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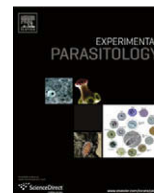

Leishmania donovani: Genetic diversity of isolates from Sudan characterized by PCR-based RAPD

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ABSTRACT

Drug unresponsiveness in patients with visceral leishmaniasis (VL) is a problem in many endemic areas. This study aimed to determine genetic diversity of *Leishmania donovani* isolates from a VL endemic area in Sudan as a possible explanation for drug unresponsiveness in some patients. Thirty clinically stibogluconate (SSG)-sensitive isolates were made SSG-unresponsive *in vitro* by gradually increasing SSG concentrations. The sensitive isolates and their SSG-unresponsive counterparts were typed using mini-circle kDNA and categorized using PCR–RAPD. All the isolates were typed as *L. donovani*, the resulting PCR–RAPD characterization of the SSG-sensitive isolates gave three distinct primary genotypes while, the SSG-unresponsive isolates showed only a single band. *L. donovani* isolates from eastern Sudan are diverse; this probably resulted from emergence of new *L. donovani* strains during epidemics due to the pressure of widespread use of antimonials.

In this communication the possible role of isolates diversity in antimonial unresponsiveness and the *in vitro* changing PCR–RAPD band pattern in SSG-unresponsive strains were discussed.

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1. Introduction

Leishmaniasis is one of the most prevalent infective protozoan diseases in developing countries where rural populations are most vulnerable. The disease phenotypes range from subclinical infection to overt visceral disease (WHO, 1996; El-Hassan and Zijlstra, 2001; Abdalla et al., 2001, 2002). Visceral leishmaniasis (VL) due to *Leishmania donovani*, has been an important health problem in Sudan since the early 1900s (Kirk and Sati, 1940; El-Hassan and Zijlstra, 2001). Parasites isolated from humans and sand flies from the endemic area were reported to be genetically diverse and belong to the *L. donovani sensu lato* cluster (Ibrahim et al., 1994). Unresponsiveness to antimonials in VL endemic areas is a major impediment to treatment (Peters et al., 1990; Ibrahim et al., 1994; Khalil et al., 1998). The mechanisms of unresponsiveness to Pentavalent antimonial compounds have not been fully elucidated. Concurrent diseases, changes in the pharmacokinetics of the drug, drug purity, uses of suboptimal concentrations and depressed immune status, have been reported as reasons for apparent resistance/treatment failure (Peters et al., 1990; Farant-Gambarelli et al., 1997; Khalil et al., 1998; Croft et al., 2006). On the other hand, *in vitro* studies have shown that drug unresponsiveness can be related to reduced drug influx/increased drug efflux or qualitative/quantitative changes in the drug targets (Gamarro et al., 1994; Essodaigui et al., 1999; Legare et al., 2001).

This study aimed to determine the genetic diversity of Sudanese *L. donovani* isolates as a possible cause for emerging sodium stibogluconate (SSG) unresponsiveness using PCR and RAPD technique.

2. Materials and methods

2.1. Ethical considerations and sample collection

The study protocol was approved by the Ethical Committee of the Institute of Endemic Diseases, University of Khartoum, Sudan. Thirty *Leishmania* isolates were collected from lymph nodes/bone marrow aspirates as part of routine parasitological investigation from patients suspected of having visceral leishmaniasis after each patient or his/her legal guardian had had signed a written informed consent to participate in this study. Reference strains of *L. donovani* were included in all tests.

2.2. Leishmania isolate cultivation

Leishmania parasites were isolated from lymph-node aspirates injected into culture bottles containing biphasic media (NNN) consisting of solid-phase agar mixed with defibrinated rabbit blood

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Table 1
Number of parasites remaining after each culture exposed to specific SSG concentrations.

SSG concentration (mg/ml)	Number of parasites remaining after exposed to specific SSG conc.
0.05	2.5×10^6 (No parasites were dead)
0.15	2×10^6
0.45	1.8×10^6
1.35	800,000
4.05	2.2×10^6
12.15	2.4×10^6
36.45	0 (All parasites were dead)

and overlaid with RPMI-1640 supplemented with 10% Foetal Calf Serum (FCS) and 1% of penicillin/streptomycin solution (10,000 U penicillin and 10 mg streptomycin). All cultures were incubated at 24 °C. The promastigote suspensions (2.5×10^6) were exposed to gradually increasing concentrations of sodium stibogluconate (SSG, Albert David Limited, Calcutta, India) for 12 h at 24 °C. Following 12-h incubation, cultures were centrifuged at 2800 rpm for 10 min at 4 °C and the parasite pellets were re-suspended in fresh medium supplemented with 20% FCS. On day 15, live parasites were expanded to early log phase ($10–15 \times 10^6$ promastigotes/ml) to be exposed again to the next higher concentration of SSG (Table 1). Control cultures were maintained in drug-free complete medium simultaneously with each batch (Hassab Elgawi, 2005).

