ACTA CYTOLOGICA

Acta Cytologica 2012;56:304–309
DOI: 10.1159/000337450

Received: December 5, 2011
Accepted after revision: February 20, 2012
Published online: April 26, 2012

Molecular Diagnosis and Species Identification of Mucosal Leishmaniasis in Iran and Correlation with Cytological Findings

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Key Words
Mucosal leishmaniasis · Nested PCR · Cytology · *Leishmania tropica* · *Leishmania major*

Abstract
Objective: Mucosal leishmaniasis (ML) is a rare destructive disease that mainly affects the mucous membranes of the mouth and nose. The etiologic agent(s) of ML are not well known in the Middle East. ***Study Design:*** Cytologic smears of ML from the mucosal lesions of 7 patients were prepared by scraping. In 2 patients with nasal lesions, exfoliative cytology was made by washing the nasal cavity. The smears were both air dried and fixed in alcohol and stained. Scrapings from the same smears were then tested for leishmanial DNA by nested PCR. ***Results:*** This study characterized 9 isolates of ML, with 7 cases identified as *Leishmania major* and 2 as *Leishmania tropica*. While 6 patients were found to be positive by the cytology technique, the nested PCR was positive for all of these samples. ***Conclusions:*** Presence of granuloma and multinucleated giant cells in the negative smears of the patients who showed clinical manifestation of ML was an important clue for diagnosis of this disease. The PCR-based method not only appears to be a precise diagnostic approach in the identification of suspected cases of ML but is also efficient in determining the species of the parasite. *L. major* and *L. tropica* can lead to ML, but they result in different cytologic features.

Leishmaniasis is a zoonotic disease caused by the genus *Leishmania* and is transmitted by sandfly vectors. It is found worldwide and is considered to be endemic in 88 countries [1]. Leishmaniasis is usually classified as cutaneous, mucocutaneous, visceral, or kala-azar, with a wide spectrum of clinical manifestations [2, 3]. It is a parasitic infection with a great geographical distribution [4]. The clinical spectrum of the disease's manifestation in Iran includes cutaneous leishmaniasis, localized leishmania lymphadenitis, diffuse multiorgan involvement (kala-azar), and rarely mucosal leishmaniasis (ML) [2, 3]. Needle aspiration biopsy of the bone marrow, lymph nodes, splenic puncture, and skin and mucosal scraping cytology for diagnosis of leishmaniasis has previously been described [2]. Nested PCR has been shown to be a highly

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Table 1. Cytology findings in 9 cases of ML caused by two species of *Leishmania*

Patient No.	FLB n	ILB n	MC %	MNG n	G n	Bh n	Mo %	L %	Eo %	Ne %	Bn %	P %
L. major												
1	–	–	–	2	6	2	3	37	–	54	4	2
2	–	–	–	1	4	1	2	24	–	62	2	5
3	–	–	–	1	3	1	–	20	–	73	1	6
4	7	–	1	–	–	–	2	33	–	62	1	1
5	30	5	1	–	–	1	2	36	1	43	1	6
6	5	2	1	3	4	2	3	33	–	58	4	2
7	88	6	1	1	1	–	3	44	1	46	2	3
L. tropica												
8	123	7	1	1	2	2	3	47	1	41	–	4
9	180	8	–	–	3	1	3	36	1	50	6	4

FLB = Free Leishman body; ILB = intrahistiocytic Leishman body; MC = mast cell; MNG = multinucleated giant cell; G = granuloma; Bh = binucleated histiocyte; Mo = monocyte; L = lymphocyte; Eo = eosinophil; Ne = neutrophil; Bn = band neutrophil; P = plasma cell.

sensitive and rapid test for the diagnosis of leishmaniasis [5]. *Leishmania (Viannia) braziliensis* has been reported as the etiologic agent of ML in Latin America [6], but the species responsible for it has not yet been reported in Iran. Herein the patients with ML for whom cytology and nested PCR helped to correctly determine ML are presented. In addition, the differential cytologic features of the species of *Leishmania* causing ML in our study are also compared. This is the first study of molecular investigation of ML in this area.

