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## Practical Approach for Typing Strains of *Leishmania infantum* by Enzyme Polymorphism: A Cross Sectional Study in Northwest of Iran

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**Abstract:** In present study, All samples collected from Kalybar and Ahar districts in Northwest of Iran from 12 patients (bone marrow aspirates), 26 dogs (splenic and hepatic aspirates) and more than 100 sand flies between years 2004-2006. All patients were clinically diagnosed to have visceral leishmaniasis. Serological profiles of all sera samples from both human and dogs were in accordance with leishmaniasis (DAT). Isoenzyme profiles of these isolates were compared with those of reference using 12 enzyme systems. *L. infantum* MON-1 is the only zymodeme present in all samples of dogs, sand flies and human. The enzymatic polymorphism is compared to that of neighboring countries (Azarbaijan, Iraq and Turkey etc.) and we concluded that the Visceral Leishmaniasis (VL) focus in northwest of Iran is evidently Mediterranean focus of zoonotic VL, which extends from Portugal and Morocco to Pakistan and the central Asian republics. Domestic dogs act as the reservoir host, where *Phlebotomus kandellakii* and *Perfiliewi ariasi* are vectors.

**Key words:** *Visceral leishmaniasis*, strain, enzyme electrophoresis, Northwest Iran

### INTRODUCTION

Protozoan parasites of the genus *Leishmania* cause a spectrum of diseases, ranging from self-limiting, self-curing Cutaneous Leishmaniasis (CL) to disseminating, fatal Visceral Leishmaniasis (VL) and they infect various mammalian hosts. *Leishmania infantum* may cause either simple CL (Rioux and Lanotte, 1990; Belhadj *et al.*, 2003), debilitating visceral leishmaniasis, or asymptomatic cases. *Leishmania* species are morphologically very similar and species identification is possible using standard biochemical methods (lectin agglutination, isoenzyme analysis, analysis of kDNA restriction fragment using different restriction endonuclease, etc.) (Sampali *et al.*, 1990). Frequently, *Leishmania* species are identified based on their geographical distribution and on clinical manifestations of the resulting disease. Currently, the universally accepted standard procedure for characterizing and identifying strains of *Leishmania* is isoenzyme analysis (Cupolillo *et al.*, 1994; Rioux *et al.*, 1985; Tibayrenc, 1979). However, this is performed only in a few laboratories and is depending on the number of enzymes examined, very labor intensive and time consuming. Unlike some species of *Leishmania*, e.g., *Leishmania tropica* and *Leishmania major*, which exhibit extensive enzymatic polymorphism (Rioux *et al.*, 1990),

the most of *L. infantum* strains isolated in Mediterranean foci belong to the sole predominant zymodeme MON-1, despite their very wide geographical distribution. (Pratlong *et al.*, 1995; Minodier *et al.*, 1997; Gallego *et al.*, 2001). The Zoonotic Visceral *leishmaniasis* (ZVL) in Iran is sporadic in almost all part of Iran and only three endemic regions were identified in Iran (provinces of Ardabil, East Azarbaijan and Fars) (Gavvani *et al.*, 2002). In the endemic region of northwest over 50% of all VL patients have been children below 2 years old and 80% have been children under 12 years (Edrissian, 1996).

As different *Leishmania* species are known to cause different clinical symptoms and may require different treatment protocols therefore identification of different species of *Leishmania* is necessary in each endemic site for providing best methods for treatment and prevention. This study carried out to characterize leishmania parasites isolated from VL patients, the reservoir host (starry and domestic dogs) and vectors, in VL endemic region of the northwest of Iran.

### MATERIALS AND METHODS

**Design:** In this cross sectional study, between 2004 and 2006 years, *Leishmania* parasites were obtained from 12

humans, 26 dogs and exceeds of sand flies from endemic area of Ahar and Kaleibar. Twelve bone marrow aspirates were obtained from patients with clinical and serological indications of VL. Patients aged were 1-30 years (3 under 1 years, 8 2-4 years, 1 exceeds of 20 years). All patients were VL endemic area habitants (Ahar and Kaleibar districts). The aspirate were examined directly for parasites to confirm infection. Nine cases had been previously diagnosed as Leishmaniasis by the detection of amastigotes in stained bone marrow smears. Domestic and starry dogs detected by clinical signs, DAT and parasitologically via microscopic examination of giemsa-stained spleen and liver aspirates. The female blood feed sand flies were directly cultivated in culture medium.

