* Thomas genus[14]. Among identified various subspecies of *T. indica*, four of them are reported from Iran, including *T. indica scansa* (in Kerman), *T. indica persica* (in Sistan), and *T. indica bailwardi* and *T. indica monticola* (in Fars and Khuzestan)[15].

Because of the proper ecological circumstances caused between reservoir hosts and vectors of Leishmaniasis in Iran, the present investigation was performed to identify *Leishmania* and *Crithidia* species involved in rodents populations of Shiraz and Kharameh (its north countryside) cities in Fars province, southern Iran, by using and comparing microscopic, cultivation and molecular methods (PCR) to have a better understanding of *T. indica* role as reservoir host of ZCL pathogens, and also develop new environmental control strategies in CL[16,17].

2. Materials and methods

2.1. Study area

Fars province is located in south of Iran, which includes 23 counties with an area of 122 400 km . Shiraz (the capital city of the province) and Kharameh (80 kilometers northeastern of Shiraz) are situated at 29 ° 59' 18" North, 52 ° 58' 37" East, and 29 ° 50'20" N, 53 ° 31'24" E, and about 1 200-1 500 m above the sea level. Recently, Shiraz and Kharameh are considered as the most important foci of cutaneous leishmaniasis in Fars province, southern Iran.

2.2. Rodent collections

Rodents' active colonies were identified in several parts of Shiraz and Kharameh cities. Afterwards, they were caught alive via wire cages located at entrances of burrows in 10 different locations of each city from March to October 2016. Traps were set in the evening and were checked the next morning. In cases of unsuccessful trapping, checking was continued at 4 hours intervals and it was conducted on average twice per week. Each time an average of 8 traps was used[1]. Rodents which were caught alive were carried to the animal laboratory cages. For recording the morphological characteristics (such as species, and sex), and finding any ulcers in their body, they were anesthetized with chloroform slightly. In this study, all applicable international, national, and/or institutional guidelines for the care and use of animals were followed, and ethical permission number IR.SUMS.REC.1395.S475 was granted for animal studies through the science and ethics committee of Shiraz University of Medical Sciences held on 20th August 2016. All performed health procedures were in accordance with the ethical standards of the Iranian institutional and/or national research committee guidelines and with the 1964 Helsinki declaration. Accordingly, rodents were euthanized by deep anesthesia and impression smears and culture sampling were prepared from ears, spleen, feet, nose, and liver. For culturing, the sampling surfaces were sterilized using 70% ethanol, Thereafter, surface cuts of 2 to 3 mm were prepared and impregnated into liquid phase of modified Novy Mac Neal Nicole (NNN) medium culture near to the flame and finally were placed in culture incubator. At least ten samples, two from each part of their bodies, were prepared for molecular, microscopic, and culture methods.

2.3. Parasites culturing

Sterilized surface cuts of ear, feet, nose, liver, and spleen were collected and transferred to the modified NNN culture medium tubes. NNN medium were slightly modified and consisted of two

phases of an overlay Locke's solution and a horse blood agar base. The specimens were inoculated into the liquid phase of the biphasic medium and incubated at (24±2) ℃. Every 2 to 3 days, the liquid phases of cultures were examined under invert microscopy. After detecting the motile promastigotes, cultures were transferred in RPMI-1640 media (Gibco, Frankfurt, Germany) containing 15% heat inactivated FCS (Sigma, cat. No. 308056), 2 mM *L*-glutamine, 100 μg/mL streptomycin (Gibco, Frankfurt, Germany), and 100 U/mL penicillin for mass cultivation. At the early stage of stationary phase, approximately 5×10^6 promastigotes/mL were harvested and used for more tests. Some of parasites were carried to Semi-solid Locke-blood agar medium and transported to 4 ℃ for more adaptation and investigation in prolonged time. Furthermore, some of the promastigotes were transferred to cryo tubes containing 5% dimethyl sulfoxide and stored in -70 ℃ and liquid nitrogen. Additionally, some promastigotes were harvested by centrifugation (10 000 *g* for 10 min) and washed twice in cold sterile PBS (pH 7.2). Parasites pellets were stored at -20 ℃ until used[18].

