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Polymerase chain reaction-based identification of New World *Leishmania* species complexes by specific kDNA probes

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Abstract

Here we define a new approach for the detection and characterisation of *Leishmania* complexes by polymerase chain reaction (PCR) and specific hybridisation. The first step consists of PCR amplification of kDNA minicircles using general kinetoplastid primers, which generate a polymorphic multi-banding pattern for all *Leishmania* species and other Kinetoplastidae. The second step is the identification of the *Leishmania* species complexes by hybridisation of the PCR products with specific kDNA probes. Polymorphic PCR-products from a genetically diverse set of *Leishmania* species were analysed by electrophoresis and the banding patterns compared with multi-locus enzyme electrophoresis (MLEE) data. The banding patterns produced by *Leishmania* species were very heterogeneous, making kDNA-PCR useful for determining closely related strains and for fingerprinting individual strains. The degree of kDNA-PCR and MLEE polymorphism was compared using UPGMA dendrograms. Three complex-specific probes were generated from major PCR bands of reference stocks belonging to the *Leishmania mexicana*, *Leishmania donovani* and *Leishmania braziliensis* complexes, and hybridisation of these probes to membrane-bound PCR products could reliably identify the strain to a complex level. A combination of kDNA-PCR fingerprinting and hybridisation with kDNA probes was found to be useful for both sensitive detection and direct identification of *Leishmania* species complexes.

Keywords: *Leishmania* genera; Kinetoplast; Polymerase chain reaction; DNA hybridisation; Phylogeny

1. Introduction

Leishmaniasis affects more than 12 million people in 88 countries of the world with 350 million

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people at risk; every year there are 1–1.5 million cases of cutaneous leishmaniasis and 500 000 cases of visceral leishmaniasis (WHO, 1997). In the New World two taxonomic groups of *Leishmania* exist, the sub-genera *Leishmania Leishmania* and *Leishmania Viannia*; the subgenus *Viannia*, which is also known as the *braziliensis* complex, includes the species *Leishmania braziliensis*, *Leishmania peruviana*, *Leishmania panamensis*, and *Leishmania guyanensis*, while the subgenus *Leishmania* may be further divided into species complexes: the *mexicana* complex *(Leishmania mexicana*, *Leishmania amazonensis*, *Leishmania garnhami*, *Leishmania aristidesi*, and *Leishmania pifanoi*), and the *donovani* complex (*Leishmania chagasi*) (Lainson and Shaw, 1987). These agents are responsible for a whole spectrum of clinical symptoms: cutaneous leishmaniasis, diffuse cutaneous leishmaniasis, muco-cutaneous leishmaniasis, and visceral leishmaniasis.

The present classification is mostly based on the geographical distribution and biological behaviour of the parasite in vectors and mammalian hosts or in culture. Genetic population studies of parasites are becoming increasingly important in the identification of *Leishmania* species (Lainson and Shaw, 1987). Construction of dendrograms from genetic marker data has shown that each complex can be considered as a separate lineage (Rioux et al., 1986; Guerrini, 1993; Soccol, 1993). Within each lineage, classification into genetic sub-groups is more difficult and commonly recognised species are not always concordant with genetic markers; for example, the *L. garnhami* species is genetically so closely related to *L. amazonensis* that they cannot be distinguished by genetic markers (Guerrini, 1993). A large survey of data in the literature reveals evidence of clonality in natural populations of many species of *Leishmania* (Tibayrenc et al., 1990; Tibayrenc, 1993; Jiménez et al., 1997). These natural clones appear to be evolving over time and dispersing over geographic locations without genetic exchange between organisms, maintaining genetically similar populations.

Several groups have proposed using the polymerase chain reaction (PCR) for detection of *Leishmania* species, due to the low sensitivity of the current detection methods (most commonly direct detection of parasites in tissue from the lesions by microscopic observation) and the difficulty in isolating parasites from reservoirs and cases of *Leishmania braziliensis* infection. Kinetoplastid species (including *Leishmania* and *Trypanosoma* species) contain an ideal target for PCR in the form of the minicircle DNA of the kinetoplast, an unusual type of mitochondrion. Minicircles are small molecules of around 750 bp in *Leishmania* species, and are present at a copy number of 10 000 molecules per cell (Barker, 1980), providing a plentiful target for PCR.

Each minicircle contains a region of conserved sequence around the origin of replication flanked on either side by a variable sequence (Fig. 1). Sets of primers designed for the detection of species complexes, generating kDNA amplification products, have been described (de Bruijn and Barker, 1992; Lopez et al., 1993; Eresh et al., 1994; Ravel et al. 1995). In this study we developed a set of primers generating a distinct and complex banding pattern for each isolate within the Kinetoplastida (*Leishmania* sp., *Trypanosoma cruzi*, *Trypanosoma rangeli*). *Leishmania* complexes can subsequently be identified by hybridisation with kDNA probes specific for the three complexes. This work was based on a large set of reference strains previously characterised by MLEE for 17 loci (Guerrini, 1993).

