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Leishmania donovani: Intraspecific Polymorphisms of Sudanese Isolates Revealed by PCR-based Analyses and DNA Sequencing

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El Tai, N. O., El Fari, M., Mauricio, I., Miles, M. A., Oskam, L., El Safi, S. H., Presber, W. H., and Schönian, G. 2001. *Leishmania donovani:* Intraspecific polymorphisms of Sudanese isolates revealed by PCR-based analyses and DNA sequencing. *Experimental Parasitology* **97,** 35–44. Four polymerase chain reaction (PCR)-based approaches were used to analyze diversity within 23 Sudanese isolates of *Leishmania donovani.* Methods compared were fingerprinting with single nonspecific primers, restriction analysis of the amplified ribosomal internal transcribed spacer (ITS) locus, single-stranded conformation polymorphism (SSCP), and sequencing of the ITS region. When PCR fingerprinting and restriction analysis of ITS were applied, highly similar fragment patterns were observed for all strains of *L. donovani* studied. The ITS1 locus gave five different SSCP profiles among the 23 Sudanese isolates, whereas the ITS2 locus was highly conserved with the exception of 1 isolate. Strains of *L. donovani* derived from other geographical areas were found to have different ITS2 patterns. SSCP analysis correlated well with results of DNA sequencing and confirmed that SSCP was able to detect genetic diversity at the level of a single nucleotide. SSCP had advantages over the other methods employed for investigation of sequence variation within the species *L. donovani.* There was no correlation between the form of clinical manifestation of the disease and the PCR fingerprinting, ITS-RFLP, or ITS-SSCP characteristics.

Index Descriptors and Abbreviations: Leishmania donovani; internal transcribed spacer (ITS); polymerase chain reaction (PCR); single-stranded conformation polymorphism (SSCP); restriction fragment length polymorphism (RFLP); PCR fingerprinting; Sudan.

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INTRODUCTION

Leishmania donovani senso lato, a member of the protozoan family Trypanosomatidae, is the etiological agent of visceral leishmaniasis (VL) in Sudan (Hoogstraal and Heyneman 1969; El- Hassan *et al.* 1995). VL is essentially a tropical disease that has forced itself upon medical attention in Sudan as an increasingly significant problem over the past decade. It has been estimated that there are likely 500,000 cases of VL per year (WHO 1998). Over 90% of cases are found in three regions, Sudan/Ethiopia/Kenya, India/Bangladesh/Nepal, and Brazil (WHO 1991, 1996), with as many as 100,000 deaths every year (Ashford *et al.* 1992). This represents only the "tip of the iceberg," as the majority of cases are asymptomatic and many symptomatic cases may not be recognized.

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The identification of the parasite and the analysis of genetic diversity are important for diagnosis, for epidemiological studies, and for taxonomic and population genetics investigations. Parasite species identification guides physicians in choosing and assessing a specific chemotherapeutic regimen. Since *Leishmania* species cannot be distinguished morphologically (Weiss 1995), alternate methods of discrimination are essential.

For epidemiological purposes, the most useful taxonomic markers have been isoenzyme profiles (Miles *et al.* 1980; Evans *et al.* 1984; WHO 1990; Andresen *et al.* 1996), however isoenzyme analysis is slow, laborious, and expensive and requires growth in *vitro* (Andresen *et al.* 1996; Noyes *et al.* 1996). Alternative techniques include monoclonal antibody typing (McMahon-Pratt and David 1981; Noyes *et al.* 1996) and DNA-based methods, such as restriction fragment length polymorphism analysis (RFLP) (Cupolillo *et al.* 1995), analysis of kinetoplast and nuclear DNA including Southern hybridization with specific DNA probes (Jackson *et al.* 1984; Beverley *et al.* 1987; Barker 1989; Van Eys *et al.* 1989, 1991), DNA fingerprinting with DNA probes complementary to repetitive DNA sequences (Macedo *et al.* 1992), PCR fingerprinting with single arbitrary primers (Williams *et al.* 1990; Tibayrenc *et al.* 1993; Pogue *et al.* 1995a,b; Schönian *et al.* 1996), and molecular karyotyping (Lighthall and Giannini 1992). Few of these methods are capable of detecting intraspecific diversity among isolates of *Leishmania.* PCR-SSCP analysis has been developed to scan genes for single base differences which could be useful as genetic markers (Orita *et al.* 1989; Gasser *et al.* 1997; Stothard *et al.* 1997).

