Molecular Characterization of *Leishmania* Species Isolated from Cutaneous Leishmaniasis in Yemen

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**Abstract**

**Background:** Cutaneous leishmaniasis (CL) is a neglected tropical disease endemic in the tropics and subtropics with a global yearly incidence of 1.5 million. Although CL is the most common form of leishmaniasis, which is responsible for 60% of DALYs lost due to tropical-cluster diseases prevalent in Yemen, available information is very limited.

**Methodology/Principal Findings:** This study was conducted to determine the molecular characterization of *Leishmania* species isolated from human cutaneous lesions in Yemen. Dermal scrapes were collected and examined for *Leishmania* amastigotes using the Giemsa staining technique. Amplification of the ribosomal internal transcribed spacer 1 (ITS-1) gene was carried out using nested PCR and subsequent sequencing. The sequences from *Leishmania* isolates were subjected to phylogenetic analysis using the neighbor-joining and maximum parsimony methods. The trees identified *Leishmania tropica* from 16 isolates which were represented by two sequence types.

**Conclusions/Significance:** The predominance of the anthroponotic species (i.e. *L. tropica*) indicates the probability of anthropogenic transmission of cutaneous leishmaniasis in Yemen. These findings will help public health authorities to build an effective control strategy taking into consideration person-to-person transmission as the main dynamic of transmission of CL.

**Materials and Methods**

**Microscopic examination**

The study was carried out on Giemsa stained smears, positive for *Leishmania* from the archives of the Central Health Laboratory, Sana’a, Yemen which is referral diagnostic laboratory under the Ministry of Health. Biodata and the geographical origin of patients...
cannot be retrieved from the records. Of the 53 patients with skin lesions suspected to be caused leishmaniasis who were referred to the Central Health Laboratory, 22 patients were positive for *Leishmania* amastigote using Giemsa stained smears and were included in the molecular analysis. The Giemsa smears were re-examined in the Department of Parasitology, Faculty of Medicine, University of Malaya, Malaysia under 1000× magnification. The smear was considered negative if amastigotes were not found after the microscopic examination of 1000 oil immersion field (OIF). Amastigote density was quantified using a semi-quantitative scale; +, 1 amastigote/whole slide to 1 amastigote/OIF in a total of at least 100 OIF; ++, 2–10 amastigotes/OIF in a total of at least 50 OIF; ++++, 11–20 amastigotes/OIF in a total of at least 50 OIF; ++++, >21 amastigotes/OIF in a total of at least 10 OIF [18].

**Ethical approval**

The study was approved by the Faculty of Medicine and Health Sciences, Sana’a University, Yemen. No information on the patients has been presented in this research.

**Polymerase chain reaction**

DNA was extracted from Giemsa stained smears positive for *Leishmania*. The slides were first cleaned with chloroform to remove oil. Fifty µl of TE buffer was transferred onto the smear and at least half of the smear was completely wiped off the slide using Whatman 1 filter paper and transferred into 1.5 ml microcentrifuge tubes. DNA was then extracted using the Qiagen DNeasy Blood Tissue Kit (Hilden, Germany) according to the manufacturer’s instructions. DNA was eluted in 100 µl and 50 µl of distilled water from slides with high and low parasitic intensity, respectively.

A Nested PCR assay was used to amplify the ribosomal internal transcribed spacer 1 (ITS-1) region as described previously [19]. Primary PCR was carried out with primers LITSR (forward: 5′-CTG GAT CAT TTT CCG ATG-3′) and LITSV (reverse: 5′-TCG CAC TT-3′) with primers LITSR and L5.8S (reverse: 5′-TGA TAC CAC TTA-3′) and the secondary PCR with primers LITSR and L5.8S (reverse: 5′-TGA TAC CAC TTA-3′). Both primary and secondary PCRs were performed in a 50 µl reaction volume containing 0.5 µM of each primer (Bio Basic Inc, Canada), 2 U of *iTaq™* plus DNA polymerase (iNtRON BIOTECHNOLOGY, Seoul, Korea), 1× of *iTaq™* plus reaction, 200 mM of each dNTPs, and 1.5 mM MgCl₂. Five µl of DNA template was used in both primary and secondary PCRs. In both amplifications, samples were incubated in the MyCycler thermal cycler (Bio-Rad, Hercules, USA) under the following conditions: denaturing step at 95°C for 2 min, followed by 40 cycles of denaturing for 20 s at 95°C, annealing for 30 s at 53°C and extension for 60 s at 72°C, followed by a final extension at 72°C for 6 min. The PCR products were subjected to electrophoresis on 2% agarose gels and stained with ethidium bromide.

**DNA sequencing and phylogenetic analysis**

The DNA was purified using the QIAquick PCR purification Kit (QIagen, Germany) according to the manufacturer’s instructions. Cycle sequencing (bidirectional) was carried out using the ABI PRISM 1 BigDye™ terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA) in a 3700 DNA Analyzer (Applied Biosystems, USA). The sequences representing all samples were aligned with previously published sequences as listed in Table 1, using the program MEGA4 (www.megasoftware.net). Similarity searches were carried out using the Basic Local Alignment Search Tool (BLAST) [20]. Neighbor-joining (NJ) and maximum parsimony (MP) analyses were performed in MEGA4.

