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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

were analyzed with 2% agarose gel and visualized under UV light to determine *Leishmania* type. The extraction of DNA and PCR assays were monitored for possible contamination using two negative controls (containing only the reagents) for every extraction series. Positive controls (*Leishmania turanica* DNA) were included to insure reliability, validity, and avoiding possible contamination. PCR inhibition control that contained both the sample DNA and purified DNA from cultured promastigotes of *L. turanica* was run along each sample. A reaction was considered positive when a band of the correct size (300–350 bp) was observed. A specimen was considered positive if it was positive by either microscopy or ITS1-PCR and defined as negative if it was negative by both.

Data analysis

ITS1-PCR assay was nominated as the reference standard. Diagnostic effectiveness (specificity, sensitivity, negative predictive values, positive predictive values) and Cohen's kappa coefficient (κ) were determined. Cohen's κ is the measure of agreement between two tests beyond that expected by chance, where 1 is perfect agreement and 0 is chance agreement.[16] Data were analyzed using four-fold tables with Fisher's exact test and multiple logistic regression with backward elimination. Odds ratios and 95% confidence intervals were calculated to evaluate the association between the *Leishmania* positivity and socioeconomic variables. Confounding was assessed using a multivariate logistic regression; variables with $p < 0.2$ in the univariate analysis were subsequently tested in a multivariate model. Data were considered statistically significant if $p < 0.05$. Statistical analysis was performed using SPSS version 17 (SPSS Inc., Chicago, IL, USA).

Results

Out of the total 250 archived slides, only 36 (14.4%) of the scanned slides for amastigotes under the bright-field microscopy were positive. Using PCR assays, *Leishmania* DNA was amplified in 50 (20%) extracted genomic DNA of the 250 archived samples. ITS1-PCR enabled identification of 50 positive samples (50/56). kDNA-PCR had six false positives samples (proved to be negative using both microscopy and ITS1-PCR; Table 1).

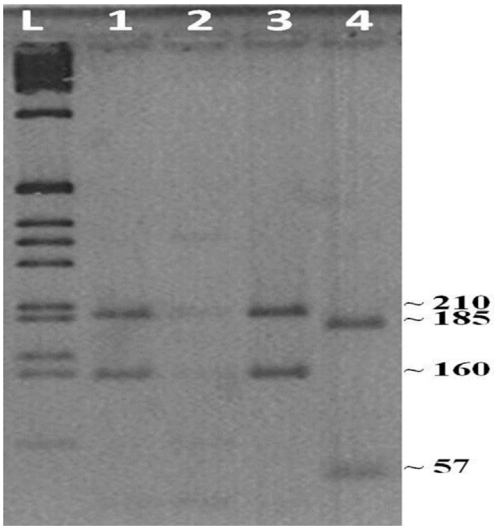

Figure 1. Agarose gel electrophoresis showing the restriction fragment length polymorphism profiles of the internal transcribed spacer 1-polymerase chain reaction products using *Hae* III restriction enzyme (Lane 1 and 3, *Leishmania major*; Lane 4, *Leishmania tropica*; and Lane 2, negative sample).

The restriction fragment length polymorphism (RFLP) profiles of the ITS1-PCR products using *Hae* III restriction enzyme PCR assays (Figure 1) detected *Leishmania* strains which included strains of *Leishmania major* (38/50, 76%) mainly from rural areas (24/108, including Asabaa, Mizdat, Alkawasem, and Alorban) and *Leishmania tropica* (12/50, 24%) were confined to urban areas (12/142, Tripoli, Gharyan, and Zantan; Figure 2). Therefore, in rural areas, the only prevalent species is *L. major*, while in urban regions *L. major* and *L. tropica* gave nearly an equal prevalence (14/142 and 12/142 respectively). Those in rural subprovinces have a 1.257 times higher risk of acquiring CL.