Here, we compare three PCR-based methods, namely PCR fingerprinting, PCR-RFLP, and PCR-SSCP with the ITS as a target, and DNA sequencing to validate the results. Data from these methods were manipulated with the aim of screening for diversity within *L. donovani.*

MATERIALS AND METHODS

Parasites. Clinical samples from inguinal lymph node or bone marrow aspirates were collected from 40 microscopically confirmed cases of VL. These patients originated from clusters of endemic villages around Gedaref in eastern Sudan. The patients were admitted to Gedaref hospital (Gedaref State, 411 km from Khartoum) between April 1997 and November 1998. Five lymph node aspirates were also collected from patients with diseases other than VL for use as negative controls. In addition to microscopic examination, each aspirate was aseptically inoculated into two tubes containing NNN or Difco 4N media. These media were prepared as described by Evans (1989). Cultures were kept at the ambient temperature (25°C) and transported to the Institut für Mikrobiologie und Hygiene, Humboldt-Universität zu Berlin, Germany for further subculturing in RPMI-1640 medium supplemented with 15% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Deoxyribonucleic acid was isolated from successful cultures as described previously by Schönian *et al.* (1996). Another 18 *L. donovani* strains used in this study were obtained from the collection of the Royal Tropical Institute, Amsterdam. Twelve of these were isolated from the same above-mentioned endemic area in eastern Sudan (Gedaref State); 6 were WHO reference strains, namely, MHOM/KE/85/NLB323, MHOM/IN/71/LRC-L51a, MHOM/CN/??/Wangjie1, MHOM/SD/75/LV139, MHOM/SD/68/1S, and MHOM/SD/62/LRC-L61

PCR fingerprinting. The core sequence of phage M13 (5′-GAG GGT GGC GGT TCT), the simple repeat sequences $(GTG)_5$ and $(GACA)_4$ that anneal to mini- and microsatellite DNA sequences and the T3B oligonucleotide (5′-AGG TCG CGG GTT CGA ATC C), derived from intergenic spacers of tRNA genes, were used as single primers for PCR fingerprinting. Amplification reactions and electrophoretic separation of the amplification products were performed as previously described (Schönian *et al.* 1996).

Amplification and restriction analysis of the ITS sequence. The whole internal transcribed spacer in the ribosomal operon was amplified using the primers LITSV (5′-ACACTCAGGTCTGTAAAC) and LITSR (5′-CTGGATCATTT-TCCGATG) as described by El Tai *et al.* (2000). Seventeen microliters of the PCR products containing the amplified ITS region were digested for 2 h with the restriction enzymes *Alu*1, *Bst*U1, *Cfo*1, *Dde*1, *Eco*R1, *Fsp*1, *Mse*1, *Msp*1, *Nde*1, and *Taq*1 under the conditions recommended by the supplier. Restriction products were subjected to electrophoresis in 1.3% metaphore agarose (FMC BioProducts, Rockland, ME) for 2 h at 80 V in 0.5 TBE buffer and visualized under UV light after staining for 15 min in ethidium bromide (0.5 μg/ml).

Amplification and SSCP analysis of ITS1 and ITS2 sequences. The ITS1 and ITS2 were separately amplified with the primer pairs L5.8S (5′-TGATACCACTTATCGCACTT-3′)/LITSR and L5.8SR (5′-AAGTGCG-ATAAGTGGTA-3′)/LITSV, respectively. Amplification reactions were performed in volumes of 50 μl; 3 μl DNA or 1 μl DNA (for nested PCR) were added to a PCR mix containing 200 μM each dNTP, 1.5 mM $MgCl_2$, 2 U *Taq* polymerase, and 25 pmol of each primer. Samples were overlaid with sterile, light mineral oil and amplified as follows: initial denaturation at 95°C for 2 min followed by 34 cycles consisting of denaturation at 95°C for 20 s, annealing at 53°C for primer pairs LITSR/LITSV, LISTR/L5.8S, and LITSV/L5.8SR or at 57°C for primer pairs LITSV/LIS2MV and L5.8SR/LIS2MR for 30 s, and extension at 72°C for 1 min. This was followed by a final extension cycle at 72°C for 6 min. Amplification products were subjected to electrophoresis in 1% agarose at 100 V in 0.5× TBE buffer (0.045 M *Tris*–borate, 1 mM EDTA) and visualized after staining for 15 min in ethidium bromide (0.5 μg/ml) under ultraviolet light. Nested PCR was performed to obtain enough PCR products for subsequent SSCP analysis or sequencing. ITS1 and ITS2 were amplified by nested PCR from previous ITS PCR products and ITS2A and B from ITS2 products.

