Simple and Direct Characterization of *Leishmania donovani* Isolates Based on Cytochrome Oxidase II Gene Sequences

Abdalla Hassan Sharief[*,1], El-Tahir Awad Gasim Khalil[2], Douglas Cameron Barker[3], Samia Ali Omer[1], Hamid Suliman Abdalla[4] and Muntasir Eltayeb Ibrahim[2]

[1]*Department of Immunology, Tropical Medicine Research Institute, National Center for Research, Khartoum, Sudan*

[2]*Department of Molecular Biology, Institute of Endemic diseases, University of Khartoum, Khartoum, Sudan*

[3]*School of Biological Sciences, Department of Pathology, University of Cambridge, UK*

[4]*Department of Parasitology, Faculty of Veterinary Medicine, University of Khartoum, Khartoum, Sudan*

Abstract: We primarily identified *Leishmania donovani* parasites from eastern Sudan using species-specific primers that amplify a whole length minicircle. Based on the amplification of a cytochrome oxidase II fragment (COII), heteroduplex analysis (HDA) was performed. In HDA, the appearance of the extra bands with molecular weights higher than 540 bp indicates the presence of mismatched bases in the selected samples. Such bands were detected when hybridization was between reference strains and clinical isolates, as well as between the reference strains themselves, while no heteroduplexes were detected between the clinical isolates. Moreover, an RFLP assay using the restriction enzyme *Ssp*1 was performed on the original 540 bp products to discern an A-G transition, which differentiates between members of the *Leishmania (L) infantum* and those of *Leishmania (L) donovani* subspecies. The proposed minicircle genes-based analysis was rapid and easy to perform method for the characterization of *Leishmania donovani* complex isolates and with a potential to be extended to characterization of other species of *Leishmania*.

Keywords: Leishmania donovani, heteroduplex analysis, COII gene, characterization, Sudan.

INTRODUCTION

Differences in clinical presentations and severity of the leishmaniases are explained by interactions between particular molecular and biologic characteristics of different *Leishmania* species, host genetics and immunity and, environmental factors [1,2]. Although it is generally accepted that major clinical forms of leishmaniasis may be caused by specific *Leishmania* species, the relationship between *Leishmania* zymodemes and clinical presentation remains unclear [3]. To date, most studies investigating the relationship between polymorphisms and clinical disease have provided little evidence for strong associations [4,5]. The method of choice for characterization of *Leishmania* is isoenzyme analysis [6] which has the disadvantages of being tedious, time consuming and requires the use of toxic substances. Other techniques for characterizing *Leishmania* species that have been developed include biochemical and molecular characterization, isoenzyme characterization, genetic typing. [7,8]. Recently PCR-based techniques have introduced and proved capability of identifying *Leishmania* species. The advantage of PCR-based characterization is sufficient to distinguish almost all medically relevant *Leishmania* species including *L. major*, *L. tropica* and *L. infantum* [9-14]. Discrimination between *L.donovani* and *L.infantum* is very much needed to type *Leishmania* isolates using a PCR-based technique that introduced for the first time. *Leishmania donovani* and *Leishmania infantum* both pertain to the *L.* (*L.*) *donovani* complex. The status of certain strains is questioned in the literature and there are no reliable discriminative markers to identify them. Molecular tools are much needed to identify diagnostic markers and to allow a better understanding of phylogenetic relationships. In this study we have developed simple and direct method of *Leishmania* characterization based on cytochrome oxidase gene sequences. This PCR discriminates between members of the *Leishmania donovani* complex with a future potential to be applied for characterizing *L.major* and *L.tropica* complexes.

MATERIALS AND METHODS

Parasites

Leishmania parasite strains were isolated from visceral leishmaniasis (VL) patients living in a cluster of villages, within VL endemic area, in eastern Sudan. Bone marrow and/or lymph node aspirates were taken and inoculated into bottles containing culture media. *L.donovani* and *L.infantum* reference strains were obtained from the Cryobank of the NIMR Outstation, Molteno Laboratory, Department of Pathology, University of Cambridge.

Genomic DNA Extraction

Promastigotes (clinical isolates) were cultured at 24^{o}C in RPMI-1640 medium containing 15% fetal calf serum.