2. Material and methods

2.1. Leishmania isolates and DNA preparation

The *Leishmania* stocks used are listed in Table 1 with geographic and host origins, and the date of isolation indicated according to WHO nomenclature. Cultures were maintained in Schneider's media at 26°C. DNA was purified by phenol–chloroform extraction, as described by Veas et al. (1991).

2.2. Polymerase chain reaction

PCR reactions for amplification of variable regions of kDNA minicircles were performed using the following primers:

L1: 5′-CCT ACC CAG AGG CCT GTC GGG-3′

L2: 5′-TAA TAT AGT GGG CCG CGC AC-3′,

purchased from Genset Laboratory (Paris, France). These primers were designed from the minicircle sequence of the *L. braziliensis* reference strain MHOM/BR/75/M2904 (de Bruijn and Barker, 1992) (Fig. 1). A restriction site (*Stu*I) was artificially introduced into the L1 oligonucleotide, and the L2 primer contains a natural *Sau*96I site. These restriction sites were used to separate the amplified variable region of the minicircle away from the oligonucleotide primers which contain part of the conserved region. The samples were amplified in 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 2 mM $MgCl_2$, 0.1% Triton X100, 0.2 mM of each deoxynucleotide triphosphate, and 1 μM of each oligonucleotide primer in a total reaction volume of 100 μl. Each reaction tube was covered with 50 μl paraffin to prevent evaporation. *Taq* DNA polymerase (2.5 U) (Promega, USA) were used for amplification, performed on a Biometra Trio thermoblock (Göttingen, Germany). Amplification took place under the following conditions: 96°C for 6 min, followed by 40 cycles of 93°C for 5 s; 58°C for 1 min; and 72°C for 1 min. Reactions were cooled at 4°C for variable times. Each run included one positive control of total DNA template and one negative

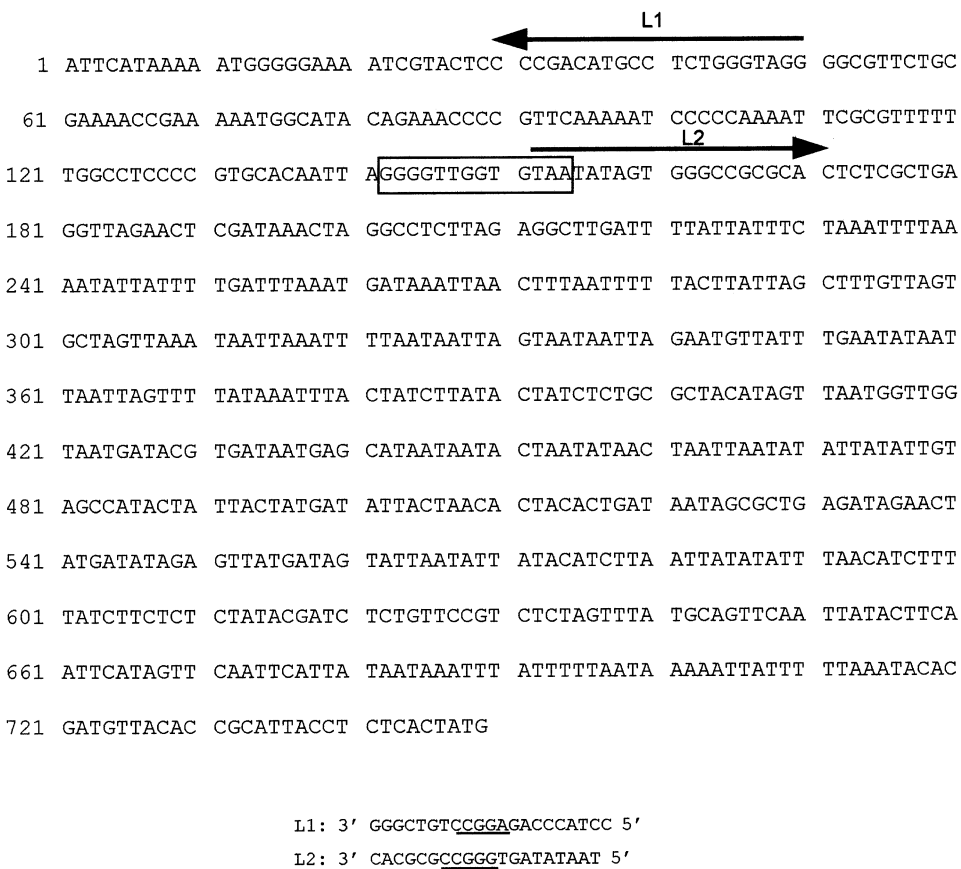

Fig. 1. Whole minicircle sequence from *L. braziliensis* M2904 (de Bruijn and Barker, 1992) showing the position of primers L1 and L2. The universal minicircle sequence (UMS) is boxed. A 145 bp conserved region runs from nt 25–170, with the variable region comprising the remainder of the minicircle. The *Sau*96I site is underlined in L2, and the artificial *Stu*1 site in L1. This minicircle would give a 610 bp product when amplified using L1 and L2.