SSCP was performed by denaturing the double-stranded DNA products as follows: 10 μl of each PCR product was added to 3 μl denaturing buffer (0.01 M EDTA, 1% SDS) and 2 μl loading buffer (80% glycerin, 0.1 M EDTA, pH 8, 10 mM Tris–HCl, pH 8, 0.1% bromophenol blue). The mixture was heated to 98°C for 15 min and immediately chilled on ice. These samples were loaded on MDE gels [37.5 ml MDE-gel

solution (FMC Bioproducts), 9 ml 10× TBE (10.8% Tris, 5.5% boric acid, 0.02 M EDTA, pH 8), 103.5 ml distilled water, 60 μl *NNNN*-tetramethylene diamine (TEMED), 600 μl 10% APS (ammonium peroxide sulfate)]. Electrophoresis was run in a cold room (8°C) using 0.5× TBE at 10 W for 15 h for the products amplified by ITS1 and ITS2 A primers and at 20 W for 15 h for the ITS2 and ITS2 B regions. After electrophoresis, the gel was fixed in 1% nitric acid for 15 min, subsequently washed in distilled water for 20 s, and then stained in 0.2% $AgNO_3$ for 25 min. After washing for 10 min in distilled water, the gel was placed in freshly prepared developing solution (3% Na carbonate with the addition of formaldehyde up to 0.05%). As soon as the bands became clearly resolved, the gel was fixed in 10% acetic acid for 5 min and dried in a Slab gel dryer (Savant-Hicksville, NY) at 80°C for 1.5 h.

ITS sequencing. ITS products that had different SSCP patterns were subjected to sequencing. For sequence analysis the two parts of ITS2, A and B (see Fig. 1), were amplified separately using the primer combinations LIS2MR (5′-AGAGTGCATGTGTGTAT-3′)/L5.8SR and LIS2MV (5′-ATACACACATGCACTCTC-3′)/LITSV, respectively. The ITS1 region was s amplified using the primer pairs L5.8S/LITSR as described above; 100-μl PCR products from the selected samples were purified from the agarose gel using QIA quick gel extraction kits (QIAGEN, Hilden, Germany) following the manufacturer's instructions. PCR cycle sequencing was performed using a thermosequenase radio-labeled terminator cycle sequencing kit (Amersham Life Science, Inc., Cleveland, OH) according to the instructions given by the supplier.

RESULTS

Forty cultures were attempted in total, of which 8 were positive. The 8 successful cultures plus the 18 from the collection of the Royal Tropical Institute, Amsterdam were positive in PCR, whereas all negative controls were negative, i.e. no contamination or inhibition was detected. Samples were scored as positive for the presence of *Leishmania* if a PCR product of approximately 1040 bp for the entire ITS amplicon was obtained. When ITS1 and ITS2 were amplified separately, amplification products of about 320 and 720 bp, respectively, were obtained. When ITS2 A and ITS2 B were amplified separately, PCR products of about 300 and 450 bp, respectively, were observed as described by El Tai *et al.* (2000). To test for sequence heterogeneity that could be detected by RFLP, the entire ITS region was cut with 10 different enzymes (results not shown), the profiles of all studied strains were, however, indistinguishable.

In total five polymorphic ITS1 SSCP patterns (Fig. 2) could be distinguished among all 26 Sudanese isolates. Three patterns, namely A, D, and J, were identified among the samples from eastern Sudan. The two isolates from Kenya and India had the same pattern, K, whereas the Chinese isolate had pattern N. The dominating pattern A was found in 18 of the 23 VL cases (78.3%) from eastern Sudan, pattern D was observed in 4 cases (17.4%), and pattern J was obtained from only 1 VL case (see Table I). ITS2 SSCP analysis also revealed five polymorphic patterns (Fig. 3). All 23 Sudanese isolates had the same pattern A with the exception of 1 sample which showed pattern J. Samples from Kenya, India, and China showed patterns X, Y, and Z, respectively (see Table I).

