

Original Article**Molecular Detection of *Leishmania major* and *L. turanica* in *Phlebotomus papatasi* and First Natural Infection of *P. salehi* to *L. major* in North-east of Iran**

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Abstract

Background: Leishmaniasis is an important public health disease in many developing countries as well in Iran. The main objective of this study was to investigate on *leishmania* infection of wild caught sand flies in an endemic focus of disease in Esfarayen district, north east of Iran.

Methods: Sand flies were collected by sticky papers and mounted in a drop of Puri's medium for species identification. Polymerase chain reaction techniques of kDNA, ITS1-rDNA, followed by restriction fragment length polymorphism were used for identification of DNA of *Leishmania* parasites within infected sand flies.

Results: Among the collected female sand flies, two species of *Phlebotomus papatasi* and *Phlebotomus salehi* were found naturally infected with *Leishmania major*. Furthermore, mixed infection of *Leishmania turanica* and *L. major* was observed in one specimen of *P. papatasi*. Sequence analysis revealed two parasite ITS1 haplotypes including three *L. major* with accession numbers: KJ425408, KJ425407, KM056403 and one *L. turanica*. (KJ425406). The haplotype of *L. major* was identical (100%) to several *L. major* sequences deposited in GenBank, including isolates from Iran, (Gen Bank accession nos. AY573187, KC505421, KJ194178) and Uzbekistan (Accession no. FN677357).

Conclusion: To our knowledge, this is the first detection of *L. major* within wild caught *P. salehi* in north- east of Iran.

Keywords: *Leishmania major*, *L. turanica*, *P. salehi*, *Phlebotomus papatasi*, Iran

Introduction

Leishmaniasis is an important public health disease in the world. The disease is endemic in more than 98 countries including Iran (WHO 2010). Cutaneous leishmaniasis (CL) is a worldwide public health and a social problem in many developing countries. Old world cutaneous leishmaniasis is present in many endemic areas of North Africa, the Mediterranean, the Middle East, the Indian

subcontinent and Central Asia. The species responsible for old world cutaneous leishmaniasis are mainly *L. major* and *L. tropica*, *L. infantum* and *L. donovani* can also cause localized CL but, are observed less frequently in the Mediterranean areas. Diffuse CL is uncommon and is caused by *L. aethiops* in Africa (Goto et al. 2010, Hotez et al. 2012).

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The incidence rate of the disease is 0.7 to 1.2 million cases all over the world (Alvar et al. 2012). The important factors that leishmaniasis is a serious public health in many countries are increasing of deforestation, urbanization, human migration, and HIV/AIDS (Desjeux 2001). The disease, almost affects the poor people, especially those with vulnerable housing and environmental conditions (Alvar et al. 2006).

At the present, zoonotic cutaneous leishmaniasis is the first important vector borne disease in Iran and is endemic in 17 out of 31 provinces of the country (Afshar et al. 2011). The annual incidence of zoonotic cutaneous leishmaniasis has gradually increased in Iran and more than 20 000 cases has been reported in 2013 (unpublished data). This increasing outbreak is in relation to human-sand fly-rodent contacts, itself probably the product of the development of irrigation schemes and the spread of human populations into the habitats of the vector and the rodents that act as reservoir hosts. The causative agent of disease is *L. major* and the sand fly species of *P. papatasi* has been reported as the most important and proven vector of disease to human in several endemic foci of Iran (Rassi et al. 2008, 2012). Esfarayen district of northern Khorassan Province, in the north east of Iran is an important focus of zoonotic cutaneous leishmaniasis due *L. major* with more than 400 new cases of CL in 2012. The main objective of this study was to detection of species of leishmania parasite in wild caught sandflies as the vector(s) of disease.

Materials and Methods

Study area

This descriptive cross sectional study was conducted in Esfarayen county, North khorasan, North east of Iran. The capital of the county is Esfarayen. At the 2006 census,

the county's population was 119,152, in 30,307 families. In general, the northern part of the Esfarayen has a temperate climate due to its proximity to mountainous areas and the south and southwest areas with hot summers and cold winters. The average annual precipitation is nine mm. The main occupations of the population are farming and raising animal.

