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Full Length Research Paper

Leishmania: Probable genetic hybrids between species in Sudanese isolates

Hamad S. H.[1], Ahmed M. Musa[1], Eltahir A. G. Khalil[1], Tamrat Abebe[2], Brima M. Younis[1], Mona E. E. Elthair[1], Ahmed M. EL-Hassan[1], Asrat Hailu[2] and Aldert Bart[3]*

[1]The Leishmaniasis Research Group, Sudan, Institute of Endemic Diseases, University of Khartoum, Sudan.
[2]Department of Microbiology and Immunology, Faculty of Medicine, Addis Ababa University, Ethiopia.
[3]Department of Medical Microbiology, Parasitology Section, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center, Amsterdam, The Netherlands.

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The horn of Africa bears the prunt of Leishmaniasis in the world where both *Phlebotomus orientalis*, the vector of *Leishmania donovani* and *Phlebotomus papatasi*, the vector of *Leishmania major*, exist. Visceral leishmaniasis (VL) caused by *L. donovani* is a major cause of morbidity and mortality in east Africa. The populations of east Africa are very dynamic in continuous movements between endemic areas of different leishmaniasis clinical forms. Molecular epidemiological studies have provided indirect evidence for genetic exchange between different strains of one Leishmania species. Only recently, genetic exchange between different Leishmania strains of one species was definitively demonstrated *in vitro*. Genetic exchange between different *Leishmania* species is probably an even rarer event. In this study, one hundred and seven *L. donovani* isolates were collected from lymph nodes/bone marrow aspirates, from patients from Sudan and Ethiopia. DNA was isolated from leishmania parasites using the High Pure PCR Template Preparation Kit. PCR products were sequenced using BigDye Terminator chemistry and analyzed using the ABI 3100 or 3730 Genetic Analyzers. Sequences were analyzed using CodonCode program (CodonCode Corporation) and MEGA. Sequences were submitted to Genbank and are accessible under accession numbers HM117696-HM117699. Our data showed hybridization between two divergent *Leishmania* species, *L. donovani* and *L. major*, possibly due to migration. Such hybridization may have clinical implications with respect to parasite fitness, vector adaptation and response to treatment.

Key words: *Leishmania donovani*, *Leishmania major*, *Leishmania infantum*, Visceral leishmaniasis (VL), leishmaniasis, *Phlebotomus orientalis*, *Phlebotomus papatasi*.

INTRODUCTION

Visceral leishmaniasis (VL) caused by *Leishmania donovani* is a major cause of morbidity and mortality in Sudan. It is principally a disease of children that is complicated in the majority of cases by a dermatosis known as post-kala-azar dermal leishmaniasis (PKDL) (Zijlstra and El-Hassan, 2001). Case detection and drug treatment is the only available method of control with emerging clinical resistance to anti-leishmanial compounds (Khalil et al., 1998). Algadarif state (Eastern Sudan) is an endemic area of VL. About a third of Algadarif population migrated from western Sudan in the mid-eighties, a known endemic focus for cutaneous leishmaniasis due to *Leishmania major*. Both *Phlebotomus orientalis*, the vector of *L. donovani* and *Phlebotomus papatasi*, the vector of *L. major*, exist in Eastern Sudan (Elnaiem et al., 1998).

The existence and the role of genetic exchange in *Leishmania* has been under debate for the last decades

*Corresponding author. E- mail: a.bart@amc.uva.nl. Tel: + 31-20-5663189. Fax: + 31-20-6979271.

Abbreviations: PKDL, post-kala-azar dermal leishmaniasis; **L.D,** leishman donovan bodies; **NNN,** Novy, Macneal, Nicolle; RPMI, Rosuell park memorial institute; **FCS,** foetal calf serum; **DNA,** deoxyribonucleic acid; **PBS,** phosphate buffered saline; **PCR,** polymerase chain reaction; **Cpb**, cysteine protease B; **MER,** mini-exon repeat; **ITS-1,** internal transcribed spacer; **UV,** ultra violet.