Table 1
Details of *Leishmania* species isolates and identification of complex based on kDNA probes

WHO species code	Species	Code	Z[a]	Identification by kDNA probes			
				PP75	M379	CG	B18
MHOM/IN(--)/61/L13	*L. donovani*	1	Z 01	+	−	−	−
MHOM/BR/79/L101	*L. chagasi*	2	Z 04	+	−	−	−
MHOM/BR/74/PP 75	*L. chagasi*	3	Z 07	+	−	−	−
MHOM/VE/76/JAP 78	*L. garnhami*	4	Z 11	−	+	−	−
IFLA/BR/67/PH8	*L. amazonensis*	5	Z 12	−	+	−	−
MHOM/BR/76/LTB 012	*L. amazonensis*	6	Z 15	−	+	−	−
MINYC/ BZ/62/ M379	*L. mexicana*	7	Z 20	−	+	−	−
MHOM/ VE/57/ LV135	*L. pifanoi*	8	Z 21	−	+	−	−
MORY/PA/68/GML3	*L. aristidesi*	9	Z 23	−	+	−	−
RTAR/SN/67/G10	*L. tarentolae*	10	Z 27	−	−	−	−
MHOM/SU/73/5 Askh	*L. major*	11	Z 29	−	−	−	−
MHOM/CO 83/REST 417	*L. panamensis*	12	Z 43	−	−	+	+
MHOM/GF/85/Lem 669	*L. guyanensis*	13	Z 50	−	−	+	+
MHOM/BR 78/M5378	*L. guyanensis*	14	Z 51	−	−	+	+
MHOM/BR/84/LTB 300	*L. braziliensis*	15	Z56	−	−	+	+
MHOM/CO/83/Lem 469	*L. braziliensis*	16	Z 58	−	−	+	+
MHOM/BO/90/CG	*L. braziliensis*	22	Z 60	−	−	+	+
MHOM/BO/90/JP	*L. braziliensis*	23	Z 60	−	−	+	+
MHOM/BO/90/JM	*L. braziliensis*	24	Z 60	−	−	+	+
MHOM/BO/90/AM	*L. braziliensis*	25	Z 60	−	−	+	+
MHOM/BO/90/EL	*L. braziliensis*	26	Z 60	−	−	+	+
MHOM/BO/90/CS	*L. braziliensis*	27	Z 60	−	−	+	+
MHOM/Br/75/M2904	*L. braziliensis*	17	Z 62	−	−	+	+
MHOM/PE/90/LH 1016	*L. braziliensis*	18	Z 64	−	−	+	+
MHOM/BO/84/LPZ 595	*L. braziliensis*	19	Z 66	−	−	+	+
MHOM/PE/90/HB44	*L. peruviana*	20	Z 78	−	−	+	+
MHOM/BR/81/M6426	*L. lainsoni*	21	Z 81	−	−	−	+
MHOM/SU/74/k–27	*L. tropica*	28	n/d	−	−	−	−

[a] *Z*, zymodeme numbers referred to in Guerrini (1993) (17 loci).

control where the DNA template was replaced by water. The sensitivity was evaluated using from 10 fg to 10 ng of *Leishmania* reference strain total DNA.

2.3. Gel electrophoresis and Southern blotting

PCR products were separated by electrophoresis on 1.5% agarose gels in 1 × TAE buffer (40 mM Tris acetate; 1 mM EDTA pH 8.0) and stained with 200 μg/l ethidium bromide. DNA was transferred from agarose gels after alkali denaturing (O.5 M NaOH, 1.5 M NaCl, twice for 15 min) on to charged nylon membranes, Hybond N+ (Amersham, UK) by vacuum blotting.

Polyacrylamide gel electrophoresis was done on 7.5% gels (10 × 7 cm) at 100 V for 90 min in a mini gel system (Bioblock Scientific, France), and stained with ethidium bromide or silver nitrate.

2.4. Probes

Five different probes were used in total: CG, M379, PP75, B18 and LTc. CG, M379 and PP75 were purified major bands of PCR products from reference isolates CG (*L. braziliensis*, 564 bp band), M379 (*L. mexicana*, 350 bp band) and PP75 (*L. chagasi*, 564 bp band), respectively. The fragments were recovered by electroelution using a 422 electroeluter device (Bio-Rad, France) according to the manufacturer's instructions, and

then digested with *Sau*96I and *Stu*I (Promega, USA). After digestion, the DNA was ethanol precipitated and resuspended in 100 μl sterilized, distilled water. The amount of DNA present was quantified by electrophoresis of sequential dilutions.

