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American tegumentary leishmaniasis: antigen-gene polymorphism, taxonomy and clinical pleomorphism

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Abstract

Multi-locus enzyme electrophoresis is the current gold standard for the genetic characterisation of *Leishmania.* However, this method is time-consuming and, more importantly, cannot be directly applied to parasites present in host tissue. PCR-based methods represent an ideal alternative but, to date, a multi-locus analysis has not been applied to the same sample. This has now been achieved with a sample of 55 neotropical isolates (*Leishmania* (*Viannia*) *braziliensis*, *L.* (*V.*) *peruviana*, *L.* (*V.*) *guyanensis*, *L.* (*V.*) *lainsoni* and *L.* (*L.*) *amazonensis*), using five different genes as targets, four of which encoded major *Leishmania* antigens (*gp63*, *Hsp70*, *H2B* and *Cpb*). Our multi-locus approach strongly supports the current taxonomy and demonstrates a highly robust method of distinguishing different strains. Within *L.* (*V.*) *braziliensis*, we did not encounter so far specific genetic differences between parasites isolated from cutaneous and mucosal lesions. Interestingly, results provided by each of the different antigen-genes in the species considered, were different, suggesting different selective pressures. Our work emphasises the need for a multi-disciplinary approach to study the clinical pleomorphism of leishmaniasis.

Keywords: Muco-cutaneous leishmaniasis; Multi-locus PCR; Antigens; Taxonomy

1. Introduction

Leishmaniasis is endemic in 88 countries on four continents, causing an estimated disease burden of 2,357,000 disability adjusted life years and 59,000 deaths per annum (WHO, 2002). This disease, caused by the protozoan parasite *Leishmania*, is characterised by a considerable clinical pleomorphism. Severity varies from self-limiting cutaneous lesions of the skin to severe mutilating mucosal manifestations or fatal visceral forms. The paradigm of the disease is that specific clinical manifestations are generally associated with particular *Leishmania* species, but within a given species, different clinical forms can be encountered. This is typically illustrated by muco-cutaneous leishmaniasis—this severe syndrome encountered in Latin America has been reported in patients infected with *Leishmania* (*Viannia*) *guyanensis* and *L.* (*V.*) *panamensis* (Thomaz-Soccol et al., 2000), but is essentially caused by one species, *L.* (*V.*) *braziliensis.* Of all patients infected with *L.* (*V.*) *braziliensis* and having developed a primary cutaneous lesion, 10% will develop a metastatic mucosal presentation during their lifetime (Campos, 1990). This is likely to be the results of interaction between host and parasite factors. With this in mind, parasite genotyping plays an essential role in both clinical and epidemiological risk assessment. Ideally, genetic characterisation tools should combine five features: (i) direct applicability on host tissues, (ii) easy, rapid and

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high throughput analyses, (iii) association with a robust taxonomy, (iv) identification of intra-species types—if any—associated with specific clinical features, and (v) fingerprinting properties for molecular tracking. The present reference method, multi-locus enzyme electrophoresis (MLEE; Rioux et al., 1990), partially addresses these criteria. MLEE constitutes the basis of current taxonomy and permits fingerprinting to a degree (through the definition of zymodemes), however, it requires parasite isolation and mass culture, and is therefore essentially limited in application to specialised reference centres. Its contribution to the understanding of clinical pleomorphism is limited.

Multi-locus PCR (MLP) analysis presents an ideal complement to MLEE. PCR can be applied directly to clinical samples, and several *Leishmania*-specific targets are available (Cupolillo et al., 1995; Marfurt et al., 2003; Victoir et al., 2003; Garcia et al., 2004), some of which allow the exploration of the genetic polymorphism of antigens. When considering the immunopathology of leishmaniasis (Chang and McGwire, 2002), an eventual link with the clinical pleomorphism of the disease might be explored with antigen-genes. However, an MLP analysis—comparing, for instance, the resolutive power of several markers on a same sample of parasites—has never been performed.

