Annals of Parasitology 2020, 66(3), 339–346
doi: 10.17420/ap6603.272

Original paper

Molecular identification of *Leishmania* species isolated from patients with cutaneous leishmaniosis in Gonbad Kavoos, northeastern of Iran using HSP70 and ITS-based PCR-RFLP

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ABSTRACT. Cutaneous leishmaniosis (CL) is mainly caused by *Leishmania major* (rural-type) and *Leishmania tropica* (urban-type). CL is a major health problem in many regions of the world, and it is associated with health complications and economic loss. The identification and differentiation of *Leishmania* species are critical because the prevention and control methods, as well as management and therapeutic strategies, are different for each type of CL. The present study aimed to identify the parasite species responsible for CL in the study area using ITS1 and HSP70-based PCR-RFLP methods. A total of 147 stained slides were prepared from samples collected from CL patients, and these slides were positive for amastigotes of *Leishmania* species on microscopic examination. Forty-three Giemsa-stained slides with 2+ to 4+ grades were selected for molecular studies for the identification of the *Leishmania* species. DNA was extracted from the selected slides for the molecular studies. The amplification of HSP70 and ITS1 genes was performed by the PCR method. The PCR products were digested with the HaeIII restriction enzyme, and banding patterns of all samples were compared with reference strains. Overall, patterns of all the samples were found to correspond to the reference strains of *L. major* based on RFLP-PCR targeting HSP70 and ITS1 genes of the parasite, demonstrating the dominance of *L. major* as the causative agent of Zoonotic cutaneous leishmaniosis (ZCL) in the study area. This area is endemic for zoonotic CL, and further studies are required to determine the reservoir and natural infection of sand flies in this county.

Keywords: cutaneous leishmaniosis, diagnosis, HSP70, ITS, PCR-RFLP, Gonbad Kavoos

Introduction

Leishmaniosis is a parasitic disease caused by the flagellated protozoa of the genus *Leishmania* and transmitted through the bites of infected female sand flies. The disease has a wide clinical spectrum. It is estimated that more than 350 million individuals are at risk of contracting the disease worldwide. There are 3 classical forms of the disease; cutaneous leishmaniosis (CL), visceral leishmaniosis (VL), and mucocutaneous leishmaniosis (MCL). CL is considered as the most common form of leishmaniosis, leading to lifelong scars, disability, and stigma among infected individuals. Annual incidence of CL has been estimated to be 600,000 to 1 million new cases worldwide, with a higher prevalence in the Mediterranean basin, the eastern Mediterranean region and Central Asia, as well as in North and South America. In the year 2018, over 85% of the global CL incident cases were reported from 10 countries, including Afghanistan, Iran, Iraq, Pakistan, Syrian, Tunisia and Algeria as well as Brazil, Colombia, and Bolivia [1,2].

Zoonotic CL (ZCL) and anthroponotic CL

(ACL) are the two major forms of cutaneous leishmaniosis, caused by *Leishmania major* (rural-type) and *Leishmania tropica* (urban-type), respectively [3].

Previous studies have indicated the distribution of CL in different areas of Iran, with an estimated 20,000 newly diagnosed cases annually. However, it has been suggested that the actual incidence may be five times higher than estimated [4,5]. In Iran, the rural CL, which causes wet lesions, is prevalent in 15 provinces, whereas the urban CL, associated with dry lesions, is distributed in almost all urban regions of the country [6]. Two sand fly genera are present in Iran, and 44 species belonging to these 2 genera have been identified. Species of the genera *Phlebotomus* are responsible for *Leishmania* transmission in Iran, including *Ph. papatasi*, *Ph. major*, *Ph. sergenti*, and *Ph. alexandri*. Among the phlebotomine sandflies, *Ph. papatasi* is considered as the main vector of the disease in Iran [7]. *Rhombomys opimus* and *Meriones libycus* are the main reservoir hosts of ZCL in Iran. *Leishmania* infection in *R. opimus* has been reported in the northeast (e.g. Golestan and Mazandaran) and central parts of Iran [8].

Diagnosis of CL is difficult due to the diverse presenting symptoms in different forms of the disease [9]. A timely and definitive diagnosis of CL is of great importance for prognostic assessment and for providing appropriate therapeutic strategy [10]. Many diagnostic methods have been applied for the diagnosis of CL, including the microscopic evaluation of direct smear, culture, molecular, and immunological methods. Microscopic evaluation is not highly sensitive because of its dependency on the technical skills of the personnel, sampling process, and the number of parasites in the smear. Parasite culture methods are not always available [9].

