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Original article

Determination of *Leishmania tropica major* infection among Sudanese patients in public hospitals of Khartoum and Omdurman provinces-Sudan

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ABSTRACT

Background: Leishmaniasis produced by flagellated protozoa return to genus Leishmania. The parasites are transmitted from human to human by bite of Phledotamine sand flies. **Objectives**: The current study is aimed to detect prevalence of *Leishmania tropica* among Sudanese patients. **Methods**: This study conducted in dermatology hospitals in Khartoum Province (Army forces hospital, Khartoum and Omdurman teaching hospitals). Sixty positive cases are obtained by repeated direct smear that were examined by PCR techniques. DB8 and AJS3 primers is used to amplify Cytochrome Oxidase II gene for identifying leishmania parasite. **Results:** Regarding to the gender, the frequency of the infection in the male is far higher (96.7%) than in females (3.3%),14(23.3%) among the accountants, students and others occupations respectively. According to duration of the infection 4(6.7%) 6(10%),10(16.7%),2(3.3%) ,16(26.7),6(10%),7(11.7%),9(15%) got the infection 1,2,3,4,5,6,10 months and one year before starting this study respectively. Considering occupation, the soldiers 36(60%) were more frequently affected by *Leishmania tropica complex*, while the distribution was 7(11.7 %,) 3(5%) ,14(23.3%) among the accountants, students and others occupations respectively. **Conclusions**: The Cytochrome Oxidase II is suitable for determining cutaneous leishmaniasis parasite, which is more common gene in KDNA. Further research is needed with more large sample size.

Introduction

Leishmaniasis an uncontrolled disease, which is occurred by flagellated protozoa of the genus Leishmania. The parasites are transmitted from human to human by bite of Phledotamine sand flies. Different types of *Leishmania* infect human leading to manifestation of three forms of leishmaniasis, *L.donovani* causes visceral leishmaniasis or Kala azar, *L.tropica*, produces cutaneous leishmaniasis and *L.braziliensis* the causative organism of mucocutaneous leishmaniasis [1]. Diagnosis is confirmed by the demonstration of the parasites in slide smears in 50-70% of cases and histological section in 70% with primers specific for *L.major*, the Polymerase chain reaction (PCR) is positive in 86% of cases [2]. Sub genus *Leishmania* complex includes

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the members of *L.donovani* Complex (*L.d.donovani*, *L.d.infantum*, L.*d.chagasi, L.d.archibaldi.)*, members of *L.tropica complex(L.tropica ,L.aethopica, L.major)* and members of *L.mexicana complex(L.mexicana, L.amazonensis ,L.pifanoi*) [3].

Leishmania has two forms amastigote form in host and promastigote in sand fly and in culture [4]. The development of *L.mexicana* (New world) *L.major, L.tropica, L.aethipoica* (Old-world) and the members of *L.donovani* group (New ,old world) is restricted to parts of the alimentary tract anterior to the pyiory (Suprapylarian leishmania) while hypoplaria Leishmania develops in the hind gut *L.brazaliensis* complex develop in the pylorius and the lumen and follow anterior migration to the *L.barazilensis* complex reviewed the development of Suprapylarian species in the sand fly [5]. While feeding infected mammalian host amastigotes are ingested by sandy fly, engorgement is quickly followed by production of peritrophic membrane which is secreted by the epithelial cell lining the mid gut of the fly. Promastigotes develop from amastigote within 24 hours under rapid division period to their transformation in 2-5 days, The transmission of the parasite is still obscure. Several studies suggest transmission by regurgitation promastigotes from blocked posterior parts of the foregut and others claim that infection occur only when the proboscis of the flies is invaded by promastigotes [6,7].

PCR based assays currently constitute the main molecular diagnostic approach of researchers and health professionals. Several distinct PCR formats are available that may broadly be classified into mid-tech, high–tech and low–tech approaches [8].

Leishmaniasis is a widely distributed disease in Sudan. Several studies have been conducted to study visceral leishmaniasis but no previous studies done to enlighten the level of cytokines in cutaneous leishmaniasis in Sudan. This study aimed to detect the gene expression of *Leishmania tropica major* in Sudan.

Material and methods

This was a descriptive laboratory-based study for isolation of *Leishmaniat tropica* in Dermatology hospitals in Khartoum and Omdurman provinces. This study was conducted in dermatology hospitals in Khartoum Teaching Hospital in the center of Khartoum, Omdurman Teaching Hospital and Army Forces Hospital in Omdurman. The study conducted during the period from November 2021-January 2023.Cutaneous leishmaniasis patients who are participated in this study are attended the outpatient of the Dermatology Departments of the hospitals with cutaneous leishmaniasis and confirmed by direct smear done by the hospital laboratory staff.

Inclusion criteria

All positive cases for *Leishmania tropica* by direct microscopy.

Exclusion criteria

patients who received antileishmania treatment before and all non-Sudanese patients.