2.4. Microscopic studies

Smear samples prepared from rodents liver, ears, feet pads, nose, and spleen were fixed by methanol, stained by 5% Giemsa, and microscopy checked for detection of *Leishmania* and/or *Crithidia* in at least 40 min times.

2.5. PCR assays

2.5.1. DNA extractions

The scraped smears of the glass slides and culture sediments of rodents were extracted by kit extraction (YTA genomic DNA extraction mini kit, Cat no. YT9030) processor; briefly, samples transferred to micro-tubes, 20 μL Proteinase K and 200 μL Lysis buffer were added to the samples, then mixed and incubated at 60 ℃ for 15 min to be completely lysed. Afterward, 200 μL absolute ethanol were added to the samples and mixed by pluse-vortexing for 30 s. Afterward, the samples mixtures containing some precipitates were carefully transferred to column micro-tubes, centrifuged for 1 min at 8 000 rpm and washed by buffers several times to remove impurities from column micro-tubes. Finally, 100 to 200 μL of elution buffer or ddH_2O were added to the membrane center of the column tubes and after 3 min, they were centrifuged for 2 min at 14 000 rpm to elute the DNA and stored at -20 ℃ for PCR assays[19].

2.5.2. Leishmania and Crithidia detections

Extracted DNAs of smears and collected culture sediments were used for *Leishmania* species detections by a sensitive modified PCR method[20]. Each 25-μL reaction mixture (total volume) contained 12 μL Ampliqon taq DNA polymerase master mix buffer (Cat No. A180301), 1 μL of each primers of forward LINR4 (5'-GGG GTT GGT GTA AAA TAG GG-3') and reverse LIN17 (5'- TTT GAA CGG GAT TTC TG-3') (with 10 pico mol concentrations), 3 μL DNA sample and 8 μL double distilled water. For PCR, Thermocycler (Eppendorf AG Master-cycler Gradient, Germany) programmed for an initial temperature of 94 ℃ for 5 min (one cycle), continued by temperatures of 94 ℃, 52 ℃, 72 ℃, for 30 s, 30 s, and 1 min, respectively (30 cycles), and followed by a final temperature of 72 ℃ for 5 min (one cycle). Finally, 5 μL of PCR products were run in 1.2% electrophoresis agarose gel which stained with ethidium bromide, and visualized with UV trans-illuminator[1]. Reference strains of *Leishmania major* (*L. major*) (MHOM/IR/54/LV39), with a band of 650 bp, would have shown the existence of *L. major* kDNA.

All extracted DNAs of rodent's tissues were selected for *Crithidia* detection, using minicircle kDNA sequences of internal transcribed spacer (ITS) gene. The specific primers were designed by GenScript online PCR primers designs tool by using gene bank information (Unpublished document). Each 25-μL reaction mixture (total volume) contained 12 μL Ampliqon master mix buffer, 1 μL of each primers of 5'-TCCATGTGCGAGGACAACGTGCT-3'and 3'-CGCGTCGTTGATGAAGTCGCT-5' (with 10 pico mol concentrations), 5 μL DNA sample and 6 μL double Distilled water. Eppendorf Master-thermocycler programmed for an initial temperature of 94 ℃ for 5 min (one cycle), followed by temperatures of 94 ℃, 55 ℃, 72 ℃, for 30 s, 1 min, and 1.5 min, respectively (30 cycles), and continued by a final temperature of 72 ℃ for 5 min (one cycle). Afterward, 5 μL of PCR products were used for electrophoresis assay. Reference strain of *Crithidia fasciculata* was used and a band of 800 bp would have shown the existence of *Crithidia* in the tested smears.

3. Results

3.1. Rodent identifications

During study on rodents from March to October 2016, 115 rodents of five species; including *T. indica* (85), *Rattus rattus* (*R. rattus*) (12), *Meriones libycus* (*M. libycus*) (9), *Mus musculus* (*M. musculus*) (7), and *Rattus norvegicus* (*R. norvegicus*) (2), were caught alive from different regions, of which, 67 (58.3%) were male and 48 (41.7%) were female.