2.3. Kinetoplast DNA amplification

Typing was done for all isolates (SSG-exposed and non-exposed). PCR was performed on total genomic DNA extracted from promastigotes using a set of species-specific primers for mini-circle kinetoplast kDNA [AJS3 5′-GGGGTTGGTGTAAAATAGGG-3′ and DBY 5′-CCAGTTTCCCGCCCCGGAG-3′]. The reaction volume was 50 µl per sample in 0.2 ml thin walled micro centrifuge tube. The mixture contained 5 µl of 10× reaction buffer (Promega, Madison WI, USA) in a final concentration of 1×, 2 µl of 20 mM dNTPs mixture (0.2 mM each of dTTP, dATP, dCTP and dGTP), 3 µl of 25 mM $MgCl_2$ (Promega, Madison WI, USA), 2.0 µl of primers mixture (1 µl of forward primer (50 µM) + 1 µl of reverse primer (50 µM), (Inqaba Biotechnical Industries (Pty) Ltd., South Africa), 0.25 µl of thermo-stable DNA polymerase (Promega, Madison WI, USA) (5 U/µl). 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 programme was run for 35 cycles: initially denaturation at 94 °C for 3 min, annealing at 64 °C for 1 min, extension at 72 °C for 1 min and denaturation at 94 °C for 30 s. A final extension cycle at 72 °C for 10 min was also included. The PCR products were stored at 4 °C for later analysis.

2.4. Gel analysis of mini-circle kDNA products

Five microliters of the mini-circle kDNA–PCR reaction product were mixed with 3 µL of 10× loading buffer (1× TBE containing 20% sucrose, 0.05% Bromophenol Blue and 0.05% xylene cyanol). The samples were loaded on 1.5% agarose gels with 1× electrophoresis buffer (TBE of 89 mM Tris–base, 89 mM boric acid, 2 mM EDTA, and pH 8.3). Three microliters of 10 mg/ml ethidium bromide stain were directly added to the agarose before pouring the gel. A DNA ladder size 100–1000 bp (Promega, Madison WI, USA), was used. Gel electrophoresis was performed at 80 V for 1 h. The gels were visualized under UV transilluminator and photographs were taken.

2.5. Random Amplified Polymorphic DNA (RAPD) procedure

The DNA of *L. donovani* sensitive and resistance isolates were subjected to Random Amplified Polymorphic DNA (RAPD) analysis. Six decamer primers (Inqaba Biotechnical Industries (Pty) Ltd., South Africa), A-12: 5′-TCGGCGATAG-3′; B-30: 5′-CATCCCCCTG-3′; IL-0875: 5′-GTCCGTGAGC-3′; A-13: 5′-CAGCACCCAC-3′; B-13: 5′-TTCCCCCGCT-3′; B-20: 5′-GGACCCTTAC-3′ were used to characterize the isolates. One microliter of 10 ng DNA was added to 25 µl reaction mixture containing 1.0 µl of 10× reaction buffer, 1.5 µl $MgCl_2$ (25 mM), 1 µl dNTPs (0.2 mM), 1 µl Taq polymerase (5 U/µl) (Promega, Madison WI, USA), 0.5 µl primers (3.2 µM), and 19 µl distilled H_2O. The reaction was carried out in a thermocycler programmed at one cycle (initial denaturation) at 94 °C for 3 min followed by 45 cycles of annealing at 34 °C for 1 min, extension at 72 °C for 2 min and denaturation at 94 °C for 30 s. A final

