

## Mixed Mucosal Leishmaniasis Infection Caused by *Leishmania tropica* and *Leishmania major*

Sadegh Shirian,<sup>a</sup> Ahmad Oryan,<sup>a</sup> Gholam Reza Hatam,<sup>b</sup> and Yahya Daneshbod<sup>c</sup>

Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran<sup>a</sup>; Basic Sciences in Infectious Diseases, Research Center, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran<sup>b</sup>; and Department of Cytopathology, Dr. Daneshbod Pathology Laboratory, Shiraz, Iran<sup>c</sup>

Mixed infections with different *Leishmania* species could explain differences in the clinical courses of these infections. On identification of *Leishmania* parasites from Iranian patients with mucosal leishmaniasis (ML), a patient with both oral and nasal lesions was found to be concomitantly infected with *Leishmania tropica* and *L. major*. Mixed infection was identified by PCR amplification of *Leishmania* kinetoplast DNA on scraping of cytological smears and histopathological sections. *L. major* and *L. tropica* were isolated from the nasal and oral lesions, respectively. These species were also confirmed by immunohistochemistry. This seems to be the first reported case of concurrent ML infection with two *Leishmania* species. It indicates that, at least in this patient, previous infection with one of these *Leishmania* species did not protect against infection with the other. This result has important implications for the development of vaccines against leishmaniasis and implies careful attention in the treatment of this infectious disease.

### CASE REPORT

A 34-year-old immunocompetent male patient presented with lesions of the mucous membranes of the nose and mouth. The patient was from Fars Province, southern Iran. He presented with a 7- and 5-month history of intranasal and oral lesions, respectively. No scar or other lesion was found in other parts of the body. On examination, there were multiple tiny erythematous lesions, varying in size from 0.1 to 0.3 cm in diameter. The nasal pyramid was edematous, and bloody crusts were observed on the inferior conchae, septum, and floor of the nasal fossa. The nasal lesions were located in the intranasal portion in the mucous membrane over the turbinates, far from the cutaneous lesions. Clinically, diffuse yellowish white erosions with grayish fibrinous membranes were seen on a reddish edematous background on the involved oral mucosa (Fig. 1).

His blood biochemistry and complete blood count were within the reference range. His hemoglobin level was 13.6 g/dl, his total leukocyte count was 6,600/mm<sup>3</sup>, his serum creatinine level was 0.8 mg/dl, and his blood urea nitrogen level was 18 mg/dl. Serological studies for human immunodeficiency virus and hepatitis B and C viruses were negative.

Tissue samples from the lesions were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at a 5- $\mu$ m thickness, and stained with hematoxylin and eosin.

Cytologic smears were prepared by scraping of the oral lesions with a scalpel. In addition, exfoliative cytology from the nasal lesions was performed by washing the nasal cavity as previously noted (7). Multiple smears were made on slides and were both air dried and alcohol fixed and then stained by the Wright method. Review of the cytologic smears and histologic sections was conducted blindly by three pathologists. Microscopic examination showed the amastigote forms of *Leishmania* (Fig. 2A and B).

The antibodies IS2-2B4 (A11; specific for *L. tropica*) and XLVI-5B8-B3 (T1; specific for *L. major*) were kindly provided by the Special Programme for Research and Training in Tropical Disease, WHO, and used as primary monoclonal antibodies (MAbs).

Sections 3  $\mu$ m thick were used for immunohistochemical



FIG 1 Diffuse erosions with pseudomembranes on edematous background on oral mucosa are visible.

(IHC) analysis. The slides were deparaffinized in xylol, rehydrated, and treated with 3% hydrogen peroxide solution for 10 min at room temperature to quench endogenous peroxidases. Antigen retrieval was conducted by microwave pretreatment (a power level of 100 for 10 min and then a power level of 20 for 20 min) using a 10-mmol/liter concentration of citrate buffer (pH 6.0). The primary antibody was applied for 1 h (diluted 1:200). Detection of the immunoreaction was achieved. The detection