The ITS1 and ITS2 PCR products of *Leishmania* isolates representing different SSCP patterns were sequenced in both directions. All the variations in the ITS1 SSCP profiles were due to the deletion of AT pairs or adenine bases from stretches in the first part of the ITS1 (first 130 nucleotides) with the exception of a Chinese isolate which has in addition a substitution at positions 27 and 89 (sequence LdN; Fig. 4).

No sequence variation was observed in the ITS2A region (positions 320–602) in all isolates. The Sudanese isolates had the same ITS2B sequence (positions 603–1042) with the exception of one sample which had a deletion of one guanine base from a G stretch at position 831 (LdJ sequence).

Isolates from Kenya and India (LdX and LdY sequences, respectively) differed in a single nucleotide, at position 762 in their ITS2 B sequence, but showed many nucleotide differences compared with the Sudanese isolates (Sequences, LdA, J, and D) and the Chinese isolate (sequence LdZ). The ITS sequence of the isolates from Kenya, India, and China were identical to those obtained previously by Schönian *et al.* (GenBank database Accession Nos. AJ000297, AJ000294, and AJ000290).

The PCR fingerprinting revealed only slight differences

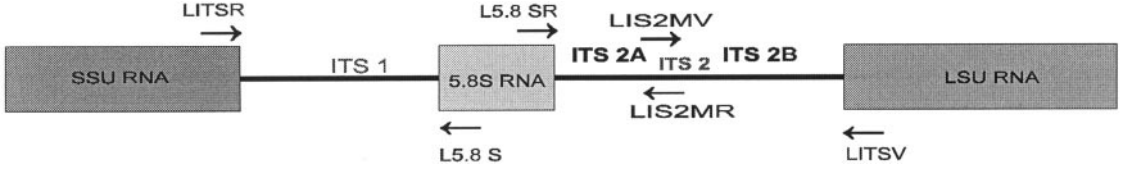

FIG. 1. The internal transcribed space (ITS) in the ribosomal operon and the position of all primers used in this study to amplify ITS sequences. Primer sequences are given in the text.

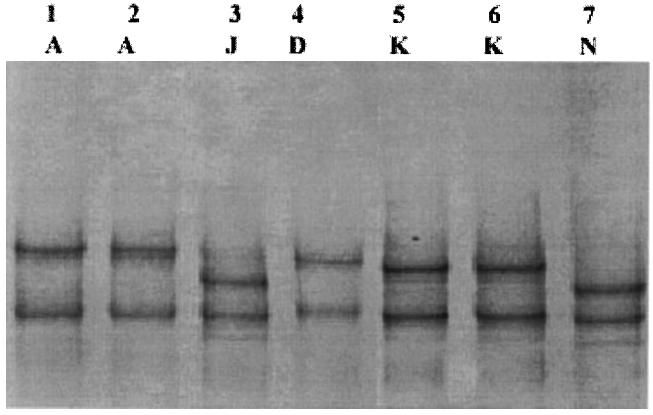

FIG. 2. SSCP analysis of ITSI regions amplified from different clinical *Leishmania donovani* isolates. Each SSCP pattern is designated by a capital letter. Lanes 1–4, Sudanese strains of *L. donovani* MHOM/SD/62/LRC-L61, MHOM/SD/98/GC1, Don 13, and Don 14; lane 5, *L. donovani* MHOM/KE/85/NLB323 (Kenya); lane 6, *L. donovani* MHOM/IN/71/LRC-L51a (India); lane 7, *L. donovani* MHOM/CN/??/ Wangjie1 (China).

TABLE I
Strains of *Leishmania donovani* Analyzed in This Study and Results of ITS Typing