The prevalence of CL among men was slightly lower than women (17.7%:22.5%) who had a 1.351 times higher risk of contracting leishmaniasis compared with men. Libyans (20.4%) were 1.668 times at a higher risk than non-Libyans (13%). House wives and students were the most frequently affected professions (30% and 22%, respectively). Children were the least affected age group (9.8%) while the middle-aged (41–60 years, 27.7%) was the most affected age group that was proved to have a 3.543 times higher risk of

Table 1 Diagnostic yield of microscopy and polymerase chain reaction assays for detection of Leishmania among the study group.

		ITS1-PCR						Total
		Positive (n = 50)			Negative (n = 200)			
		kDNA PCR			kDNA PCR			
		Positive	Negative	Total	Positive	Negative	Total	
Microscopy	Positive	36 (14.4)	0 (0)	36 (14.4)	0 (0)	0 (0)	0 (0)	36 (14.4)
	Negative	14 (5.6)	0 (0)	14 (5.6)	6 (2.4)	194 (77.6)	200 (80.0)	214 (85.6)
	Total	50 (20.0)	0 (0)	50 (20.0)	6 (2.4)	194 (77.6)	200 (80.0)	250 (100.0)

Data are presented as n (%).
kDNA = kinetoplast-DNA; ITS1 = internal transcribed spacer 1; PCR = polymerase chain reaction.

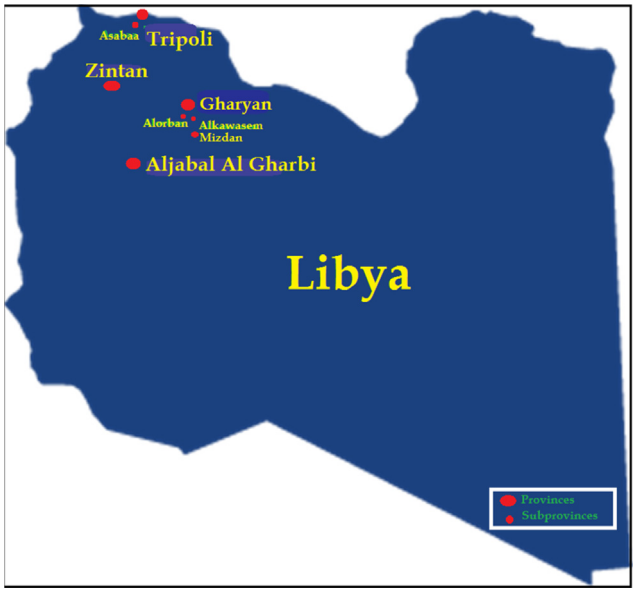

Figure 2. Libyan map showing the current study geographic distribution of cutanoeus leishmaniasis in different localities.

acquiring CL followed by the adolescents age group (20.9%; Tables 2 and 3). Nominating ITS1-PCR as the reference standard in this study, kDNA-PCR showed the highest sensitivity (100%), followed by the ITS1-PCR (91.0%), while microscopy had the least sensitivity of 72%. Kappa test of agreement between microscopy and ITS1-PCR revealed a substantial agreement (0.88) between the two methods. All assays were 100% specific.

Discussion

CL has not been fully documented in Libya.[1] The present study aimed to detect *Leishmania* amastigotes microscopically and *Leishmania* DNA molecularly using kDNA and ITS1-PCR assays from smeared cutaneous lesions in addition to studying different sociodemographical aspects of the reported cases from some provinces of Libya.

Our study showed that *Leishmania* DNA could be amplified from old microscopic slides that were stored for more than 4 years. Moreover, we employed ITS1-PCR-RFLP analysis that revealed the presence of two co-existing species causing CL in Libya; *L. major* and *L. tropica*. We proved that ITS1-PCR as a diagnostic test on skin scraping has the potential of overcoming darkly-stained areas that are difficult to examine by microscopy in addition to recognizing traces or sheds of leishmanial-DNA which are unattainable by microscopy.

