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Molecular detection of *Critidia fasciculata* and other blood parasites in *Rhombomis opimus* from northern Iran as endemic area

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ABSTRACT

Human blood parasites are one of the most critical infections in human that transmit by vectors. Reservoirs of the parasites are crucially important in the epidemiology and control. In the current study isolated parasites from a *Rhombomys Optimus* (*R. opimus*), rodent confirms that *Crithidia* is a zoonotic parasitic disease. This study aimed to find the high-risk areas of this infection by considering the distribution of reservoirs and human infection. In this study, 148 rodents from an endemic focus of Gonbad-e-Qabus city in Golestan province were trapped and then killed ethically and direct smear and culture in Novy- MacNal-Nicolle medium (NNN) were taken and finally, results were confirmed by Polymerase Chain Reaction (PCR) and Sequencing method. Out of 148 rodent, 97 (65.54%) rodent were male and 51 (34.45%) were female ($P < 0.05$). and in smear and culture were found 8 (5.40%) *T. lewisi*, 6 (4.05%) *L. major*, and 2 (1.35%) *Crithidia* spp. Based on the time; 40 (27.02%), 50 (33.78%), 38 (25.67%), and 20 (13.51%) rodents were trapped in spring, summer, fall, and winter, respectively. Due to northeastern Iran (Gonbad-e-Qabus) being the endemic focus of cutaneous leishmaniasis (CL), it should be noted that the reservoirs of this disease may also be contaminated with *Leishmania* spp, and *Crithidia*. Results showed that *R. opimus* are the important reservoirs of CL in northeastern of Iran. Important foci of the diseases in almost all areas of Iran are dispersed. Therefore, reliable methods to control mice are essentially needed.

1. Introduction

Rodentia is one of the largest orders of warm-blooded mammals that are widely distributed throughout the world. These animals cause a wide variety of disorders with medical and veterinary importance (Azizi et al., 2011). CL are the most common form of leishmaniasis (Mirzapour et al., 2019). It is a serious health problem and the disease is endemic in 20

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provinces in Iran with an annual incidence of 20,000-60,000 cases (Askarian et al., 2012; Sarkari et al., 2014) leishmaniasis is one of the most neglected tropical diseases (NTDs) which has received little attention and resources despite its serious impacts on both economic developments and quality of life (Relman and Choffnes, 2011). It is one of the most important vector-borne diseases and public health problems in the world that is transmitted by sandflies to human and other animals (Alageel et al., 2016).

Iran is facing both forms of CL and visceral leishmaniasis (VL) and *L. tropica, L. major* and *L. turanica* are the most prevalent species of *Leishmania* in rodents as reservoir host (Akhoundi et al., 2013). Although *L. turanica* is not a pathogen for humans but, it may have a co-infection with *L. major* and have a positive effect on its pathogenicity. *L. tropica* and *L. major* are the main cause of Old World CL (OWCL) that is spread through the bite of different species of phlebotomine sandflies (Mohebali et al., 2004). Rassi et al., (2008) reported that *L. major* is the predominant species of CL in these areas using PCR against rDNA loci of the parasite (Rassi et al., 2006). Also, they indicated that *P. papatasi* and *R. opimus* are the main vector and reservoirs for circulating of the parasite between human and reservoirs, respectively. The main CL foci are located in the north-eastern Iran (Gonbad-e-Qabus County) and the incidence and prevalence rates of Zoonotic CL (ZCL) have recently had a sharp increase and outspread in the world. *L. major* is the causing agent for CL and *P. papatasi* is the main vector for this disease. Wild rodents are identified as reservoir host for ZCL in Iran. One of these rodents is *R. opimus*, which is a potential focus of CL in this area. Harboring these rodents in the vicinity of the villages also poses a potential danger to the native inhabitants. These rodents can also be the reservoir of other parasites, such as *Crithidia.* Super Infection of CL and co-infection caused by *L. major* and *L. tropica* to *Crithidia fasciculata* (*C. fasciculata*) was reported in Iran (Ghobakhloo et al., 2019; Mirzapour et al., 2019).