By combining three targets of previously reported PCR assays (*rDNA ITS*, Cupolillo et al., 1995; *gp63*, Victoir et al., 2003; *Hsp70*, Garcia et al., 2004) to two new ones (histone 2B (*H2B*) and cysteine proteinase B (*Cpb*)), this study presents the MLP analysis of 55 *Leishmania* isolates from patients with American tegumentary leishmaniasis. Taxonomic validity of MLP analysis was assessed by comparison with previously reported MLEE results (Bañuls, 1998). Exploration of a possible link between antigen-gene polymorphism and clinical pleomorphism was performed on 20 *L.* (*V.*) *braziliensis* isolates, 9 of which originated from mucosal lesions, 11 from cutaneous ones.

2. Materials and methods

2.1. Patients and samples

Fifty five *Leishmania* strains (Table 1) were isolated from (i) 51 patients from Peru and Bolivia, (ii) 2 reservoir animals, and (iii) 2 sand fly vectors. Species identification (*L.* (*V.*) *braziliensis*, *L.* (*V.*) *peruviana*, *L.* (*V.*) *guyanensis, L.* (*V.*) *lainsoni* and *L.* (*L.*) *amazonensis*) was performed by multi-locus enzyme electrophoresis (Bañuls, 1998). The 20 *L.* (*V.*) *braziliensis* strains were isolated between 1993 and 1994 in two Amazonian foci of Peru (Pilcopata) and Bolivia (Isibore Secure): 11 strains came from patients with simple cutaneous lesions and 9 from patients with mucosal lesions. Informed consent was obtained from all patients and research protocols complied with respective institutional policies as well as with the Helsinki Declaration. The cryopreserved *Leishmania* strains were grown first in Tobie blood agar medium (Tobie et al., 1950) at 26 °C and then mass cultures were grown in HO-MEM supplemented medium. The parasites were harvested by centrifugation at the late log phase and DNA purification was performed using DNAzol (Gibco, Merelbeke, Belgium) reagents according to the manufacturer instructions.

2.2. PCR assays

Five PCR assays were used. Three are reported elsewhere: *rDNA ITS* PCR (Cupolillo et al., 1995), *gp63* PCR (Victoir et al., 2003) and *Hsp70* PCR (Garcia et al., 2004). Two assays were developed for targeting the coding regions of the following genes: histone 2B (*H2B*) and cysteine proteinase B (*Cpb*). Using sequences reported in GenBank, primers were designed using Primer Premier 5.0™: for *H2B*, (sense) 5′-CGG TGC GCC TTG TGC TGC-3′ and (antisense) 5′-AGC CTT CGT GCC CTC AGC-3′ (respectively, at positions 543–560 and 1503–1520 on *L. enriettii* reference sequence M38215) and for *Cpb*, (sense) 5′-TGT GCT ATT CGA GGA GTT CAA-3′ and (anti-sense) 5′-TTA CCC TCA GGA ATC ACT TTG T-3′ (respectively, at positions 106–126 and 1254–1275 on reference *L.* (*V.*) *braziliensis* sequence; Lanfranco, 2003). The PCR mix (50 μl) contained: 10 ng DNA, 1× buffer, 0.5 mM (final) $MgCl_2$, 200 μmol of dNTPs mix, 20 pmol of each primer and 1.5 U *Taq* DNA polymerase (Eurogentec, Seraing, Belgium); for *H2B*, 0.75 mM $MgCl_2$ and 0.25% (final) DMSO were used. Thermal cycling parameters of the two new PCR assays were—*H2B* PCR: (i) initial denaturation at 94 °C for 5 min, (ii) 33 cycles consisting in denaturation at 94 °C for 30 s, annealing at 61 °C for 1 min, and extension at 72 °C for 2 min, followed by (iii) a final extension at 72 °C for 8 min; *Cpb* PCR: (i) initial denaturation at 94 °C for 5 min, (ii) 33 cycles consisting in denaturation at 94 °C for 30 s, annealing at 53 °C for 1 min, and extension at 72 °C for 3 min, followed by (iii) a final extension at 72 °C for 8 min. PCR products and negative controls, were analysed on 2% agarose gel and remaining amplification products were stored at 4 °C for further analysis.