Materials and Methods

Nine patients with mucosal lesions and without a history of immunosuppressed disease presented from different urban areas of the Fars and Kerman Provinces in the south and southeast of Iran were involved in this study. Of these patients, 5 had oral lesions. Of these 5 patients, 1 was known to have oral leishmaniasis with recurrence of oral lesions, 1 had lower lip lesions, and 3 had nasal lesions. The patient with laryngeal leishmaniasis had recurrence of prior oral lesions.

Scraping cytology was performed, using a scalpel, in 5 cases of oral leishmaniasis. A cytobrush was used in 1 case of nasal mucosa ML and in 1 case of laryngeal ML. In 2 patients with nasal lesions, exfoliative cytology was made by washing the nasal cavity [7]. Multiple smears were made on slides and were both air dried and alcohol fixed and stained by Wright and Papanicolaou stains, respectively. Review of the cytologic smears was conducted blindly by 4 pathologists and was scored according to table 1 by differentiation of different types of inflammatory cells (band and segmented neutrophils, lymphocytes, plasma cells, eosinophils, monocytes, mast cells, multinucleated giant cells, and binucleated histiocytes), free or intrahistiocytic Leishman-Donovan bodies (LDBs), and granulomatous reaction. Each pathologist was asked to study five fields with ×40 objective magnification from each slide and to calculate the average LDB counts.

DNA Extraction

The entire smear was scraped off the slide with a sterile scalpel so that the total DNA in the smear could be extracted by digestion, in a 1.5-ml microcentrifuge tube, with 200 μl lysis buffer [50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1% (v/v) Tween 20] containing 8.5 μl of a proteinase-K solution that had 19 mg enzyme/ml [8]. The tube was incubated at 37°C overnight, and then 200 μl phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) was added to it. The tube was then shaken vigorously and centrifuged at 6,000 *g* for 10 min. The resultant supernatant solution was transferred to another tube and mixed with 400 μl absolute ethanol. The precipitated DNA was centrifuged (at 6,000 *g* for 20 min), dried, dissolved in 50 μl ultrapure water [produced in a Purelab® UHQ system (Siemens Water Technologies, Warrendale, Pa., USA)], and stored at 4°C [9] before use in the PCR-based assay.

Nested PCR Assay

Variable segments on the minicircles of kinetoplast DNA from the *Leishmania* species present in the smear scraping were amplified with two rounds of nested PCR [9]. 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 they were LiR (TCG CAG AAC GCC CCT) and 13Z (ACT GGG GGT TGG TGT AAA ATAG) [9]. Each 25-μl first-round reaction mixture contained 5 μl template DNA, 200 μM of each deoxynucleoside triphosphate (Roche, Penzberg, Germany), 1.5 mM $MgCl_2$, 1.0 U Taq polymerase, 50 mM Tris-HCl (pH 7.6), 10 mM CSB1XR, and 10 mM CSB2XF. The thermocycler used (Eppendorf AG, Humburg, Germany) was set for 5 min at

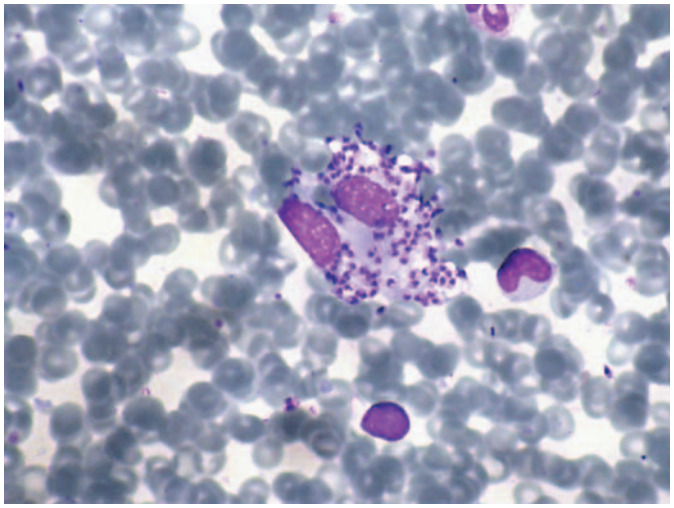

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Fig. 1. Macrophages loaded with Leishman bodies. Wright stain. ×100.