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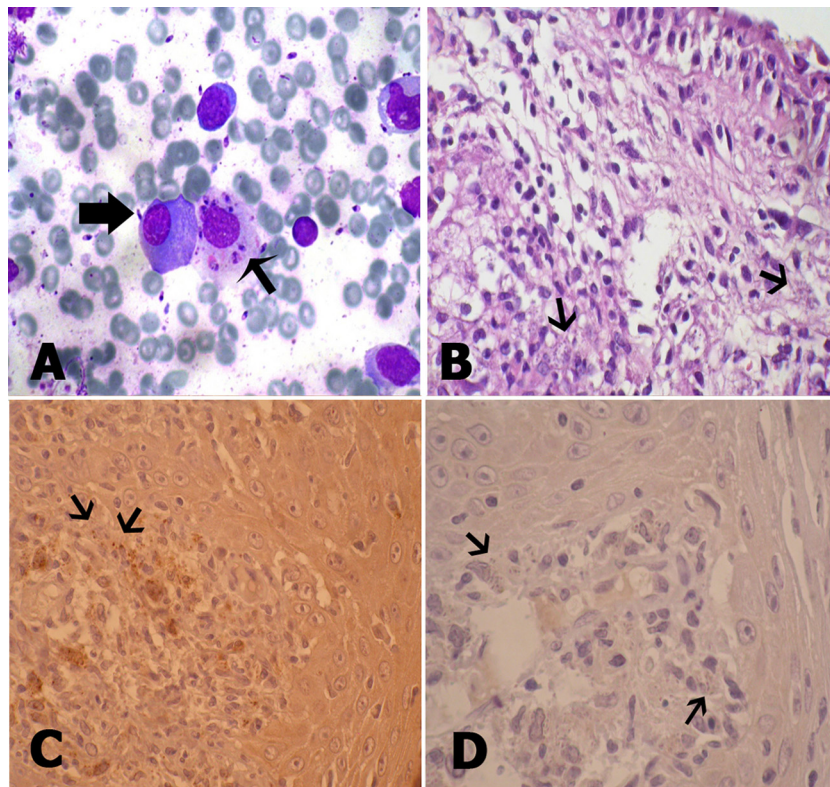
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Address correspondence to Sadegh Shirian, shirian@shirazu.ac.ir.

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**FIG 2** (A) Plasma cell (thick arrow) and macrophage loaded with *Leishmania* bodies associated with free *Leishmania* bodies (thin arrow) (Wright stain; magnification,  $\times 200$ ). (B) Massive infiltration of macrophages loaded with *Leishmania* bodies in the submucosal area of the respiratory epithelium (arrows) (hematoxylin and eosin staining; magnification,  $\times 200$ ). (C) Oral lesions showing immunoreactivity to *L. tropica*-specific MAb (arrows) (IHC; magnification,  $\times 100$ ). (D) Negative IHC staining of oral lesions for *L. major* (arrows) (magnification,  $\times 100$ ).

system used was Envision+ (DakoCytomation), and development was done with diaminobenzidine (DakoCytomation). 3,3'-Diaminobenzidine–hydrogen peroxide was used as the chromogen, and hematoxylin was used as the counterstain. The nasal lesions showed immunoreactivity to the *L. major*-specific MAb, and the oral lesions showed immunoreactivity to the *L. tropica*-specific MAb (Fig. 2C), while IHC staining of the nasal lesions with the *L. tropica* MAb and IHC staining of the oral lesions for *L. major* were negative (Fig. 2D).

To identify the *Leishmania*-specific DNA, the entire smear was scraped off the slide with a sterile scalpel and the phenol-chloroform-isoamyl alcohol extraction method was used as previously described to extract the DNAs (23). The DNA samples were dissolved in 50  $\mu$ l of deionized distilled water and stored at 4°C. Variable segments of the minicircles of the kinetoplast DNA from the *Leishmania* species present in the smear scrapings were amplified by two nested PCR rounds. The primers for the first round were CSB1XR (ATT TTT CGC GAT TTT CGC AGA ACG) and CSB2XF (CGA GTA GCA GAA ACT CCC GTT CA), and those used for the second round were LiR (TCG CAG AAC GCC CCT) and 13Z (ACT GGG GGT TGG TGT AAA ATAG) (23). The products of the second-round PCR were loaded onto a 1.5% agarose gel. As positive controls, the DNA extracted from promastigote cultures of the reference strains of *L. infantum* (MCAN/IR/97/LON490) were run on each gel. Extravasation cysts (oral mucocelles) from 10 patients were used as negative controls. Negative controls in which ultrapure water replaced the template DNA

were also run. A 560-bp fragment of *L. major*-specific kinetoplast minicircle DNA was amplified from the nasal lesions by the second-round PCR assay, whereas a 750-bp fragment of *L. tropica*-specific kinetoplast minicircle DNA was amplified from the oral lesions by the second-round PCR assay (Fig. 3).

The patient was treated by intravenous infusion of amphotericin B at 1 mg/kg/day for 14 days, and resolution of the lesions started 1 week after the treatment started.

**Ethics statement.** The Ethics Committee of the Faculty of Medical School, University of Shiraz Medical Sciences and the Institutional Review Board of the Dr. Daneshbod Laboratory approved this study, and we obtained written informed consent from the patient.