Sand fly collection

Based on prevalence of disease with positive human cases, four villages of Kalatereza, Esmaeilabad, Kalatehshor and Hosseinabad were selected. Sand flies were collected biweekly from indoors (e.g. bedroom, guest bedroom, toilet, and stable) as well as outdoors (wall cracks and crevices and animal burrows) by using sticky paper (30 papers for indoors and 30 papers for outdoors per village) during July–October 2013. All traps were installed at sunset and collected near sunrise. The sand fly specimens were washed in 96 % ethanol alcohol to get rid of the sticky materials and to preserve them. Dissection of preserved sand flies was done in phosphate buffered saline (PBS) solution. The terminal segments of the abdomen containing the spermatheca and the heads of females were removed and mounted in a drop of Puri's medium and identified to species level using light microscope and key of Theodor and Mesghali, 1964. The remains of the bodies of the sand flies were kept individually in 96 % alcohol and stored at -20 °C for molecular analysis.

DNA extraction

DNA of the specimens was extracted using the Bioneer Genomic DNA Extraction Kit Cat. No.K-3032 Lot.No.1204D, (North Korea), according to the manufacturer's instructions. Extraction was carried out on the remaining body of the individual sand fly and stored at 4 °C. Double distilled water as a negative control and DNA from *L. major*

and *L. tropica*, provided to the Iranian Institute of Pasteur by the World Health Organization, were used as positive controls.

DNA amplification and PCR-RFLPs

Primary examination for infection of sand flies with *Leishmania* species was performed using nested-polymerase chain reaction (PCR) against the mini circle kinetoplast (k)-DNA using the following primers (Noyes et al. 1998) CSB2XF (forward): 5'-C/GA/GTA/GCAGAAAC/TCCCGTTCA-3' (20bp), CSB1XR(reverse): 5'-ATTTTTTCG/CGA/TTTT/C GCAGAACG-3' (20 bp), 13Z (forward): 5'-ACTGGGGGTTGGTGTAATAATAG-3' (22 bp), LIR (reverse): 5'-TCGCAGAACGCC-CCT-3' (15 bp).

Positive samples against kDNA were tested against the ribosomal internal transcribed spacer 1 (ITS1) region using the primers LITSR (5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCAC TT-3') followed by digestion with *Hae* III (El Tai et al. 2000). The PCR products were run along with a 100 bp ladder on 1.2 % agarose gel containing ethidium bromide for 1 h at 80 V. The gel was observed on an ultraviolet (UV) transilluminator and then digital photographs were prepared. Parasites were identified by comparison with positive controls of *L. infantum*, *L. major* and *L. tropica* and molecular weight markers. We added 2 μ L *Hae* III to the ITS1 PCR products (20 μ L) at 37 °C for 12 h with conditions recommended by the supplier (Fermentas, Germany). The restriction fragments were subjected to electrophoresis in 3 % agarose gel containing ethidium bromide (0.5 μ g/ mL) for 3 h at 65 V and observed on a UV trans illuminator (Hide and Banuls 2006, Oshaghi et al. 2009).

Results

In total 2305 sand flies comprising five species (3 *Phlebotomus* and 2 *Sergentomyia*)