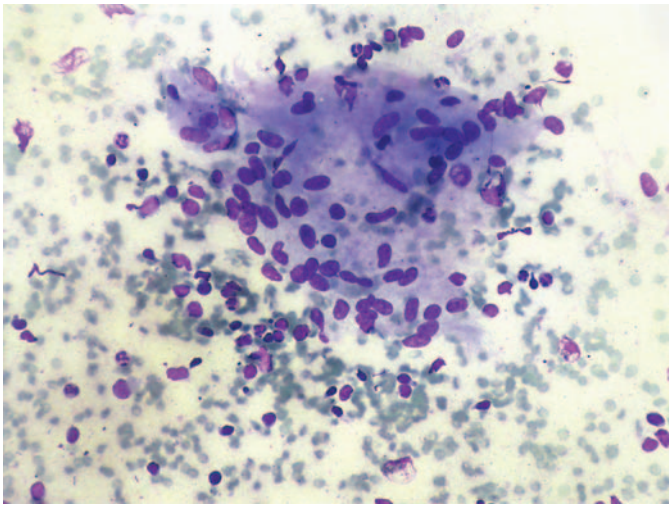

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Fig. 2. Granuloma with scattered Leishman bodies. Wright stain. ×40.

94°C, followed by 30 cycles, each of 30 s at 94°C, 1 min at 55°C, and 1.5 min at 72°C, and then a final extension for 5 min at 72°C. The product of the first round of PCR was diluted 1:9 with ultrapure water and then 1 μl of this dilution was used as the template for the second round of PCR, which used the same conditions and reaction mixture as the first round except that LiR and 13Z were used as the primers. A 5-μl sample of the second-round product was subjected to electrophoresis in 1.5% (w/w) agarose gel, stained with ethidium bromide, and visualized by ultraviolet transillumination [10]. The size of each detected amplicon was estimated by comparison with a 100- to 1,500-bp molecular-weight 'ladder' (Roche) run on the same gel. As positive controls, the DNA extracted from the promastigote cultures of the reference strains of *Leishmania major* (MHOM/IR/54/LV39) and *Leishmania tropica* (MHOM/IR/89/ARA2) was run on each gel [11]. These strains, which were routinely maintained on Novy-MacNeal-Nicolle medium, were transferred to Roswell Park Memorial Institute medium 1640 (Invitrogen, Carlsbad, Calif., USA) enriched with 20% (v/v) fetal calf serum (Invitrogen) prior to extraction of their DNA. Extravasation oral mucoceles from 10 patients and ultrapure water were used as the negative controls.

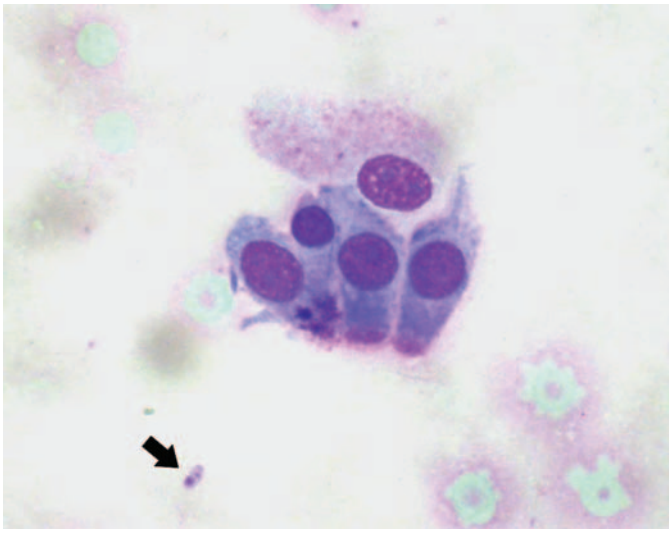

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Fig. 3. Respiratory epithelium with one free Leishman body in its vicinity (arrow). Wright stain. ×100.

Results

The clinical and cytological data of 9 patients, i.e. 7 men and 2 women, with an age range of 21 to 45 years are explained. The cytologic findings consisted of macrophages loaded with LDBs (fig. 1), multinucleated giant cells (fig. 2), binucleated histiocyte cells (Reed-Sternberg-like) loaded with LDBs, free LDBs (fig. 3), LDBs in the vicinity of the respiratory epithelium in nasal smears, and atypical intracytoplasmic LDBs with ballooning changes together with acute and chronic inflammatory cells. The cytologic findings and scoring of the ML together with species identification are summarized in table 1.