PCR-based methods are rapid and highly sensitive techniques and are commonly used, but the field deployment of these methods can be very expensive [11]. The ITS-ribosomal DNA (rDNA) region, glucose-6-phosphate dehydrogenase (G6PD), heat shock protein 70 (HSP70), mini-exon, and kinetoplastic DNA (kDNA) are commonly used as PCR targets for the diagnosis of *Leishmania* species [12–17].

PCR-based method targeting the ITS-ribosomal DNA gene is capable of detecting *Leishmania* species even with a low number of parasites in the sample [17]. A PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) method can provide a timely and definitive diagnosis of CL, which is necessary for choosing the appropriate management and therapeutic strategies for the disease treatment. Although microscopic examination is the easiest diagnostic method, the remarkably low sensitivity makes it inefficient [17]. PCR-based RFLP-HSP70 method is also capable of differentiating *Leishmania* species due to its low level of genetic variation [12,18]. Therefore, we used the PCR-RFLP method targeting the HSP70 and ITS sequences for the identification of *Leishmania* species in Gonbad Kavoos in Golestan Province in Iran.

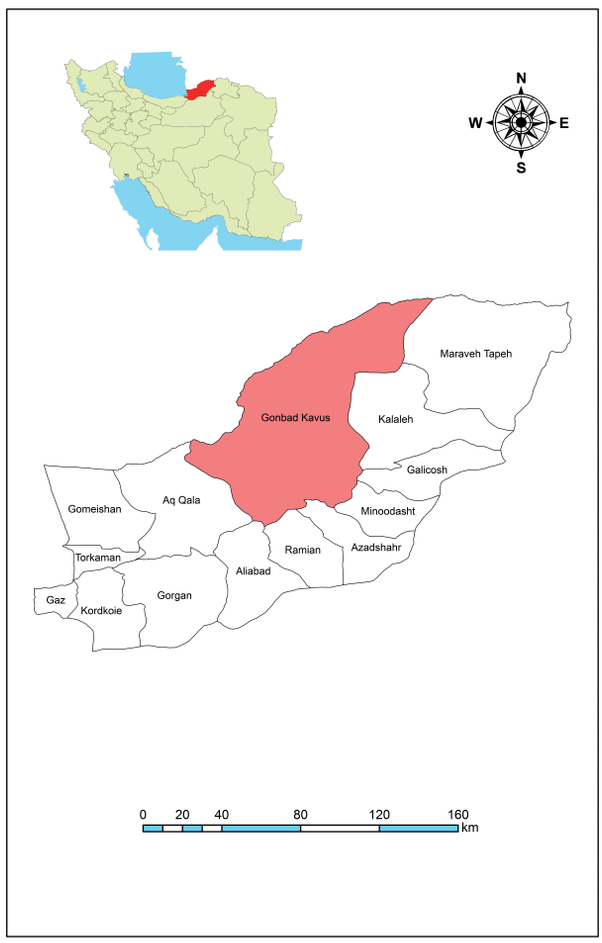

Figure 1. Map of Gonbad Kavoos county, Golestan province, Iran

Materials and Methods

Study area. The present study was performed in Gonbad Kavoos County (37°15′00″N; 55°10′02″E) in Golestan Province in the northeast of Iran. Gonbad Kavoos County is located in the east of the Caspian Sea with an elevation of 38 m above the sea level (Fig. 1). This area is characterized by two main climates; mild mountainous climate in the southern

and eastern parts and semi-arid and semi-desert climates in the northern regions. Overall, the climate in this area is described as a warm temperate climate with a mean annual temperature of 17.8ºC and a mean annual rainfall of 363 mm. Agriculture and animal husbandry are the most common occupations among the inhabitants of this city. This region is considered as an endemic focus of CL.

Sample collection and microscopic evaluation. Slides were prepared from the skin lesions of CL patients referred to the *Leishmania* Research Laboratory in the Health Center of Gonbad Kavoos in Golestan province in Iran during the spring of 2015. Giemsa-stained slides were prepared and examined under a light microscope at ×1000 magnification. After Giemsa-staining, 147 slides containing the amastigote form of *Leishmania* were selected. The Giemsa-stained slides containing amastigotes were transferred to the Leishmaniasis Laboratory of the Department of Parasitology and Mycology in the School of Public Health of Tehran University of Medical Sciences for molecular evaluation. In this study, Giemsa-stained slides were evaluated microscopically and were classified as 1+, 2+, 3+, 4+, 5+ or 6+ grade based on the WHO basic laboratory procedures [19]. Of the 147 slides examined, no grades 5 and 6 (hyper-infection) were seen. Forty-three (43) slides classified as grade 2+, 3+, or 4+ were selected for DNA extraction. For the remaining slides, either parasites were not seen or had grade 1+ and were not considered to be suitable for DNA extraction (Table 1).

