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Cutaneous and post kala-azar dermal leishmaniasis caused by *Leishmania infantum* in endemic areas of visceral leishmaniasis, northwestern Iran 2002–2011: a case series

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Visceral leishmaniasis (VL) is endemic in Northwest and southern Iran. Reports of cutaneous leishmaniasis (CL) in Northwest areas are rare, and its etiological agents are unknown. In the current study, we report six CL and two post kala-azar dermal leishmaniasis (PKDL) cases caused by *Leishmania infantum* from endemic areas of VL in the Northwest. Smears were made from skin lesions of 30 suspected patients in 2002–2011, and CL was determined by microscopy or culture. *Leishmania* spp. were identified by nested-PCR assay. The disease was confirmed in 20 out of 30 (66%) suspected patients by parasitological examinations. *L. infantum* was identified in eight and *Leishmania major* in 12 CL cases by nested-PCR. Cutaneous leishmaniasis patients infected with *L. major* had the history of travel to CL endemic areas. *L. infantum* antibodies were detected by direct agglutination test (DAT) at titers of 1:3200 in two cases with history of VL. Results of this study indicated that *L. infantum* is a causative agent of CL as well as PKDL in the VL endemic areas.

Keywords: Cutaneous leishmaniasis, Visceral leishmaniasis, *Leishmania infantum*, Post kala-azar dermal leishmaniasis, Nested-PCR, Iran

Introduction

Leishmaniasis occurs in 98 countries, varying considerably in severity and importance.¹ Reports indicate 350–400 million people at risk and about 12 million victims worldwide.^{1,2} Leishmaniasis has been clinically classified into at least three primary categories including visceral (VL), cutaneous (CL), and mucocutaneous forms.^{2,3}

Iran is an important focus of CL and VL in the Middle East.^{2,4} Rarely, mucosal and lymphatic leishmaniasis have also been reported in the country. Cutaneous leishmaniasis caused by *Leishmania major* and *Leishmania tropica* is a major health problem in Iran with incidence increasing in recent years.^{4,5} *Leishmania infantum* infections have been reported in canines,^{6,7} humans,⁸ *Phlebotomus*

kandelakii,⁹ *Phlebotomus major*,¹⁰ and *P. perfiliewi transcaucasicus*^{11,12} but rarely in rodents.¹³ In recent years, cases of skin lesions similar to those in CL have been reported in northwestern (Ardabil and East Azerbaijan provinces) and southern parts (Fars province) of Iran, and the etiological agents are being sought.^{14,15} This study was conducted to determine parasitological, serological, and molecular aspects of CL in endemic areas of VL in Iran.

Materials and Methods

Sampling and patients

From April 2002 to August 2011, samples were collected from 30 suspected CL patients from Ardabil and East Azerbaijan provinces in Northwest Iran. The samples were collected by trained health workers from all suspected CL patients who had skin lesions for at least a duration of 2 weeks. Smears were prepared from the margins of scraped lesions. Fine-needle aspiration was performed with a sterile syringe

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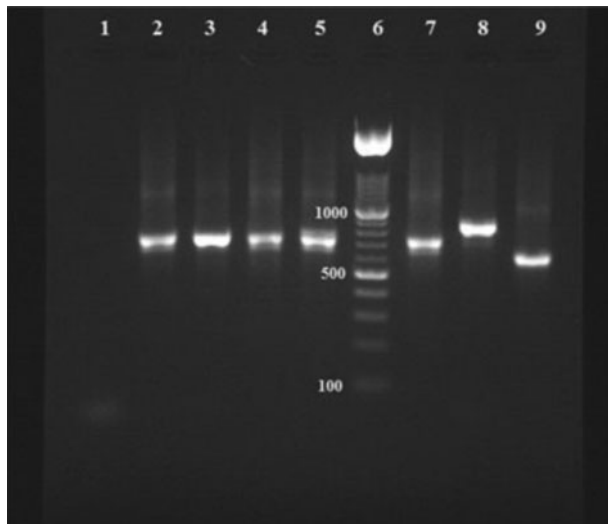