(Tibayrenc et al., 1990, 1991, 2002). Although, the consensus seems that on a population level, *Leishmania* is essentially clonal, this does not rule out the possibility of genetic exchange (Banuls et al., 1999, 2002; Tibayrenc and Ayala, 1999). There have been reports that suggested hybridization within species (Chargui et al., 2009; Lukes et al., 2007; Schwenkenbecher et al., 2006), but only recently, this was demonstrated in the vector, experimentally (Akopyants et al., 2009). Several studies reported the isolation of New World strains that have been characterized as putative hybrids between different, though closely related, *Leishmania* subgenus *Viannia* species (Banuls et al., 1997; Belli et al., 1994; Delgado et al., 1997; Dujardin et al., 1995; Nolder et al., 2007). In the Old World, a hybrid between the closely related *L. infantum* and *L. donovani* was found (Hide and Banuls 2006). A remarkable study reported genetic hybridization between *L .infantum* and *L. major* among isolates from immunocompromised patients in Portugal (Ravel et al., 2006). Although, this isolate has been characterised extensively, including its enhanced transmission potential and fitness (Volf et al., 2007), it is puzzling how a hybrid arose in a country where only *L. infantum* is endemic.

In this study, we provide evidence for genetic hybrids between two divergent *Leishmania* species, *L. donovani* and *L. major*, among isolates from Eastern Sudan.

MATERIALS AND METHODS

Leishmania isolates

107 *Leishmania* isolates were collected from lymph nodes/bone marrow aspirates, from patients from different geographical areas in Sudan and Ethiopia. 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 *Leishmania* were included in all tests.

Isolation of parasites and cultivation

Lymph-node/bone marrow aspirates 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 min. 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 or clinical samples using the High Pure PCR Template Preparation Kit, (Roche), according to the instruction of the manufacturer.

PCR amplification

PCR was performed as described previously for the targets cpb (Hide et al., 2006), spliced leader RNA or mini-exon repeat (MER) sequences. MER) (Marfurt et al., 2003), ribosomal DNA internal transcribed spacer (ITS-1) sequences (Schonian et al., 2003), and repetitive DNA sequences (LEG) (Piarroux et al., 1995). Amplification was checked by 1% agarose gel electrophoresis followed by ethidium bromide staining and UV visualization.

DNA sequencing and data analysis

PCR products were sequenced using BigDye Terminator chemistry (Applied Biosystems) and analyzed using the ABI 3100 or 3730 Genetic Analyzers according to the manufacturer's instructions. Sequences were analyzed using CodonCode program (CodonCode Corporation) and MEGA (Tamura et al., 2007). Sequences were submitted to Genbank and are accessible under accession numbers HM117696-HM117699.

RESULTS

During an epidemiological study of East African visceral leishmaniasis, 107 clinical isolates were characterized by RFLP of ITS, MER, and the LEG repeat PCR products and size of cpb gene. *V*isceral *leishmaniasis* is caused by *L. donovani* and *L. infantum*, which are closely related. By cpb gene size, all isolates were identified as *L. donovani.* To our surprise, for the three other markers we found three isolates that had unexpected RFLP patterns, similar to those of L. major. To confirm these findings, sequence analyses were performed. These confirmed that for these markers, sequences from three isolates were more similar to *L. major* than to *L. donovani* or *L. infantum*.

Isolates 1559, S3, and 12.15 g contained LEG sequences similar to L. major (Figure 1). Isolate ROB1 also showed a MER sequence similar to L. major, and also, its ITS sequence was similar to that of *L. major*. The individual sequence traces showed no evidence of mixed signals at the polymorphic positions.

DISCUSSION

The possibility of contamination with *L. major* amplicons, DNA or isolates is highly unlikely, as; i) for each DNA preparation, only a minority of markers showed *L. major*-like alleles, whereas others were *L. donovani*-complex like, ii) different markers showed *L. major*-like patterns, iii) the most abundant *L. major*-like marker, MER, had polymorphisms not previously encountered among 89 *L. major* strains typed in the (adjacent) clinical laboratory. It

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L.donovani|AY185121 ATGGGCGAAG GGGGACGGCG GGAGCGGCAG AGAGAGCGCG GGCACACAGC  [ 50]
SLEG22              .t........ ....a..... .......... .......... ..........  [ 50]
L.major|L42501      .......... ........T. .......G.A .....CGA.. .....GTG..  [ 50]
LEG_12.15g          .t........ ....a...T. .......G.A .....CGA.. .....GTG..  [ 50]
LEG_S3              .t........ ....a...T. .......G.A .....CGA.. .....GTG..  [ 50]
L.tropica|L42495    ...C...... ....G...T. .......G.. .....C.... .....GTG..  [ 50]
LEG_1559            a......... ....a...T. .......G.. .....C.... .....GTG..  [ 50]

