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ORIGINAL ARTICLE

Molecular prevalence and estimated risk of cutaneous leishmaniasis in Libya

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Abstract *Background/Purpose:* Cutanoeus leishmaniasis (CL) is an endemic disease in the Mediterranean area including Libya. The aim of the present study is to detect the prevalent *Leishmania* species obtained from smeared cutaneous lesions in addition to studying the diverse sociodemographic risk factors of the reported cases from different provinces of Libya.
Methods: A total of 250 archived microscopic slides from clinically suspected cases of CL attending the leishmaniasis clinic in the Dermatology Department, Tripoli Central Hospital, Tripoli, Libya, were microscopically examined. *Leishmania*-DNA was amplified using combined polymerase chain reaction (PCR) targeting kinetoplast-DNA (kDNA) and ribosomal internal transcribed spacer 1 (ITS1)-DNA with restriction fragment length polymorphism analysis for direct *Leishmania* species identification.
Results: Using kDNA and ITS1-PCR, 22.5% and 20% of cases were positive, respectively. Only 14.4% of cases were positive using microscopy. Nominating ITS1-PCR as the reference standard, kDNA-PCR assay was highly sensitive while microscopy was 100% specific but of limited sensitivity (72%) with a substantial agreement and an overall accuracy of 94.4%. *Leishmania major* and *Leishmania tropica* were the predominant species reported from the north-western provinces including Tripoli, Zintan, and Gharyan with their related subprovinces; Asabaa, Mizdan, Alkawasem, and Alorban. CL prevailed more among men and residents of rural areas. House wives and students were the most affected professions. Children were the least affected, while the middle-aged were the most affected age group.
Conclusion: *L. major* and *L. tropica* are the predominant species in the north-western regions of Libya. ITS1-PCR-restriction fragment length polymorphism assay offered a sensitive, specific, and faster diagnostic method especially with negative parasitologic examination.

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Introduction

Cutaneous leishmaniasis (CL) is well-known in the northwestern areas of Libya.[1] Precise diagnosis and characterization of *Leishmania* species to observe clinical consequences is important for satisfactory treatment and assessment of prognosis and epidemiological hazards in CL.[2] The sensitivity of conventional methods including microscopic examination has been reported to range from 17% to 83% for CL depending on clinical symptoms, parasite species, technical proficiency, and other factors.[3] Studies proved a higher sensitivity of polymerase chain reaction (PCR) in *Leishmania* DNA detection in clinical specimens compared with conventional methods. Besides, the added advantage of pointing to recent parasitic contact, in case of inability to isolate *Leishmania* amastigotes from the patient's material.[4–7] Unfortunately, there is no standardized diagnostic procedure that is used for evaluating leishmaniasis. Nevertheless, PCR-based methods using either nuclear DNA markers—the coding and intergenic noncoding areas of the internal transcribed spacer (ITS) regions[8] or kinetoplast DNA (kDNA)[9]—are frequently used. Principally, the diagnosis of CL is based on clinical symptoms and direct detection of amastigotes in smears or aspirates from skin lesions. This technique is of low sensitivity, as it depends on the number of parasites available in addition to the skills of the microscopist.[10,11] *In vitro* culture is considered a gold standard assessment. However, it is deficient for species differentiations as cultures may require extensive periods to get adequate parasites for species description. Furthermore, various *Leishmania* species are not uniformly cultured; culture contamination is probable with heterogenecity in usefulness of different growth media.[11,12] Recently, molecular methods offer one more promising tool for leishmaniasis diagnosis.[13] Even though prior studies were performed in Libya, there is a considerable lack of knowledge about the true prevalence of *Leishmania* species. This led us to employ PCR assays aiming to detect and genetically differentiate *Leishmania* species from archived microscopic slides in addition to elucidating the estimated risk factors for CL strains isolated from different districts of Libya.

Methods

Study design

A cross-sectional study was carried out to detect the molecular prevalence of *Leishmania*. Cases were diagnosed by physicians at the Dermatology Department, Tripoli Central Hospital, Tripoli, Libya. A case was defined by having at least one *Leishmania* lesion or scar. The study group included 250 individuals. Data were collected using structured questionnaires included socioeconomic indicators (age, sex, occupation, education, and time of living in the district). Parents of young children provided consent and responded to questionnaires. Data collection was conducted during the period from August 2010 to August 2011.

Sample collection

A total of 250 archived slides were prepared from smeared skin lesions of CL from referred patients to Dermatology Department, Tripoli Central Hospital, Tripoli, Libya (during the period from August 2010 to August 2011) from seven different areas of CL including Tripoli, Gharyan, Zantan Al-Kawasem, Asabaa, Alorban, and Mizdan. Smeared slides were archived either Giemsa stained or unstained. Patients' related demographic and clinical data were collected and archived.

Microscopy

Slides and corresponding patients' data were made accessible for this study. Received unstained slides (72/250) were stained with Giemsa stain. All slides were microscopically examined (3—5 fields/slide) for the presence of *Leishmania* amastigotes using light microscopy at 400× magnification. Positive slides were stored and kept for molecular study.

PCR assays and molecular characterization of *Leishmania*

The DNA extraction was carried out by scraping off the tissue material adherent to the slide using 200-μL lysis buffer and transferred to a sterile labeled tube. Genomic DNA was extracted and purified using Thermo Scientific Gene JET Whole Blood Genomic DNA Purification Mini Kit (Thermo Scientific fermentas, & Lithuania, Cat No # K0781 & Lot No 00118770) according to the manufacturer's protocol. To molecularly identify *Leishmania* parasite, it was necessary to amplify 120 bp and 300—350 bp using two sets of primers: the first targeting kDNA (13A: 5′-GTGGGGGAGGGGCGTTCT-3′, 13B: 5′-ATTTTCCACCAACCCCCAGTT-3′) with the expected product size of 120bp.[14] The second targeting ITS1 (LITSR: 5′-CTGGATCATTTTCCGATG-3′, L5.8S: 5′-TGATACCACTTATC GCACTT-3′) had the expected product size of 300—350bp.[15] In a 25-μL volume containing 2.5-μL genomic extracted DNA, 12.5-μL PCR hot-start master mix kit (Thermo Scientific Fermentas, lot no. #K1051) and 2 μL of each forward and reverse primer was added. The PCR products were electrophoresed in 1.5% agarose gels then stained with ethidium bromide, visualized under UV light, and photographed. ITS-1 PCR products were digested using *Hae* III restriction enzyme according to manufacturer's instructions and the fragments

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