By cytologic examination, 6 out of 9 patients were reported to have leishmaniasis and the other 3 patients were suggestive for leishmaniasis, whereas nested PCR was positive for all of these samples (9/9). The 3 negative pa-

tients at cytologic examination had *L. major* and both cases of *L. tropica* were positive in both cytologic and PCR examinations. The ultrapure water, used as a negative control, showed negative results when analyzed by nested PCR. Seven (77.7%) of the smears yielded the 560-bp product indicative of *L. major*, the other two (22.3%) yielded the 750-bp second-round amplicon indicative of *L. tropica,* and no amplicon was detected in the negative-control samples (fig. 4). The mean percentage of neutrophils was 63.0% (range 54.0–73.0), 52.3% (range 43.0–62.0), and 56.8% (43.0–73.0) among the cytologically negative, the LDB-positive, and the nested PCR-positive cases caused by *L. major,* respectively. The mean percentage of lymphocytes was 36.5% (range 33.0–44.0), 27.0% (range 20.0–37.0), and 32.4% (range 20.0–44.0) in cases with LDBs, in cases without LDBs, and in the nested PCR-positive cases caused by *L. major,* respectively. As shown in table 1 the mean percentage of neutrophils and lymphocytes for *L. tropica* was 45.5% (range 41.0–50.0) and 41.5% (range 36.0–47.0), respectively. In 3 patients with negative cytology but positive nested PCR, granuloma and multinucleated giant cells of histiocytic origin were dectected. The number of LDBs in the smears of cases caused by *L. tropica* was higher than the number in those caused by *L. major.*

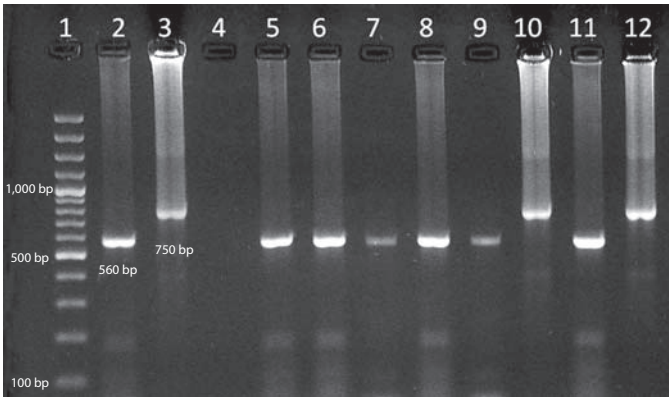

Fig. 4. Results of the electrophoresis of the products of the nested-PCR-based amplification of DNA extracted from the stained smears. The 12 lanes contain a molecular-weight 'ladder' (lane 1), the products from reference strains of *L. major* (lane 2) and *L. tropica* (lane 3), a negative control (lane 4), and test samples identified as *L. major* (lanes 5, 6, 7, 8, 9, and 11) and *L. tropica* (lanes 10 and 12).

Discussion

Leishmaniasis is a disease that is increasing in the northern hemisphere as a result of tourism and armed conflict in tropical regions [12]. Leishmaniasis is particularly endemic in the southern and southeastern regions of Iran in which the Fars and Kerman provinces are located [13]. ML is an important but rare disease in the world, even in endemic areas such as Iran [3]. It is characterized by a severe inflammatory reaction and tissue damage, with the presence of few parasites in the lesion. Demonstration of the parasite is necessary for the diagnosis of leishmaniasis [14]. However, the kinetoplasts are not visible in the histology sections (hematoxylin and eosin staining method), and the organism can be easily mistaken for toxoplasmosis, fungal elements, histoplasmosis, tingible bodies, or artefacts [2]. Detection of the parasites in the tissue sections in cases that have been infected with few parasites is histologically difficult since the cytopathologic findings in those cases may be mistaken for those of nonspecific chronic inflammation and/or granulomatous reaction [15]. Cytologically, the complete form of an LDB, a nucleus, and a kinetoplast can be seen. Although there were no LDBs at the cytological level in the lesions of 3 patients in the present study, the smears of these patients demonstrated several granulomas and the diagnosis was confirmed by the nested PCR. In patients with an intense inflammatory response, partially in treated and parasite-free patients, cytology may be suggestive or inconclusive for leishmaniasis [3, 14]. Thus, the presence of granulomatous reaction and multinucleated giant cells in the negative smears of patients who showed clinical manifestation of ML, even in those who did not demonstrate LDBs, was an important clue for diagnosis of this disease.