Table 1. Number of selected Giemsa-stained slides for molecular evaluation

Selected samples	Grade
4	2+ (1-10 parasite/100 field)
17	3+ (1-10 parasite/10 field)
22	4+ (1-10 parasite/field)
43	Total

DNA extraction. The Giemsa-stained slides were first kept overnight in absolute ethanol and then dried at laboratory temperature. DNA was extracted from the 43 Giemsa-stained slides using High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany), according the manufacturer's protocol. The extracted DNA samples were then stored at -20ºC until use.

HSP70–PCR-RFLP. The amplification of HSP70 was performed by applying specific primers as in a previous study: HSP70 sen (5´-GACGGTGCCTGCCTACTTCAA-3´) and HSP70 ant (5´-CCGCCCATGCTCTGGTACATC-3´) [12]. The 50 µl PCR reaction contained 4 µl genomic DNA (20-30 ng), 2 µl of each forward and reverse primers (10 pmol), 20 µl master mix 1x (SinaClon, Iran), and 22 µl of double distilled water. Amplification was done by using the MyCycler Thermal Cycler (BioRad, USA) based on the protocol described by Nemati et al. (20): initial denaturation cycle for the PCR reactions was performed at 94ºC for 5 min, followed by 30 cycles; at 94ºC for 5 min, 61ºC for 1 min and 72ºC for 1 min, and a further extension step at 72ºC for 5 min. Then, 5 µl of each PCR amplification product was run on a 1.5% agarose gel and stained with Safe-Red staining and visualized under UV light. The PCR products of *Leishmania* species isolates were eventually considered as positive if the reaction produced about 1420 bp fragment for the HSP70 gene. The HSP70 gene amplicons were digested with the restriction endonuclease HaeIII (Fermentas, Life Sciences, Germany), according to the manufacturer's instruction.

First, restriction sites for the HaeIII enzyme within the HSP70 sequences of different reference species were searched using Restriction Mapper 3 (www.restrictionmapper.org).Theoretically, fragments from the HSP70 gene of different reference species digested with HaeIII would be expected to make different patterns as follows: *L. major* (2, 34, 40, 41, 47, 99, 152, 246, 307, and 351 bp), *L. tropica* (8, 13, 21, 40, 41, 80, 99, 150, 246, 338, and 354 bp), and *L. infantum* (13, 40, 41, 47, 53, 80, 99, 152, 246, 307, and 338 bp). Following HaeIII digestion, the products were run on 3% agarose gel and then visualized using Safe-Red staining under UV light. Finally, the parasite species were identified using HSP70 banding patterns. Iranian reference strains, *L. tropica* (MHOM/IR/02 /Mash10, Acc. No. EF653267), *L. major* (MRHO /IR/11/GOL-2, Acc. No.JN860745), and *L. infantum* (MCAN/IR/07 /Mash-ir1, Acc. No. EU810776) were used as positive controls for matching the banding patterns of the amplicons [21].

ITS1–PCR-RFLP. The amplification of ITS1 gene was performed by applying the primers used for ITS-ribosomal DNA fragment amplification (LITSR 5′-CTGGATCATTTTCCGATG-3′ and L5.8S 5′-TGATACCACTTATCGCACTT-3′) as reported by Dávila and Momen [22]. The 50 µl PCR reaction contained 4 µl genomic DNA (20–30 ng), 2

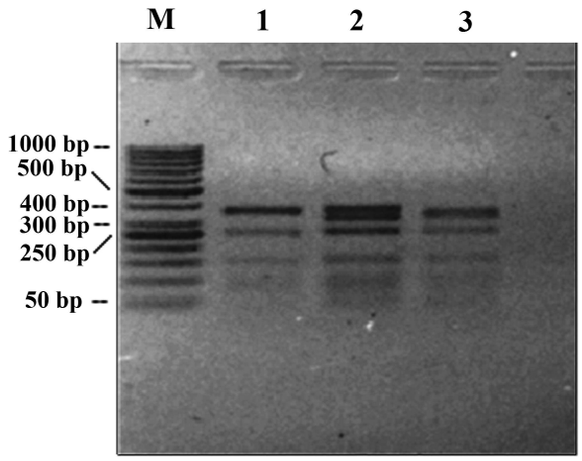

Figure 2. HSP70 PCR-RFLP profiles obtained from patient's sample and *Leishmania* reference strains digestion with HaeIII enzyme. Lane 1: standard *L. tropica*; lane 2: patients sample (*L. major*); lane 3: standard *L. infantum*; M: 50 bp DNA ladder.