L.donovani|AY185121 GACGTCCGTG GAAAGAAAAA AAGAGAAGAC AACGCGTATT CCCTTCTGCT  [100]
SLEG22              .......... .......... .......... .......... ..........  [100]
L.major|L42501      C...C..... .......CG. ..--.T...A G......... .....T....  [100]
LEG_12.15g          C...C..... .......CG. ..-..T...A G......... .....T....  [100]
LEG_S3              C...C..... .......CG. ..-..T...A G......... .....T....  [100]
L.tropica|L42495    .......... ...CC..... ..-..C...A G...A..... .....T....  [100]
LEG_1559            .......... ...CC..... ..-..C...A G...A..... .....T....  [100]

L.donovani|AY185121 AATGTGTACC CGCCTCTCTG CCACAGATCA CGAGGTCAGC TCCACTCCAC  [150]
SLEG22              .......... .......... .......... .......... ..........  [150]
L.major|L42501      G......G.. .......... .......... ....C..... ..........  [150]
LEG_12.15g          G......G.. .......... .......... ....C..... ..........  [150]
LEG_S3              G......G.. .......... .......... ....C..... ..........  [150]
L.tropica|L42495    G......GA. .A........ .......... ....C..... ...C......  [150]
LEG_1559            G......GA. .A........ .......... ....C..... ...C......  [150]

L.donovani|AY185121 CCTAACGCCT CCCCCGCGCA GCCCTGTCAC ACGCT [185]
SLEG22              .......... .......... .......... ..... [185]
L.major|L42501      .......... .......A.G .......... .G... [185]
LEG_12.15g          .......... .......A.G .......... .G... [185]
LEG_S3              .......... .......A.G .......... .G... [185]
L.tropica|L42495    .......... .........G .......... .G... [185]
LEG_1559            .......... .........G .......... .G... [185]
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Figure 1. Alignment of LEG sequences of *L. donovani*, *L. major* and *L. tropica* reference sequences and sequences for strains S3, 12.15g and 1559. For comparison, the sequence obtained for one of the other *L. donovani* strains is given.

should be stressed that this does not imply that the isolates concerned are *L. major*, rather *L. donovani* strains that for some markers have *L. major*-like characteristics. The most plausible explanations that we present are: a subpopulation of *L. donovani* complex strains in Sudan has recently acquired certain alleles from *L. major*, or these alleles have already been present in the *L. donovani* population, but were not detected previously because only limited numbers of isolates were extensively investigated from this area. It should be noted that the putative hybrids are not heterozygous isolates in the strict sense, which is probably due to the fact that the relevant markers are non-coding. The first possibility, recent genetic exchange between *L. donovani* and *L. major*, is supported by the suggestion of Lukes et al., 2007, who suggested a high frequency of genetic exchange among *Leishmania* strains in Sudan, even if their prevailing mode of reproduction is clonally. If genetic exchange takes place, and crosses species borders, as suggested by our and other's data, this will be relevant with respect to future drug resistance development. If drug resistance develops, and genetic exchange takes place, resistance is expected to spread faster. It would be interesting to explore this possibility further, to clarify the extent and the mechanism of genetic exchange between these parasites. The second possibility, which implies bias in previous strain collection, could also account for the *L. infantum- L. major* hybrids reported from Portugal as *L. major* is not known to occur in this country, Ravel *et al.,* 2006. Finally, our data show that reliance on single markers is apparently not sufficient for species determination of isolates from this geographical area, this has implications for laboratories that rely on single markers for diagnosis in settings where different

Leishmania species can be encountered.

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REFERENCES

Akopyants NS, Kimblin N, Secundino N, Patrick R., Peters N, Lawyer P (2009). Demonstration of genetic exchange during cyclical development of Leishmania in the sand fly vector. Science, 324: 265-268.

Banuls AL, Guerrini F, Le PF, Barrera C, Espinel I, Guderian R (1997). Evidence for hybridization by multilocus enzyme electrophoresis and random amplified polymorphic DNA between Leishmania braziliensis and Leishmania panamensis/guyanensis in Ecuador. J. Eukaryot. Microbiol., 44: 408-411.

Banuls AL, Hide M, Tibayrenc M (1999). Molecular epidemiology and evolutionary genetics of Leischmania parasites. Int. J. Parasitol., 29: 1137-1147.

Banuls AL, Hide M, Tibayrenc M (2002). Evolutionary genetics and molecular diagnosis of Leishmania species. Trans. R. Soc. Trop. Med. Hyg., 96(1): S9-13.

Belli AA, Miles MA, Kelly JM (1994). A putative Leishmania panamensis/Leishmania braziliensis hybrid is a causative agent of human cutaneous leishmaniasis in Nicaragua. Parasitology, 109(Pt 4): 435-442.

