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Correlation between clinical responses with the drug-susceptibility of parasites in Iranian cutaneous leishmaniasis caused by *Leishmania major*

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Abstract. Reviews have shown increasing number of Iranian patients with cutaneous leishmaniasis (CL) who are unresponsive to pentavalent antimonial compounds such as meglumine antimoniate (Glucantime, MA). The present investigation aims to determine the correlation between clinical responses (healing, or non-healing) with susceptibility of *Leishmania* parasites to glucantime. Initially, *in vitro* susceptibility of *Leishmania* parasites was carried out on 93 isolates using macrophage models. Identification of these species was also performed by molecular methods including Nested-PCR and PCR-RFLP. The f indicated that total isolated were *L. major*. A significant association between the clinical outcome and the *in vitro* effective concentration 50% (EC_{50}) values was observed. *Leishmania* derived from patients with non-healing lesions had EC_{50} values at least 3-fold higher than parasites isolated from lesions of healing patients. By molecular methods, patterns for both sensitive and resistant samples demonstrated restriction band which is related to *L. major*. The obtained findings in the present study demonstrated that MA-resistant *L. major* field isolates are now frequent in Iran. Such studies help to find strategies for rapidly diagnosing resistance in order to improve the clinical management of CL.

INTRODUCTION

Leishmaniasis is a prevalent parasitic disease found in 98 countries in 5 continents, causing 20,000 to 40,000 deaths per year (WHO, 2010). The disease has three main types of manifestations including visceral, cutaneous, and mucocutaneous leishmaniasis. Cutaneous leishmaniasis (CL) as most common form of leishmaniasis has an occurrence rate of 0.7 to 1.2 million cases per year (Desjeux, 2004, Kheirandish *et al.*, 2012). The disease normally considered by chronic skin lesions and permanent scars in the infected area (Ezatpour *et al.*, 2015).

At the present, both epidemiological forms of CL are present in Iran; anthroponotic CL (ACL) and zoonotic CL (ZCL) which caused by *Leishmania tropica* and *L. major*, respectively (Mahmoudvand *et al.*, 2014a). Today, there is no safe and useful vaccine existing and chemotherapy is the main method for management of CL (Noazin *et al.*, 2019; Mahmoudvand *et al.*, 2015).

Pentavalent antimonial combinations such as meglumine antimoniate and sodium stibogluconate are first-line antileishmanial agents that have been applied clinically from last decades in the world (Firooz *et al*., 2006). In Iran, the first-choice antileishmanial drug for the treatment of all forms of leishmaniasis is meglumine antimoniate (Glucantime®, MA) (Shirzadi & Gouya 2010). According to the WHO reports, occurrence of the parasite resistance to these drugs is increasing in some parts of the world (WHO, 2010). Currently, it has been demonstrated that primary glucantime-resistant *L. tropica* isolates are now frequent in Iran; whereas the failure of antimonial treatment was particularly reported in 16% of ZCL in Iran (Hadighi *et al*., 2009; Mohebali *et al*., 2007; Mahmoudzadeh-Niknam *et al*., 2012). However, according to the best of our knowledge there is no study on Iranian Glucantim-resistance *L. major* parasites. Therefore, the present study aims to evaluate the correlation between the clinical responses with the drug-susceptibility of parasites in Iranian cutaneous leishmaniasis caused by *L. major* using macrophage model and molecular analysis.

MATERIALS AND METHODS

Ethical statement

This study was reviewed and approved by the Ethics Committees of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

Patients

Leishmania parasites were isolated from the lesions of 93 patients infected with cutaneous leishmaniasis. Patients of between 18 and 60 years of age and residing in Esfahan, Kashan, Shiraz cities (as the main endemic area of ZCL) who were willing to participate were included in the study. Patients were treated according to the physician's and/or the patient's decision either systemically, intralesionally, or, for a small number of patients, with both regimens. No interference with common practice implemented in the treatment strategy was made through this study. Successful treatment was described as complete re-epithelialization of all lesions with no relapse within 3 months of follow up.