B18 is a 525 bp partial minicircle from M2904 (*L. braziliensis*) cloned into a PUC8 vector: it contains the conserved region of the minicircle and is specific for the subgenus *Viannia* (Barker et al., 1986; Barker, 1989).

LTc is a mixture of PCR products from SO34 cl4 and MN *T. cruzi* reference stocks, amplified with L1 and L2. These stocks are from two different lineages of the *T. cruzi* taxon. The PCR products were digested with *Sau*96I and *Stu*I as above, and used directly for labelling.

2.5. Labelling and hybridisation conditions

Probes CG, M379 and PP75 were labelled using the enhanced chemiluminescence gene detection system (ECL), according to the manufacturer's instructions (Amersham, UK). Hybridisation was performed at 42°C overnight in a rotating oven (Appligen, France.). The membranes were washed twice to a stringency of 0.1 × SSC. Two exposures were performed (1 and 30 min) on autoradiography film (HyperfilmTM-MP, Amersham, UK).

The B18 probe was labelled with α[^{32}P]dATP using the Prime-It II random priming kit (Stratagene, USA). Hybridisation took place at 65°C overnight, and the filters were washed to a stringency of 0.1 × SSC for 15 min at 65°C. Autoradiographic film was exposed overnight at − 70°C.

2.6. Phylogenetics

Each reproducible kDNA-PCR gel band was coded, starting with 1 for the largest DNA fragment, so that each stock was represented by a set of numbers. Similarly, for each enzyme system each band is coded. For estimation of divergence and clustering, Jaccard's distance (Jaccard, 1908) was used. It is estimated as follows: $D_j = 1 - (C/2 N - C)$ where C is the number of bands common to both genotypes to be compared, and N is the total number of observed bands in the two compared genotypes. The UPGMA method (unweighted pair-group method with arithmetic averages; Sokal and Sneath (1963)) was used to cluster the zymodemes or the kDNA-PCR profiles together according to their Jaccard's distances. Dendrograms were computed using Mac Dendro software (Thioulouse, 1989).

3. Results

3.1. Sensitivity and specificity of kDNA-PCR

For all of the 28 *Leishmania* isolates studied, the primers L1 and L2 generated multi-sized products ranging from 100–1300 bp, amplified from the variable regions of kDNA minicircles (Fig. 2a). The banding patterns were reproducible when the annealing temperature of the PCR was raised from 58 to 61 and 64°C. The PCR was sensitive enough to detect 1–100 parasites depending on the *Leishmania* complex: the minimal amount of total DNA used as template was 0.1, 10 and 100 pg from stocks CG, PH8 and PP75, respectively. Hybridisation was found to increase the sensitivity 10-fold. Primers L1 and L2 were not specific to *Leishmania* species, amplifying all other Kinetoplastidae tested: 12 stocks of *T. cruzi*, four stocks of *T. rangeli* and two stocks of *T. brucei* (Fig. 3).

To ensure that L1 and L2 did not amplify human DNA, DNA was extracted from non-infected patients using the method of Wincker et al. (1994). The quality of the DNA was confirmed by amplification using β-globin primers. No product was observed when this DNA was used as template in a L1L2 PCR reaction. Similarly, hamster DNA was extracted from non-infected and infected animals; an amplification product with L1 and L2 was observed only from the infected animals. FASTA searches (Pearson and Lipman, 1988) of EMBL and Genbank using the primer sequences showed significant homology only to *Leishmania* species from the subgenus *Viannia*, with only very weak homology to some human unique sequences. The kDNA nature of the amplified products was confirmed both from strains of the subgenus *Viannia* by hybridisation with

B18, which is a cloned minicircle fragment, and from *T. cruzi* strains: the L1L2 PCR product from *T. cruzi* recognised the same bands of *Hin*f1- and *Eco*R1-digested total *T. cruzi* DNA as variable region kDNA probes previously used (Veas et al., 1991).

3.2. PCR-polymorphism of Leishmania isolates

Agarose electrophoresis of PCR products revealed polymorphism among *Leishmania* species. All isolates from the subgenus *Viannia* and the *L. lainsoni* species presented a similar profile characterised by a pack of bands of around 610 bp (Fig. 2a). The other *Leishmania* species fail to show specific patterns. Acrylamide electrophoresis gave a clearer separation of PCR products (Fig. 4) and was used to assess the relationship between the 28 *Leishmania* strains by constructing a UPGMA dendrogram (Fig. 5b), and comparing it with a similar dendrogram constructed from MLEE data (Fig. 5a). The topologies of the two dendrograms are similar.

