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Original Article

In Vitro Infectivity of *Leishmania major* Isolated from Patients with Different Clinical Forms of Cutaneous Leishmaniasis and Its Association with Parasite Zymodemes

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Abstract

Background: The aim of this study was to characterize the *Leishmania* parasites isolated from cutaneous leishmaniasis (CL) patients in Fars Province in Iran and to compare the potential infectivity of the isolates in macrophage cell line. Moreover, attempt was made to find out the association between parasite infectivity and their zymodemes.

Methods: Twenty samples were taken from the skin lesion of CL patients. The samples were cultured in biphasic media followed by mass cultivation in RPMI medium. Each isolate was tested for the activity of the 5 enzymes including glucose phosphate isomerase (GPI), malate dehydrogenase (MDH), nucleoside hydrolase 1 & 2 (NH1 & NH2), and phosphoglucomutase (PGM). The enzymatic profiles of the isolates were compared with WHO reference strains. Specific PCR (primers: LIN17 & LIN R4) and RAPD-PCR were used as complementary methods for characterization of the isolates.

Results: Isoenzyme electrophoresis showed that all of the isolates were *L. major*. PCR with LIN17 and LIN R4 and RAPD-PCR with AB-07 primers further determined the isolates as *L. major*. Results of macrophage infectivity experiment, using J774 cell line, showed that the most virulent isolates were related to Z1 with 63% macrophage infectivity rate. A well correlation was found between the infectivity rate of the isolates and type of ulcer. Those isolates with high infectivity rate were involved in more severe, ulcerative or erythematose lesions in CL patients.

Conclusion: The most invasive isolates might be a good candidate for immunological studies and for vaccine development.

Key words: *Leishmania major*, Macrophage infectivity, Isoenzyme, PCR, Iran

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Introduction

Leishmaniasis is a parasitic disease affecting more than 12 million people in 88 countries (66 Old World and 22 New World countries), with 350 million more at risk. Leishmaniasis has several different clinical features from ulcerative skin lesion and destructive mucocutaneous forms to disseminated visceral infection (1). The infection is caused by about twenty distinct *Leishmania* species.

The few species of *Leishmania* produce cutaneous leishmaniasis (CL), in Iran and Middle East are primarily *L. major* and *L. tropica* (2-4). Motazedian et al., isolated *L. tropica* and *L. major* from CL patients in four provinces and *L. tropica* from three provinces in Iran (4). Furthermore, in areas endemic for CL in Iran, *L. major* has been isolated from 4 species of rodents: *Rhombomys opimus*, *Meriones libycus*, *Tatera indica* and *Mer. Hurrianae* (5). Cases of cutaneous leishmaniasis due to *L. infantum* have also been reported in a few studies in Iran (6).

The two main causative agents of CL, *L. major* and *L. tropica*, induce variable disease expression in CL patients in endemic areas (7). This diversity has also been shown in naturally or experimentally in-

fectured animals. Parasite properties (infectivity, virulence) and host factors regulate various disease expressions. Clinical manifestations and yet basic clinical features vary further by parasite species within the endemic region. (1). Atypical presentation of CL is common in CL endemic areas in Iran (8).

When the parasite enters the mammalian host, it undergoes a transformation process, which results in small, round and non-flagellated amastigote form. Amastigotes replicate inside the macrophage, are finally released from the macrophage, and attack other macrophages. Inside the macrophage, the parasite also resists the killing activity of the macrophage. Biochemical features of the parasite play a pivotal role in parasite survival inside the macrophages. One of these features is parasite's zymodemes.

The aim of this study was to characterize the *Leishmania* parasites isolated from CL patients in Fars Province in south of Iran. The study also aimed to compare the potential infectivity of *Leishmania* parasite isolated from CL patients in macrophage cell line and to find out any association between parasite infectivity and their zymodemes.

Materials and Methods

Leishmania parasite

Leishmania parasites were isolated from 20 patients suspected of having CL and referred to Department of Parasitology and Mycology at Shiraz University of Medical Sciences, Shiraz, Iran. The clinical pictures of smear positive lesions were compatible with rural form of leishmaniasis. An enriched modification of Evans Tobies medium was used for parasite isolation. Mass cultivation of the isolates was performed using RPMI medium containing 15% fetal calf serum (Gibco).