During the last several decades, researchers have described rare cases of patients co-infected with both *Leishmania* and other groups of protozoan parasites that usually infect insects, including *Crithidia* spp. In this study isolated parasites from a *R. opimus*, rodent confirms that *Crithidia* spp. parasites also can infect people. The aim of this study was to find the high-risk areas of this infection by considering the distribution of reservoirs and human infection.

2. Materials and Methods

2.1. Study area

This study was conducted in Golestan Province (37°15′28.9″N 55°10′8.4″E min elevation and population of approximately 152000) from northeastern of Iran from September 2019 to December 2020. Golestan province has dry and hot climate the average daily temperature is 15 to 25°C in summer and 3 to 21 °C in winter (The main annual temperature 18/6 °C) The average yearly precipitation is very low (230 mm). The inhabitants are primarily farmers, and the fields are wheat, barley and field workers and laborers (Figure 1).

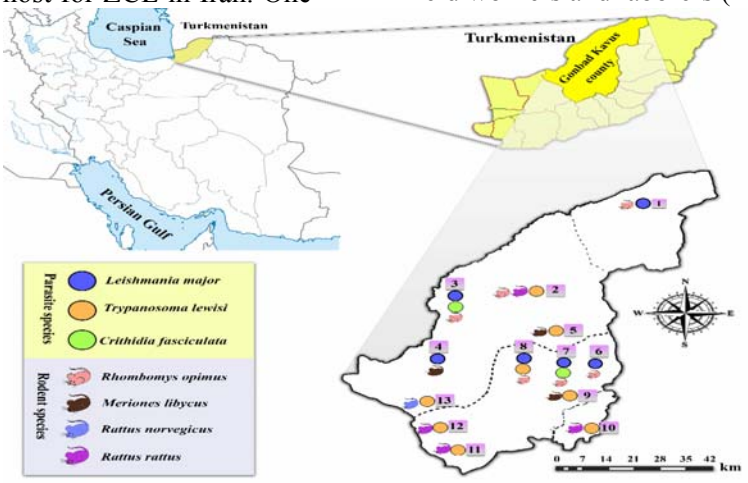

Figure 1. The Location of Golestan province (northeastern of Iran).

2.2. Ethical consideration

Ethical approval was granted by the joint Ethical Committees of Mazndaran University of medical sciences and Mazndaran Leishmaniasis Research Center (Ethic No. IRMAZUMS3280),

2.3. Samples collection

One hundreds forty eight of rodents including 10 *Merinus libycus*, 29 *R. opimus*, 61 *Rattus rattus*, 28 *Rattus norvegicus,* 5 *Allactaga elater* and 15 *Pygeretmus pumilio* were trapped with Shrman trap nearby the human sites. The rodent detection criteria were based on Dr. Jamshid Darwish's. Trapped rodents were killed ethically (Intramuscular injection Ketamine 500 mg) and touch smears were obtained from the–spleen, liver, Servitude of rodents auricle and Snout and smears were stained using Giemsa staining and microscopically (Zeiss; Germany) inspected under 1000x of magnification with immersion oil to determine the presence of blood parasites. Due to the high probability of parasite presence in the auricle and snout specimens, these specimens were also cultured in NNN culture medium which was made with 10% defibrinated rabbit blood and 1% Pen-Strep (Penicillin & Streptomycin)

2.4. DNA extraction

DNA was directly extracted from the slides containing smears and from NNN medium using phenol chloroform isoamide method (Barbier, Chabikwa et al. 2019). 200 µl of the culture medium containing parasite were mixed by 200 µl of the lysing solution and 20 µl of proteinase K in 2ml microtube and incubated at 56 ºC for 12h. After incubation time, the microtubes were centrifuged at 15000 RPM for 15 min and the upper solution was transferred to a new microtube. Absolutely cold ethanol were added, gently mixed and then centrifuged at 15000RPM for 15 min. The supernatant was removed and 70 µl of alcohol 70% to resulting precipitate and centrifuge at 15000 RPM for 15 min. The supernatant was removed and leave the microtube in a clean place until the remaining alcohol evaporates and finally add 50 µl sterile distilled water and the extracted DNA was stored at -20°C for further molecular characterization.