All patients were examined and then selected considering epidemiological risk factor for cutaneous leishmaniasis as well as signs of the disease. Lesion and the adjacent normal looking skin around them cleaned and disinfected. Skin biopsies of 4 mm in diameter was taken aseptically from the border of the ulcer using disposable scalpel blade. The blade was turned 90 degrees and scarped along the cut edge of the incision to remove and pick up skin tissue which was divided into two parts. One part was used for smear and one was stored at -20℃ for DNA extraction later used for PCR analysis [9]. The smear was prepared by smearing the biopsy on glass microscopic slide. After the smear was dried completed, it was fixed with absolute methanol, allowed to dry again and then stained with Giemsa stain. The amastigotes were found within macrophages. The presence of intra cytoplasmic, kinetoplast confirm the identification after staining, the cytoplasm appeared light blue, the nucleus and kinetoplast appeared red to purple. In very early and old lesion, very few organisms were presented.

Biopsy tissue was collected directly in lysis buffer, DNA was isolated according to the user manual and kept at-20℃.

Kinetoplast DNA (KDNA) was performed for the diagnosis of *Cutaneous leishmania* and characterization of causative species. Negative control tubes that received 2 µl of water instead of DNA extract was induced in each PCR run to detect any amplican contamination. DNA was extracted from each biopsy sample according to the manufactures protocol of pathogen extraction. DNA was analyzed by PCR using primer specific for *leishmania tropica complex*. Then positive samples were subject to PCR using *Leishmania tropica complex* specific primers to amplify KDNA **(Table 1)**.

Data was analyzed using percentage and frequencies.

Table 1. The oligonucleotides primer of reverse and forwarded for each specific primer for PCR

Oligonucleatide	DNA sequence	Primer Length	TM	Expected Size
AJS3	**F**-5-GGGGGTTGGTGTAGGGC-3	17bp	65.0℃	800
DB8	**R**-5-CCAGTTTCCCGCCCCG-3	16bp	70.0℃	

Ethical consideration

The study was approved by the ethical committees of the National Ribat University before starting the study. Also, permission was obtained from government officials for initiation of the study. A consent was taken from each patient.

Results

Out of 65 samples examined the true positive cases were 60(92.3), While there are 5(7.7%) false positive result, the sixty positive cases are subject of further study **(Table 2)**. Regarding the age the frequency of the disease was illustrated in **Table 3**.

Regarding to the gender, the frequency of the infection in the male is far higher (96.7%) than in females (3.3%) **(Table 4)** ,14(23.3%) among the accountants, students and others occupations respectively **(Table 5)**.

Regarding to the residence the frequency of cutaneous leishmaniasis was pointed out in **Table 6**.

According to duration of the infection 4(6.7%)6(10%),10(16.7%),2(3.3%),16(26.7),6(10%) ,7(11.7%),9(15%) got the infection 1,2,3,4,5,6,10 months and one year before starting this study respectively **(Table 7)**. According to occupation the soldiers 36(60%) were more frequently affected by *Leishmania tropica complex,* while the distribution was 7(11.7 %,) 3(5%) ,14(23.3%) among the accountants, students and others occupations respectively **(Table 5)**.

Table 2. Accuracy of the hospitals laboratories results compared to that of study

Number of cases Examined		Hospital lab Results
65	True	60(92.3)
	False	5(7.7%)

Table 3. Frequency of patients with Cutaneous leishmaniasis according to age

Age group (years)	Frequency (%)
13-20	5 (8.3)
20-27	16 (26.7)
27-30	22 (36.7)
30-40	14 (23.3)
40-47	1(1.7)
47-65	2 (3.3)
Total	60 (100)

Table 4. Frequency of patients with Cutaneous leishmaniasis according to gender

Gender	Frequency (%)
Males	58 (96.7)
Females	2 (3.3)
Total	60 (100%)

Table 5. Frequency of patients with Cutaneous leishmaniasis according to occupation

Occupation	Frequency (%)
Soldier	36 (60.0)
Accountant	7 (11.7)
Student	3 (5.0)
Others	14 (23.3)
Total	60 (100%)

Table 6. Frequency of patients with Cutaneous leishmaniasis according to residence

Residence	Frequency (%)
Khartoum North Province	5 (8.3)
Khartoum	3 (5.0)
Omdurman province	8 (13.3)
Kordofan states	4 (6.7)
Darfour states	21 (35.0)
Aljazeera state	2 (3.3)
White Nile state	1(1.1)
Others Sudan states	16 (26.7)
Total	60 (100%)

Table 7. Frequency of patients with Cutaneous leishmaniasis according to duration of the infection

1st sign of disease (month)	No of patient	%
Before 1 month	4	6.7
Before 2 months	6	10.0
Before 3 months	10	16.7
Before 4 months	2	3.3
Before 5 months	16	26.7
Before 6 months	6	10.0
Before 10 months	7	11.7
Before 12 months	9	15.0
Total	60	100%

Discussion

Main findings of the present study revealed that out of 65 samples examined the true positive cases were 60(92.3), While there are 5(7.7%) false positive result. Regarding to the gender, the frequency of the infection in the male is far higher (96.7%) than in females (3.3%) ,14(23.3%). According to duration of the infection 4(6.7%) 6(10%),10(16.7%),2(3.3%),16(26.7),6(10%), 7(11.7%),9(15%) got the infection 1,2,3,4,5,6,10 months and one year before starting this study respectively. According to occupation the soldiers 36(60%) were more frequently affected by *Leishmania tropica complex*, while the distribution was 7(11.7 %,) 3(5%) ,14(23.3%) among the accountants, students and others occupations respectively.