*Address correspondence to this author at the Tropical Medicine Research Institute, National Centre for Research, Khartoum, P.O. Box 1304, Sudan; Tel: + 249 183 777501; Fax: + 249 183 781845;
E-mail: abdullasharief@postmaster.co.uk

1874-3153/11

Parasites were harvested at a density of 2 x10^7 parasites/ml, pelleted at 2,000 *g* for 8 min at 4°C, washed 3 times in phosphate-buffered balanced salt solution (PBSS) pH 7.4, and then re-suspended in 500 ml PBSS. The DNA was extracted using salt extraction method according to Miller [15]. Briefly, parasite pellet was digested with 20 mg/ml proteinase K (400 µg/ml final concentration) was added and mixed well. The suspension was incubated at 55-65°C overnight, after which 300 µl of 6 M NaCl (NaCl saturated H_2O) was added. Samples were vortexed for 30 seconds at maximum speed, and tubes spun down for 30 minutes at 10,000 *g*. The supernatant was transferred to fresh tubes. An equal volume of isopropanol was added to each sample, mixed well, and samples were stored at -20°C for 1 h. Samples were then centrifuged for 20 min, 4°C, at 10 000 *g*. The pellet was washed with 70% ethanol, dried and finally re-suspended in 300-500 µl sterile dH_2O. Genomic DNA (5-15 ng) in 10 µl of ddH_2O was used for amplification of *Leishmania* genomic DNA.

Amplification of *Leishmania* DNA

Clinical isolates were characterized based on the size of their minicircle DNA as previously described [16]. 10 ng of parasite DNA was amplified using two primers (AJS3 and DBY). The primer position is on the conserved region of the minicircle DNA and encompassing part of the sequence of origin of replication. In amplification, these primers yielded a whole length minicircle sequence, which differs between the different *Leishmania* species. Another PCR was performed, in which the COII gene was targeted. In this PCR procedure 10 ng of genomic DNA was added to 46 µl of the PCR mix, which consisted of 3 µl l of each primer. The optimal conditions for COII amplification were 3 µl (15 pmol/µl) of each primer (forward: 5' GGCATAAATCCATG TAAGA 3'; reverse: 5' TGGCTTTTATATTATCATTTT 3'), 1U Taq polymerase, 2 µl dNTPs, 4 µl PCR buffer, 3 µl of 25mM MgCl2 and 31 µl of PCR H2O. PCR amplification was performed with a DNA thermocycler (PTC-100, MJ Research, Inc., Watertown, MA) with an initial denaturation step (4 minutes at 94°C), then 40 cycles of 94°C for 30 seconds, 45°C for 30 seconds (annealing), and 72°C for 2 minutes (extension).

The amplification reactions were analyzed by agarose gel electrophoresis, followed by ethidium bromide staining and visualization under UV light.

Heteroduplex Analysis of *Leishmania* Amplified DNA

Six ul of the COII amplification reaction of each *Leishmania* strain was transferred into 0.5 ml PCR tube. An equal volume of amplification reaction from the reference strains was added into the same tube. The two DNAs were mixed together and then the tubes were placed in the thermocyler. All *Leishmania* DNAs were tested against DNA of *L.infantum* and *L.donovani* reference strains and against each other. The tubes passed through different temperature intervals (94°C for 5 minutes, 4°C for 5 minutes) and finally left for 1 hour at 68oC. Heteroduplex reactions were run in 6% polyacrylamide gel supplemented with 3% (w/v) urea. The gel was pre-run for 30 minutes before samples loading, and then the gel was allowed to run 3 hours in a low-temperature atmosphere. The gel was silver-stained and bands were visualized under white light illumination.

Restriction Fragment Length Polymorphism (RFLP)

Five µl of the amplification reaction was digested with the enzyme *Ssp*1 according to the manufacturer's instructions (BioLabs Inc. USA). The product of the digestion was loaded onto 2% agarose gel stained with ethidium bromide. Electrophoretic analysis was carried out in polyacrylamide gel for 90 minutes after which the gel was visualized.

RESULTS

Prior to HDA, all samples were subjected to DNA amplification using species-specific primers (AJS3-DB8) that amplify a whole length minicircle gene [7]. DNAs yielded bands of a molecular weight of 800 bp, which corresponds to the *L.donovani* complex minicircle size, thus confirming the identity of the parasites analyzed (Fig. **1**). Reference strains are already characterized by isoenzymes as well as by DNA analysis. As well, HDA yielded extra slow moving bands with a MW of about 630-640 bp (Fig. **2**). The PCR amplification of COII with two maxicircle primers, yielded bands

