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Original

Genetic variation among Sudanese *Leishmania donovani*: origin and evolution

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Abstract

Introduction: Leishmaniasis is a geographically widespread severe disease, with an increasing incidence of two million cases per year, and 350 million people from 88 countries at risk. The causative agents are species of *Leishmania*, a protozoan flagellate. Visceral leishmaniasis, the most severe form of the disease, lethal if untreated, is caused by species of the *Leishmania donovani* complex. These species are morphologically indistinguishable but have been identified by molecular methods**.** This study aimed to explore intra specific diversity among Sudanese *L. donovani* strains and compare it to Ethiopian and Indian strains.

Methods: In this study Random Amplified Polymorphic DNA (RAPD) was used to detect intra specific diversity for the *Leishmania donovani* in ninety five *L.donovani* isolates collected from eastern Sudan.

Results& Discussion: this study found three different genotypes of Sudanese strains. The similarity between Sudanese strains and Ethiopian and Indian reference strains was measured. Diversity among Sudanese genotypes and the detection of one genotype closely related to the Indian and Ethiopian genotype: led to an evolutionary hypothesis for the origin and dispersal of the species. This proposes that the genus *Leishmania* may have originated in eastern Sudan.

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Conclusion: *Leishmania donovani* isolates from Sudan are genetically diverse. This parasite could be the ancestor of the *leishmania* parasites, and its distribution started from Sudan to all of the worlds.

Keywords:genetic distance, genetic diversity, *Leishmania donovani*, PCR-RAPD.

Introduction

Visceral Leishmaniasis (VL, Kala-azar) is a deadly parasitic disease caused by infection with protozoan parasites of the *Leishmania donovani* complex in east Africa and the Indian subcontinent and *Leishmania infantum* in Europe, North Africa and Latin America [1], this parasites transmitted by bites of phlebotomine sand flies (Diptera: Psychodidae) of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World [2, 3].

In Sudan, the main endemic region of kalaazar occurs in a wide belt extending from the east-central Sudanese-Ethiopian border to the west up to the White Nile [4, 5, 6].

The evolutionary relationships within the genus *Leishmania* and its origins are the source of ongoing debate, reflected in conflicting phylogenetic and biogeographic reconstructions. Noyes [7] has renewed the hypothesis of a Neotropical origin for the genus using arguments mainly based on the published gene sequence phylogenies. This hypothesis has been contested by Kerr [8] who instead proposed a Palearctic origin for *Leishmania* and suggested that the genus was only introduced into the Neotropics during the Pliocene after the formation of the Panamanian land bridge about 3 million years ago.

The supercontinent hypothesis reflects much better the available molecular phylogenetic data and was recently corroborated by phylogenomic reconstruction using new bioinformatics methods [9]. PCR was successfully amplified *L.donovani* DNA in ancient Egyptian and Christian Nubian mummies dating 4,000 years [10], this may support the hypothesis of East African origin. Molecular trees, fossil records, historical events and discoveries which associated with biogeographical, entomological and ecological evidence need to be consolidated to support some of the hypotheses regarding the origin of *Leishmania* and the resulting in human disease.

Molecular techniques such as PCR-restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD, and single-strand conformation polymorphism analyses(SSCP) have been used to demonstrate the genetic variability within and between different *Leishmania* species including *L. donovani* complex also they appear a partial correlation between genetic diversity and geographic origin [11, 12, 13]. In 2010 Gelanew [14] and co-worker found

a remarkably high genetic diversity among the East African strains of *L. donovani* which grouped into two genetically and geographically distinct populations comprising parasites from South Ethiopia and Kenya, and those from North Ethiopia and Sudan.

Several questions have marked the history and evolution of visceral leishmaniasis (VL) in East Africa, and Africa as the origin of migration of leishmania parasite.

In this paper we measured the similarity between the Sudanese *Leishmania* genotypes and Ethiopian, Indian reference strains as indicator to the origin of *Leishmania* parasites.

