Mixed mucosal leishmaniasis infection caused by Leishmania tropica and Leishmania major and brief literature review

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Abstract

Mixed infection by different *Leishmania* species could explain differences in the clinical course 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 *L. tropica* and *L. major*. Mixed infection was identified by PCR amplification of *Leishmania* kinetoplast DNA on scraping of cytological smears and histopathological sections. *Leishmania 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 infection of ML with two *Leishmania* species. It indicates that, at least in this patient, previous infection with a one of these *Leishmania* species did not protect against another. This result has important implications in development of vaccines against leishmaniasis, and implies careful attention in treatment of this infectious disease.

Case description and methods

A 34-year-old immunocompetent male subject presented with lesions on the mucous membranes of nose and mouth. The patient was from Fars Province, southern Iran. He presented with a 7 and 5 months 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 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 reference range. His hemoglobin was 13.6 g/dl, total leukocyte count 6600/mm$^3$, serum creatinine 0.8 mg/dl, and blood urea nitrogen was 18 mg/dl. Serological studies for human immunodeficiency virus and hepatitis B and C virus were negative.

Tissue samples from the lesions were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5-µm in thickness, and stained with haematoxylin and eosin. The cytologic smears were prepared by scraping from 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 Wright method. Review of the cytologic smears and histologic sections were conducted blindly by three pathologists. Microscopic examination showed the amastigote forms of *Leishmania* (Fig. 2 A, B).

The following antibodies that were kindly provided by Special Programme for Research and Training in Tropical Disease (TDR), WHO: IS2-2B4 (A11) specific for *L. tropica*, and XLVI-5B8- B3 (T1) specific for *L. major*, each was used as a primary monoclonal antibody. Sections of 3 µm in thickness were used for immunohistochemical (IHC) analysis. The slides were deparaffinized in xylol, rehydrated, and treated with 3% hydrogen peroxide solution for 10 minutes at room temperature to quench endogenous peroxides. The antigen retrieval was conducted by pre-treatment with microwaving (power 100 for 10 min and then power 20 for 20 min) using a 10-mmol/L concentration of citrate buffer (pH 6.0). The primary antibody was applied for 1 hour (diluted 1:200). Detection of the immunoreaction was achieved. The detection system used was Envision+(DakoCytomation) and developed with diaminobenzidine (DakoCytomation). 3,3′-diaminobenzidine–hydrogen peroxide was applied as the chromogen and hematoxylin was used as the counterstain. The nasal lesions showed immunoreactivity for specific monoclonal antibodies (mAb) of *L. major* and the oral lesions...
for *L. tropica* (Fig. 2C), while the IHC staining of the nasal lesions with *L. tropica* mAb and IHC staining of the oral lesions for *L. major* was negative (Fig. 2D).

To identify the *Leishmania*-specific DNA, the entire smear was scraped off the slide with a sterile scalpel, and as previously described, the phenol-chloroform-isoemil alcohol extraction method was used to extract the DNAs (23). The DNA samples were dissolved in 50 μl deionized distilled water and stored at 4 °C. Variable segments on the minicircles of the kinetoplast DNA from the *Leishmania* species present in the smear scrapings were amplified with two rounds of nested PCR. 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 for the second round were LiR (TCG CAG AAC GCC CCT) and 13Z (ACT GGG GGT TGG TGT AAA ATAG) (23). The PCR products of the second-round of the PCR were loaded onto a 1.5% agarose gel. As the positive controls, the DNA extracted from the promastigote cultures of the reference strains of *L. infantum* (MCAN/IR/97/LON490) were run on each gel. Extravasation oral mucocele from 10 patients were used as the negative controls. The negative controls, ultrapure water replaced the template DNA, were also run.

The fragment of *L. major* specific kinetoplast mini-circle DNA, 560 bp in length, was amplified from the nasal, whereas the fragment of *L. tropica*, 750 bp in length, was amplified from the oral lesions by the second-round PCR assay, respectively (Fig. 3).