Parasite and cell culture

The parasites were collected from the margin of the lesions of patients that revealed a significant level of unresponsiveness to MA among other patients. Lesions were in general ulcerative (58%), and 69% of patients had multiple lesions. Parasites grown in NNN medium and subcultured in RPMI 1640 medium supplemented with 15% fetal calf serum (FCS), 100U/mL penicillin, and 100 µg/mL streptomycin (Gibco/ BRL).

Glucantim susceptibility assay

The murine macrophage cell line (J774-A1) was obtained from Pasteur Institute of Iran (Tehran, Iran). The cells were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS at 37°C in 5% CO_2. The susceptibility of intracellular amastigote to MA was carried out according to the method described elsewhere (Mahmoudvand *et al*., 2014b,c,d; Kheirandish *et al*., 2016). Briefly, before adding the macrophages to the plates, 1 cm^2 cover slips were placed in the wells of 6-chamber slides (Lab-Tek, Nalge Nunc International NY, USA). In the next step, 200 µL of macrophage cells (10^5/mL) were incubated at 37°C in 5% CO_2 for 2 h in DMEM. Then 200 uL of promastigotes (10^6/mL) in stationary phase were added to murine macrophages, so that proportion of *Leishmania*/macrophage was 10:1 and incubated again in a similar condition for 24 h. Free parasites were removed by washing with RPMI 1640 medium and the infected macrophages were treated with 50 µL of various concentrations of MA (2, 4, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70 µg/ mL) at 37°C in 5% CO_2 for 72 h. The selection of doses was obtained based on the preliminary screening tests). At the end, the dried slides were fixed with methanol, stained by Giemsa and observed under a light microscope. Also, the macrophages containing amastigotes without glucantime and those with no parasite and glucantime were considered positive and negative controls, respectively. The susceptibility of

intracellular amastigotes to glucantime was evaluated by counting the number of amastigotes in each macrophage by examining 100 macrophages in comparison with those obtained with positive control (Saedi Dezaki *et al*., 2016). The EC_{50} is defined as the concentration of glucantime that reduces the survival of *Leishmania* parasites by 50% which were measured for all the tested drugs by Probit test in SPSS software.

Identification of *Leishmania* parasites

DNA extraction

DNA extraction was performed on promastigotes obtained from RPMI 1640 medium. Briefly, 6 x 10^6 cultured promastigotes were harvested by centrifugation (2,000 rpm) at 4°C for 10 minutes and washed three times in cold sterile PBS (pH = 7.2). DNA was extracted by High Pure PCR template preparation Kit (Roche, Germany) according to manufacturer's instruction. Finally DNA was resuspended in 200 µL of elution buffer, 1 µL of DNA was used as template in the PCR reaction. Quality and quantity of extracted DNA was analyzed by agarose gel electrophoresis (1%) and spectrophotometry, respectively (Kheirandish *et al*., 2013).

PCR Analysis of the Internal Transcribed Spacer 1 (ITS1)

The samples were analyzed for ITS1 PCR using 400 nM primers: LITSR: 5'-CTGGATCATTTTCCGATG-3' and L5.8S 5'-TGATACCACTTATCGCACTT-3' (Molbiol-Germany). Amplification reactions were performed in volumes of 25 µL. All PCR assays were optimized with regard to annealing temperature, to concentrations of primers, and to cycling protocols. For all experiments, 5 µL of isolated DNA was added to the PCR mixture (the mixture contained 0.2 µL. Taq DNA Polymerase, 0.2 µL dNTPs, 0.2 µL $MgCl_2$, and 1+1 p^{mol}/µL each primer and each individual PCR experiment included at least one positive control*: L. tropica*; *L. major* (1 µL of DNA of standard strain) and one negative control (1 µL of nuclease-free water). The cycling conditions were 94°C, 5 min (1 cycle); 94°C, 30 sec (35 cycles), 49°C, 30 sec (35 cycles); 72°C, 45 sec (35 cycles), 72°C, 5 min (1 cycle). Amplification products were subjected to electrophoresis in 1.5% agarose at 75 V in 1x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) buffer, stained with ethidium bromide (5 µL/100 mL), and visualized and photographed using a UV trans-illuminator (Zarean *et al.*, 2015).