Materials and Methods

Ethical considerations and sample collection

The study protocol was approved by the Ethical Committee of the Institute of Endemic Diseases, University of Khartoum, Sudan. 95 *Leishmania* isolates were collected from lymph nodes/bone marrow aspirates, from patients from El-gadarif area in eastern Sudan. This was part of a routine investigative procedure for the diagnosis of visceral leishmaniasis. Part of the aspirate was smeared onto slides and stained with Giemsa stain and examined for the presence of Leishman Donovan bodies (LD bodies). Reference strains of Indian (DD8) and Ethiopian (TU3) *Leishmania donovani* were included in all tests (reference strains were donated from Pasteur Institute of IRAN) .

Isolation of parasites and cultivation

Parasites collected from VL patients were injected into culture bottles containing biphasic media (NNN) consisting of solid-phase agar mixed with defibrinated rabbit blood and overlaid with RPMI-1640 supplemented with 10% Foetal Calf Serum (FCS) and 1% of penicillin/streptomycin solution (10,000 units penicillin and 10 mg streptomycin). All cultures were incubated at 24°C and examined daily. After the promastigotes were built up, they were transferred into a 50 ml tissue culture flasks containing RPMI-1640 supplemented with 10% FCS and 1% penicillin/ streptomycin solution.

Preparation of total genomic DNA:

Late-log phase promastigotes were harvested from cultures by centrifugation at 2000 rpm and 4°C for 10 minutes. The pellet was then washed twice with cold Phosphate

Buffered Saline (PBS), pH 7.5 and stored at –20°C until used. DNA was isolated from leishmania parasites using the ZR Genomic DNA™-Tissue MiniPrep Kit (Zymo Research), according to the instruction of the manufacturer.

PCR amplification:

PCR was performed for minicircle kDNA to detect the leishmania species using a set of primers [AJS3 GGGGTTGGTGTAAAATAGGGand DBY CCAGTTTCCCGCCCCGGAG]. The reaction volume was 50 µl per sample in 0.2 ml thin walled micro centrifuge tube. The mixture contained 5 µl of 10X reaction buffer (Promega, Madison WI, USA) in a final concentration of 1X, 2 µl of 20 mM dNTPs mixture (0.2 mM each of dTTP, dATP, dCTP and dGTP), 3 µl of 25 mM MgCl2 (Promega, Madison WI, USA), 2.0 µl of primers mixture (1 µl of forward primer + 1 µl of reverse primer (50 mM) (Inqaba Biotechnical Industries (Pty) Ltd., South Africa), 0.25 µl of thermo-stable DNA polymerase (Promega, Madison WI, USA) (5 U/ µl) was added To each tube, 3 µl of template DNA were added, and the PCR mixture was completed to 50 µl with double distilled water. The PCR program was run for 35 cycles: initially denaturation at 94ºC for 3 min, denaturation at 94ºC for 30 s, annealing at 64ºC for 1 min, extension at 72ºC for 1 min and a final extension cycle at 72ºC for 10 min was also included. Amplification was checked by 1.5% agarose gel electrophoresis followed UV visualization.

PCR-RAPD

RAPD amplification was done using 11 single short primers, the primers were selected randomly from literature review with the requirement that their C+G contents are 60% to 70% (as the Leishmania genomes CG rich) and that they have no self-complementary ends.(Table 1). Each 25 µl RAPD reaction contained 5ng of genomic DNA, 3 mM $MgCl_2$, 25 pmol of primer, 20 mM dNTPs mixture and 0.5 units of *Taq* polymerase in the appropriate buffer. The amplification cycle was 94°C for Three minutes; then 35 cycles at 94°C for one minute, 42°C for one minute, and 72°C for two minutes; and at 72°C for 10 minutes. The RAPD products were separated by electrophoresis on ethidium bromide-stained 1.5% agarose gels in 0.5× Tris-acetate-EDTA buffer (20 mM Tris-acetate, 0.5 mM EDTA) at 80 V for two hours.