2.3. Restriction fragment length polymorphism analysis of PCR products (PCR-RFLP)

After PCR amplification, PCR products were ethanol-precipitated, dried and resuspended in 20 μl of water. In order to determine the concentration of the PCR products after precipitation, 1 μl was analysed on 2% agarose gel together with a ladder designed for DNA quantification (MBI-Fermentas, St. Leon-Rot, Germany). PCR products were separately digested overnight in a total volume of 10 μl, with 10 U of each restriction enzyme, as recommended by the manufacturer (Eurogentec, MBI-Fermentas, New England Biolab). Restriction enzymes were selected among those found to cut more than three times in sequences reported in GenBank. The following enzymes were used:

Table 1
Stocks used in present study: species identification is based on MLEE typing

Species	International code	Origin	Pathology
L. (*V.*) *braziliensis*	MHOM/BO/94/CUM29	Bolivia	M
	MHOM/BO/94/CUM43	Bolivia	M
	MHOM/BO/94/CUM45	Bolivia	M
	MHOM/BO/94/CUM49	Bolivia	M
	MHOM/BO/94/CUM52	Bolivia	M
	MHOM/BO/94/CUM68	Bolivia	M
	MHOM/BO/94/CUM152	Bolivia	M
	MHOM/PE/93/LC2143	Peru	C
	MHOM/PE/93/LC2176	Peru	C
	MHOM/PE/93/LC2177	Peru	C
	MHOM/PE/94/LC2320	Peru	M
	MHOM/PE/94/LC2368	Peru	M
	MHOM/BO/94/CUM41	Bolivia	C
	MHOM/BO/94/CUM153	Bolivia	C
	MHOM/BO/94/CUM42	Bolivia	C
	MHOM/BO/93/LC2123	Peru	C
	MHOM/BO/94/CUM97	Bolivia	C
	MHOM/PE/94/LC2355	Peru	C
	MHOM/PE/94/LC2284	Peru	C
	MHOM/PE/94/LC2367	Peru	C
L. (*V.*) *peruviana*	MHOM/PE/90/HB22	Peru	C
	MHOM/PE/90/HB44	Peru	C
	MHOM/PE/90/HB67	Peru	C
	MHOM/PE/90/HB83	Peru	C
	MHOM/PE/90/LCA09	Peru	C
	MHOM/PE/90/LH249	Peru	C
	MHOM/PE/90/LH827	Peru	C
	MHOM/PE/90/LC1015	Peru	C
	MHOM/PE/90/LCA04	Peru	C
L. (*V.*) *guyanensis*	MHOM/PE/91/LC1446	Peru	C
	MHOM/PE/91/LC1447	Peru	C
	MHOM/PE/91/LC1448	Peru	C
	MHOM/PE/94/LC2309	Peru	C
	MHOM/PE/00/LC2797	Peru	C
	MHOM/BR/75/M5378	Brazil	C
	MHOM/GF/85/LEM699	French Guyana	C
	IPRN/PE/00/Lp52	Peru	*Lu. peruensis*
	MHOM/PE/90/LH941	Peru	C
	MHOM/PE/00/LH705	Peru	C
L. (*V.*) *panamensis*	MHOM/PA/71/LS94	Panama	C
	MCHO/PA/00/M4039	Panama	*Choloepus*
L. (*V.*) *lainsoni*	MHOM/BO/95/CUM71	Bolivia	C
	MHOM/BO/94/CUM78	Bolivia	C
	MHOM/BO/94/CUM88	Bolivia	C
	MHOM/BO/95/CUM129	Bolivia	C
	MHOM/PE/92/LC1581	Peru	M
	MHOM/PE/00/LH619	Peru	C
	MHOM/PE/93/LC2029	Peru	C
	MHOM/PE/93/LC2190	Peru	C
	MHOM/PE/91/LH1154	Peru	C
	MHOM/PE/91/LH762	Peru	C
L. (*L.*) *amazonensis*	MHOM/BO/00/CEN001	Bolivia	C
	MHOM/BO/00/CEN018	Bolivia	C
	MPRO/BR/77/LV78	Brazil	*Proechimys*
	IFLA/BR/67/PH8	Brazil	*Lu. flaviscutellata*
	MHOM/BR/73/M2269	Brazil	C