**Cultivation of parasites:** The aspirated biological materials from human and animals (12 human and 26 dogs and sand flies) were oculted into sloppy Evans culture medium (Evans *et al.*, 1984) at 24°C for growth and isolation of Leishmania parasites. Impression smears were made for direct microscopic observation after fixation and staining. Due to the non-aseptic conditions of isolation in the field, despite the addition of antifungal (100 mg mL<sup>-1</sup> 5-fluorocytosin) and anti-bacterial agents (50 µg mL<sup>-1</sup> gentamycin) to the media, fungal and bacterial contamination of the cultures led to only 21 out of 26 microscopically confirmed infected dogs yielding positive culture. Hence, only 21 isolates were obtained from dogs cultures were examined for at least 6 weeks before being discarded if negative. Only three sand flies out of all of them were grown in NNN media. The resultant cultures were maintained by serial passage (maximum 4 passages) in sloppy medium. Cultures were grown in the laboratory in London and were cryopreserved by standard procedures (Evans *et al.*, 1989) in liquid nitrogen for subsequent isoenzyme characterization. The samples were interred into the World Health Organization International Reference center Cryo-bank which is maintained by the Department of Infectious and Tropical Disease, London School of Hygiene and Tropical Medicine.

**Bulk cultivation of promastigotes:** Frozen stabilities of the 36 isolate Leishmania parasites were thawed and cultured in NNN medium and transferred to commercially available Minimum Essential Medium α MEM modification (catalogue No M 0644 Sigma), with ribonucleosides, Deoxy ribonucleosides L-glutamine at 24°C. The α MEM medium was supplemented with 548 mg L-glutamine, 3 g D-glucose, 5 mg folic acid, 2 mg D-biotin, 11 g of sodium bicarbonate 50 µg mL<sup>-1</sup> and gentamycin to minimize bacterial contamination (Evans *et al.*, 1984). The pH of the medium was adjusted to 7.5 and 10% heat inactivated fetal calf serum (56°C for 30 min) was added prior to use.

**Extraction of soluble enzymes:** Soluble enzymes were extracts prepared for electrophoresis by standard methods. Promastigotes were harvested at the end of logarithmic phase of the growth and the number of organisms adjusted to 1-1.5×10<sup>7</sup> mL<sup>-1</sup>. 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 by re-suspension and re-centrifugation in cold-proline balanced salt solution. Then these re-suspended in an enzyme stabilizing solution 2 mm Dithiothritol (DTT), 2mm E-aminocaproic Acid (ACA) and 2 mm EDTA (PH 7.0). The organisms were crashed by three cycles of freezing and thawing in liquid nitrogen and after high-speed centrifugation (>13000 g), 15 µg beads were prepared from the supernatant by dropping into liquid nitrogen. The beads then stored in liquid nitrogen. Thin-layer and starch gel electrophoresis methods was carried out as described by Evans *et al.* (1989). The enzyme profiles of the new isolates were compared with WHO reference strains.

**Enzyme electrophoresis:** Analysis 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. The enzymes studied were Alanine Amiono Transferase (ALAT) EC.2.6.1.2, Aspartate Amino Transferase (ASAT) EC.2.6.1.1, Superoxide Dismutase (SOD) EC.1.15.1.1, estrase (ES) EC.3.1.1.1, nucleoside hydrolase (NH) EC.3.2.2.2, mannose phosphate isomerase (MPI) EC.5.3.1.8, Glucose Phosphate Isomerase (GPI) EC.5.3.1.9, malate dehydrogenase (MDH), EC 1.1.1.37 6-phosphogluconate dehydrogenase (6PGD) EC1.1.1.44), phosphoglucomotase (PGM) EC.2.7.5.1, prolineimino peptidase (PEP-D) EC.3.4.11.5 and Pyruvate Kinase (PK) EC.2.7.1.40.

## RESULTS

Direct Agglutination Test (DAT) was positive in all of the cases examined (dogs and human) excepted sand flies. The isoenzyme profiles of the Leishmania organisms isolated from the bone marrow of the twelve patients with kala-azar, twenty one isolates from dogs and three isolates of sand flies were all indistinguishable from the World Health Organization reference strain of *L. infantum* (MHOM/IN/80/IPTI) zymodeme LON49. They were clearly distinguishable from the profiles of *L. donovani* (MHOM/TN/80/DDT) zymodeme LON41, *L. major* (MHOM/SU/73/5ASKH) zymodem LON1 and *L. tropica* (MHOM/SU74/K27) zymodem LON12. The reference

strains and examined isolated Leishmania strains from Iran are shown in Table 1. All 36 strains of leishmania isolated from human VL patients, dogs and sand flies are shown in Table 2 in north west of Iran were therefore identified as *L. infantum* zymodeme LON49. Various enzyme banding pattern of NH are presented in Fig. 1.