µl of each forward and revers primers (10 pmol), 20 µl master mix 1x (SinaClon, Iran), and 22 µl double distilled water. The amplification was performed in MyCycler Thermal Cycler (BioRad, USA) by the following program: 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 49°C for 30 s, 72°C for 45 s, and a final extension step 72°C for 5 min. Then 5 µl of each PCR amplification products was run on a 1.5% agarose gel and stained with Safe-Red staining and visualized under UV light. Positive PCR products of *Leishmania* species isolates were eventually considered as 300–350 bp for the ITS1 gene.

ITS1-PCR amplicons were digested with the HaeIII restriction endonucleases (Fermentas, Life Sciences, Germany), based on the manufacturer's instructions. First, restriction sites for the HaeIII enzyme within ITS1 sequences of different reference species were searched using Restriction Mapper 3 (www.restrictionmapper.org). Theoretically, fragments from ITS1 gene of different reference species digested with HaeIII would be expected to create different patterns as follows: *L. major* (140 and 220 bp), *L. tropica* (50, 60, and 200 bp), and *L. infantum* (60, 80, and 200 bp). The digestion products were run on 3% agarose gel and then visualized under UV light after staining with Safe-Red. Amplicons banding patterns of ITS1 fragments were identified by comparing them with reference strains.

Results

The HSP70-PCR reactions produced 1420 bp fragments for the HSP70 region in all the 43 *Leishmania* isolates. The results of HSP70 PCR-RFLP method indicate that the banding patterns of all the samples were identical to the reference strains of *L. major* species. The reference strains exhibited 3 different electrophoretic patterns after HSP70 gene digestion with HaeIII according to the expected profile of the restriction map (Fig. 2).

A 300-350 bp fragment was released from ITS1-PCR fragment of the genus *Leishmania*. The results of ITS1 PCR-RFLP method demonstrate that the patterns of all the samples were identical to the reference strains of *L. major* and were shown two pieces with size 220 and 140 bp, whereas the digested reference strains exhibited bands of 200, 60, and 50 bp for *L. tropica* and 200, 80, and 60 bp for *L. infantum* based on the ITS1 region (Fig. 3).

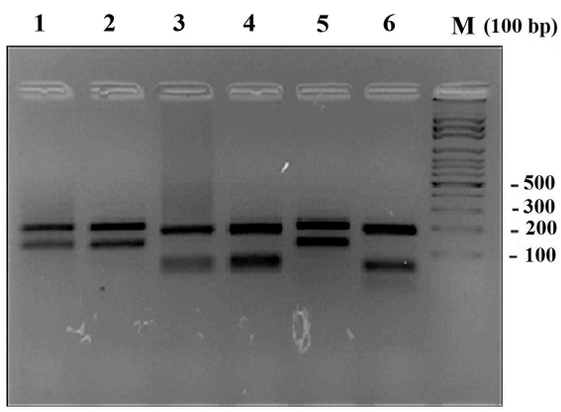

Figure 3. ITS1 PCR-RFLP profiles obtained from patient's sample and *Leishmania* reference strains digestion with HaeIII enzyme. Lanes 1 and 2: patients samples; lanes 3 and 4: standard *L. infantum*; lane 5: standard *L. major*; lane 6: standard *L. tropica*; M: 100 bp DNA ladder (Fermentas).

Overall, patterns of all the samples corresponded to the reference strains of *L. major* based on the RFLP-PCR targeting HSP70 and ITS1 genes, demonstrating dominance of *L. major* as causative agent of ZCL in study area.

Discussion

Identification of the epidemiological status and the parasite species of CL are of great importance for developing control measures and therapeutic strategies in endemic areas such as Iran [23,24]. In

the present study, *Leishmania* DNA was extracted from Giemsa-stained slides prepared from skin lesions of CL patients. Identification of the *Leishmania* species was performed using the PCR-RFLP method targeting the HSP70 and ITS genes.