3.2. Microscopic findings

Totally, 490 slide samples from five different tissues belonging to 115 rodents (115, 100, 85, 100, and 90 samples of ears, liver, nose, feet, and spleens, respectively) were checked for *Leishmania* and/or *Crithidia* detections. As there were no reliable differential criteria between *Leishmania* and *Crithidia* detections in microscopic method, consequently, the slides checked for both parasites together, and finally positive slides were confirmed by PCR assays. The results revealed that 5.3% of slides (26/490) were found positive in microscopic method, of which 24.7% (21/85) of the *T. indica* and 11.1% (1/9) of the *M. libycus* were microscopic positive for *Leishmania* and/or *Crithidia* parasites (Figure 1). Furthermore, none slide smears of *R. rattus*, *R. norvegicus*, and *M. musculus* were found positive in microscopic studies.

Overall, 24 out of 490 positive slides were belonged to the *T. indica* organs, of which 3.5% of ears, 6.0% of feet, 3.5% of noses, 6.0% of

livers, and 7.8% of spleens were microscopic infected to parasites. In addition, 2 slides of one *M. libycus* belonged to Kharameh focus (1% of each organs of ear and liver) were positive in microscopic investigations (Table 1).

3.3. Culture findings

Because of no accurate differential criteria between *Leishmania* and *Crithidia* detections and due to preparation of the same culture media for both parasites, they were checked together in cultivation method, and finally were confirmed by molecular methods. Some cultures media were infected, so they were removed from the study. Totally, 37 out of 227 cultures media (16.3%) were positive, 33 samples belonged to the *T. indica*, of which 14.3% of ears, 9% of feet, 5.7% of noses, 9% of livers, 18% of spleens were cultural infected to parasites. Furthermore, 2 positive media were isolated from organs of ear and liver (1% of each organ), and were belonged to two of *M. libycus* caught from Kharameh focus. Overall, 41.17% (35/85) and 22.2% (2/9) of *T. indica* and *M. libycus* were found positive in microscopic and cultivation methods, respectively (Table 1).

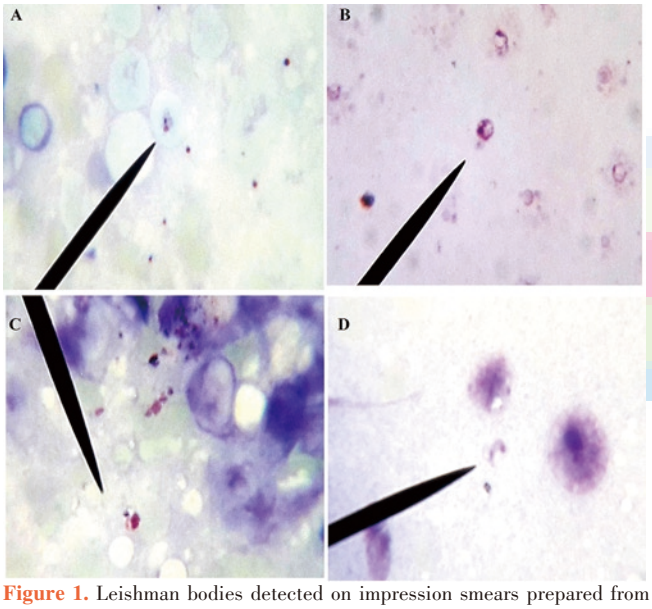

Figure 1. Leishman bodies detected on impression smears prepared from spleen (A) and liver (B) of *T. indica*, and ear (C) and liver (D) of *M. libycus* caught from Kharameh, 2016.

3.4. PCR findings

3.4.1. Leishmania detection

In PCR assays, *L. major* kDNA was detected in rodents' organs of *T. indica*, *M. libycus*, and *M. musculus*. The samples of the *R. rattus* and *R. norvegicus* were negative for *Leishmania* spp. parasite (Figure 2A). Of 49.56% (57/115) PCR positive samples, 63.53% (54/85) samples belonged to the *T. indica*, of which 61.18% (52/85)) were infected to *L. major* (Table 1).

3.4.2. Crithidia detection

Crithidia kDNA was detected in foot-pad, and spleen samples of 2.35% (2/85) of *T. indica* caught from Kharameh focus (Figure 2B). In addition, *M. libycus*, *M. musculus* *R. rattus* and *R. norvegicus* were checked for *Crithidia*, but the samples prepared from these species were negative for the parasite (Table 1).