Enzyme extraction

The cultures of the parasites were harvested at the end of logarithmic phase of the growth and the numbers of organisms were adjusted to $1-1.5 \times 10^7$ cell/ml. The cultures were centrifuged at 2000 g for 20 min at 4 °C. The supernatant was discarded and the pellet of promastigotes washed three times in cold-proline balanced salt solution. To the washed pellet of promastigotes, an equal volume of hypotonic aqueous solution of enzyme stabilizers (1 mM ϵ -amino-n-caproic acid, 1 mM dithiothreitol and 1 mM EDTA, Sigma) was

added and mixed thoroughly. After five cycles of freezing and thawing, the extract was centrifuged at 30000×g for 30 min at 4 °C, supernatant was aliquot and stored at -70 °C until use.

Isoenzyme electrophoresis

Isoenzyme electrophoresis was performed using discontinuous polyacrylamide gel electrophoresis (PAGE). Electrophoresis was performed using 3% of stacking gel and 7.5% of separating gel, and a stacking buffer composed of Tris/HCl (pH 6.7), a resolving buffer of Tris/HCl (pH 8.9), a tank buffer of Tris/HCl (pH 8.3), run under a constant current of 2 mA/well for 150 min. Each isolate from CL patients was tested for the activity of 5 enzymes including glucose phosphate isomerase (GPI), malate dehydrogenase (MDH), nucleoside hydrolase 1 and 2 (NH1 & NH2) and phosphoglucomutase (PGM). The enzymatic profiles of the isolates were compared with six WHO reference strains (Table 1).

Macrophage infectivity rate assessment

J774 cell line was maintained at 37 °C with 5% CO₂ and normally subcultured every 1-2 days in RPMI medium (9), containing 10% FCS, 2 mM L-glutamine and 100 µg/ml gentamicin sulfate. To assess the infectivity rate of each isolate, flasks of monolayer J774 cell line were prepared and inoculated with stationary phase (7-day-old culture) of each isolate, in a ratio of 10 parasites per macrophage. After overnight incubation at 32 °C, the cells were washed several times to remove all free parasites from the flasks. The cells were incubated for another 48 h in 32 °C with 5% CO₂. Microscopic slides were prepared from each cell suspension and stained by Giemsa. One hundred cells were counted at oil immersion magnification of

microscope and the ratio of the infected cell (those containing promastigote and amastigote) to non-infected cell (those without parasite) and intensity of infection (mean of parasite number in infected cells) was determined.

PCR and RAPD-PCR

PCR for characterization of parasites was performed by using LIN17 and LIN R4 primers and RAPD-PCR was used using AB1-07 primers as described before (4). The DNA of each cultivated sample was extracted by digestion in lysis buffer. The precipitate of the cultivated sample was added to 100 µL of double distilled water (DDW) and 100 µL of phenol: chloroform (1:1 V/V). The sample was centrifuged at 10000×g for 5 min and then the DNA in the supernatant solution was mixed with phenol/chloroform/isoamylalcohol (25:24:1 V/V) and again was centrifuged at 10000×g for 5 min. Then, sodium acetate (3M, pH=5.2) was added to the supernatant solution and the DNA was precipitated with 400 µL of pure ethanol, re-suspended in 100 µL of DDW, desiccated and stored at -20°C until use. Concentration of the extracted DNA was estimated by measurement of optical density at 260 nm.

Reactions were done in a final volume of 25 µl, containing 1× PCR buffer, 2 mM MgCl₂, 1.5 µl of each primer LINR4 (5'-GGG GTT GTT GTA AAA TAG GG-3'), reverse LINR17 (5'-TTT GAA CGG GAT TTC TG-3'), and 2U of Taq DNA polymerase. DNA was first denatured at 94 °C for 3 min and then cycled. The cycles were: 94 °C for 1 min, 65 °C for 30 s and 72 °C for 1 min. Amplified products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized by ultraviolet light.