Seven primers had a sufficient number of scorable amplicons and good reproducibility, tested by repeating most reactions, although profiles were reproducible. High-quality DNA was always used because degradation of DNA altered amplification profiles. The RAPD profiles were manually scored as presence or absence bands. Then the jaccard's similarity coefficient was used to detect the similarity between strains. Jaccard's coefficient was calculated by the following formula [15]:

$$Sj = \frac{a}{a+b+c}$$

Where a is the sum of agreements (+ +), while b and c represent the sums of absent/present combinations (i.e. +/_, and _/+, respectively).

Results

Detection of Lesihmania species:

All Sudanese strains showed similar band patterns to the reference L. donovani strains (Indian strain DD8, and Ethiopian strain TU3) with an 800 bp band size Fig 1.

RAPR-PCR profiles of the *Leishmania donovani* isolates

Isolated DNA from 95 *leishmania donovani* promastigotes strains characterized by minicircle kDNA, and the reference strains of *L.donovani* species (DD8 Indian strain and TU3 Ethiopian strain), were screened by eleven of RAPD primers.

Not all random primers could efficiently and specifically amplify portions of the genome of the parasites.

Three primers were differentiated between the Sudanese *L.donovani* isolates and the Indian and Ethiopian reference strains, four primers (A13, M13/pUC, OPA2 and OPA9) give same band patterns in all isolates.

A-12 primer divided the Sudanese strains to three different genotype, genotype 1 was common between the Sudanese isolates, Jaccard's similarity between this genotype and Ethiopian reference strains (TU3) was 0.16. Genotype two was closely related to Ethiopian reference strain (JSC=1), while genotype 3 appeared 0.4 of similarity with the Ethiopian reference strain genotype.

Similarity of Indian *Leishmania donovani* reference strain genotype with the Sudanese genotype 1 was 0.4, 0.3 for genotypes 2 and 0.16 for genotype 3.

Table 2 showed the similarity between three Sudanese genotypes. The Jaccard's similarity coefficient between DD8 the

Indian strain and TU3 Ethiopian strain was 0.3.

Figure 2 showed the phylogenetic tree for the Sudanese strains and Indian and Ethiopian reference strain using A-12 primer.

The results of Univ mini primer showed there was no differences between the Indian and Ethiopian strains (JSC=1). And also this primer divided the Sudanese strains to three genotypes, genotype 1 which is not common (only 15 isolates) was similar to Indian and Ethiopian strains (JSC= 1), while the similarity for the genotype 2 was 0.5, and 0.6 for genotype 3. The similarity between the Sudanese genotypes was appeared in (table 3). Figure 3 explain the phylogenetic relationship between the Indian and Ethiopian reference strains.

IL-0875 primer divided the Sudanese strains into two genotype; JSC for genotype two with Ethiopian reference strain genotype was 1, and 0.75 for genotype 1.The similarity between this genotypes and Indian reference strains was 0.8 and 0.6 respectively. And the JSC between two Sudanese genotype was 0.75. Fig 4 showed the phylogenetic relationship between the Sudanese and Indian and Ethiopian genotype.

Other RAPD primers failed to amplify *leishmania* isolates and reference strains.

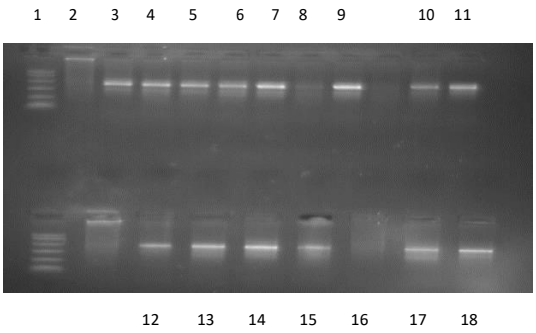

Fig 1: Band patterns of the study isolates using Kinetoplast DNA & species-specific minicircle primers (AJS3 & DBY) PCR and electrophoresis in 1.5% Agarose gel. Lane 1 Mwt DNA maker, Lane 2 in the first and second row negative control, Lane 3 Indian reference strain (DD8), lane 4 Ethiopian reference strain, Lane 5-18 Sudanese stains

Table 1: List of 11 random primers used in RAPD analysis.