Table 1

Leishmania/*Crithidia* infections of the examined samples consistent with the investigation methods and examined organs of rodents in Shiraz and Kharameh, Fars province, southern Iran.

Rodents´ organs	Microscopic method		Cultivation method		PCR method	
	Slides* (No.)	Positive (No.)	Cultured** (No.)	Positive (No.)	*Leihmania* positive	*Crithidia* positive
Ear	115	4 (3.5%)	42	6 (14.3%)	7/150 (4.5%)	0/150 (0%)
Foot	100	6 (6.0%)	50	10 (20.0%)	21/150 (14%)	1/150 (0.7%)
Nose	85	3 (3.5%)	35	2 (5.7%)	4/110 (3.6%)	0/110 (0%)
Liver	100	6 (6.0%)	50	10 (20.0%)	13/150 (8.7%)	0/150 (0%)
Spleen	90	7 (7.8%)	50	9 (18.0%)	12/140 (8.6%)	1/140 (0.7%)
Total	490	26/490 (5.3%)	227	37/227 (16.3%)	57/700 (8.1%)	2/700 (0.3%)

*Some slides were missed during the investigation.

**Some cultures media were infected, so removed from the culture study.

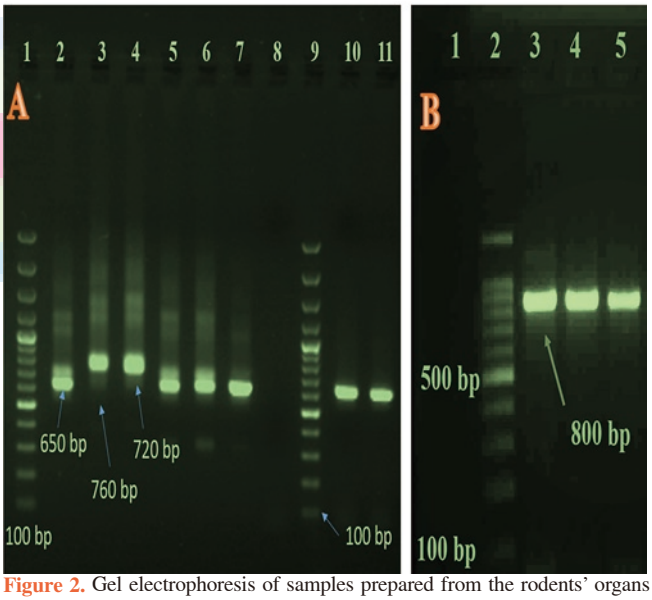

Figure 2. Gel electrophoresis of samples prepared from the rodents' organs caught from urban areas of Shiraz and Kharameh during 2016.

A: PCR based products of *Leishmania* detections; The bands correspond to molecular weight marker (Lanes 1 and 9), Reference strains of *L. major* (650 bp) (Lane 2), *Leishmania tropica* (760 bp) (Lane 3), *Leishmania infantum* (720 bp) (Lane 4), foot, and liver sample of *T. indica*, respectively (Lanes 5 and 6), ear sample of *M. libycus* (Lane 7), and double distilled water as a negative control (Lane 8), in Kharameh focus, liver and spleen sample of *M. musculus*, respectively (Lanes 10 and 11), in Shiraz focus.

B: PCR based products of *Crithidia* detections; double distilled water as a negative control (Lane 1), molecular weight marker (Lane 2), Reference strains of *Crithidia* sp. (Lane 3), foot and spleen of *T. indica*, respectively (Lanes 4 and 5).

4. Discussion

In Iran, Fars province is a considerable endemic focus of ZCL and new studies indicated that the prevalence of the disease has increased recently[21,22]. In this study, five species of rodents were caught (*T. indica*, *R. rattus*, *M. libycus*, *M. musculus*, and *R. norvegicus*). The largest number of infested rodents was related to *T. indica* in several villages of Kharameh, which the largest number of *T. indica* was captured within or out of human residual places. Their breeding places consisted of agricultural lands which products such as wheat, corn, or alfalfa were planted. This species was mostly found in countryside's regions, especially in rural regions of Kharameh, and was not trapped in Shiraz focus. The outcomes of the microscopic and/or cultural, and molecular assays revealed that 41.17% and 45.3% of *T. indica* were positive for *L. major*, respectively. It should be noted that this study included the first detection of *Crithidia* sp. from Iranian *T. indica* caught in Kharameh region, where ZCL has recently occurred.