No.	WHO code or lab code	Origin	Zymodeme	Pathology	SSCP ITS1 pattern	SSCP ITS2 pattern	Seq. type ITS1 and ITS2
1	MHOM/SD/98/GC1	eastern Sudan	n.d.	VL	A	A	LdA, LdA
2	MHOM/SD/98/GC2	eastern Sudan	n.d.	VL	A	A	LdA, LdA
3	MHOM/SD/98/GC3	eastern Sudan	n.d.	VL	A	A	LdA, LdA
4	MHOM/SD/98/GC4	eastern Sudan	n.d.	VL	A	A	LdA, LdA
5	MHOM/SD/98/GC5	eastern Sudan	n.d.	VL	A	A	LdA, LdA
6	MHOM/SD/98/GC6	eastern Sudan	n.d.	VL	A	A	LdA, LdA
7	MHOM/SD/98/GC7	eastern Sudan	n.d.	VL	A	A	LdA, LdA
8	MHOM/SD/98/GC8	eastern Sudan	n.d.	VL	A	A	LdA, LdA
9	DON 12	eastern Sudan	MON-30	VL	A	A	LdA, LdA
10	DON 13	eastern Sudan	MON-82	VL	J	J	LdJ, LdJ
11	DON 14	eastern Sudan	MON-82	VL	D	A	LdD, LdA
12	DON 15	eastern Sudan	MON-30	VL	A	A	LdA, LdA
13	DON 16	eastern Sudan	MON-30	VL	A	A	LdA, LdA
14	DON 17	eastern Sudan	MON-18	VL	D	A	LdD, LdA
15	DON 18*	eastern Sudan	MON-30	PKDL	D	A	LdD, LdA
16	DON 19*	eastern Sudan	MON-30	PKDL	A	A	LdA, LdA
17	DON 20	eastern Sudan	MON-30	VL	A	A	LdA, LdA
18	DON 21	eastern Sudan	MON-82	VL	A	A	LdA, LdA
19	DON 22	eastern Sudan	MON-30	PKDL	A	A	LdA, LdA
20	DON 23	eastern Sudan	MON-18	PKDL	D	A	LdD, LdA
21	MHOM/SD/75/LV139	Sudan	n.d.	CL	A	A	LdA, LdA
22	MHOM/SD/62/LRC-L61	Sudan	n.d.	??	A	A	LdA, LdA
23	MHOM/SD/68/1S	Sudan	n.d.	VL	A	A	LdA, LdA
24	MHOM/KE/85/NLB323	Kenya	n.d.	??	K	X	LdK, LdX
25	MHOM/IN/71/LRC-L51a	India	n.d.	VL	K	Y	LdK, LdY
26	MHOM/CN/??/Wangjie1	China	MON-35	VL	N	Z	LdN, LdZ

Note. VL, visceral leishmaniasis; PKDL: post Kala-azar dermal leishmaniasis; CL: cutaneous leishmaniasis; n.d: not done.
* Isolated from different body sites of the same patient.

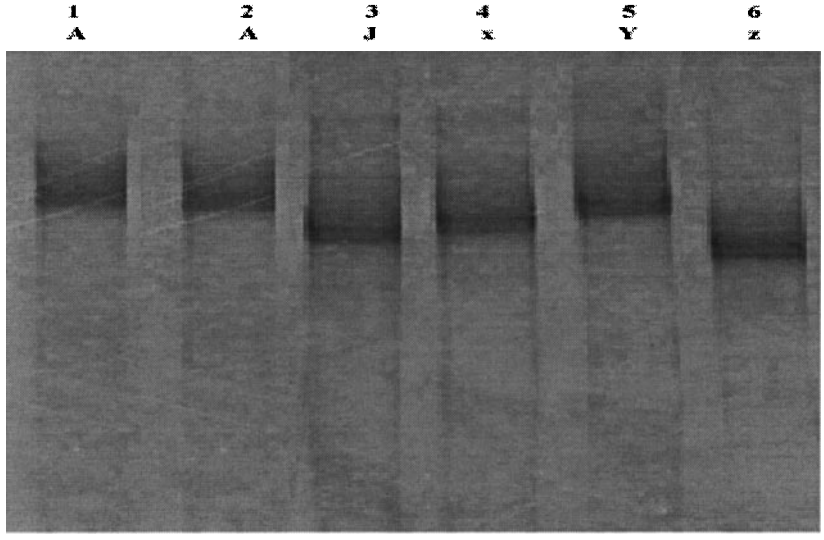

FIG. 3. SSCP analysis of ITS2 regions amplified from different clinical *Leishmania donovani* isolates. Each SSCP pattern is designated by a capital letter. Lanes 1–3, Sudanese strains of *L. donovani* MHOM/SD/62/LRC-L61, MHOM/SD/98/GC1, and Don 13; lane 4, *L. donovani* MHOM/KE/85/NLB323 (Kenya); lane 5, *L. donovani* MHOM/IN/71/LRC-L51a (India); lane 6, *L. donovani* MHOM/CN/??/Wangjie1 (China).