Results

Sixteen of the twenty isolates from the CL patients were characterized by isoenzyme analysis comparing with six WHO reference strains. By analyzing 5 soluble isoenzymes, five different zymodemes (Z1-Z5) were determined (Table 2). Most of the isolates (75%) had Z1 zymodem. Comparing the isoenzyme profile of the isolates with those of WHO reference, it was found that all isolates had isoenzyme profiles consistent with WHO reference strain of *L. major* (MHOM/SU/73/5ASKH) (Fig.1). For further characterization of the isolates and to confirm the finding of isoenzyme analysis, PCR with LIN17 and LIN R4 and RAPD-PCR with AB-07 primers were used. Results of PCR and RAPD-PCR were consistent with those obtained with isoenzyme analysis (Fig. 2 & 3). To determine the potential in vitro

infectivity of the isolates and to find out any association between parasite's infectivity and their zymodemes, J774 macrophage cell line was infected with metacyclic form of the isolates and the infectivity rate was assessed microscopically (Fig. 4). Results of macrophage infectivity experiment showed that the most virulent isolate (one of isolates) was related to Z1 zymodem with 63% macrophage infectivity rate (Table 3). A variation in the in vitro macrophage infectivity rate of *Leishmania* isolated from patients with different clinical forms (Fig. 5) was noted where the parasite isolated from the more severe forms of CL showed the higher degree of infectivity. The result demonstrated an association between the infectivity rate and ability of parasite to cause severe and ulcerative lesions in the hosts.

Table 1: WHO reference strains of *Leishmania* used in this study

International WHO code	Species and zymodemes
MHOM/IN/80/DD8	1- <i>L. donovani</i> MON-2
MHOM/TN /80/IPT1	2- <i>L. infantum</i> MON-1
MHOM/SU/73/5ASKH	3- <i>L. major</i> MON-4
MHOM/SU/74/K27	4- <i>L. tropica</i> MON-60
MHOM/IR/95/LEM3119	5- <i>L. tropica</i> MON-39
MHOM/FR/97/LEM3336	5- <i>L. infantum</i> MON-29

Table 2: Determined zymodemes and their pattern numbers in *Leishmania* parasites isolated from CL patients, using five enzymatic systems

Zymodem No.	Enzyme pattern number					No. of isolates	Final diagnosis
	MDH	GPI	PGM	NH1	NH2		
Z1	P1	P1	P1	P2	P1	12	<i>L. major</i>
Z2	P2	P2	P1	P2	P1	1	<i>L. major</i>
Z3	P2	P1	P1	P2	P1	2	<i>L. major</i>
Z4	P1	P1	P2	P1	P1	1	<i>L. major</i>

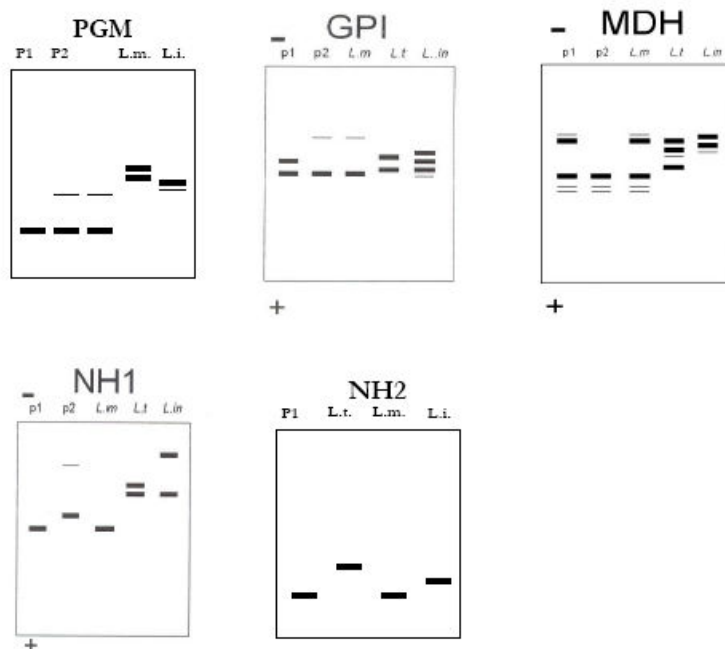


Fig. 1: Electrophoretic profiles obtained with soluble extracts of *Leishmania* promastigotes for five enzymatic systems. Each isolates were compared with the reference strain of *L. tropica* (L.t), the reference strain of *L. major* (L.m) and the reference strain of *L. infantum* (L.i). (+) is anode and (-) is cathode. P1 and P2 are pattern number of studied isolates.