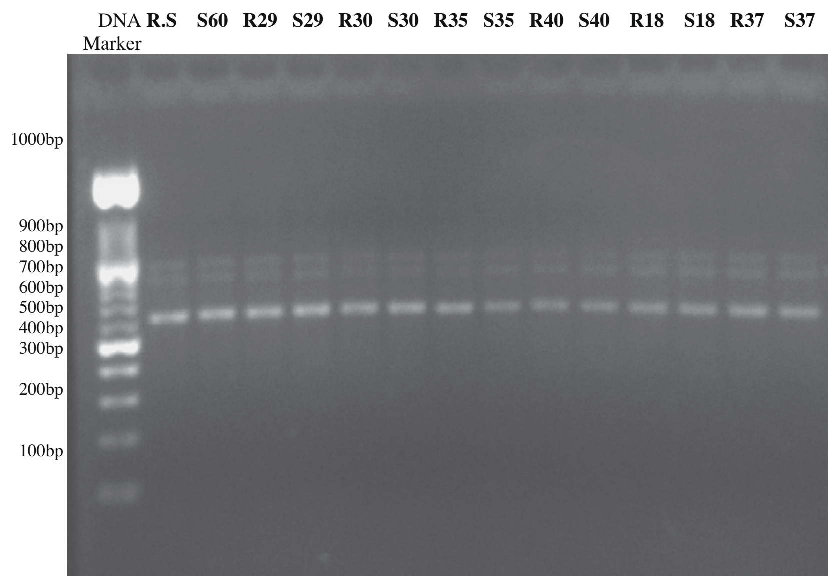

Fig. 1. Band patterns of the study isolates (sensitive/resistant isolates) using Kinetoplast DNA and species-specific mini-circle primers (AJS3 and DBY) PCR and electrophoresis in 1.5% agarose gel. Lane 1 Mwt DNA marker. RS = reference strain. DNA R.S S35 R35 S30 R30 S29 R29 100 −ve marker.

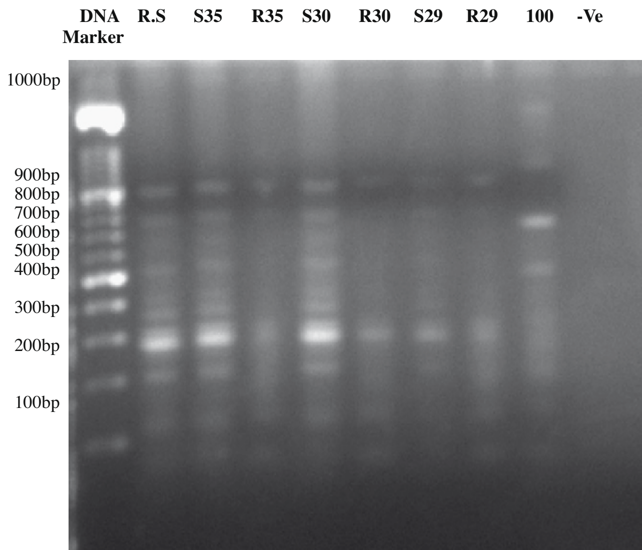

Fig. 2. PCR–RAPD analysis of the study isolates. The RAPD band patterns was generated using A-12 primer, and electrophoresis in 2% agarose gel.

extension cycle at 72 °C for 10 min was also included. The products were stored at 4 °C for later analysis.

2.6. Gel analysis of RAPD products

Five microliters of the RAPD–PCR reaction product were mixed with 3 μl of 10× loading buffer. The samples were loaded on 2% agarose gels with 1× electrophoresis buffer. Three microliters of 10 mg/ml ethidium bromide stain were directly added before pouring the gel. A DNA ladder size 100–1000 bp (Promega, Madison WI, USA), was included for identification of RAPD bands. Gel electrophoresis was performed at 50 V for 3 h. The gels were visualized under UV transilluminator and photographs were taken.

2.7. Data analysis

RAPD band patterns were compared within and between isolates (SSG-sensitive/resistant). Only reproducible bands that could

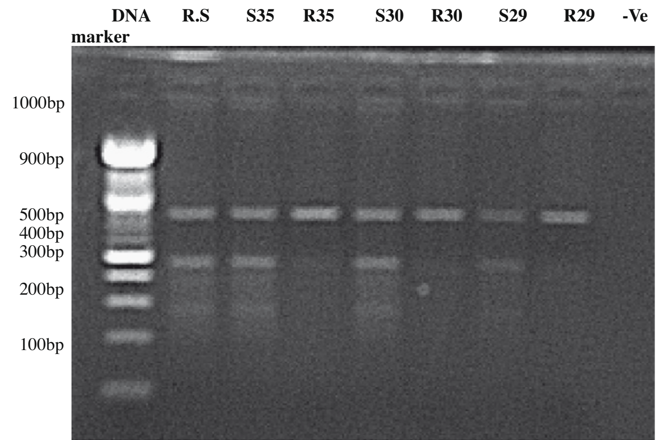

Fig. 4. PCR–RAPD analysis of the study isolates. The RAPD band patterns was generated using IL-0875 primer, and electrophoresis in 2% agarose gel.