in profiles of all the *L. donovani* strains studied using the M13 and T3B primers (results not shown). When the $(GTG)_5$ primer was used, *L. donovani* from India showed a unique profile (Fig. 5, lane 5). The fingerprint of the Chinese strain (Fig. 5, lane 6) was very similar to those of the Sudanese strains; however, the high-molecular-weight bands appeared very faint, which might be due to partial degradation or lower concentration of DNA in this sample. With primer $(GACA)_4$ identical fingerprinting patterns were obtained for all isolates from Sudan with the exception of the one ITS2 pattern J isolate (Fig. 6, lane 3). This isolate differed slightly from the other Sudanese isolates as did the isolate from China (Fig. 6, lane 6). The Kenyan and the Indian isolates (Fig. 6, lanes 4 and 5, respectively) showed the same profiles with this primer.

DISCUSSION

We assessed intraspecific DNA polymorphisms among 23 isolates of *L. donovani* from eastern Sudan, 1 from Kenya, 1 from India, and 1 from China using four PCR-based approaches as discriminating tools to detect polymorphisms.

Intraspecific variation in SSCP banding patterns was clearly observed in the ITS1 region, with 3 profiles detected among Sudan isolates, with profile A being dominant (78.3%), and 2 ITS1-SSCP profiles observed among the samples from Kenya, India, and China. This corroborates the results of a previous study in which 11 polymorphic SSCP patterns were identified among 63 clinical samples of *L. donovani* from eastern Sudan, with pattern A being predominant (76.1%), On the other hand, no variation was observed in the ITS2 region among the 63 studied cases from Sudan (El Tai *et al.* 2000). In this study, all but 1 of the 23 Sudanese samples showed again the same ITS2 SSCP pattern (A). The ITS2 sequence seems to be highly conserved in all isolates of *L. donovani* from Sudan, with the exception of only 1 sample. In contrast, ITS1 regions appear to undergo rapid evolutionary changes and may vary among populations of the same species of the same geographical origin. Isolates of the same species (*L. donovani*), but of different origin, showed varying ITS2 SSCP patterns. We may conclude that ITS2 SSCP pattern analysis could be a useful method for differentiation of *L. donovani* strains from different geographical areas. To consolidate this observation, more samples from different geographical origins are required.

DNA fragments showing different SSCP patterns were further characterized by radioactive cycle sequencing to determine the underlying nucleotide polymorphisms. Different patterns in the ITS1 region were mainly due to deletion of adenine residues from A stretches or AT pairs (Fig. 4). This may be attributed to the weak AT double bond. The results

Sequence Type	Strain (n)	Accession number	position 26-35 (ITS1)	position 60-90 (ITS1)	position 123-131 (ITS1)
LdA	18	AJ276258	CAAAAAAAAC	TATATATATATATGTAGGCCTTTCCCACATA	AAAAAAAAG
LdJ	1	AJ276260	CAAAAAAAAC	TATATATAT....GTAGGCCTTTCCCACATA	AAAAAAAAG
LdD	4	AJ276259	CAAAAAAAAC	TATATATATAT..GTAGGCCTTTCCCACATA	AAAAAAAAG
LdK	2	AJ000297	CAAAAAAAAC	TATATATATAT..GTAGGCCTTTCCCACATA	AAAAAAA.G
LdN	1	AJ000294	CCAAAAAA.C	TATATATAT....GTAGGCCTTTCCCACACA	AAAAAAA.G
			* *	**** *	*

Sequence Type	strain (n)	Accession number	position 735-738(ITS2)	Position 760-763(ITS2)	position 825-862(ITS2)	position 932-941(ITS2)
LdA	22	AJ276258	GGGG	CCAG	GGGGGGGAGGTGGGTGTGGGTGGTGTGTGG.....C	ATATATATAT
LdJ	1	AJ276260	GGGG	CCAG	GGGGGG.AGGTGGGTGTGGGTGGTGTGTGG.....C	ATATATATAT
LdX	1	AJ000297	GGTG	CCCG	GGGGGG.AGGTGG.T..GGGTG.TGGGTGGTGTGTGGC	ATATATATAT
LdY	1	AJ000290	GGTG	CCAG	GGGGGG.AGGTGG.T..GGGTG.TGGGTGGTGTGTGGC	ATATATATAT
LdZ	1	AJ000294	GGGG	CCAG	GGGGGG.AGGTGGGTGTGGGTGGTGTGTGG.....C	ATATAT....
			*	*	* * ** * *******	****

FIG. 4. Alignment of the variable parts of the ITS (1 and 2) sequences amplified from *Leishmania donovani* isolates representing the different SSCP patterns (LdA, LdD, LdJ, LdK, LdN LdX, LdY, LdZ). Dots represent absence of nucleotide. The complete ITS sequences were submitted to the EMBL data bank; the accession numbers of each sequence type are given in the figure.