PCR has proved to have great potential in the detection and diagnosis of various infection by different parasite species in number of clinical

samples and in epidemiological studies, largely with application of PCR [9, 10, 11, 12].

Approximately 20 Leishmania species are known to cause cutaneous, mucocutaneous, and visceral disorders in humans. Identification of the causative species in infected individuals is important for appropriate treatment and a favorable prognosis because infecting species are known to be the major determinant of clinical manifestations and may affect treatments for leishmaniasis. Although Leishmania species have been conventionally identified by multilocus enzyme electrophoresis, genetic analysis targeting kinetoplast and nuclear DNA (KDNA and NDNA, respectively) is now widely used for this purpose [13].

PCR-Restriction Fragment Length Polymorphism (RFLP) analyses targeting multiple nuclear genes were established for the simple and practical identification of Leishmania species without using expensive equipment. This method was applied to 92 clinical samples collected at 33 sites in 14 provinces of Ecuador, which have been identified at the species level by the kinetoplast cytochrome b (cyt b) gene sequence analysis, and the results obtained by the two analyses were compared. The result demonstrated that genetically complex Leishmania strains are present in Ecuador [14].

Leishmania tropica was isolated from the clinical patients of cutaneous leishmaniasis in rural community of Kohat district in Khyber Pakhtunkhwa province and was identified through PCR, microscopy, and culture techniques. A total of 113 samples from the clinical patients were examined through PCR, microscopy, and culture which showed 87.61% (99/113), 53.98% (61/113), and 46.90% (53/113) prevalence. During the study, 186 bp *Leishmania tropica* was identified through PCR. Thus, the sensitivity of PCR is very high as compared to the conventional techniques [15].

Echchakery *et al.*, PCR constitutes a complementary method for the identification of the causal species. The results indicate that both the *L. tropica* (dominant) and *L. infantum* are the causative agents of CL in the Marrakesh-Safi region [16].

L. major was considered the causative agent of leishmaniasis in the region, but the identification of a non-native *L. tropica* revealed the importance of further isolation of Leishmania parasites following molecular analyses and confirmation, and also revealed the importance of further isolation of Leishmania parasites from patients of the field areas who do not have easily access to health care centers for specialized treatment strategies [17].

A multitude of laboratory techniques for the detection of Leishmania have been developed over the past few decades, and although many have drawbacks, several of them show promise, particularly molecular methods like polymerase chain reaction [18].

PCR followed by a simple restriction fragment length polymorphism (RFLP) analysis using HaeIII endonuclease indicated that Leishmania major was responsible for all CL infections in the study area [19].

Next-generation sequencing (NGS) was used to investigate the genetic diversity of *Leishmania tropica* in the sand fly vector, targeting the internal transcribed spacer 1 (ITS1) of the genus Leishmania. Bioinformatics analyses were conducted using Galaxy, the median-joining haplotype network produced a total of 11 active haplotypes. In conclusion, *L. tropica* from sand flies in Palestine is monophyletic that assembled in one main phylogroup and one haplotype [20].

Leishmania RNA virus (LRV) is a dsRNA virus member of the Totiviridae family, which was first detected in the promastigotes of *Leishmania braziliensis guyanensis*. A recent study investigated possible changes in the predominant and causative strains of CL and screening the LRV1 and LRV2 species genome from Leishmania species isolated from the lesions of patients. The findings after performed molecular techniques showed that presence of a significant number of LRV2 in isolated samples, as well as the recognition of LRV1 in one of the Old World leishmaniasis species, which is a new result, could pave the way for investigating further aspects of this disease and successful treatment strategies in future studies [21].

The practical implications for the present case report revealed that PCR has proved to have great potential in the detection and diagnosis of various infection by different parasite species in number of clinical samples.

Limitations of the study

All study population was from residential area in Khartoum State, the study neglects the other Sudan States.

Conclusion:

The Cytochrome Oxidase II gene of the cutaneous leishmaniasis parasite is more common gene in KDNA. The present study recommends further research with more large sample size.

List of abbreviations

Cyt b: Cytochrome b

KDNA: Kinetoplast DNA

LRV: Leishmania RNA virus

NDNA: nuclear DNA

NGS: Next-generation sequencing

PCR: Polymerase chain reaction

RFLP: Restriction Fragment Length Polymorphism

Conflicts of interest
None.

Financial disclosure
None.

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