OPA2	5'-TGCCGAGCTG-3'
*OPA5	5'-AGGGGTCTTG-3'

*OPA7	5'-GAAACGGGTG-3'
*OPA8	5'-GTGACGTAGG-3'
OPA9	5'-GGGTAACGCC-3'
*OPA10	5'-GTGATCGCAG-3'
M13/pUC Forward Sequencing Primer	5'-CGCCAGGGTTTTCCCAGTCACGA-3'
Universal Minicircle Sequence	5'-GGGGTTGGTGTA-3'
5'- TCGGCGATAG-3'	
5'- CAGCACCCAC-3'	
IL-0875	5'- GTCCGTGAGC-3'

*These primers did not amplify leishmania isolates and reference strains.

Table 2: Jaccrd's similarity coefficient between three Sudanese genotypes (using A12 primer)

Sudanese genotypes	JSC
Genotype 1 and genotype 2	0.16
Genotype 2 and genotype 3	0.4
Genotype 1 and genotype 3	0.66

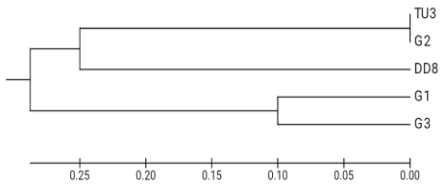

Fig 2: The relationship between the Sudanese strains (G1, G2, G3) and Indian (DD8) and Ethiopian (TU3) reference strain using A-12 primer.

Table 3: Jaccrd's similarity coefficient between three Sudanese genotypes (using Univ mini primer)

Sudanese genotypes	JSC
Genotype 1 and genotype 2	0.5
Genotype 2 and genotype 3	0.3
Genotype 1 and genotype 3	0.6

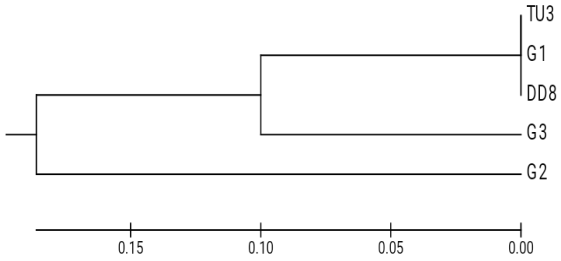

Fig 3: Phylogenetic tree for the Sudanese strains (G1, G2, G3) and Indian (DD8) and Ethiopian (TU3) reference strain (using univ mini primer).

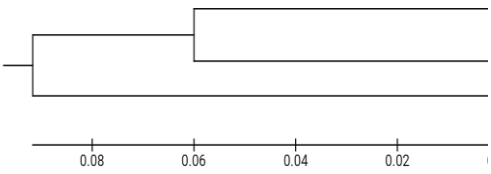

Fig 4: phylogenetic tree for the Sudanese strains (G1, G2) and Indian (DD8) and Ethiopian (TU3) reference strain using IL-0875 primer.

Discussion

Leishmnai donovani parasites that cause visceral leishmaniasis are very diverse especially in East Africa [16]. Several trends have emerged from dozens of analyses. They include a partial correlation between genetic diversity and geographic origin [17], some flexibility in host specificity, hybrid genotypes and mixed infections of strains were assigned to different species [18, 19].

A number of methods have been applied to or developed in order to study genetic diversity and relationships within *Leishmania.*Among these, Random amplified polymorphic DNA (RAPD) was used to detect intra- specific diversity for the *Leishmania donovani* complex [11]. Therefore, eleven RAPD primers were used in the present study for elucidating the genetic diversity among the Sudanese *L.donovani* strains, and for comparing the similarity between these strains and the Indian and Ethiopian *L.donovani* reference strains.