The MLEE dendrogram clusters the isolates in principal lineages as previously reported (Guerrini, 1993). The three stocks from the *donovani* complex are clustered together, as are those from the *mexicana* complex (*L. amazonensis*, *L. mexi-*

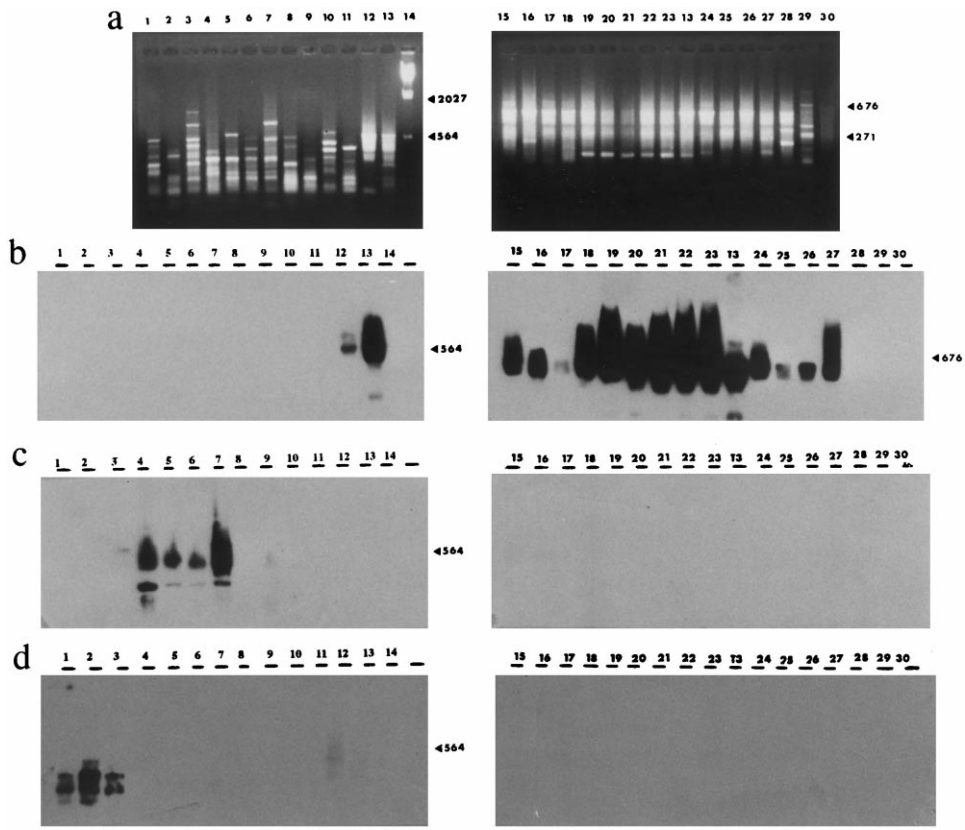

Fig. 2. (a) Ethidium bromide stained 1.5% agarose gel containing kDNA PCR products from *Leishmania* strains. (b, c, d) Hybridisation with *Leishmania* complex specifc probes (CG, M379, and PP75, respectively). Lanes 1–3: *L. donovani* complex (L13, L101 and PP75); lanes 4–7 and 9: *L. mexicana* complex (JAP 78, PH8, LTB 012, M379 and GML3); lane 8: *Leishmania* sp.; lane 10: *L. tarentolae* (G10); lane 11: *L. major* (5 Askh); lanes 12–13, 15–27: *braziliensis* complex (subgenus *Viannia*) (REST 417, CG, Lem 669, M5378, LTB 300, Lem 469, JP, AM, EL, JM, CS, GC, M2904, LH 1016, LPZ 595 and HB44); lane 28: *L. lainsoni* (M6426); lane 29: *L. tropica* (k27); lanes 14 and 30: molecular weight markers λ *Hin*dIII and Puc Ras I. NB: Amplification of GML3 (lane 9) was subsequently repeated, giving a much stronger product, which gave a strong signal when hybridised with probe M379.

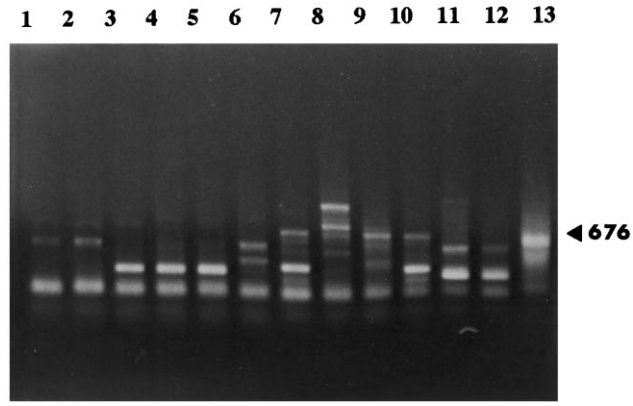

Fig. 3. Ethidium bromide stained 1.5% agarose gel containing kDNA PCR products from Kinetoplastidae strains. Lanes 1–6: *T. cruzi* strains (OPS21, Cuica, CBB, MN, Tpk1 and Tehuentepec); lanes 7–10: *T. rangeli* strains (Basel, Riera, 2008 and ITMAP 1140); lanes 11 and 12: *T. brucei* strains (Biyamina and Eatro 1125); and lane 13: *L. peruviana* (HB44).