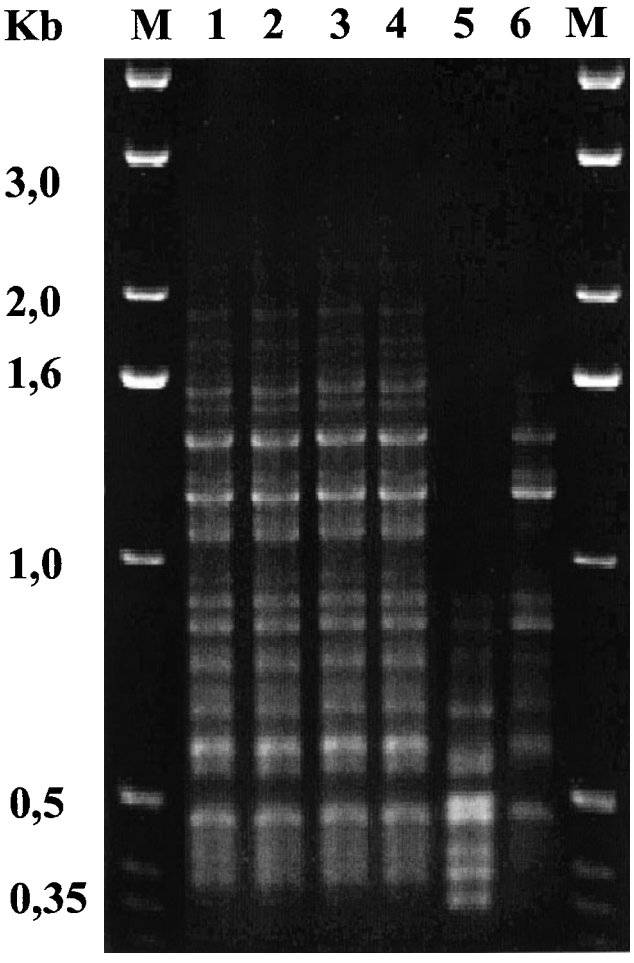

FIG. 5. Results of the PCR fingerprinting using the $(GTG)_5$ primer. Lanes 1–3, Sudanese strains of *Leishmania donovani* MHOM/SD/62/LRC-L61, MHOM/SD/98/GC1, and Don 13; lane 4; *L. donovani* MHOM/KE/85/NLB323 (Kenya); lane 5, *L. donovani* MHOM/IN/71/LRC-L51a (India); lane 6, *L. donovani* MHOM/CN/??/Wangjie1 (China). M.; molecular size (weight) standard.

obtained by SSCP for ITS2 were also confirmed by DNA sequencing.

No intraspecific variation was observed in the RFLP patterns when the amplified ITS regions of different strains of *L. donovani, L. infantum,* and *L. infantum* (syn. *L. chagasi*) were digested with a panel of different frequently cutting restriction enzymes (data not shown). However, patterns of all other species of *Leishmania* were different, allowing identification of unknown isolates at the species level. This may be explained by the fact that sequence variation may go undetected by RFLP analysis since the restriction enzymes survey only a subset of the total variable sites (Stothard *et al.* 1997).

In contrast to our observations, considerable heterogeneity was found within the ITS RFLP of strains of *L. tropica* and *L. aethiopica* (Schönian *et al.* 2001, 2000) and of new world species of *Leishmania,* whereas different species showed different levels of variation (Guevara *et al.* 1992; Cupolillo *et al.* 1995).

When PCR fingerprinting was done, highly similar PCR profiles were observed in all tested isolates (Figs. 5 and 6), thus confirming previous results (Schönian *et al.* 1996; Oskam *et al.* 1998). This may be attributed to the fact that the fingerprinting technique is less discriminatory because mutations can be detected only if they affect primer binding sites or if they lead to bigger insertions/deletions that would change the fragment size.