Our results were supported by Kochar et al[17] who explained the minor product of direct microscopy as it requires the presence of a somewhat high number of viable or integral microorganisms which are absent in the chronic phase of CL. Similarly, in a previous study by Kazemi-Rad et al,[18] Giemsa-stained slides could be utilized as archived specimens for recognizing *Leishmania* species using PCR-RFLP. Yet, in Iran, *Leishmania* species were identified from archived Giemsa-stained slides for the diagnosis of CL by PCR, but species of *Leishmania* had not been microscopically detected.[19]

Owing to the low sensitivity of microscopy, we could not determine the true specificity of kDNA-PCR assay. One attractive approach is a composite reference standard of Alonzo and Pepe[20] which enabled the evaluation of kDNA and calculation of positive cases to estimate the risk factors. Therefore, we used ITS1-PCR (resolver test) as an additional test to microscopy. Composite reference standard considered a specimen positive if it was negative by either microscopy or ITS1-PCR assay and defined as negative if the specimen was negative by both. In the present work, most of slides that were positive by ITS1-PCR were also microscopy positive (36/50), achieving a substantial agreement with ITS1-PCR and an overall accuracy of 94.4%. Also, we reported that PCR had the highest diagnostic yield as ITS1-PCR revealed a higher sensitivity compared with microscopy (72% sensitivity and 93.5% negative predictive values), detecting an additional 5.6% positive cases than microscopy.

Table 2 Studied variable distribution using univariate and multivariate logistic regression analysis among internal transcribed spacer 1-polymerase chain reaction positive cases.

		Cases (N = 250) (%)	Univariate analysis		Multivariate model[a]	
			OR (CI)	p	OR (CI)	p
Sex	Men (n = 130)	23 (17.7)	Reference category	0.342		
	Women (n = 120)	27 (22.5)	1.351 (0.725–2.515)			
Age group (y)	Child (n = 41)	4 (9.8)	Reference category	–		
	Adolescence (n = 43)	9 (20.9)	2.449 (0.690–8.688)	0.157	0.50 (0.1–2.56)	0.403
	20–40 (n = 85)	16 (18.8)	2.145 (0.668–6.885)	0.192	1.30 (0.3–5.69)	0.725
	41–60 (n = 65)	18 (27.7)	3.543 (1.104–11.367)	0.027	1.17 (0.29–4.72)	0.828
	>60 (n = 16)	3 (18.8)	2.135 (0.420–10.839)	0.388		
Nationality	Libyan (n = 235)	48 (20.4)	1.668 (0.364–7.645)	0.506		
	Non-Libyan (n = 15)	2 (13.3)	Reference category			
Residence	Rural (n = 108)	24 (22.2)	1.257 (0.684–2.374)	0.444		
	Urban (n = 142)	26 (18.3)	Reference category			

[a] Only age variable with $p < 0.2$ has been included in the multivariate model analysis. Confidence intervals and p values derived from univariate and multivariate logistic regression models.
CI = confidence interval; OR = adjusted odds ratio.

Table 3 Profession distribution among polymerase chain reaction positive cases.

Profession	Frequency within PCR+ cases	% within PCR+ cases	*p*
Contractor ($n = 1$)	1	2	0.235
Worker ($n = 5$)	1	2	
Employee ($n = 27$)	5	10	
Military ($n = 4$)	1	2	
Freelance ($n = 12$)	2	4	
House wife ($n = 59$)	15	30	
Child ($n = 30$)	3	6	
Cooker ($n = 2$)	2	4	
Driver ($n = 7$)	1	2	
Pilot ($n = 1$)	1	2	
Retired ($n = 8$)	1	2	
Student ($n = 66$)	11	22	
Teacher ($n = 27$)	5	10	
Welder ($n = 1$)	1	2	

PCR = polymerase chain reaction.