cana, *L. pifanoi*, and *L. garnhami* species). The stocks from the subgenus *Viannia* (the *braziliensis* complex) are separated into two groups corresponding to *L. panamensis* and *L. guyanensis* species on the one hand (Fig. 5a, isolates numbered 12–14) and *L. braziliensis* and *L. peruviana* on the other (Fig. 5a, isolates numbered 15–20 and 22–27). These two groups are more closely related to each other than to isolates from the other complexes. *L. lainsoni* is most closely related to species of the subgenus *Viannia*.

The kDNA-PCR dendrogram shows large genetic distances between the different isolates. Isolates belonging to the subgenus *Viannia* (*braziliensis* complex) or *mexicana* complex are closely related to other isolates in their complex, but within the complexes the topology is different to that observed from MLEE data. It is worth noting that the isolates belonging to zymodeme 60 (Table 1) are distinguished by kDNA-PCR.

3.3. Complex specificity of the three Leishmania kDNA probes

High stringency hybridisation results using the CG probe (from *L. braziliensis*) to Southern blots of PCR products is presented in Fig. 2b. The probe hybridised only to bands of around 600 bp in size from the isolates belonging to the subgenus *Viannia* (Table 1).

The B18 probe hybridised the to same set of isolates as CG but also to the *L. lainsoni* isolate (Table 1), again recognising only bands of around 600 bp in size (data not shown).

Hybridisation of probe M379 (*L. mexicana*) was limited to *L. amazonensis*, *L. pifanoi*, *L. mexicana*, *L. garnhami* and *L. aristidesi* (Fig. 2c, Table 1). This probe recognised two bands: one of around 600 bp, and another of around 200–300

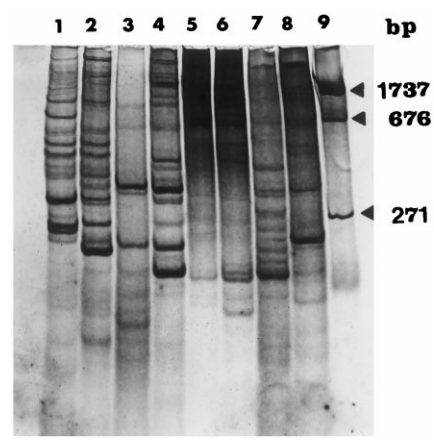

Fig. 4. Ethidium bromide stained 7.5% acrylamide gel showing kDNA PCR products from *Leishmania* strains. Lane 1: PP75 *L. chagasi*; lane 2: L13 *L. donovani*; lane 3: M379 *L. mexicana*; lane 4: M6426 *L. lainsoni*; lane 5: GML3 *L. aristedesi*; lane 6: M2904 *L. braziliensis*; lane 7: M5378 *L. guyanensis*; lane 8: G10 *L. tarentolae*; and lane 9: molecular weight markers Puc 19/Ras I.