2.5. Amplification of DNA (PCR)

The DNA extracted in the previous step was amplified according to the following protocol. PCR conditions - A volume of 2 µL of extracted DNA with 1 µL of each forward primer (LITSR (5′-CTGGATCATTTTCCGATG-3′) and reverse primer L5.8S (5′TGATACCACTTATCGCA CTT-3′), master mix 12.5 µL (PCR master mix) and 8.5 µL of DW in a final volume of 25 µL. The program of PCR amplification protocol was Initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 30 s annealing at 53°C for 45 s, and extension at 72°C for 1 min, with a final extension of 72°C for 3 min. In order to determine the species and confirm the species, the sequence was performed by Sanger method and the resulting genes were registered in the gene bank with Accession numbers (Accession numbers: MT674939, MT674943, MT674944, MT674940, MT674941, MT674942, MW115869, MW115870).

2.6. Data analysis

The obtained results are analyzed using descriptive statistics and Chi-square and Fisher exact tests and SPSS ver-16 software. A p-value less-than 0.05 will be considered statistically significant.

3. Results

Out of 148 rodent, 97 (65.54%) rodent were male and 51 (34.45%) were female (P <0.05). 8 (5.4%) *T. lewisi*, 6 (4.05%) *L. major* and 2 (1.35%) *Crithidia* spp. was detected in smear and culture medium (Figure 2). Based on the time 40 (27.02%), 50 (33.78%), 38 (25.67%) and 20 (13.51%) rodents were trapped in spring, summer, fall and winter, respectively.

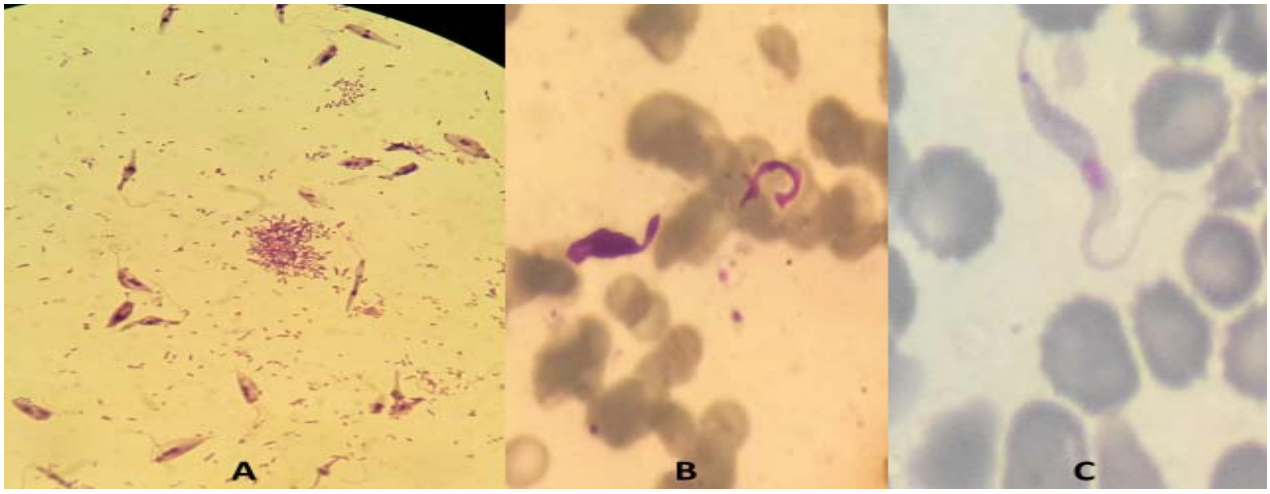

Figure 2. Microscopic image of rodent parasites. *A. Crithidia* Spp. in NNN medium culture, B. L. major from rodent ear lesion and C, T. lewisi in rodent Peripheral blood smears.

Table1. The prevalence of rodent blood parasites in different sexes and hosts in Golestan province, northern Iran.