We can conclude that SSCP is advantageous relative to both PCR-RFLP and PCR fingerprinting approaches for the detection of sequence variation in rRNA genes within the *L. donovani* species. The SSCP technique was able to detect genetic diversity that differed by only one nucleotide (Fig. 4, sequences LdX and Ld Y).

In addition, ITS-SSCP is a technique that can be performed easily and rapidly without prior cultivation of the parasite, unlike PCR fingerprinting. Samples collected in the field where cultures are vulnerable to contamination can be analyzed directly by PCR-SSCP methods in specialized laboratories. Thus, SSCP can be an excellent method for the screening of genetic polymorphisms in *Leishmania* because it can be used with low parasite numbers and contaminated samples. Samples showing different SSCP patterns could be subsequently sequenced.

In this study, of 40 culture attempts in the field only 8 were successful. This was due to problems with contamination (aseptic techniques in the field were not ideal as in the equipped laboratory), problems with the conservation of adequate media, and problems with maintenance of the optimal temperature under field conditions. Simultaneously, we were able to apply ITS-SSCP for these 8 samples directly from collections obtained by spotting of the lymph node or bone marrow aspirates on filter papers. Although SSCP has been used to detect sequence variation in the ITS region of *L. donovani,* this method could be a powerful analytical tool also for other parasitic organisms. SSCP can be used for the detection of strain-specific polymorphisms not only in the ITS region but also in any already published sequence and thus lessen the dependence on reference laboratories for identification of *Leishmania* strains.

No correlation was, however, discerned between the PCR-SSCP polymorphic pattern and the clinical manifestation of the human disease (4 post kala-azar dermal leishmaniasis

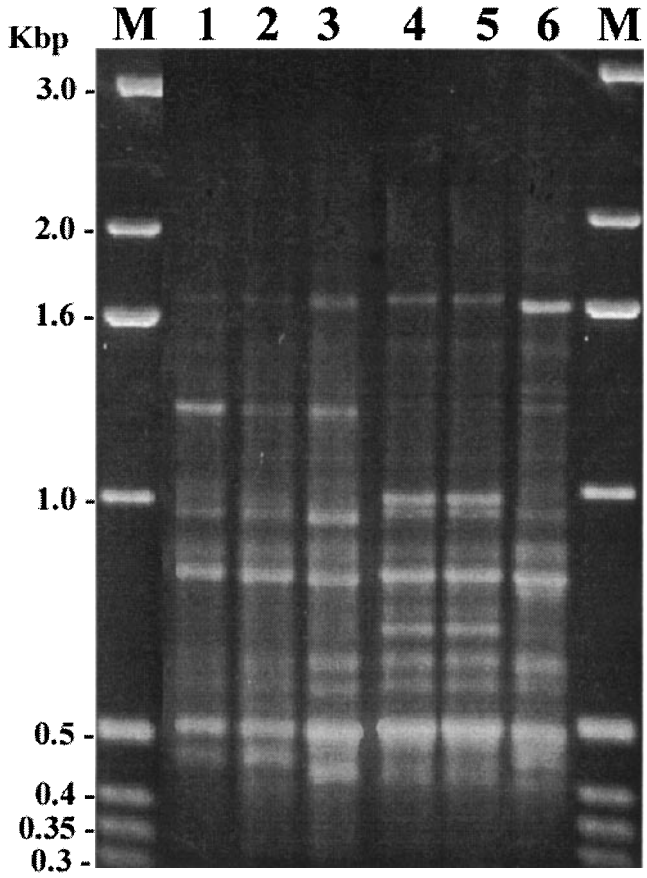

FIG. 6. Results of the PCR fingerprinting using the $(GACA)_4$ primer. Lanes 1–3, Sudanese strains of *Leishmania donovani* (MHOM/SD/62/LRC-L61, MHOM/SD/98/GC1, and Don 13; lane 4, *L. donovani* MHOM/KE/85/NLB323 (Kenya); lane 5, *L. donovani* MHOM/IN/71/LRC-L51a (India); lane 6, *L. donovani* MHOM/CN/??/Wangjie1 (China). M., molecular size (weight) standard.

(PKDL), 19 VL, and 1 cutaneous leishmaniasis (CL) patients). It would be interesting to determine whether *L. donovani* causing mucosal leishmaniasis, PKDL, and VL in Sudan could be correlated with specific SSCP patterns, maybe a relevant gene could be used as a target in further study.

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