C and M represent cutaneous and muco-cutaneous leishmaniasis, respectively.

rDNA ITS (*Bsh*1236I and *Hinc*6I), *gp63* (*Hinc*II, *Sal*I, *Apa*lI and *Apa*I), *H2B* (*Taq*I, *Hae*III and *Asu*I), *Hsp70* (*Hae*III) and *Cpb* (*Bsh*1236I, *Pst*I and *Taq*I). Reactions were stopped with EDTA (0.5 M, pH 8.0). PCR-RFLP products were analysed separately by capillary electrophoresis (Agilent 2100 bioanalyzer system, Agilent Technologies, Karlsruhe, Germany) in a microchip device (DNA 1000 LabChip, Caliper Technologies, Mountain View, CA, USA). This system was selected not only for its high sensitivity and discriminatory power (Panaro et al., 2000), but also because it requires only 1 μl of restriction products for loading electrophoretic chips (versus 18 μl in agarose gel). All PCR-RFLP assays were performed in duplicate and redigested in order to avoid misleading results caused by incomplete restriction cleavage.

2.4. Phenetic analysis

A character-matrix was created manually by reporting all possible PCR-RFLP fragments in the sample studied. Then, for each strain, presence (1) or absence (0) of bands was scored. We did not take into consideration differences of intensity among restriction fragments of a given amplicon: this phenomenon is common in tandemly repeated genes consisting of sequence variants present in different copy number (Victoir et al., 1998). These matrices were processed for phenetic analyses, with the following programs of PHYLIP version 3.6 package (Felsenstein, University of Washington, 2002): RESTDIST (restriction fragments distance, modification of Nei and Li restriction fragments distance method; Nei and Li, 1979), UPGMA (unweighted pair group method with arithmetic averages), CONSENSE (majority rule consensus), and SEQBOOT (bootstrap analysis). Analysis of restriction fragments was also done using REAP version 4.0, a package which takes into account related molecular factors such as cut site sequence (McElroy et al., 1992). The bootstrap analyses were performed for 1000 replications to estimate the robustness of the nodes. Drawing of dendrograms was performed with the DRAWGRAM program (PHYLIP). Analyses were done on (i) individual matrices built-up from each of the five sequences and (ii) global matrices gathering the five sets of PCR-RFLP data. The Newick format (http://evolution.genetics.washington.edu/phylip/newicktree.html) was used to summarise and compare the different trees in Table 2: each tree is specified by nested pairs of parentheses, enclosing names and separated by commas; the pattern of the parentheses indicates the pattern of the tree by having each pair of parentheses enclose all the members of a monophyletic group.

3. Results

3.1. PCR-RFLP assays

The five PCR-RFLP assays were applied to all 55 isolates. Assays targeting *rDNA ITS* as well as *gp63* and *Hsp70* genes gave similar results as described elsewhere, i.e. polymorphic patterns made of one or more fragments (Cupolillo et al., 1995; Victoir et al., 2003; Garcia et al., 2004). With respect to the two new assays (*H2b* and *Cpb*), amplicons were the size expected from the reported sequences: 980 bp for *H2b* and 1050 bp for *Cpb*. No amplification was obtained with the *Cpb* primers here used in *L.* (*V.*) *lainsoni* and *L.* (*L.*) *amazonensis*; this was probably due to variation in the *Cpb* priming site(s) of these two species. After cleavage with restriction enzymes, one or more fragments were obtained. The sum of the restriction fragments size was similar to the amplicon's size, demonstrating completeness of digestion.