Figure 1 Nested-PCR-based amplification of kDNA extracted from Giemsa-stained lesion smears; lane 1: negative control; lanes 2, 3, 4, and 5: positive samples of CL patients due to *Leishmania infantum*; lane 6: 100–1500 bp molecular-weight ladder (Roche); lanes 7, 8, and 9: positive control of *L. infantum* (680 bp); *L. tropica* (750 bp); and *Leishmania major* (560 bp), respectively.

with saline (0.1 ml) and a 26-gauge needle. The needle was inserted into the outer border of the lesion, then saline was injected and the tissue fluid was aspirated back into the needle. Then the materials were air dried and fixed with 100% methanol prior, and stained with 10% Giemsa. Examinations (culture and microscopy) were used to confirm CL. Amastigote forms of *Leishmania* spp. were determined under light microscopy at high magnification (1000×). Fluid materials from skin lesions was cultured in NNN and RMP11640.¹⁶ Only one case was positive in culture and the other cases (19) were microscopically positive. This study was reviewed and approved by the ethical committee of the Center of Diseases Control (CDC), health deputy, Ministry of Health and Medical Education, Islamic Republic of Iran.

Serology

Finger prick blood samples (~50 ul) were taken by sterile lancets, and sera were separated immediately by centrifugation. The titer of anti-*L. infantum* antibodies were detected by the direct agglutination test (DAT).^{8,17,18}

Molecular study

Smears wiped off with the xylol and paper tissue were then scraped with a sterile scalpel and the entire DNA in the smear was extracted by digestion, in a 1.5 ml micro tube with 200 µl lysis buffer. DNA was extracted by standard protocols with a DNA extraction and purification kit (Qiagen, Germany).^{19–21} The DNA samples were stored at 4°C. Nested-PCR was conducted on the 20 confirmed CL cases, following the method described by Ghasemian *et al.*,²⁰ using



Figure 2 Post kala-azar dermal leishmaniasis (PKDL) due to *Leishmania infantum*, DAT positive and history of visceral leishmaniasis (VL) from Ardabil province.

two pairs of special primers of kDNA (external and internal primers). The primers CSB1XR and CSB2XF for the first round, and LiR and 13Z for the second, were used to amplify the variable segments on minicircles of kDNA from *Leishmania* parasites.

After PCR amplification, amplicons (PCR products) of the second round were analyzed on 2% (w/w) agarose gel under UV light. DNA extracted from promastigote cultures of reference strains of *L. infantum* (MCAN/IR/07/Moheb-gh.), *L. major* (MHOM/IR/75/ER), and *L. tropica* (MHOM/IR/02/Mash10) were run on each gel as positive controls. Negative controls (the products of PCR in which ultrapure water replaced the template DNA) were also run. The size of each amplicon detected was estimated by comparison with a 100–1500 bp molecular-weight ladder (Roche) run on the same gel (Fig. 1).^{19–21}

Results

Twenty of the 30 (66%) samples were positive for *Leishmania* spp. The positive smears were examined by nested-PCR, and *L. infantum* was identified as the causative agent in eight children aged ≤5 years (Table 1). *Leishmania major* was indicated as the agent in the remaining 12 patients. Cases with *L. major* had a history of travel to endemic regions of zoonotic CL in Iran. Post kala-azar dermal leishmaniasis (PKDL) cases caused by *L. infantum* were identified in two boys lesser than 5 years of age with a history of VL (~1.5 years ago) who showed anti-*L. infantum* antibodies at titers of 1:3200.