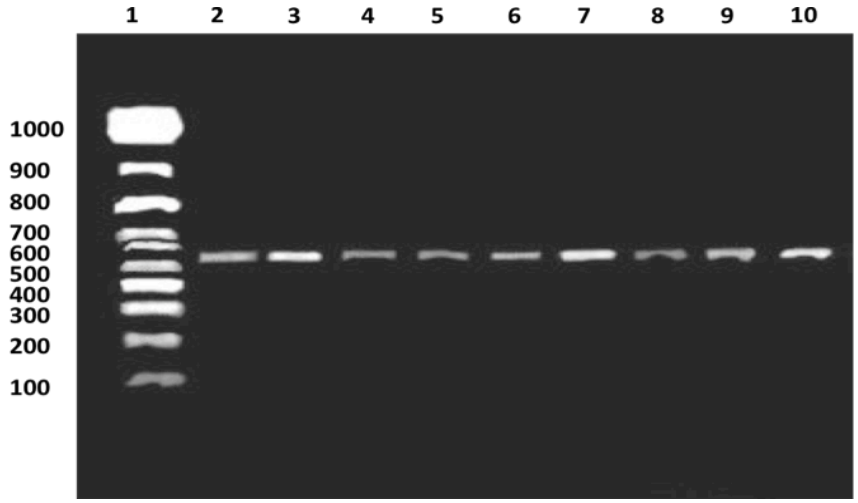

Fig. (1). PCR result of field isolates in addition to a reference strains using species-specific minicircle primers. The PCR product size is approximately 540 bp. Lane 1: 1kb marker (ladder); Lane 2: REBEL02; Lane 3: LEM307; Lanes 4-10: field isolates from Sudan.

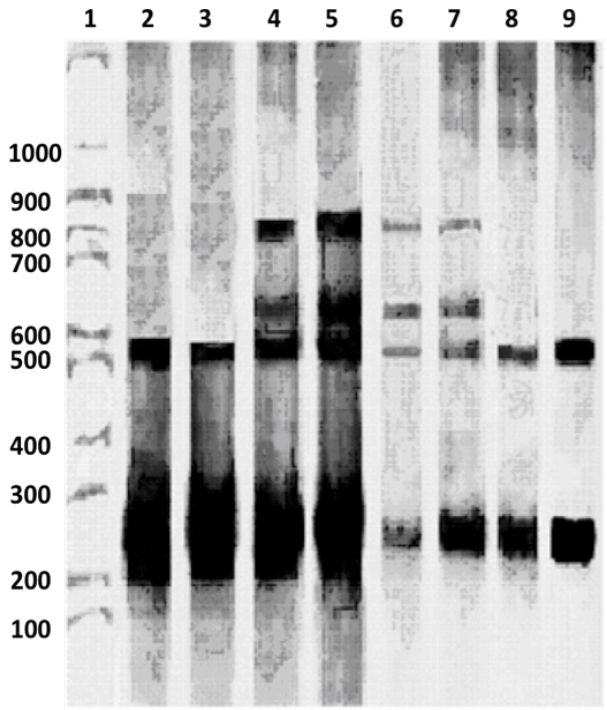

Fig. (2). Results of heteroduplex analysis of 2 *L.d..infantum* and 3 *L.dononvani* isolates from Sudan. Lane 1: 1Kb DNA marker; Lane 2: *L.donovani* 2+ *L.donovani* 3; Lane 3: *L.donovani* 1 *L.donovani* 2; Lane 4: LEM307+ *L.donovani* 3; Lane 5: LEM307+ *L.dononvani* 2; Lane 6: *L.donovani* 2 + REB2; Lane 7: LEM307+REB2; Lane 8: REB2; Lane 9: LEM307.

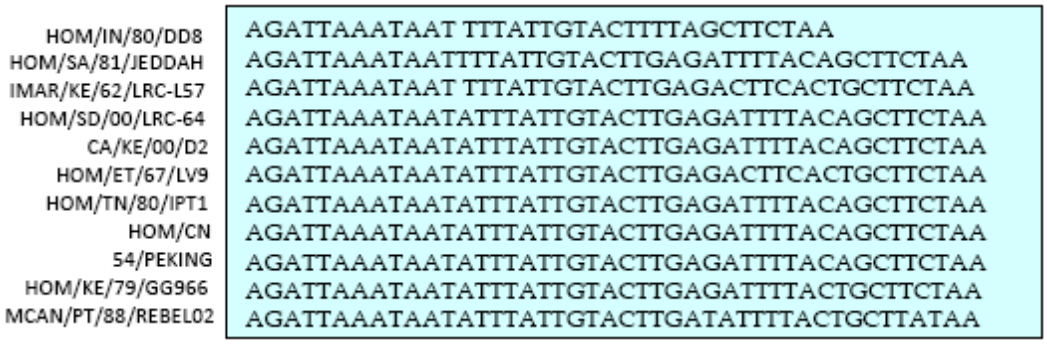

Fig. (3). Partial sequence of the COII gene from the various strains investigated by *Ibrahim and Barker* indicating the position of the polymorphic site that differentiates between members of the *L.donovani* and *L. infantum* subspecies. This G to A transition creates a two recognition sites for the enzyme S*sp1* in the infantum *versus* a single site already present downstream for the donovani.

of approximately 540 bp, corresponding to the main portion of the COII gene of the *L.donovani* complex (Fig. **2**). Based on a known A to G transition within the COII gene that differentiates between members of the *L.donovani* and *L.infantum* (Fig. **3**), we designed a RFLP assay using the enzyme *Ssp1* that readily differentiates between members of the two subspecies. The *Ssp1* cleaved the 540 bp *Leishmania* DNA PCR product at a single recognition site in the case of *L.donovani* and two recognition sites in the case of *L.infantum*, thus yielding different restriction size products (Fig. **4**).