Chargui N, Amro A, Haouas N, Schonian G, Babba H, Schmidt S (2009). Population structure of Tunisian Leishmania infantum and evidence for the existence of hybrids and gene flow between genetically different populations. Int. J. Parasitol., 39: 801-811.

Delgado O, Cupolillo E, Bonfante-Garrido R, Silva S, Belfort E, Grimaldi JG (1997). Cutaneous leishmaniasis in Venezuela caused by infection with a new hybrid between Leishmania (Viannia) braziliensis and L. (V.) guyanensis. Mem. Inst. Oswaldo. Cruz., 92: 581-582.

Dujardin JC, Banuls AL, Llanos-Cuentas A, Alvarez E, DeDoncker S, Jacquet D, Arevalo J, Tibayrenc M (1995). Putative Leishmania hybrids in the Eastern Andean valley of Huanuco, Peru. Acta. Trop., 59: 293-307.

Elnaiem DA, Connor SJ, Thomson MC, Hassan MM, Hassan HK, Aboud MA (1998). Environmental determinants of the distribution of Phlebotomus orientalis in Sudan. Ann. Trop. Med. Parasitol., 92: 877-887.

Hide M, Banuls AL (2006). Species-specific PCR assay for L. infantum/L. donovani discrimination. Acta. Trop., 100: 241-245.

Khalil EA, El Hassan AM, Zijlstra EE, Hashim FA, Ibrahim ME, Ghalib HW (1998). Treatment of *V. leishmaniasis* with sodium stibogluconate in Sudan: management of those who do not respond. Ann. Trop. Med. Parasitol., 92: 151-158.

Lukes J, Mauricio IL, Schonian G, Dujardin JC, Soteriadou K, Dedet JP (2007). Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. Proc. Natl. Acad. Sci, U S A., 104: 9375-9380.

Marfurt J, Nasereddin A, Niederwieser I, Jaffe CL, Beck HP, Felger I (2003). Identification and differentiation of Leishmania species in clinical samples by PCR amplification of the miniexon sequence and subsequent restriction fragment length polymorphism analysis. J. Clin. Microbiol., 41: 3147-3153.

Nolder D, Roncal N, Davies CR, Llanos-Cuentas A, Miles MA (2007). Multiple hybrid genotypes of Leishmania (viannia) in a focus of mucocutaneous Leishmaniasis. Am. J. Trop. Med. Hyg., 76: 573-578.

Piarroux R, Fontes M, Perasso R, Gambarelli F, Joblet C, Dumon H (1995). Phylogenetic relationships between Old World Leishmania strains revealed by analysis of a repetitive DNA sequence. Mol. Biochem. Parasitol., 73: 249-252.

Ravel C, Cortes S, Pratlong F, Morio F, Dedet JP, Campino L (2006). First report of genetic hybrids between two very divergent Leishmania species: Leishmania infantum and Leishmania major. Int. J. Parasitol., 36: 1383-1388.

Schonian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HD, Presber W (2003). PCR diagnosis and characterization of Leishmania in local and imported clinical samples. Diagn. Microbiol. Infect. Dis., 47: 349-358.

Schwenkenbecher JM, Wirth T, Schnur LF, Jaffe CL, Schallig H, Al-Jawabreh A (2006). Microsatellite analysis reveals genetic structure of Leishmania tropica. Int. J. Parasitol., 36: 237-246.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol., 24: 1596-1599.

Tibayrenc M, Ayala FJ (1991). Towards a population genetics of microorganisms: The clonal theory of parasitic protozoa. Parasitol. Today, 7: 228-232.

Tibayrenc M, Ayala FJ (1999). Evolutionary genetics of Trypanosoma and Leishmania. Microbes. Infect., 1: 465-472.

Tibayrenc M, Ayala FJ (2002). The clonal theory of parasitic protozoa: 12 years on. Trends. Parasitol., 18: 405-410.

Tibayrenc M, Kjellberg F, Ayala FJ (1990). A clonal theory of parasitic protozoa: the population structures of Entamoeba, Giardia, Leishmania, Naegleria, Plasmodium, Trichomonas, and Trypanosoma and their medical and taxonomical consequences. Proc. Natl. Acad. Sci. U S A., 87: 2414-2418.

Volf P, Benkova I, Myskova J, Sadlova J, Campino L, Ravel C (2007). Increased transmission potential of Leishmania major/Leishmania infantum hybrids. Int. J. Parasitol., 37: 589-593.

Zijlstra EE, El-Hassan AM (2001). Leishmaniasis in Sudan. Visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg., 95(1): S27-S58.