The nested PCR is a sensitive test for the detection of low amounts of leishmanial DNA in tissues and is the method of choice for leishmaniasis diagnosis since the conventional methods are not sufficiently sensitive [16]. However, though the causative agents of ML have been reported from various continents of the world *(Leishmania infantum, Leishmania donovani, Leishmania viania, Leishmania amazonensis,* and *L. tropica)* [17–23], they have not been determined yet in Iran and this is the first study of molecular investigation of ML in some endemic areas of this country. In addition, based on our knowledge, there are only 3 reports worldwide in which cytology has been conducted as the diagnostic method for ML identification [2, 3, 18]. In one of those three studies, the investigators tried to specify different species of *Leishmania (L. donovani)* that are responsible for ML develop-

ment, and this is the first study of cytologic findings of ML caused by *L. major* and *L. tropica*.

The present study indicated that both *L. major* and *L. tropica* can lead to ML, but they result in different cytologic features (table 1). The number of LDBs in the smears of patients with ML caused by *L. tropica* was greater than the number in those caused by *L. major,* whereas the percentage of neutrophils in the smears of patients with ML caused by *L. tropica* was lower than the percentage in those caused by *L. major*. It has been stated that *L. major* can result in multiple lesions in patients with cutaneous leishmaniasis and the lesions are accompanied by marked inflammation and crusting, whereas in patients with *L. tropica* the lesions develop more slowly than those caused by *L. major* [24]. Cytopathological diagnosis of uncomplicated lesions of leishmaniasis is usually easy. However, diagnosis of this disease in conditions such as late stages of leishmaniasis when granulomas develop, and when infection with multiple opportunistic organisms takes place in the same lesion, may be difficult. This may explain why there were more LDBs in some smears of the present study than in others.

The presence of higher neutrophil and lower lymphocyte numbers in the cytologically negative cases could indicate that the lesions of these patients were possibly in the earlier stages and the cytopathologic changes due to leishmaniasis had not yet completely developed in these patients, or the parasites were mistaken for fragments of the polymorphonuclear cells. The presence of a higher percentage of neutrophils and a lower percentage of lymphocyte constituents in cases caused by *L. major* could indicate that these lesions were still in the acute phase of inflammation, or the acute phase of inflammation lasts longer compared to that of cases caused by *L. tropica*. An inverse relationship between the number of LDBs and the percentage of lymphocytes and neutrophils has previously been reported in the lesions of patients with leishmaniasis [25]. Except for the lymphocyte percentage, the differential count of neutrophils and multinucleated giant cells in the present study were in agreement with those that have been reported earlier for cutaneous leishmaniasis [25–27].

In conclusion, based on the results of the present study, the presence of granulomatous reaction and multinucleated giant cells in the negative smears of patients who showed clinical manifestation of ML, even in those who did not demonstrate LDBs, was an important suggestive clue for the diagnosis of this disease. The PCR-based method not only appears to be a useful and more precise diagnostic approach in the identification of suspected cases of ML with negative cytology but is also efficient in determining the species of the parasite. Since cytology is inexpensive and easy to perform, it is the preferred primary approach; however, PCR is indicated as the second-line approach in negative cytology or for species identification. Both *L. major* and *L. tropica* were the causative agents of ML, but they resulted in different cytologic features.

Acknowledgements

The authors would like to thank the authorities of the Veterinary School of Shiraz University and the Medical School of Shiraz University of Medical Sciences, and the Institute of Experimental Pathology, University of Münster, for their support. They also would like to thank Drs. M. Davarmanesh, M.M. Davarpanah, M. Kalantari, and Prof. J. Brosius, T.S. Rozhdestvensky and G. Randau from the Institute of Experimental Pathology, Münster, Germany, for their help and advice.

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