Mucosal leishmaniasis (ML) is a rare disease in the world, even in areas where it is endemic, such as Iran (6, 25). The importance of ML is due to the severity of its clinical lesions, poor response to traditional antimony therapy, and destruction of the nasal architecture with gross facial alterations (17). ML is a form of tegumentary leishmaniasis that has been shown to be associated with *L. braziliensis* and *L. panamensis* and less frequently with *L. amazonensis*, although it has been reported in infections caused by other New-World *Leishmania* species, such as *L. guyanensis* (13). A few patients in the Old World with ML infections caused by *L. infantum*, *L. tropica*, and *L. major* have been described (16, 18, 21,



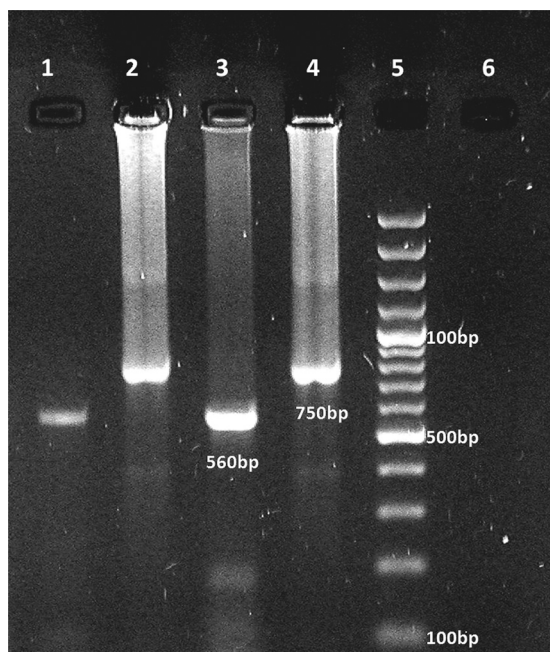


FIG 3 Electrophoresis of products of nested-PCR-based amplification of DNA extracted from stained smears. The six lanes contained the products from reference strains of *L. tropica* (lane 4) and *L. major* (lane 3), a negative-control test sample (lane 6), oral and nasal lesions due to *L. tropica* and *L. major* (lanes 1 and 2, respectively), and a molecular size ladder (lane 5).

25). The patient in the present study had nasal lesions caused by *L. major*, but *L. tropica* was isolated from his oral lesions too. The association between ML and previous or active skin lesions is widely accepted, as both forms can originate from a single species (13). It has also been demonstrated that localization of the parasites in the mucous membranes of the nasal, oral, and pharyngeal areas occurs as a result of migration of *Leishmania* via the lymphatic system or because of hematogenous dissemination of amastigotes from the skin of 5% of patients with cutaneous leishmaniasis (CL) (14). Oral involvement is unusual, and in most cases it becomes evident several years after the resolution of the original cutaneous lesions (22).

Sporadic *L. major* and *L. tropica* infections have occasionally been reported in patients with ML in Afghanistan, Saudi Arabia, and Sudan (5, 9, 11). Fars Province, a region in southern Iran, is a classical focus of CL, and the previous studies have consistently documented the etiologic agents to be *L. tropica* and *L. major* in urban and rural areas, respectively (4, 12). Mixed infections with different *Leishmania* species could explain differences between the clinical courses of these infections, as well as resistant cases (1). We have presented the first report of coinfection with *L. major* and *L. tropica* isolated from a patient with ML. In the sub-Andean region of Bolivia, coinfection with *L. amazonensis* and *L. infantum*/*L. chagasi* has been identified in a patient with diffuse CL (20). In the suburban district of Campo Grande, Municipality of Rio de Janeiro, Brazil, *L. donovani* and *L. braziliensis* have been isolated from the bone marrow and forehead of a patient with concurrent asymptomatic visceral leishmaniasis (VL) and typical CL (24). There are data indicating that concomitant natural infection with *L. donovani* and *L. major* has occurred in humans with CL and VL (2, 15). Mixed infections have also been observed in sand flies and

dogs (8, 10). Antoniou et al. indicated that the VL form may occur because of mixed infection with different strains of *L. infantum* (3). Such reservoirs are exposed to large numbers of sand fly bites, which increases the possibility of infection with different strains or species of the parasite. Moreover, mixed infections of the same macrophage with different species of *Leishmania* have been shown to be experimentally possible (1). On the basis of PCR and IHC results, concomitant or mixed mucosal infection with two *Leishmania* species can occur in immunocompetent subjects. This seems to be the first described case of concurrent or mixed ML infection with *L. major* and *L. tropica*. *L. major* was isolated from the nasal lesion that occurred 2 months earlier than the oral lesion from which *L. tropica* was isolated, and this can indicate that, at least in this patient, a previous infection with *L. major* did not protect against *L. tropica*. On the other hand, it has previously been reported that *L. tropica* primary infection was not efficient in reducing the parasite load of the spleen in the secondary *L. major* infection (19). This result has important implications for the development of vaccines against leishmaniasis and emphasizes attention to the diagnosis and treatment of mixed ML infections.

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