were collected and identified. They included: *Phlebotomus papatasi* (43.43%), *P. salehi* (0.74%), *P. causicus* (2.33%), *Sergentomyia sintoni* (42.12%), and *S. dentata* (11.28%). Among the collected sand flies, a total of 390 females of sand flies were surveyed to find *Leishmania* parasites. They were *P. papatasi* (200/390), *P. salehi* (17/390), *S. sintoni* (120/390) and *S. dentata* (53/390). All specimens of *P. causicus* were male. Our results showed only 3 out of *P. papatasi* (1.5%) and 1 out of *P. salehi* (5.88%) were positive to *L. major*, while one specimens of *P. papatasi* (0.5%) were found mix infection with *L. turanica* and *L. major*. This was observed in the kDNA nested-PCR amplification assays where a ~560 bp PCR band was produced. This length of PCR in the system is assigned to *L. major* (Fig. 1). Their abdominal stages were either gravid or empty indicating there was enough time for the parasites to develop and transform to promastigote, the infective form. Further analyses showed that they were positive against ITS1 locus and produced a band of ~340 bp in gel electrophoresis. Also, ITS1 PCR-RFLP analysis by *Hae*III revealed the fragments of 220 and 140 bp for infected sand flies which are characteristic of *L. major*. The diagnostic fragments are 200 and 60 bp for *L. tropica* and 200, 80 and 60 bp for *L. infantum/L. donovani* (Figs. 2, 3).

Sequence analysis revealed two parasite ITS1 haplotypes including three *L. major* with accession numbers: KJ425408, KJ425407, KM052753 and one *L. turanica* (KJ425406). The three specimens of *L. major* were 100 % identical, although they were isolated from different species of *P. papatasi*, and *P. salehi*. The haplotype of *L. major* was identical (100 %) to several *L. major* sequences deposited in GenBank, including isolates from Iran, (GenBank accession Nos. AY573187, KC505421, KJ194178) and Uzbekistan (Accession No.FN677357). Also it was found to be 99 % similar to *L. mexicana venezuelensis*

from Mexico (Accession No. F339752).

The haplotype of *L. turanica* was found to be identical (100%) to that of isolates of *L. turanica* from Central Asia, including Iran (GenBank accession No. EF413079), Mongolia (Accession No. AJ272380), Turkmenistan, (Accession Nos. AJ272379 and AJ272381), and Kazakhstan (Accession No. AJ272382). Furthermore was found to be 99 % similar to *L. gerbilli* from China (Accession No. HQ 830351).

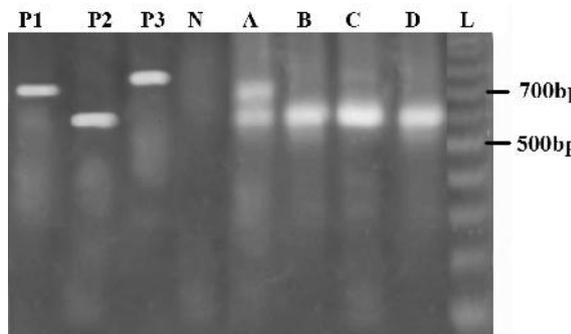


Fig. 1. kDNA nested PCR amplification (560 bp). *L. major* in *P. papatasi* (Lane B, C), *L. major* in *P. salehi* (LaneD), Mixed infection of *L. major* and *L.turanica* in *P. papatasi* (lane A), Positive control of *L. tropica* (Lane P3, 720 bp), Positive control of *L. major* (Lane P2), Positive control of *L. infantum* (680 bp, LaneP1), Negative control (Lane N) and (L) 100 bp molecular weight marker (Fermentase)

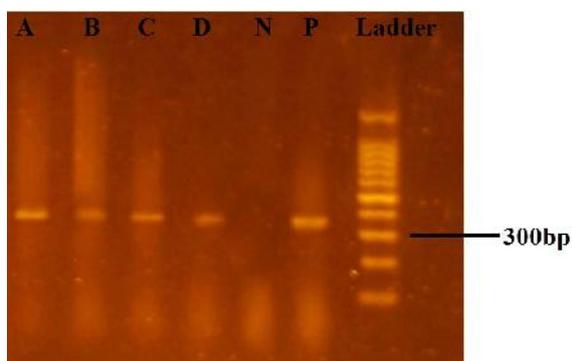


Fig. 2. ITS1 amplification of *L. major* in *P. papatasi* (Lane B,C) and *P. salehi* (Lane D), Mixed infection of *L. major* and *L. turanica* in *P. papatasi* (lane A), positive control of *L. major* (P), Negative control (Lane N) and (L)100 bp molecular weight marker (Fermentase)