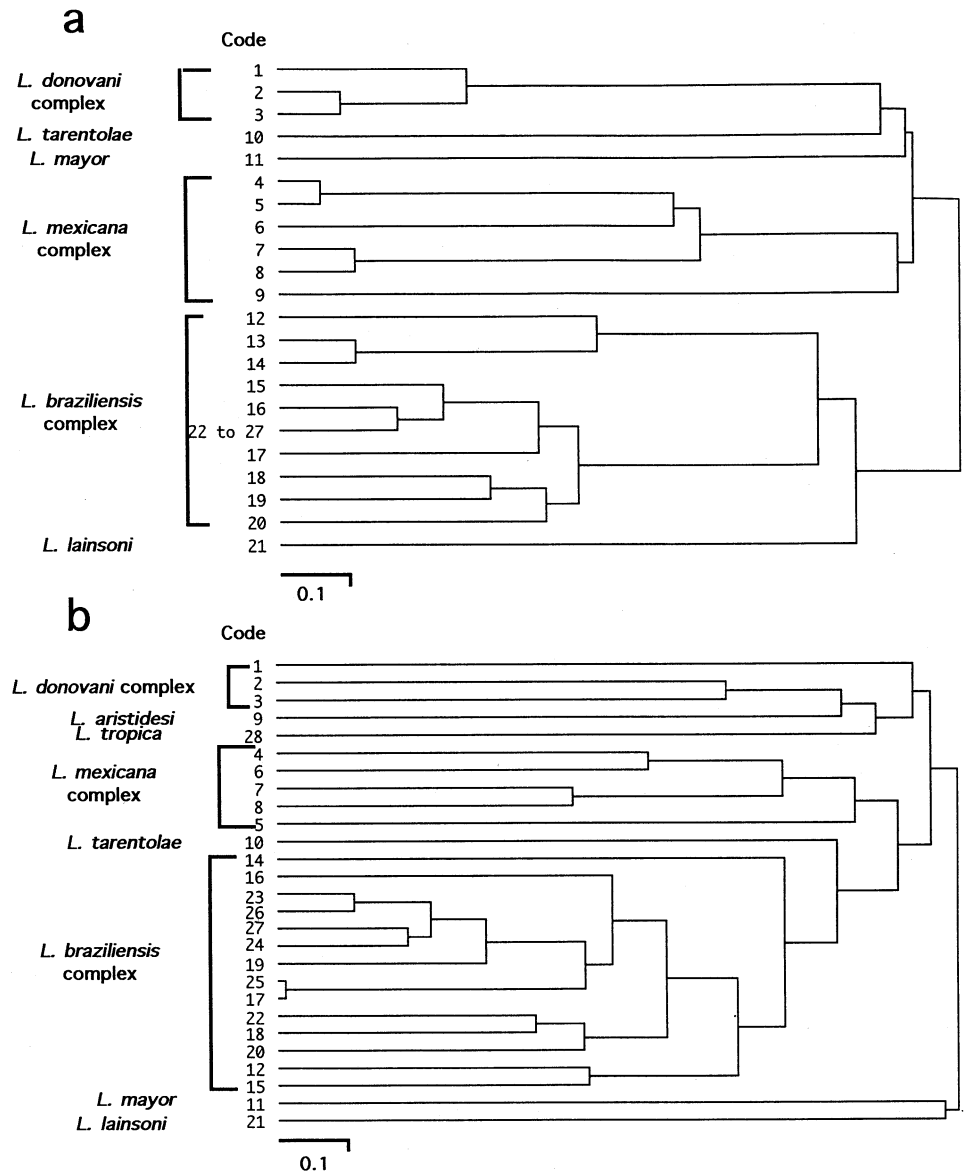

Fig. 5. Dendrograms constructed using the unweighted pair group method with arithmetic mean from a Jaccard distance matrix, based on (a) a multilocus enzyme electrophoresis study, and (b) kDNA-PCR patterns. The numbers at the extremities of the branches refer to strain codes indicated in Table 1.

bp. High stringency hybridisation of probe PP75 (*L. chagasi*) to Southern blots of PCR products was limited to small bands from the three isolates belonging to the *donovani* complex (Fig. 2d). Probes CG, B18, M379 and PP75 do not hybridise to PCR products from *T. cruzi*, *T. rangeli* and *T. brucei* sp. isolates.

Interestingly, one sample (*Leishmania* sp., Fig. 2a, lane 8) gave a small PCR product which was not hybridised by any of the probes. This sample, from Paraguay, was subsequently found not to be related to *L. braziliensis*, *L. mexicana*, *L. amazonensis* or *L. donovani* by isoenzyme analysis at eight loci, although it was most similar to mem-

bers of the *Leishmania* subgenus. It remains as yet unidentified.

4. Discussion

The prognosis of human leishmaniasis depends in part on the species of the infecting parasite. In field situations the isolation of *Leishmania* cultures for their species characterisation is not always possible, especially since often very few parasites are present in lesions, blood, skin, organs and sandflies. Moreover, isolation and culture protocols can select for particular clones from a heterogeneous population. It is highly desirable to develop sensitive molecular tools that are able to identify and type *Leishmania* directly.

Several studies have used PCR to amplify parasite DNA in biological samples using species- or complex-specific primers (de Bruijn and Barker, 1992; Arevalo et al., 1993; Guevara et al., 1992; Eresh et al., 1994). In some geographical areas more than one *Leishmania* species may be present, and to screen a single sample with multiple PCRs is expensive and introduces a major risk of contamination (false positives). Thus a general PCR avoiding population selection followed by secondary typing with probes is useful to discriminate *Leishmania* in the field (Rodgers et al., 1990; Degrave et al., 1994). Here, we use a general Kinetoplastida PCR to generate polymorphism among Kinetoplastidae followed by *Leishmania* species-complex discrimination using kDNA probes, an approach involving only one PCR reaction which can be used to identify a wide range of species.