DISCUSSION

We employed two characterization approaches in a consecutive manner to obtain maximum information on the infra-specific variation of *L.donovani* species complex, HDA and restriction fragment length polymorphism (RFLP). The formation of the heteroduplex or mismatched bases in the Sudanese *Leishmania* DNAs, in spite of the small number of tested isolates, highlights the sequence difference of the COII gene of these samples compared to reference strains. No mismatches were detected when the field strains were tested against each other, thus reaffirming the complete homology of the COII sequence of the clinical isolates. In contrast, the reference strains themselves produced heteroduplexes, indicating that they differ in their gene sequence by fewer bases (Fig. **2**). The results indicated that all isolates tested were in fact *L.donovani*, which is in conformity with previous findings from the area [7,8]. Independent reports based on isoenzyme typing, however, claim the presence of *L.infantum* in an area north of our field

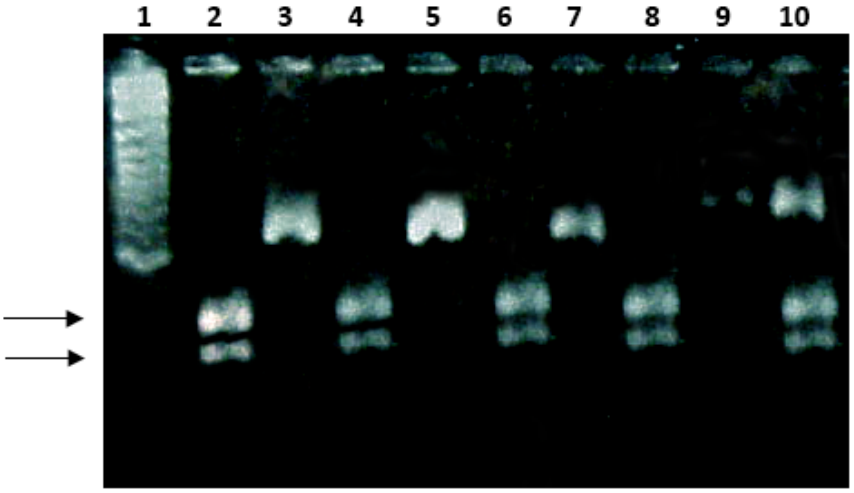

Fig. (4). RFLP using the enzyme Ssp1 on *L.infantum* reference strains (Lanes 2 and 3); *L.donovani* reference strains (Lanes 4 and 5); *L. donovani* field isolates (Lanes 6-10). Lane 1 is 1Kb DNA marker. The Ssp1 cleaves a 540 bp PCR product at a single recognition site in the case of *L.donovani* (upper arrow) and two recognition sites in the case of *L.infantum*, thus yielding different restriction size products (lower arrow).

site [17]. HDA is sensitive in detecting sequence differences down to a single base substitution [18, 19]. However, other similar techniques for identifying single base substitutions are available like single strand conformation polymorphism (SSCP) and DNA sequencing [20]. Data on species-specific PCR assay for *L.infantum/L.donovani* discrimination has recently been reported [21]. HDA is a reasonable choice due to its relatively straightforward procedure. The presence of extra bands, rather than the main gene fragment, is taken as an evidence for difference in the DNA sequence by at least a single base pair. The two methods of HDA and RFLP could be applied separately or in a complementary fashion depending on the objective set by the investigator. We believe that this is a simple, rapid and easy to perform method for characterization of *L.donovani* complex strains and with a potential to be extended to other *Leishmania* species.

ACKNOWLEDGEMENTS

This work was financially supported by the Wellcome Trust and The TDR/WHO grant No. M8/181/4/S.496. We thank Dr. Jill Seaman from the MSF-Holland her help in lymph node and bone marrow aspirates and for providing the clinical data.

ABBREVIATIONS

HAD = Heteroduplex analysis

RFLP = Restriction fragment length polymorphism

COII = Cytochrome oxidase

VL = Visceral leishmaniasis

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Received: September 20, 2010 Revised: October 14, 2010 Accepted: October 29, 2010