Their skin lesions caused by *L. infantum* were single, relatively ulcerative, occurred on the face, and persisted for about 1 year (Fig. 2). On average, the

Table 1 Characteristics of patients with *Leishmania infantum* infections

| Patient | Age (year) | Sex | History of visceral leishmaniasis (VL) | No. of lesions | Location of lesions | Type of lesions | Treatment of Lesions* | Cured/failure | Diagnosis | | |
|---------|------------|--------|--|----------------|---------------------|-----------------|-----------------------|---------------|----------------|----------|--------------------|
| | | | | | | | | | Parasitology** | Serology | Nested-PCR |
| 1 | 2 | Male | Negative | 1 | Face | Nodule | Yes | Cured | 1+ | Negative | <i>L. infantum</i> |
| 2 | 1 | Female | Negative | 1 | Face | Ulcer | Yes | Cured | 4+ | Negative | <i>L. infantum</i> |
| 3 | 4 | Male | Positive | 1 | Face | Ulcer | Yes | Cured | 2+ | 1:3200 | <i>L. infantum</i> |
| 4 | 4 | Female | Positive | 1 | Face | Ulcer | Yes | Cured | 4+ | 1:3200 | <i>L. infantum</i> |
| 5 | 5 | Male | Negative | 1 | Face | Ulcer | Yes | Cured | 2+ | Negative | <i>L. infantum</i> |
| 6 | 3 | Male | Negative | 1 | Face | Ulcer | Yes | Cured | 2+ | Negative | <i>L. infantum</i> |
| 7 | 4 | Female | Negative | 1 | Face | Ulcer | Yes | Cured | 3+ | Negative | <i>L. infantum</i> |
| 8 | 1 | Male | Negative | 1 | Face | Ulcer | Yes | Cured | 2+ | Negative | <i>L. infantum</i> |

* Treatment with meglumine antimoniate (Glucantime®) drug (20 mg/kg for 14–28 days IM and/or intra lesions).

**Parasitemia grade.

Bold indicates innovations.

amastigote forms of *L. infantum* were smaller than *L. major*, usually less than 3 µm and they were not vacuolated. Both cases of PKDL were successfully treated by meglumine antimoniate (Glucantime®, Rorer Rhone-Poulenc Specia, Paris, France) administered intramuscularly at 20 mg/kg body weight daily for 28 days.

Discussion

Leishmaniasis is an emerging disease in immunosuppressed patients and travelers with a wide clinical spectrum. Clinical forms of leishmaniasis in developed and non-endemic countries have increased sharply over the past decade.¹ This can be associated with increasing international tourism, military operations, and immigration from endemic countries.¹ Post kala-azar dermal leishmaniasis due to *Leishmania donovani* is prevalent in India and the Sudan, while PKDL caused by *L. infantum* is rare, with few reported cases. Dereure *et al.* identified *L. infantum* from a PKDL case,²² and an additional report of PKDL caused by *L. infantum* occurring 13 months after a diagnosis of VL was confirmed by molecular methods in an AIDS patient.²³ Stark *et al.* reported the first case of PKDL due to *L. infantum* in a human immunodeficiency virus type 1-infected patient in Australia.²⁴

From 2002–2011, we confirmed eight CL cases caused by *L. infantum*, two of which had a history of VL. Previously, three cases of PKDL of unknown causative agent were reported from Northwest Iran by Mohebbali *et al.*¹⁴ Five cases of PKDL of unknown etiology have been reported from Shiraz City in southern Iran where VL is endemic.¹⁵ All the PKDL cases due to *L. infantum* in the present study showed a history of VL 5 years earlier and were DAT positive. Post kala-azar dermal leishmaniasis caused by *L. donovani* in India and Sudan have been reported in at least 10–15% of VL cases.² Ulcerative lesions are rare in Indian PKDL, because the lesions are usually closed and present as macular or popular, and nodular shapes,²⁵ while skin lesions in the

present study were usually open, restricted to the face, and of nearly a year duration.

Leishmania infantum has been previously reported as a causative agent of CL in the Middle East. Most cases of CL in Tunisia are associated with *L. infantum* named sporadic cutaneous leishmaniasis and CL caused by *L. infantum* was reported from Italy.^{26–28}

Our findings indicate that *L. infantum* is a causative agent of CL and PKDL in VL endemic areas of Iran. International travels to Mediterranean countries are suggested as a risk factor for leishmaniasis. Leishmaniasis should be considered in patients presenting with a clinical symptoms consistent with the disease and a history of travel to an endemic area, even if the travel was several months or years earlier.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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