PCR-RFLP

Restriction fragment length polymorphism (RFLP) analysis of the ITS1 amplicons was performed on the ITS1 amplicons, obtained from 2 samples and the reference strain, using the restriction enzyme HaeIII (BsuRI-Fermentase). PCR products (8–15 µL) were digested with Hae III enzyme, according to the manufacturer's instructions. The amplicons of about 300–350 bp were analyzed on 3% agarose gels and the restriction fragments on 4% agarose gels by electrophoresis at 75 V in 1X Tris-acetate-EDTA buffer (0.04 M Tris acetate and 1 mM EDTA, pH 8) and visualized by UV light after being stained with ethidium bromide (0.3 µg/mL). The Gene Ruler DNA ladder Mix (Fermentas, MBI) was used as the DNA molecular marker.

Nested- PCR

External primers CSB1XR (ATTTTTCG CGATTTTCGCAGAACG) and CSB2XF (CGAGTAGCAGAAACTCCCGTTCA) and the internal primers, 13Z (ACTGGGGG TTGGTGTAAAATAG) and LiR (TCGCAG AACGCCCCT) were used to amplify the minicircle variable kDNA. Two *Leishmania* species produced the amplified fragments of about 750 and 560 bp for *L. tropica* and *L. major*, respectively (Zarean *et al.*, 2015, Rasti *et al.*, 2016). Amplification reactions visualized in 1.5% Agarose Gel Electrophoresis, using a 100 bp DNA ladder.

Statistical analysis

Data analysis was carried out by using SPSS statistical package version 17.0 (SPSS Inc., Chicago, IL, USA). Differences between test and control groups were analyzed by *t*-

test. In addition, $p<0.05$ was considered statistically significant.

RESULTS

Patients

From 93 patients with parasitological proven CL and with no previous history of glucantime treatment, we successfully cultivated 79 isolates. We observed that 12.9% of patients (12/93) did not respond to the first course of glucantime treatment. The findings demonstrated a considerable association between the clinical manifestation and susceptibility values.

MA susceptibility assay

The results showed that 67 patients who responded to glucantime treatment were infected with parasites with EC_{50} values of less than 10 µg/mL ($P< 0.001$); whereas the 12 non-responders were infected with parasites that represent higher EC_{50} to glucantime ratios (Table 1). The obtained findings revealed that *Leishmania* isolates from unresponsive patients were significantly (at least three times) less susceptible than *Leishmania* from responsive patients ($P< 0.001$).

Identification of *Leishmania* isolates

PCR product of the Internal Transcribed Spacer1 (ITS1) Region of Genomic DNA samples collected from sensitive and resistant to treatment with Glucantime is shown in Figure 1. The ITS1- PCR was further identified by digestion with the restriction enzyme HaeIII. As result of digestion with HaeIII, ITS1-PCR amplicon yielded 140 and 210 bp fragments which are corresponding with *L. major* patterns (Fig. 2). By Nested-PCR, patterns for both sensitive and resistant samples analyzed presented restriction band (560 bp) (Fig. 3) which is correlated to *L. major*.

Table 1: Relationship between clinical outcome and *in vitro* susceptibility testing in mouse-derived macrophages of *Leishmania* cells isolated from patients with ZCL

Clinical outcome	*In vitro* susceptibility tests EC_{50} (µg/mL)
Healing (n = 67)	<10 (n = 67); mean = 5.12 ± 1.3
Non-healing (n = 12)	10-25 (n = 11); mean = 16.2 ± 2.9 >25 (n = 1); mean = 25.6 ± 3.7