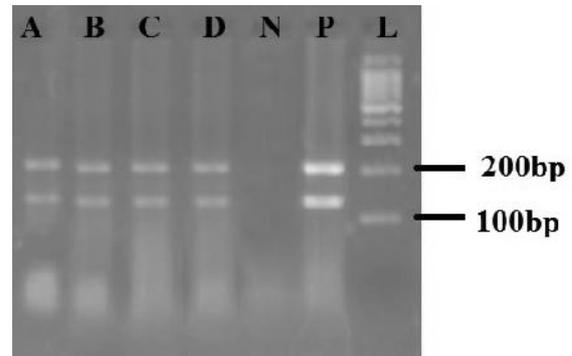


Fig. 3. PCR-RFLP analysis of ITS1 region for identification of Leishmania species using HaeIII. (L) 100 bp molecular weight marker, (P), positive control of *L. major*, (N) negative control, (A,B,C) samples of infected *P. papatasi* to *L. major*, (D) Infected *P. salehi* to *L. major*

Discussion

Due to the nature of zoonotic infections, the challenge of elucidating the structure of ecological systems are highly complex and is very important for the effective application of control measures (Reithinger et al. 2007).

Cutaneous leishmaniasis (CL) is an old endemic public health problem in Iran and more than of 80 % cases are caused by *L. major*, the zoonotic (ZCL) form of disease (Rassi et al. 2006, 2008, 2011a, 2011b).The incidence of CL in Northern Khorassan Province has been reported to be around 400 per 100,000 in 2013 (Unpublished data). It is more disabling disease with several endemic foci in north east of Iran.

Entomo-parasitological survey with epidemiological data are very important for control planning against leishmaniasis disease (Rassi et al. 2006). Natural infection of wild caught sand flies with the same leishmania parasites in human and their anthropophily, indicate the capacity of them as the vectors (Killick-kendrick 1990). Molecular techniques is highly sensitive to detection of leishmania parasites in sand flies and commonly used in Iran and other countries (Rassi et al. 2011b,

2012b, Killick-kendrick 1990, De Bruijn et al. 1992, Mukherje et al. 1997).

Based on animal reservoir host of ZCL, the great gerbil of *Rhombomys opimus* and sand fly species of *P. papatasi* are the main reservoir and vector in transmission of parasite to human in central and north east of Iran. At the present study, high density of *P. papatasi* and its natural infection with *L. major* is attributed to the fact that this species plays a major role as a principally vector in the region. *Phlebotomus papatasi* is known as a restricted vector and specifically is able to support only the development of *L. major* (Dobson et al. 2010).

In Iran this species is the most predominant sand fly in and around the burrows of great gerbils as well as human places. According the results of current study we found mix infection of *L. turanica* and *L. major* in one specimens of *P. papatasi*. This finding is incompatible with the concept of restriction transmission of *L. major* by the species but is congruent with the findings of Strelkova et al. 1996, Parvizi and Ready, 2008 and Bakhshi et al. 2013 demonstrating possible transmission of both *L. major* /*L. turanica* by *P. papatasi* (Strelkova et al. 1996, Parvizi and Ready 2008, Bakhshi et al. 2013). Detection of *L. major* in one specimen of *P. salehi* was another finding of this study.

Although the population and infection rate of this species to *L. major* appeared to be low, our results confirmed the studies of other scientists in south and south east of Iran (Azizi et al. 2012, Kassiri et al. 2012).

Conclusion

Since *P. salehi* specimens were only collected from rodent burrows and this species with *P. papatasi* appear to occur sympatrically and simultaneously in a few ZCL foci of Iran, indicating that the *P.*

papatasi has the main vector's role in transmission of *leishmania* parasite to human (Killick-kendrick 1990) and *P. salehi* is a secondary maintenance vector in the transmission cycle of infection between humans and rodents in Iran (Killick-kendrick 1999).

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