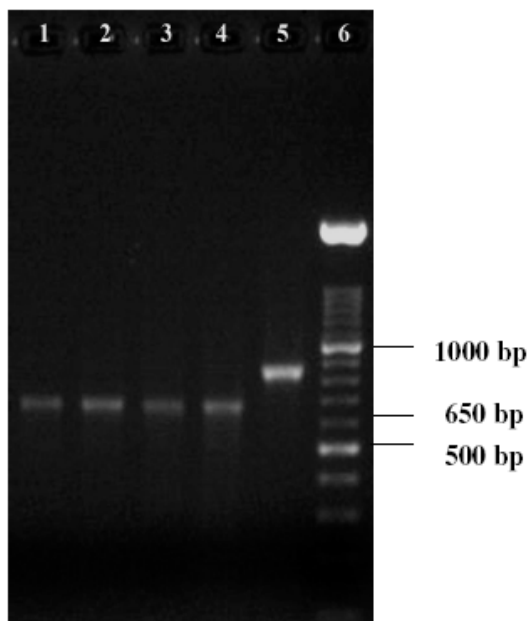


Fig. 2: PCR profiles obtained from *Leishmania* reference and the isolates with the LIN17 and LIN R4 primers. Lane 1-3: isolated samples from the CL patients, lane 4: *L. major* (650 bp), Lane 5: *L. tropica* (760 bp), Lane 6: molecular marker

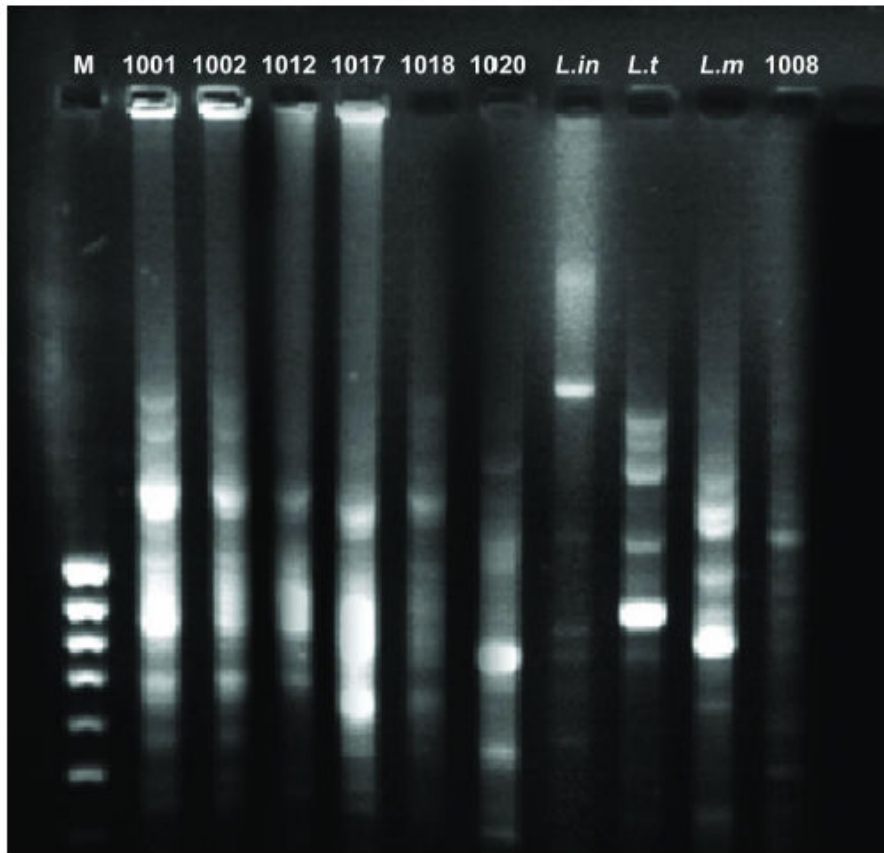


Fig. 3: PCR profiles obtained from RAPD-PCR on *Leishmania* reference and the isolates with the AB1-07 primers. Lane 1 molecular marker, Lane 2-7 and 11 isolated samples from the CL patients, lane 8: *L. infantum*, Lane 9: *L. tropica* and Lane 10: *L. major* reference strains