Variable		All trapped rodent No. (%)	*Trypanosoma* spp. No. (%)	*Leishmania* spp. No. (%)	*Crithidia* sp p. No.
Merinus libycus		10 (6.75%)	2 (25.00%)	1 (16.66%)	0
R. opimus		29 (19.59%)	1 (12.5%)	5 (83.33%)	2
Rattus rattus		61 (41.21%)	4 (50.00%)	0(0.00%)	0
Rattus norvegicus		28 (18.91%)	1 (12.5%)	0 (0.00%)	0
Other rodents		20 (13.54)	0 (0.00%)	0 (0.00%)	0
Sex	Male	97 (65.54%)	5 (62.5%)	5 (83.33%)	1
	Female	51 (34.45%)	3 (37.5%)	1 (16.66%)	1

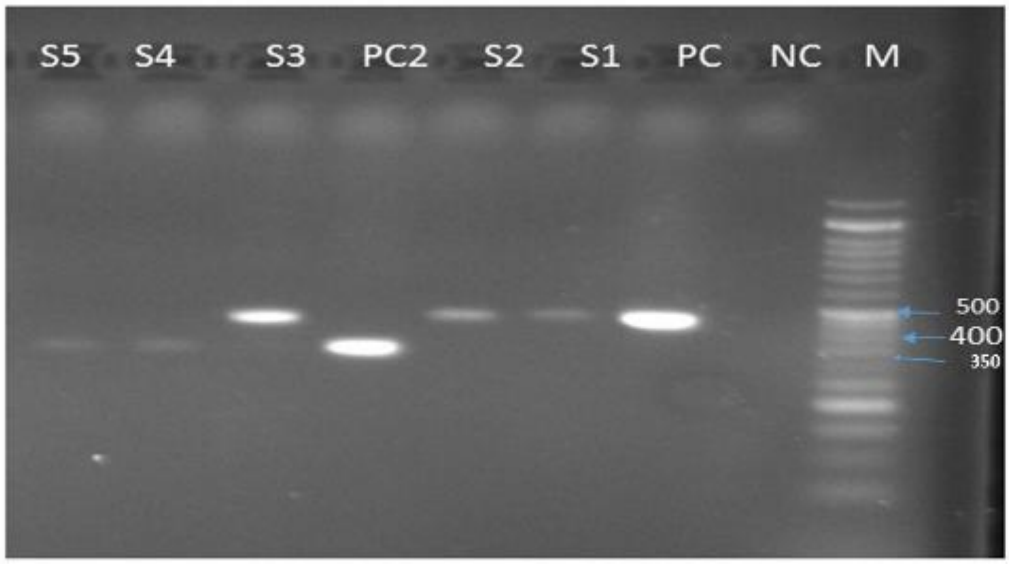

Figure 3. A sample of PCR products from amplification of ITS in *leishmania* genus, Lane M is a 1Kb plus ladder, lane NC is negative control , lane PC is positive control (*Crithidia fasciculata*) lanes S1 and S2 rodents samples isolate (*Crithidia* spp 450bp),lane PC2 is positive control *(L. major*, friedlin strain) S4 and S5 *L. major* isolated from *R. opimus* (350bp)

4. Discussion

Rodents are prominent invasive species, and are as reservoirs for many zoonotic infectious diseases such as many helminthic and protozoan parasitic infections. In the current study, the blood parasitic infections of rodents from northern areas of Iran were assessed and the results indicated that *Trypanosome*, *Leishmania*, and *Crithidia* are the most prevalent infections. Natural infections by various *Leishmania* species have been repeatedly reported in domestic, peridomestic, and wild animals, which dogs and rodents being the most commonly investigated animals and traditionally considered reservoirs (Wardle et al., 2011).
For instance, they might lead to novel parasite-host combinations and have dramatic effects on the dynamics of diseases that affect wildlife, livestock, and/or humans (Young et al., 2017).

Disease emergence events associated with exotic pathogens imported by animal invaders have already been reported (Dunn and Hatcher, 2015). The dynamics of endemic diseases could also be affected by invasive species that act as novel hosts, or could negatively affect native host species. Human activities such as deforestation, agricultural development and settlements near forested areas, and domestication of animals are reasons for the occurrence of both zoonotic and anthroponotic transmissions of leishmaniasis (Tsegaw et al., 2013).