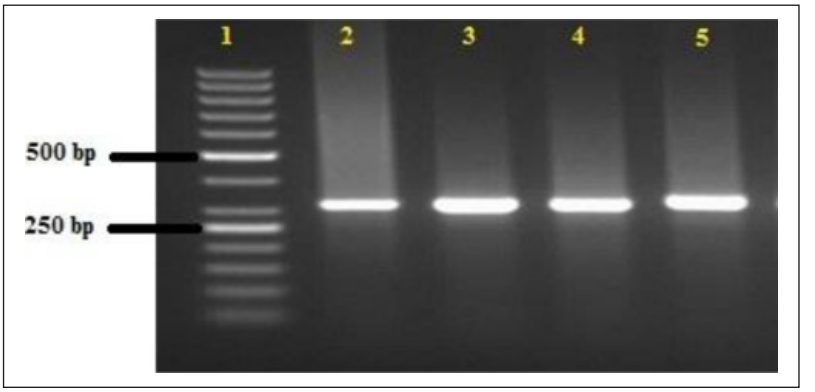

Figure 1. Agarose (1.5%) gel electrophoresis of Internal Transcribed Spacer (ITS 1) PCR with sensitive and resistant isolates and standard strains stained with ethidium bromide. **Lane 1:** DNA size marker 50 bp, **Lane 2:** *L. major* (positive control), **Lane 3:** Antimony sensitive *L. major* isolate, **Lane 4:** *L. tropica* (positive control), **Lane 5:** Antimony resistance *L. major* isolate.

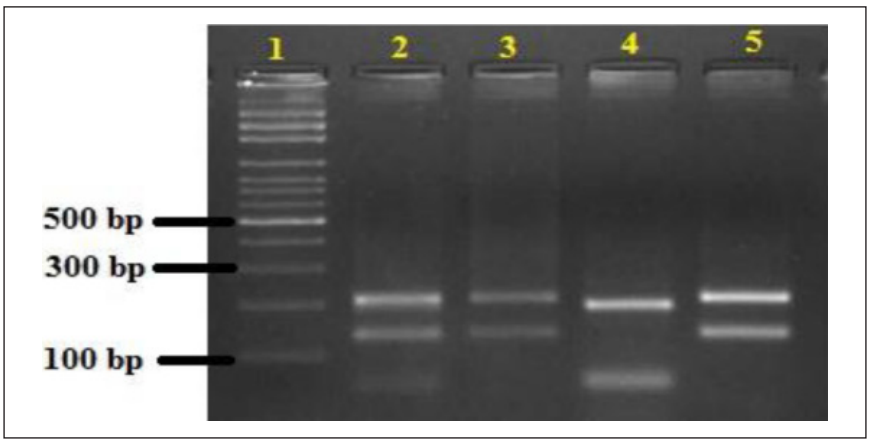

Figure 2. Agarose (3%) gel electrophoresis of Internal Transcribed Spacer (ITS 1) PCR with sensitive and resistant isolates and standard strains after digestion with HaeIII enzyme and stained with ethidium bromide. **Lane 1:** DNA size marker 100 bp, **Lane 2:** *L. major* (positive control), **Lane 3:** Antimony sensitive *L. major* isolate, **Lane 4:** *L. tropica* (positive control), **Lane 5:** Antimony resistance *L. major* isolate.