be obtained from the same DNA sample in two or more different daily runs with well-marked amplified bands were scored. For each genotype (cluster) the presence and absence of fragments (bands) were recorded as +ve or −ve, respectively. A pair-wised comparison of banding patterns was evaluated by the Jaccard's similarity coefficient (Jaccard, 1901). Jaccard's coefficient was calculated by the following formula:

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

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

3. Results

3.1. Induction/augmentation and selection of sodium stibogluconate-unresponsive L. donovani isolates

Thirty *Leishmania* isolates were successfully grown and passed through increasing concentrations of SSG. The drug concentrations that inhibited 50% (IC50) and 90% (IC90) of parasite growth of the

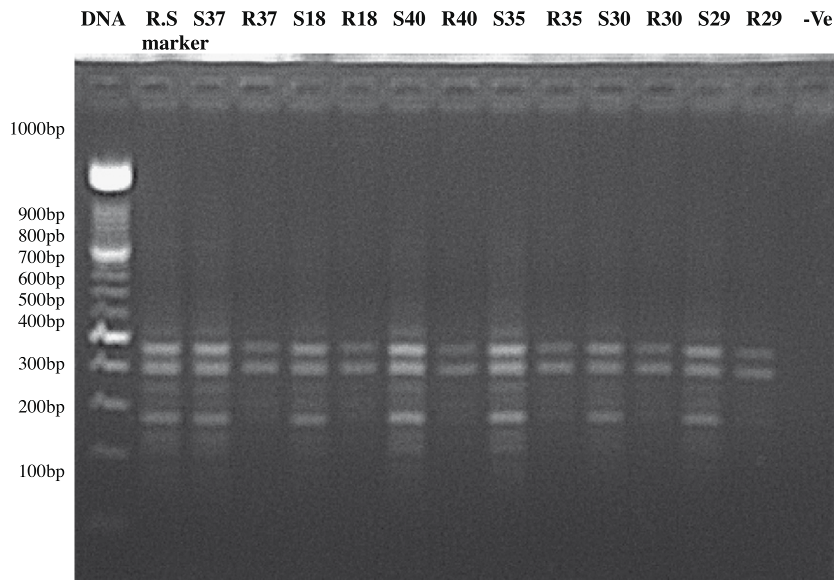

Fig. 3. PCR–RAPD analysis of the study isolates. The RAPD band patterns was generated using A-13 primer, and electrophoresis in 2% agarose gel.

wild type isolates were found to be 0.4 ± 0.04 and 0.8 ± 0.1 mg/ml, respectively. Following successful induction/augmentation of resistance/unresponsiveness in all isolates, the IC50 and the IC90 were found to be 50 and 35-fold higher than those of the wild type, respectively, ($p < 0.002$).

3.2. PCR-based (kDNA) species characterization of the study isolates

SSG-sensitive and resistant isolates showed similar patterns to the reference *L. donovani* strain with an 800 bp size band (Fig. 1).

3.3. PCR–Random Amplified Polymorphic DNA (RAPD analysis) results

The PCR–RAPD bands pattern analysis showed common, consistent and reproducible bands that were present in all the *Leishmania* isolates (both sensitive and resistant) with all primers (i.e. species bands). These bands sizes were 290 bp for A-12 primer; 400 and 590 bp for A-13 primer and 890 bp for IL-0875 primer (Figs. 2–4). The A-12 primer band patterns for the SSG-sensitive isolates showed three different clusters (primary genotypes/clusters) with similar species band and additionally one or more bands that were absent in other clusters. The first primary genotype showed a band pattern identical to that of the reference strain RS (Fig. 2).

The initial and final scoring for the three primary genotypes and the Jaccard's similarity coefficient are shown in Tables 2–4. The other primers (A-13 and IL-0875) failed to detect any genetic diversity between the SSG-sensitive isolates i.e. all study isolates have similar patterns (Figs. 3 and 4). Band patterns of SSG-unresponsive strains for the three primers showed the disappearance of bands when compared to SSG-sensitive band patterns i.e. band patterns were identical for each primer (Figs. 2–4). Primers B-13, B-30 and B-20 failed to amplify parasite DNA.

Table 2
Bands scoring for the study isolates.