Several species of wild, domestic, and synanthropic mammals have been recorded as hosts and/or reservoirs of *Leishmania* spp. in different parts of the world. Rock hyraxes, rodents, mongoose, dogs, cats, foxes, jackals, wolves, bats, primates, armadillos, and other domestic animals are among the multihost reservoirs to maintain transmission of leishmaniasis in different areas. *L. tropica*, *L. major*, and *L.turanica* are the most prevalent species of *Leishmania* spp. in rodents. Although *L. turanica* is not pathogen for humans, it may have a co-infection with *L. major* and have positive effect on its pathogenicity (Dereure et al., 2000; Rohousova et al., 2015).

L. tropica and *L. major* are the main cause of Old World CL (OWCL) that is spread through the bite of different species of phlebotomine sandflies (Campino et al., 2013). Rassi et al., (2008) reported that *L.major* is the predominant species of CL in these areas using PCR. Also, they indicated that *P. papatasi* and *R. opimus* are the main vector and reservoir for circulating of the parasite between human and reservoirs and maintenance of the parasite source in Golestan province, respectively *T. gambiense* and *T.lewisi* are reported from different kinds of rodents that

T. lewisi such as one of the most prevalent protozoan that is not pathogenic for human (Cohen et al., 2019).

Another finding of current study similar to many former studies is that the *R.opimus* (83.3%) and *M. libycus* (16.7%) are the principal reservoir hosts for CL in these areas with 83.3 and 16.7% of infectivity, respectively. In current study, 5.40% of the assessed rodents were infected with *T. lewisi*. In a similar study in Southwestern Iran, Seifollahi et al., (2016) reported that 11.54% of the studied rodents were infected by *T.lewisi* (Seifollahi et al., 2016). According to the previous works *R. opimus* is primary reservoir host for *L. major* in Isfahan and Khorassan provinces in Iran as other foci of disease (Nilforoushzadeh et al., 2014; Jalali et al., 2021).

The complexity of *leishmania* transmission lays in its involvement of various mammalian hosts, ranging from small rodents to big domestic animals, as reservoir hosts. Human imposed environmental changes result in the modification of the micro-ecology of the parasite, the vector, and the reservoir host favoring the higher transmission of leishmaniasis in areas. *T. cruzi*, *T. brucei* and *T. gambiense* are the main pathogenic species for humans and cause Chagas' disease, sleeping sickness, and African animal trypanosomiasis, or nagana in livestock respectively, as well as other species considered to be non-pathogenic for humans. Ghobakhloo et al., isolated of *Crithidia* spp. from lesions of immunocompetent patients with suspected CL in Iran with PCR method using specific primers based on the sequence of GAPDH(Glyceraldehyde-3-Phosphate Dehydrogenase) gene (Ghobakhloo et al., 2019).

Conclusion

It is important to study the infection in rodents because many diseases of this animal are common to humans and study in this area can elucidate important information about the region's pathogenic reserves. And also identification and detection of this infection are important because they are carriers in the environment and can cause human injury the results of this study and other similar studies can help health professionals to do better for control and prevention of zoonotic diseases.

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Declarations

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Conflict of interests

The authors declare that there is no conflict of interest.

Statement of availability of data and materials

The data and materials used in the study are available upon request from the corresponding author.

Authors' contributions

Conceptualization: [Hajar Ziaei Hezarjaribi, Mahdi Fakhar, Mitra Sharbatkhori, Hossein Ghalehnoei], Study design: [Yousef Moghaddam, Hajar Ziaei Hezarjaribi, Mahdi Fakhar, Bahman Rahimi Esboei], Formal analysis and investigation: [Yousef Moghaddam, Abdol Sattar Pagheh, Reza Saberi, Eisa Nazar, Bahman Rahimi Esboei], Writing - review and editing: [Yousef Moghaddam, Hajar Ziaei Hezarjaribi, Mahdi Fakhar, Abdol Sattar Pagheh, Reza Saberi, Mitra Sharbatkhori, Hossein Ghalehnoei, Eisa Nazar, Bahman Rahimi Esboei], Funding acquisition: [Hajar Ziaei Hezarjaribi, Mahdi Fakhar].

Ethical approval

This study was approved by Mazandaran University of Medical Sciences, Sari, Iran (IR.MAZUMS.REC.1399.7970).

Consent for publication:

The authors of current study were declared that there is not any conflict of interest and are agree to publish this work in the Journal of Acta Parasitologica.

Refereces

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