Table 2
PCR-RFLP analysis of non-coding and antigen-coding sequences

Target	Species (order of branching)
RDNA ITS (2)	((((*Lb*, (*Lp*, *Lb*)), *Lg*), *Ll*), *La*)
gp63 (4)	((((*Lb*, *La*), *Lp*), *Lg*), *Ll*)
H2B (3)	(((*Lb*, *Lp*), (*Lg*, *Ll*)), *La*)
Hsp70 (1)	((*Lb* + *Lp*), ((*Lg*, *La*), *Ll*))
Cpb (3)	(*Lb*, (*Lg*, *Lp*))
ML-4	((((*Lb*, *Lp*), *Lg*), *Ll*), *La*)
ML-5	((*Lb*, *Lp*), *Lg*)

Target	Polymorphism in each species							
	Total	*Lb*	*Lb*C	*Lb*M	*Lp*	*Lg*	*Ll*	*La*
RDNA ITS (2)	0.24	**0.35**	*0.45*	*0.33*	0.1	0.1	0.3	0.2
gp63 (4)	0.18	0.1	*0.18*	*0.11*	0.1	0.3	0.2	**0.4**
H2B (3)	0.36	0.3	*0.36*	*0.55*	0.4	0.1	**0.7**	0.4
Hsp70 (1)	0.09	0.05	*0.18*	*0.11*	**0.1**	**0.1**	**0.1**	**0.1**
Cpb (3)	0.41	**0.57**	*0.63*	*0.77*	0.2	0.3	–	–
MLP-4	0.53	**0.55**	*0.73*	*0.66*	0.4	0.3	0.8	0.6
MLP-5	0.72	**0.95**	*0.91*	*1*	0.5	0.5	–	–

Summary of the features observed on dendrograms built-up from single-locus (number of restriction enzymes between parentheses) or multi-locus (MLP-4 and MLP-5, without and with *Cpb*, respectively) data. Species: *Lb*, *L.* (*V.*) *braziliensis* (C and M, from cutaneous and mucosal patients respectively); *Lp*, *L.* (*V.*) *peruviana*; *Lg*, *L.* (*V.*) *guyanensis*; *Ll*, *L.* (*L.*) *lainsoni*; *La*, *L.* (*V.*) *amazonensis*. Order of branching: see Section 2. Polymorphism in each species is calculated by dividing the number of different genotypes encountered in a given species by the number of strains analysed (bold, maximum value among species studies; underlined, maximum values among C and M *Lb* isolates).

3.2. Phenetic analysis

In order to obtain a comprehensive view of the genetic polymorphism within the sample studied, PCR-RFLP data were processed by phenetic analysis. Three types of analyses were performed, and results were similar when using RFLP data analysis softwares of PHYLIP and REAP.

First, PCR-RFLP patterns obtained from each target sequence were analysed individually (see summary in Table 2) and the following features observed:

(i) The five species separated individually in dendrograms built-up from *gp63* and *H2B* data, *Hsp70* did not allow *L.* (*V.*) *braziliensis* to be distinguished from *L.* (*V.*) *peruviana*, whilst two *L.* (*V.*) *braziliensis* isolates were

clustered together with *L.* (*V.*) *peruviana* in *rDNA ITS*-based dendrograms.

(ii) The relative branching of the different species varied according to the marker used. For instance, *L.* (*L.*) *amazonensis* identified separately from all species in the *H2B*-based tree, while it clustered together with *L.* (*V.*) *guyanensis* in the *Hsp70*-based dendrogram or together with *L.* (*V.*) *braziliensis* after *gp63* analysis. Similarly, *L.* (*V.*) *peruviana* clustered together with *L.* (*V.*) *braziliensis* in both trees built-up from *H2B* and *Hsp70* data, whilst it was grouped with *L.* (*V.*) *guyanensis* by *Cpb* analysis. With the exception of the two *L.* (*V.*) *braziliensis* isolates clustering with *L.* (*V.*) *peruviana*, the *rDNA ITS* tree was the only tree showing similar relationships among species as the reference MLEE tree (Bañuls, 1998) as well as the MLP trees described here.