In the present study, HSP70 PCR-RFLP and ITS PCR-RFLP methods were used to identify *Leishmania* species in clinical isolates from lesions of CL patients. All samples were initially confirmed under microscope as having amastigotes of *Leishmania* species. Banding patterns of all the samples corresponded to the reference strains of *L. major*, indicating that *L. major* is the predominant *Leishmania* species in Gonbad Kavoos, an endemic focus of cutaneous leishmaniasis.

Based on the data presented in the literatures, conventional and molecular techniques have been used for the identification of by targeting different gene fragments such as ITS-rDNA [24–26], HSP70 [16,27,28], the cysteine protease B [29], G6PD, mini-exon, kDNA [15], and N-acetyl glucosamine-1-phosphate transferase gene (NAGT gene) [21]. Semi-nested PCR of kDNA has also been used for the identification of sand fly species [30].

Among the many target genes, the HSP70 has a highly conserved sequence, which can be applied for the identification of *Leishmania* species via sequencing [18,27]. HSP70 PCR-RFLP has been also described as a valuable diagnostic method for *Leishmania* species differentiation due to its low level of genetic variation compared with other gene fragments such as the ITS1-rDNA and glucose-6-phosphate dehydrogenase (G6PD) [12,18].

ITS-PCR of DNA samples extracted from Giemsa-stained slides is commonly used as a favorable technique in endemic regions of Iran for the identification of *Leishmania* species without the need for cultivation or injection of parasites into laboratory animals [17,31]. The PCR-RFLP technique has been previously described as a specific and sensitive method for the identification of polymorphisms and microorganisms [32,33]. ITS1-PCR technique using the RFLP method was capable of identifying *L. major* in all evaluated samples.

Evidence indicates that *L. major* is distributed in different areas of Iran, especially in Gonbad Kavoos County in Golestan province. Our data is in line with a previous study conducted in Gonbad Kavoos County in 2009, which reported that *L. major* was the predominant species among all isolates in the county [34]. Gonbad Kavoos County is an endemic area located near other endemic areas of CL in the Golestan Province (e.g., Kalaleh, Minoo Dasht, Marave Tappe) and North Khorasan province. *L. major* was the only identified species in Kalaleh County, an endemic area with a high prevalence of CL [35].

The wide spread of CL in this geographical region is linked to the ecological distribution of the disease vectors and reservoirs, as well as environmental variables and their interaction with the vectors of ZCL. The distribution of *Ph. papatasi* has been reported in difference regions of the country. *Leishmania* species has been isolated from *Ph. papatasi* in most regions of Golestan province, where spatial distributions of phlebotomine sand flies have been mapped using data obtained from 1930–2012 [36]. It has been demonstrated that the activity of phlebotomine sand flies ends during the cold season. The activity of phlebotomine sand flies has been reported to occur between May and September in different provinces in Iran. Previous studies have reported that the rate of CL increases from July and peaks in November, and declines in March in Golestan province [36,37]. Another study reported a peak of cases in October and November, which coincides with the occurrence of the second peak of the sand flies in September [38], when autumn occurrence is expected in Golestan province, after 1 to 2 months of incubation period of the *Leishmania* species [39].

Moreover, the highest incidence of CL cases was reported in Korand village in Gonbad Kavoos in Golestan province. This village has a large number of rodents' active burrows [40]. In the last decade, the incidence of rural CL cases has elevated in this Province, especially in the last three years (2014 to 2017). This is linked to increased presence of gerbils, increased vicinity of wild rodents to villages, and increased population and CL carriers, as well as climate changes and increased public health attention CL [37,40].

The public health importance of rural CL and urban CL have been recently highlighted by previous studies conducted in different areas of Golestan province [41]. However, these studies did not report the predominant species in each study area, separately.

It is worth mentioning that the results of the present study have some limitations. The accuracy of the techniques used in this study depends on the sample size, such that large sample size yields a higher accuracy. PCR-RFLP method targeting

HSP70 and ITS genes can provide a favorable technique for the identification of *Leishmania* species using DNA samples from Giemsa-stained slides. In the present study, *L. major* was found to be the predominant species among all isolates of CL in Gonbad Kavoos County in Golestan province in Iran. This area is endemic for ZCL, and further studies are needed to determine the reservoir and natural infection of sand flies in this County.

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Received 15 February 2020
Accepted 07 May 2020