**Results**

Microscopic examination detected *Leishmania* amastigotes in 22 smears of the 53 smears collected from patients with suspected cutaneous leishmaniasis who attended the Central Health Laboratory. Amastigote density was ++++, ++++, ++ and + in 27%, 14%, 14% and 45% of the samples, respectively. Of the 22 microscopically positive smears for *Leishmania* species, amplicons (~350 bp) were produced from 17 samples (77%). Sixteen of these amplicons were successfully sequenced in both directions except one. Sequence analysis divided the samples into two groups; group 1 contained eleven isolates (2, 3, 5, 6, 7, 8, 9, 10, 14, 16, and 17) and had identical sequences and group 2 consisted of five isolates (4, 11, 20, 29, and 21) and had identical sequences. Polymorphism between the two sequence types was detected in 4 positions (4 SNP). For phylogenetic analysis, two sequence types representing all 16 isolates and five reference sequences obtained from GenBank representing *L. donovani*, *L. infantum*, *L. major*, *L. aethiopica* and *L. tropica* (table 1) were multiple aligned. The inferred phylogenetic trees based on Neighbor-Joining (Figure 1) and Maximum Parsimony (Figure 2) were concordant in topology with strong support. In the NJ method, *Leishmania* isolates were grouped with *L. tropica* in one cluster (98% bootstrap), while the MP method

**Table 1. Sequences of *Leishmania* species used in the phylogenetic analysis.**

| Isolate                  | GenBank accession number* |
|-------------------------|---------------------------|
| Isolates (2, 3, 5, 6, 7, 8, 9, 10, 14, 16, and 17) | GU561644                  |
| Isolates (4, 11, 20, 29, and 21)               | GU561643                  |
| *L. tropica*            | FJ948456.1                |
| *L. aethiopica*         | GQ323259                  |
| *L. infantum*           | GQ323259                  |
| *L. major*              | GQ323261.1                |

*Accession numbers in bold represent sequences from this study.*

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NJ analyses were performed with distances calculated with the Kimura 2-parameter [21]. Unweighted parsimony analyses were performed using the Close-Neighbor-Interchange algorithm [22]. To evaluate the support for inferred topologies, bootstrapping [23] was carried out using 1000 replicates.

![Figure 1. Phylogenetic analysis using Neighbor-joining Method.](https://doi.org/10.1371/journal.pone.0012879.g001)
northern Yemen. Of these, four CL cases were characterized using RFLP involving 155 CL cases originated from 10 governorates of Yemen in 1989 [24]. Almost two decades later, Khatri and his colleagues detected Leishmania amastigotes in 128 cases of CL from northern Yemen. Of these, four CL cases were characterized using the isoenzyme electrophoresis technique and L. tropica was identified in all these cases [14]. In their study, 49% of skin smears were highly positive compared to 27% of CL cases in our study.

However, L. tropica was not the only species identified to be the causative agent of CL. In 1993, L. donovani was isolated from a cutaneous lesion in a tourist from southern France who visited Yemen for two weeks in 1992 [25]. He was reported to have been bitten repeatedly by sandflies. The patient did not present any signs and symptoms of VL although L. donovani is more commonly associated with VL. Again, a recent study carried out using PCR-RFLP involving 155 CL cases originated from 10 governorates of northern Yemen highlighted the possibility of CL being caused by more than one species of Leishmania. This study detected L. tropica in 133 cases (85.80%), L. infantum in 17 cases (10.97%) and L. donovani in 3 cases (2.3%) [8]. Nonetheless, based on findings from these studies in Yemen, L. tropica is still the predominant species responsible for CL. The predominance of L. tropica as a causative agent of CL has been reported from Saudi Arabia [26].

Although L. tropica most commonly causes CL, it has been isolated from VL cases [27,28]. The isolation of L. tropica from human cases of VL among the US soldiers returning from Operation Desert Storm in the Gulf countries [15,16,29] raised a question as to whether L. tropica could manifest in the form of VL in Yemen. The potential of this species to cause VL, the most virulent form of leishmaniasis, will exacerbate the situation in Yemen since this form of the disease may respond poorly to different therapeutic regimens [8].

L. tropica is commonly stated to be anthroponotic [30], although zoonotic transmission has been reported from Greece [31], Kenya [32], Jordan [33] and Saudi Arabia [26]. In Yemen, a survey of reservoir hosts has not been performed to date based on the assumption that CL is usually an anthroponotic infection. Moreover, not much is known about the vector of L. tropica. However, Phlebotomus sergenti, the most common vector of L. tropica [30], was detected in an entomological collection from Taiz governorate in 1951 [34].

Leishmaniasis is a neglected disease in Yemen. The true incidence is not well reflected as only a few published documents are available. The disease burden is underestimated as there is no national reporting system in place and no active case detection is being carried out. Furthermore, there are no systematic national efforts to control the disease. Since there is a scarcity of research done on Leishmania in Yemen, further investigations on aspects of the type of vectors, the vectors’ behaviour and feeding preference, insecticide susceptibility, the presence of reservoir hosts, risk factors of acquiring the infection and drug resistance are crucial for effective control strategies to be formulated.

This study is the first study in Yemen to identify the Leishmania species causing CL based on sequencing the ITS locus. Previous research carried out by Khatri et al. [8] was based on PCR-RFLP. Building a GenBank database on Leishmania in Yemen could be useful for future comparison studies. However, findings from the current study are limited by the small sample size and the lack of the geographical information on Leishmania cases.

The findings of this present study and the previous studies conclude that CL is most commonly caused by L. tropica suggesting the anthroponotic transmission of CL in Yemen. Leishmaniasis is a neglected disease and does not receive much attention from public health authorities. An effective strategy for the control of leishmaniasis should be developed, embedded in the national health plans and harmonized with operational, entomological and epidemiological research to ensure its continuing effectiveness. An effective surveillance system is imperative to monitor cases and improve disease awareness among medical personnel.

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Author Contributions

Conceived and designed the experiments: MAKM YALL AAA RM. Performed the experiments: MAKM HMAM AMAM NOMBS. Analyzed the data: MAKM YALL. Contributed reagents/materials/analysis tools: MAKM. Wrote the paper: MAKM HMAM YALL AAA RM.

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