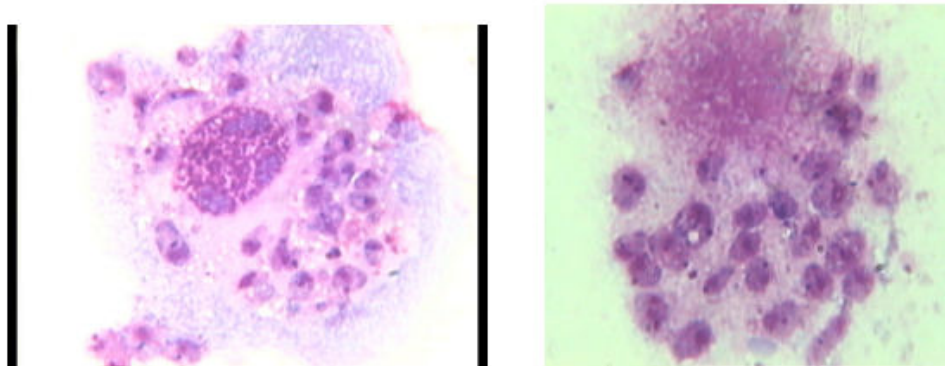


Fig. 4: In vitro infection of macrophages (J774 cell line) with *Leishmania* parasite isolated from CL patients

Table 3: Macrophage infectivity rate of *Leishmania* parasite, with different zymodemes, isolated from CL patients

Zymodem No.	No of isolates	Macrophage infectivity rate (%)
Z1	12	63
Z2	1	59
Z3	2	10
Z4	1	40



Fig. 5: Patients with different clinical forms of CL. *Leishmania* parasite were isolated from these patients and evaluated for their in vitro infectivity of macrophage cell line (J774)

Discussion

Cutaneous leishmaniasis is a common parasitic disease in Iran and zoonotic cutaneous leishmaniasis (ZCL) is a major and increasing public health problem in 11 of the 30 provinces of Iran. One of the objectives of the current study was to characterize the *Leishmania* parasites isolated from CL patients in Fars province in Iran. This was done by using molecular and isoenzyme methods.

The enzymes MDH, NH [1 & 2], PGM, and GPI could differentiate *L. major* and *L. tropica* from *L. infantum* though the enzymes MDH, NH and GPI were found to be more efficient in characterizing these organisms (10-15). In our study, we used six enzymatic systems to characterize the *Leishmania* spp. isolated from CL patients. Our findings showed that all the isolates had a profile pattern similar to those refer-

ences of WHO for *L. major*. This is expected since *L. major* is the most common causative agents of CL in Fars province in Iran. Molecular analysis using *Leishmania* specific PCR and RAPD-PCR confirmed the findings of the isoenzyme analysis.

In recent years, CL due to *L. major* has appeared in areas where the dominant causative agents of CL showed to be *L. tropica*. This seems to be the result of the construction of buildings near colonies of rodent burrows and traveling to ZCL foci of Iran. In the current study, we have found that different strains of *L. major* isolated from CL patients show different rates of infection in macrophage cell line. Findings of this study revealed that the parasite with zymodem 1 isoenzyme pattern (Z1) has the most infectivity rate as compared to those with other zymodemes. Further studies are

required to characterize further the parasites with Z1 isoenzyme pattern. Those parasites, which contain this zymodem, might be a good candidate for vaccine investigation. Differences *in vitro* infectivity rate have been found in human macrophage cell line U937 in different species of *L. viannia* (16). Furthermore, differences in the infectivity of New World *Leishmania* species in BALB/c mice peritoneal macrophages have been reported by Campos *et al.* They found that the *L. (V.) braziliensis* from mucocutaneous leishmaniasis, the more severe form of the disease, showed the highest infection index compared with those of other species (17).

In our study, an association was found between the infectivity rate of the isolates and type of ulcer in CL patients. Those isolates with high infectivity rate were involved in more severe and ulcerative or erythematic lesions. This is in agreement with Gomes *et al.* study who found that *Leishmania* isolated from patients with different clinical forms of human CL have different rates of infectivity for mice peritoneal macrophages. They reported that strains from the more severe forms of American cutaneous leishmaniasis showed the highest degree of infectivity and lowest level of NO production (18). These findings are in keeping with those reported by Balestieri *et al.*, where they found that *Leishmania* with high pathogenicity for human had a greater degree of resistance to anti-microbial effect of nitric oxide in the host (19).

In conclusion, our findings demonstrated the variation in the infectivity of *L. major* isolated from CL cases in Iran. Further studies are required for a more comprehensive understanding of these diversities.

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The authors declare that they have no conflicts of interest.

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