Table 1: WHO reference *Leishmania* species used for isoenzyme characterization

Code	Clinical condition	Species	Zymodeme
MHOM/TN/80/IPT1	VL	<i>L. infantum</i>	LON-49
MHOM/IN/80/DD8	VL	<i>L. donovani</i>	LON-41
MCAN/IT/76/ DORA	VL	<i>L. donovani</i>	LON-50
MHOM/ET/67/ LV9	VL	<i>L. donovani</i>	LON-46
MHOM/SU/74/K27	ACL	<i>L. tropica</i>	LON-12
MHOM/SU/73/5ASKH	ZCL	<i>L. major</i>	LON-1

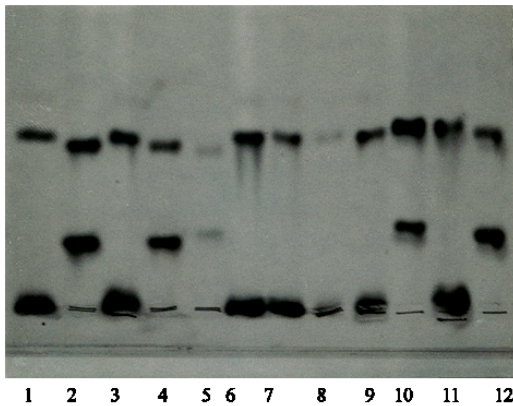


Fig. 1: Photograph of isoenzyme characterisation of VL isolates on Thin Layer Starch Gel Electrophoresis after staining for NH (a)

- 1- MHOM/TN/80/IPT1
- 2- MHOM/SU/74/K27
- 3- MHOM/IN/80/DD8
- 4- MHOM/SU/73/5ASKH
- 5- MHOM/IR/95/HajL1
- 6- MHOM/IR/95/Maz-6
- 7- MHOM/IR/95/Maz-9
- 8- MHOM/IR/96/Maz-10
- 9- MCAN/IR/96/Maz-14
- 10- MHOM/IR/96/YaghibiL2
- 11- MCAN/IR/96/Maz-15
- 12- MHOM/IR/95/Edris

No. 1, 2, 3 and 4 are reference strains  
 No. 6, 7 and 8 are isolated from bone marrow of patients with VL disease  
 No. 9 and 11 are isolated from spleen or liver of dogs with VL disease  
 No. 5, 10 and 12 are isolated from CL cases from Iranian patients

Table 2: *Leishmania* species and strains isolated from human, dogs and sand flies in northwest of Iran (2004-2006)

Location	No. tested	No. Were grown in NNN	<i>Leishmania</i> species identified	<i>Leishmania</i> strains identified
<b>Ahar</b>				
Human	8	8	<i>L. infantum</i>	LON49
Stary dogs	5	3	<i>L. infantum</i>	LON49
Domestic dogs	9	8	<i>L. infantum</i>	LON49
Sand flies	>100	2	<i>L. infantum</i>	LON49
<b>Kaleibar</b>				
Human	4	4	<i>L. infantum</i>	LON49
Stary dogs	4	4	<i>L. infantum</i>	LON49
Domestic dogs	8	6	<i>L. infantum</i>	LON49
Sand flies	>100	1	<i>L. infantum</i>	LON49

## DISCUSSION

We have developed an isoenzyme analysis method that was able to characterize and distinguish closely related strains of *L. infantum* collected in Ahar and Kalybar in northwest of Iran. The usefulness of isoenzyme analysis, which is still the gold standard, for epidemiological and taxonomic studies (Shamsuzzaman *et al.*, 2000) has been widely proven. This study included parasites from all of the hosts, reservoirs and vectors. Between 2004 and 2006, thirty six strains from humans, dogs and sand flies were studied by isoenzyme analysis. The enzymatic polymorphism is compared to that of neighboring countries (Azarbaijan, Iraq and Turkey) and we concluded that the VL focus in northwest of Iran is evidently Mediterranean focus of zoonotic VL, which extends from Portugal and Morocco to Pakistan and the central Asian republics. The same zymodeme of *L. infantum* (MON-1~LON49) which was identified in human VL case in northwest of Iran has been found in human VL patients from, Iraq (Aljeboori and Evans, 1980), Israel (Ya'ari *et al.*, 2004), Turkey (Sakru *et al.*, 2007), Central Asia e.g., Azarbijan republic (Tagi-zad *et al.*, 1989), Mediterranean region (Gramiccia *et al.*, 1989), Greece (Maltezou *et al.*, 2000), Portugal (Abranches *et al.*, 1993; Campino *et al.*, 2006), Georgia (Bardzhadze and Safianova, 1999). In one study from the south part of Iran *Leishmania tropica* was distinguished as a causative agent of VL (Alborzi *et al.*, 2006). All strains had been preserved in the International Leishmania Cryo-bank of London. The canine strains were obtained during epidemiological investigations carried out in the valleys where the human cases had been detected the domestic dog is incontestably the reservoir host of human VL due to zymodeme MON-1 (Gavani *et al.*, 2002; Mohebbali *et al.*, 2001). A review of the literature for the years 1970 to 2007 showed that VL is not only sole predominant but also zoonotic in this foci (Nadim and Faghih, 1968; Nadim and Seyedi Rashti, 1971; Ardehali *et al.*, 1980). Entomological surveys carried

out in this focus led to the demonstration of *Phlebotomus kandellakii* and *Perfiliewi ariasi* as vectors of *L. infantum* MON-1.

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