In Asia, *T. indica* is recorded from Iran, Iraq, Turkey, Pakistan, Syria, Afghanistan, Sri Lanka, India, Kuwait, Nepal, and Bangladesh[10]. However, other genus of *Tatera*, such as *Tatera lataste*, has been reported and widespread from some parts of Middle East and Pakistan subcontinent[23]. In Iran, *T. indica*, *Rhombomys opimus*, *M. libycus*, and *Meriones hurrianae* have been reported as the main "reservoir" hosts of ZCL in several endemic foci of Iran[6]. In addition, *Gerbillus nanus*, and *Nesokia indica* have been reported as the accidental, main, or probable "reservoir" hosts of ZCL in different parts of Iran[1,24]. In Fars province, *T. indica*, *Meriones persicus*, and *M. libycus* are reported positive for *L. major* in different foci of Marvdasht, Zarqan, Fasa, Estahban, and Jahrom[25-27]. In addition, *M. musculus*, *R. rattus*, and *R. norvegicus* have also been found positive to *L. major* in the province, which may explain the considerable increase of ZCL in some urban areas of the country in the recent years[28,29]. *T. indica* along with other rodents (such as *Rhombomys opimus*, *Meriones persicus*, and *M. libycus*) served and confirmed as *Leishmania* spp. reservoir hosts in different regions of the country. Furthermore, they were found to be infected with *L. major* in different provinces of Iran including Khuzestan, and Fars[15,26].

In the recent investigation, the most number of trapped rodents (73.9%) were *T. indica*. Based on findings obtained in other regions of Fars province, this result was predictable. Besides, despite the wild life, this species is able to adapt itself to live in/out of human residential houses, consequently, the transmission cycle of the ZCL infection was facilitated in the studied regions.

In the current study, *Crithidia* sp. was detected in foot-pad and spleen of *T. indica*, which was introduced as a new reservoir host of ZCL in southern regions of Iran. However, the presence and role of *Crithidia* in leishmaniasis infection in human has remained considerable[5,30].

In this investigation, *M. libycus* and *M. musculus* were also found infected with *L. major* in Kharameh and Shiraz rural areas, respectively. *M. libycus* mostly were captured on the edge of agricultural lands, where the man-made canals carried out water to lands. This species was not trapped in Shiraz, but was founded in Shiraz countryside villages. Living places of *M. musculus* was limited to human residual places, and only captured within the houses in both foci of Shiraz and Kharameh.

R. rattus and *R. norvegicus* were trapped in Shiraz city and mostly captured on the sidelines of sewages, gardens and parks. These species were not found in rural areas of Kharameh. In our laboratorial studies, no *Leishmania* and/or *Crithidia* parasites were detected in Rattus species in Shiraz foci.

Different experimental methods, such as biochemical, molecular, and immunological methods, have been used for *leishmania* detection, but, accurately detection of parasites species on the smears of rodent tissues needs a specific and sensitive PCR. In this investigation, using the PCR method was very helpful to confirm the parasitological and/or cultural finding. Besides, the missed parasites, which were not recognized by microscopic (because of low parasite rates) and/or cultivation methods (un-adaptation or unavoided infection in culture media), were detected in PCR assays.

In conclusion, considering the role of rodents in ZCL epidemiological aspects, the present study results revealed that *T. indica* was a main host of *L. major* in the ZCL focus of Kharameh County. Additionally, *M. libycus* and *M. musculus* played significant roles in the maintenance of the leishmania agent in urban areas of Kharameh and Shiraz. Also, *Crithidia* parasite was isolated from Iranian *T. indica* for the first time. Therefore, further studies are required to be conducted on the role of this species along with *Leishmania* species in the maintenance, transmission, and epidemiologic cycle of ZCL.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgments

This research was financially supported by the Research Vice-chancellor of Shiraz University of Medical Sciences and extracted from the results of an approved Ph.D. student thesis (No: 94-01-104-10873) conducted by the author, Mr. Mohsen Kalantari.

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