The PCR-RAPD band patterns of the wild isolates could clearly demonstrate that *L.donovani* isolates that causes VL in Sudan are genetically diverse. Different genotypes (clusters) could be identified with different primers. The variety of drug responses may result from this diversity within *L.donovani* isolates [17, 20].

univ mini RAPD primer which is a 10 decamer oilgonucleotides, were designed to monitor the minicircle gene, detected a commongenotype in Sudanese, Ethiopian and Indian strains. And the similarity between the other Sudanese genotype and India and Ethiopian strains was 0.5 and 0.6. Accordingly the minicircle kDNA gene size band might differentiate between the leishmania species, but cannot detect the intra-specific variation within the genes.

Using Jaccard's similarity coefficient, this study found that one of Sudanese genotype was closely related to the Ethiopia strain (JSC=1), while it is very distant from Indian strain, this may support the divided of East African *L.donovani* strains into two genetically and geographically distinct populations comprising parasites from South Ethiopia and Kenya, and those from North

Ethiopia and Sudan [14, 21]. This two genetic population of parasites corresponding to the different vector species in this area, in Eastern Sudan and North Ethiopia VL transmitted by *Phlebotomus orientalis* , while in Kenya and South Ethiopia VL transmitted by *P. martini* and *P. celiae [1].*

This study also seek to elucidate the evolutionary history of the genus *Leishmania*, a parasitic protozoan of great public health significance. Our analysis represents one of the most extensive attempts to examine intra- and inter- specific genetic diversity in a group of parasites. It has been proposed that the genus *Leishmania* first appeared in the Old World [10, 22, 23]. Sudan is one of the highly endemic countries for visceral leishmaniasis or kala-azar, which is thought to have originated in East Africa and later spread to the Indian subcontinent and New World [23, 24]. This is supported by presence of phylogenetic relationship between Sudanese genotypes and Indian and Ethiopian reference strains characterized in this study. Also Ibrahim [25] reported that the analysis of the mitochondrial DNA has shown that the disease in Sudan is caused by a single mitochondrial haplotype found in parasites isolated from animals and humans during the 196Os, 1980s and 1990s in both eastern and southern Sudan. This haplotype is quite different from that of all other parasites world-wide.

An African origin for the visceralizing species of the *L.donovani* complex has also been argued by Ashford [26] and Ngure [27]. They have suggested an ancient cluster derived from an ancestral root stock in the Sudan from which all other forms of the complex have derived. In this study,we proved that the common *L. donovani*genotype in eastern Sudan are different from Ethiopian and Indian strain, and there is a genotype closely related to Ethiopia strain, also we found one genotype common in Sudanese, Ethiopian and Indian strains. This may have been due to the introduction of the Sudanese strains to India by the slave trade from Africa to India [23, 28, 29]. By study the ITS gene, Kuhls and co-worker [30], reported three distinct groups of strains in East Africa: two groups from Sudan/Ethiopia and one group from Kenya. One of the Sudanese groups is more closely related to the Indian/Kenyan strains than to the other group from Sudan. Ancestral parasites of the *L. donovani* Indian reference strain DD8 may have arrived with early migrations through Arabia from Africa as the isolate from Arabia (Jeddah) that shows an intermediate haplotype between the Indian and the Sudanese isolates [29]. Man is believed to have originated in Africa and it is reasonable to consider that anthroponotic parasites such as members of the *L. tropica* and (*L. donovani*) complex which have evolved with him may also have originated there[23]. The migration started from Sudan to Ethiopia then India through Kenya, this explain the similarity of Indian and Kenyan strain and Kenyan and south

Ethiopia strains that are different from the Sudanese and north Ethiopian strains. The present study revealed the presence of remarkable genetic heterogeneity among East African strains of *L. donovani*. This study also sheds some light on understanding of the population structure and reproductive pattern of East African *L. donovani*. This information, together with future epidemiological and population genetic studies will be very useful to design parasite-targeted control strategies to eradicate VL in East Africa.

Conclusion & Recommendation(s)

In conclusion*: Leishmania donovani* isolates from Sudan are genetically diverse. This parasite could be the ancestor of the *leishmania* donovani parasites, and migrated from Sudan to all the worlds.

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