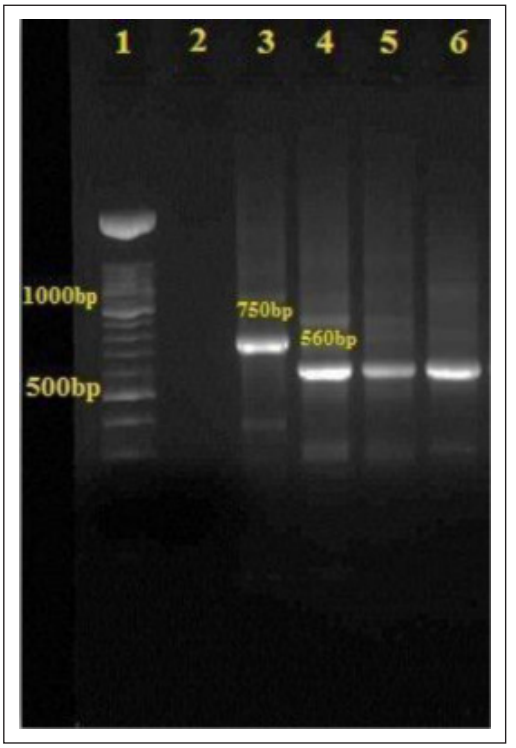

Figure 3. Agarose (1.5%) gel electrophoresis of Internal Transcribed Spacer (ITS 1) PCR with sensitive and resistant isolates and standard strains. **Lane 1:** DNA size marker 100bp, **Lane 2:** Negative control, **Lane 3:** *L. tropica* (positive control standard = 750 bp), **Lane 4:** *L. major* (positive control = 560 bp), **Lanes 5,6:** *L. major* isolates obtained from skin lesions of the patients sensitive and resistant to Glucantime® respectively.

DISCUSSION

Cutaneous leishmaniasis is considered an important public health and social problem in many part of the world particularly in the countries of the Middle East. The control strategy efforts are hampered by diverse ecology of species of sand fly vector and reservoir host. In fact, the variety of clinical and epidemiological forms makes it difficult to use a single measure, globally (Alvar *et al.*, 2012).

There are various drugs available for the treatment of CL. Although using the first-line drugs including pentavalent antimonials and sodium stibogluconate (SSG) exhibited some problems such as prolonged systemic therapy, high toxicity; however they have remained effective and available in endemic countries (Desjeux, 2004; Mahmoudvand *et al.*, 2016a,b). To maintain their effectiveness the drug concentration and duration of therapy have had to increase over the years, indicating that the parasites have been slowly acquiring mutations leading to reduced susceptibility to the drugs.

The ZCL cycle caused by *L. major* is a serious and increasing public health problem in rural areas of the Isfahan and Fars provinces, Iran (Mahmoudzadeh-Niknam *et al.*, 2012). Epidemiological evidences demonstrated that an increasing proportion

of patients with ZCL are failing glucantime therapy. To evaluate whether resistant isolates from patients of these provinces, we measured the EC_{50} of 93 isolates derived from patients before initiation of treatment. The intracellular amastigote model in a macrophage cell line is appropriate for evaluating the activity of anti-*Leishmania* drugs and identification of the resistant parasites can lead to the treatment failure. This approach was used to study the susceptibility of *L. major* isolates to glucantime. However, the susceptibility assay for promastigote forms of *Leishmania* could not be used to predict clinical outcome (Zarean *et al.*, 2015).

Our findings revealed that a significant association between the clinical outcome and the susceptibility levels of parasites. Moreover, the isolated parasites from non-healing patients were characterized using PCR-RFLP and Nested-PCR and were identified to be closely related to parasites isolated from susceptible patients. Previously, Hadighi *et al.* (2006) have demonstrated the first report of proven resistant parasites contributing to treatment failure for CL and shows that primary glucantime -resistant *L. tropica* field isolates are now frequent in Iran.

Based on susceptibility data obtained in the present study, there are two possible resistance forms, one leading to an intermediate level of resistance and the other leading to a high level of resistance. Studies have shown that failure in glucantime treatment in *Leishmania* has been assigned to various factors other than resistant parasites, such as immunological status of host, suboptimal treatment, and pharmacokinetic properties; however but it is now clear that an increase in resistance can lead to treatment failure for CL (Croft 2001, Sundar *et al.*, 2001). On the most likely mechanisms of resistance is inhibiting glutathione biosynthesis, a backbone of trypanothione, which previously proven can lead to antimony resistance reversal *in vitro* and *in vivo* (Carter *et al.*, 2003). Furthermore, there are some genes whose expressions were significantly changed in glucantime-resistant *Leishmania* isolates might be implicated in the natural antimony resistance compared to the sensitive isolate genes (Reynolds *et al.*, 2006). However, future studies are require to clear a precise understanding of the resistance mechanisms in field isolates, which should

CONCLUSION

The obtained findings in the present study demonstrated that similar to MA-resistance *L. tropica* isolates, glucantime -resistant *L. major* field isolates are now frequent in Iran. This investigation suggests a strategy of using drug combinations to reverse drug resistance in the eld. Such studies lead to find strategies for rapidly diagnosing resistance in order to improve the clinical management of CL.

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