Primers L1 and L2 were designed from the minicircle sequence of M2904 *L. braziliensis* (Fig. 1). Bearing in mind that minicircles from the *braziliensis* complex are approximately 750 bp in size, the product of L1L2 amplification should be around 610 bp in size; matching the set of bands shown in Fig. 2a. The kDNA nature of amplified products was confirmed by hybridisation to a cloned minicircle fragment, probe B18 (Table 1).

A search of GenBank revealed that the primers share sequence homology only with minicircle sequences from the subgenus *Viannia*. This correlates well with the higher sensitivity of L1 and L2 for species of this subgenus. The highly polymorphic nature of the PCR products is rationalised by hybridisation: only some of the amplified bands are detected by kDNA probes. This does not mean to say that the remaining bands are not of *Leishmania* kinetoplast or nuclear DNA origin; when primers L1 and L2 were tested on human and hamster DNA, no product was observed, implying that all products are amplified from parasite DNA. In this way, L1 and L2 act almost as RAPD primers, producing a fingerprint of the DNA of each parasite strain, the complex of which can then be identified by hybridisation.

This fingerprint may be due to annealing of the primers at multiple sites around minicircles; indeed we know that minicircles from *T. cruzi* have four conserved regions equally spaced around the minicircle (Degrave et al., 1988). In addition, the minicircle network of kinetoplastid organisms is often comprised of a number of different sequence classes, ranging from one in *T. evansi* (Borst et al., 1987) to an estimated 300 in *T. brucei* (Steinert and Van Assel, 1980). Thus, a multiple-sized PCR product may be the result of the primers annealing to different positions on each minicircle sequence class. The less polymorphic PCR product generated from species of the *braziliensis* complex reflects the remarkable homogeneity of the minicircle sequence over the primer annealing sites in these species (Brewster and Barker, unpublished observations).

The degree of polymorphism of the PCR product was used to relate species: species from the same complex showed similar, but not identical banding patterns. When the products were analysed in greater detail using polyacrylamide gel electrophoresis, extensive intra-specific and intra-zymodeme polymorphism was observed, and this was confirmed by phylogenetic analysis. The identification of individual strains is frequently required to infer the presence of the same pathogenic agent in different hosts; the technique is useful for vector and reservoir studies, and for the analysis of relapsed leishmaniasis in patients.

Several authors class *L. lainsoni* as belonging to the subgenus *Viannia* (Eresh et al., 1995).

Whether or not this is justified remains debatable: the CG probe hybridised to all strains of the *braziliensis* complex except *L. lainsoni* species, but B18 hybridised to all strains of the *braziliensis* complex including *L. lainsoni*. Isoenzyme analysis shows that *L. lainsoni* is genetically distant to other members of the *Viannia* subgenus (Guerrini, 1993; Soccol, 1993) and the kDNA-PCR pattern is also different to all other strains tested (Fig. 4b), results which favour the theory that this species is substantially different to other species of the subgenus *Viannia* (Eresh et al., 1995).

The M379 probe hybridised to strains from the *mexicana* complex including *L. aristidesi*, which is genetically distant to other members of the complex. This parasite is not a human pathogen and its taxonomic position is not well established. Similarly, the PP75 probe hybridised only to strains of the *donovani* complex, although more extensive testing is necessary to confirm the specificity of this probe. It is worth noting that numerous kDNA minicircle probes have been developed to type complexes and species of *Leishmania* (Barker and Butcher, 1983; Barker, 1990; Degrave et al., 1994; Ravel et al., 1995; Fernandes et al., 1996). These probes should be tested on our kDNA-PCR products and may be able to discriminate the species within each complex.

Kinetoplast DNA is a useful target for PCR due to minicircle abundance. The high degree of sequence heterogeneity makes minicircle sequence data useful for taxonomic purposes. PCR and subsequent typing with kDNA probes have been applied to identify vectors and reservoirs and to type parasites in biopsy tissue and the blood of patients (Perez et al., 1994; Vasconcelos et al., 1994; Ravel et al., 1995; Passos et al., 1996). Primers L1 and L2 are being tested on blood samples from a preliminary set of patients with active cutaneous lesions from a focus of *mexicana* complex *Leishmania* infection in Bolivia. Five out of six blood samples gave products with similar multi-banding patterns, which did not resemble the characteristic pattern of the subgenus *Viannia*. Similarly, multi-banding patterns were obtained from blood samples of mammals captured in the *mexicana* focus (15/39) and from tissues (liver, spleen and skin) of a *Dasypus novemcinctus* from the Alto Beni region. Hybridisation is in progress, and the study of reservoirs has been extended to another Bolivian focus.

The use of the L1 and L2 primers in the search for reservoirs offers an important auxiliary tool that allows the identification of both *T. cruzi* and *Leishmania* species. In most Bolivian foci of *Leishmania*, *T. cruzi* is also present. This tool allows rapid screening of multiple samples and facilitates large scale epidemiological studies.

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