It is noteworthy that despite including PCR inhibition and extraction controls in every experiment to avoid false negative results, 50 samples (20%) were only successfully amplified and characterized using PCR-RFLP while the remaining specimens were possibly inhibited due to the presence of impurities in the DNA extracts, which were not sufficiently eliminated during DNA purification process, or to degraded DNA. This explains the negative results of those patients presenting with CL lesions. Nevertheless, compared with microscopy that revealed positivity in 14.4% of the scanned slides, our results showed that kDNA and ITS1-PCR-based techniques offer sensitive diagnostic methods similar to that adopted by the World Health Organization for visceral leishmaniasis.[21]

Certain sociodemographic and environmental aspects as sex, age, behavioral, and occupational activities of peoples should be considered during CL intervention approaches.[22,23] In the present study, the association between *Leishmania* infection and sociobehavioral characters (sex, age, nationality, residence, and profession) of the study individuals was studied. We found that all CL cases originated from the north-western provinces of the country entirely. However, our results revealed additional described foci of CL to those described previously by Amro et al,[24] including Tripoli, Zantan, and Gharyan with their related subprovinces Asabaa, Mizdan Alkawasem, and Alorban. The prevalence of CL among men was slightly lower than women (17.7%:22.5%) who had 1.351 times higher risk of contracting leishmaniasis compared with men. This could be attributed to the fact that Libyan women have a habit of sitting outside in the evenings and working in farms in the afternoon increasing their exposure to sand flies. In agreement with our results, several studies ascertained that women rather than men were chiefly affected. The authors elucidated that women are more exposed to insect bites than male patients because most dairy farm workers were women.[25–28] In contrast to these reports, other studies[24,29,30] mentioned that men are at more risk for CL due to their habit of sleeping outdoors. Also, we reported that children were the least affected age group while the most affected age group was the middle-age group (41–60 years; 27.7%) with a mean age of 31.08 years. This age group proved to have a 3.543 times higher risk of acquiring CL.

Partially in agreement with the present study at Wasit governorate, similar findings were reported where a high rate of infection (40%) in adults (31–60 years) while, in the young adults (13–30 years), children (1–12 years), and old (above 60 years) it was 35%, 15%, and 10%, respectively.[31] Also, we reported that acquisition of CL was higher in residents of rural (22.2%) than urban (18.3%) areas. This could be elucidated by the presence of suitable environmental conditions for spread of reservoir hosts and vectors. What is more is that the rural subprovinces had 1.257 times higher risk of *Leishmania* compared with the urban areas which was explained by Sabra and Hala[32] who reported that the presence of rodents and sand flies making these subprovinces proper environment for *Leishmania* to spread. Our study revealed that around 94% of the study populations were Libyans and 6% were non-Libyans. Libyans had the highest rate of CL (20.4%) compared to non-Libyans (13.3%) and the estimated risk for having CL in Libyans was 1.668 times than non-Libyans which could be due to non-Libyans may be immigrants from non-endemic areas of *Leishmania*. CL is considered an occupational illness[30] which is in compliance to the present study that, professions necessitate the outdoor activities were considered as risk factors for exposure to CL infection. House-wives and students were the most frequently affected (30% and 22%, respectively). This may be explained by engagement in farm activities and staying outdoors for a part of the night. In conclusion, CL is considered a health problem in the north-western provinces of Libya with particular insight to the reported sub-provinces described in the current study. This seems essential for applying effective prevention and control strategies for the population at risk. Moreover, ITS1-PCR as compared to microscopy, offered a sensitive, specific and faster method for Giemsa-stained smears, especially for those with negative parasitological results. However, the prospective of introducing ITS1-PCR and PCR-based methods to be more economically and practically achievable in endemic districts remains a challenge.

Conflicts of interest

All authors declare that they have no conflicts of interest.

Acknowledgments

The study design and procedures were approved by the Dermatology Department, Tripoli Central Hospital, Faculty of Medicine, Tripoli University, Libya, and an ethical approval was received from the Deanship of higher education and scientific researches, Faculty of Medicine, Cairo University.

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