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

**Ethics Statement**

The Ethics Committee of the Faculty of Medical School, University of Shiraz Medical Sciences and the authors' institutional review board of Dr. Daneshbod Laboratory approved the study and the authors group collected written informed consent from the patient.
Mucosal leishmaniasis is a rare disease in the world, even in endemic areas 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). Mucosal leishmaniasis is a form of tegumentary leishmaniasis that has been shown to be associated with \textit{L. braziliensis}, \textit{L. panamensis} and less frequently with \textit{L. amazonensis}, although it has been reported in infections caused by other new word \textit{Leishmania} species, such as \textit{L. guyanensis} (13). A few patients with ML have been described in the old world in infections caused by \textit{L. infantum}, \textit{L. tropica}, and \textit{L. major} (16,18,21,25).

The patient in the present study had nasal lesions caused by \textit{L. major}, but \textit{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 be originated from a single species (13). It has also been demonstrated that localization of the parasites in the mucous membrane of nasal, oral, and pharyngeal areas occurs as a result of migration of \textit{Leishmania} via lymphatic or it is due to the hematogenous dissemination of the amastigotes from the skin of 5% of the patients affected with CL (14). Oral involvement is unusual and in most cases it becomes evident after several years of resolution of the original cutaneous lesions (22).

Sporadic \textit{L. major} and \textit{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 agent to be \textit{L. tropica} and \textit{L. major} in urban and rural areas, respectively (4,12).

Mixed infections by different \textit{Leishmania} species could explain differences in the clinical course of these infections as well as resistant cases (1). We presented the first report of co-infection by \textit{L. major} and \textit{L. tropica} isolated from a patient with ML. In the sub-Andean region of Bolivia, co-infection by \textit{L. amazonensis} and \textit{L. infantum}/\textit{L. chagasi} have been
identified in a patient with diffuse cutaneous leishmaniasis (20). In the suburban district of Campo Grande, Municipality of Rio de Janeiro (Brazil) *L. donovani* and *L. braziliensis* have been isolated from bone marrow and forehead of a patient with concurrent asymptomatic 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. (2004) indicated that the VL form may occur due to mixed infection by different strains of the *L. infantum* (3). Such reservoirs are exposed to a large numbers of sand fly bites, which increases the possibility of infection by different strains or species of the parasite. Moreover, mixed infections of the same macrophage by different species of *Leishmania* have been shown to be experimentally possible (1). Based on the results of PCR and IHC, concomitant or mixed mucosal infection can occur in immunocompetent subjects with two *Leishmania* species. This seems to be the first described case of concurrent or mixed infection of ML with *L. major* and *L. tropica*. *Leishmania major* was isolated from the nasal lesion that occurred two 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 development of vaccines against leishmaniasis and emphasizes attention to mixed ML infection in diagnosis and treatment.

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References


Figure legends

Fig. 1: Diffuse yellowish white erosions with grayish pseudomembranes on reddish edematous background on oral mucosa.

Fig. 2A, Plasma cell (thick arrow) and macrophage loaded with leishman bodies associated with free leishman bodies (thin arrow) (Wright stain, X200). B, Massive infiltration of macrophages loaded with leishman bodies in submucosal area of the respiratory epithelium (arrows) (H & E, X200). C, the oral lesions showed immunoreactivity for specific monoclonal antibodies of L. tropica (IHC, X100). D, the IHC staining of the oral lesions for L. major was negative (IHC, X100).

Fig. 3: The results of the electrophoresis of the products of the nested-PCR-based amplification of DNA extracted from the stained smears. The six lanes contained the products from reference strains of Leishmania tropica (lane 4), Leishmania major (lane 3) and a negative control (lane 6), test sample, oral and nasal lesions, identified as L. tropica and L. major (lanes 1 and 2, respectively) and a molecular-weight ‘ladder’ (lane 5).