Samples ID	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	JSC
30	+	+	+	+	+	+	+	1
35	+	+	+	+	+	+	+	1
18	+	+	+	+	+	+	+	1
37	+	+	+	+	+	+	+	1
44	+	+	+	+	+	+	+	1
33	+	+	+	+	+	+	+	1
19	+	+	+	+	+	+	+	1
42	+	+	+	+	+	+	+	1
46	+	+	+	+	+	+	+	1
51	+	+	+	+	+	+	+	1
53	+	+	+	+	+	+	+	1
70	+	+	+	+	+	+	+	1
75	+	+	+	+	+	+	+	1
80	+	+	+	+	+	+	+	1
82	+	+	+	+	+	+	+	1
11	+	+	+	+	+	+	+	1
47	+	+	+	+	+	+	+	1
31	+	+	+	+	+	+	+	1
36	+	+	+	+	+	+	+	1
54	+	+	+	+	+	+	+	1
56	+	+	+	+	+	+	+	1
60	+	+	+	+	+	+	+	1
64	+	+	+	+	+	+	+	1
67	+	+	+	+	+	+	+	1
68	+	+	+	+	+	+	+	1
32	+	+	+	+	+	+	+	1
29	+	–	–	+	+	+	+	0.7
28	+	–	–	+	+	+	+	0.7
40	+	+	–	–	–	–	–	0.3
27	+	+	–	–	–	–	–	0.3

Table 3
Primary genotypes (clusters) of the study isolates based on PCR–RAPD band patterns.

	Genotype 1	Genotype 2	Genotype 3
JSC**	1	0.7	0.3
No. of strains	$n = 26$	$n = 2$	$n = 2$
No. of common bands	7	5	2

** JSC = Jaccard's similarity coefficient.

Table 4
Jaccard's similarity coefficients within and between strains sensitive and resistant for each primer (A-12, A-13 and IL-0875).

Primer	Within sensitive isolates	Within resistant isolates	Between sensitive and resistant isolates
A-12	0.78	0.98	0.7
A-13	0.9	0.9	0.616
IL-0875	1.0	1.0	0.33

4. Discussion

Antimonial-unresponsive visceral leishmaniasis is a major problem in the Indian subcontinent and is predicted to increase markedly as a result of HIV/AIDS infections in other parts of the world especially Africa and southern Europe.

Drug unresponsive promastigotes were used in this experiment for two reasons: promastigotes are inherently partially resistant to Pentavalent antimonials with the mechanism of unresponsiveness already in place and secondly, the use of promastigotes eliminates the problem of starting with a heterogeneous parasite population. Such a heterogenous initial population may lack the necessary characteristics for evaluating the genetic and biochemical changes that accompany acquisition of drug unresponsiveness in parasites. But here emerges a potential problem in the interpretation of results, for using a parasite stage that may not be directly referable to pathogenicity in the host (Peters, 1985).

In this study, we proved that all wild *L. donovani* isolates from eastern Sudan are genetically diverse (heterogeneic) and that under drug pressure an SSG-unresponsive and homogenous promastigote population can be produced. Abdalla and his colleagues reported similar findings from Nuba Mountains in southern–western Sudan (Abdalla et al., 2003).

The uniformity of the band patterns of the SSG-unresponsive isolates produced with A-13 and IL-0875 primers may offer some explanation for the emergence/dominance of non-responsive isolates in nature. It is probable that the experimental isolates down regulated some genes that normally enhance the anti-leishmanial effect of the drug during adaptation to the escalating SSG concentration. But, the possibility that the genetic change as a direct result of exposure to drug pressure seems to be unlikely, because the parasite has to undergo a number of genetic changes. Alternatively, unresponsiveness may develop in a stepwise manner at some point during multiple exposures to SSG depending on the rate of elimination of the SSG-sensitive strains and enhancement of survival of an initially tolerant parasite strains. We still believe that the difference in the PCR–RAPD band pattern between SSG-sensitive and unresponsive isolates needs further clarification.

The study results showed that whatever mechanisms operated to induce SSG-unresponsiveness was completely abolished by increasing the SSG concentration to 36.45 mg/ml.

In conclusion; *L. donovani* isolates from eastern Sudan are genetically diverse with an SSG-resistant clone that is initially present and expands with the use of SSG (genetic selection). The SSG resistance probably develops in the field over time as a result of exposure to low SSG concentrations. Care should be taken to

ensure that the initial treatment is adequate to prevent induction/selection of resistant *Leishmania* strains.

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