(iii) The level of genetic polymorphism within each species was calculated by dividing the number of different genotypes by the number of strains of that species. This was variable according to the marker used. For instance, *L.* (*V.*) *guyanensis* isolates were three times more polymorphic than *L.* (*V.*) *braziliensis* with respect to their *gp63* genes, whilst they were less polymorphic with respect to the *Cpb* (two times) and *H2B* (three times) genes. A similar trend was observed within *L.* (*V.*) *braziliensis*: cutaneous strains were slightly more polymorphic with respect to *rDNA ITS*, *gp63* and *Hsp70*, while *H2B* and *Cpb* polymorphism was higher among mucosal strains.

(iv) Within *L.* (*V.*) *braziliensis*, mucosal strains were scattered in each dendrogram.

Secondly, data of the different assays were processed together (MLP). This was possible for the five species with four sequences only (MLP-4, without *Cpb).* On the corresponding dendrogram (Fig. 1), the five species were distinguished individually (all with bootstrap values of 100%), branching in the following order (starting from the closest): *L.* (*V.*) *braziliensis*, *L.* (*V.*) *peruviana*, *L.* (*V.*) *guyanensis*, *L.* (*V.*) *lainsoni* and *L.* (*L.*) *amazonensis.* All branchings were robust (bootstrap values higher than 99%), except the one between *L.* (*V.*) *guyanensis* and the couple *L.* (*V.*) *braziliensis–L.* (*V.*) *peruviana* (66%); the latter bootstrap value was still higher than one reported with MLEE (41%, Bañuls, 1998). Twenty-nine different genotypes were encountered among the 55 strains analysed (total polymorphism of 53%). Within *L.* (*V.*) *braziliensis*, mucosal strains were scattered. When *Cpb* data were included (MLP-5, for 40 strains of three species only, Fig. 2), *L.* (*V.*) *braziliensis*, *L.* (*V.*) *peruviana* and *L.* (*V.*) *guyanensis* remained individualised in the same branching order, and polymorphism was higher: 0.72 for the whole sample and 0.95 within *L.* (*V.*) *braziliensis* (0.95). As in MLP-4, there was no clustering of mucosal strains within *L.* (*V.*) *braziliensis*, but a trend of geographical structuring was observed, with a major cluster containing

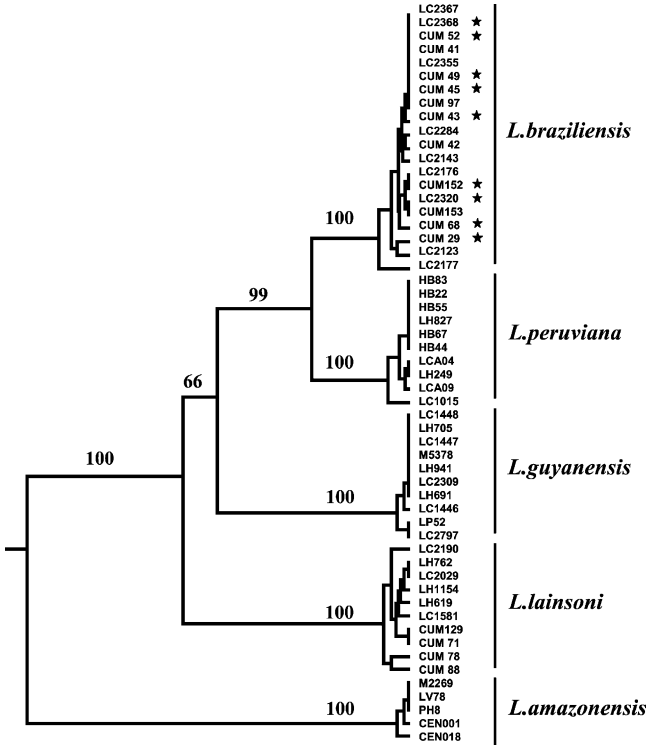

Fig. 1. Genetic polymorphism after PCR-RFLP analysis of *rDNA ITS*, *gp63*, *H2B* and *Hsp70*. Bootstrap values (% from 1000 replicates) are shown above the branches. See Table 1 for origin of the isolates. Stars represent mucosal isolates.

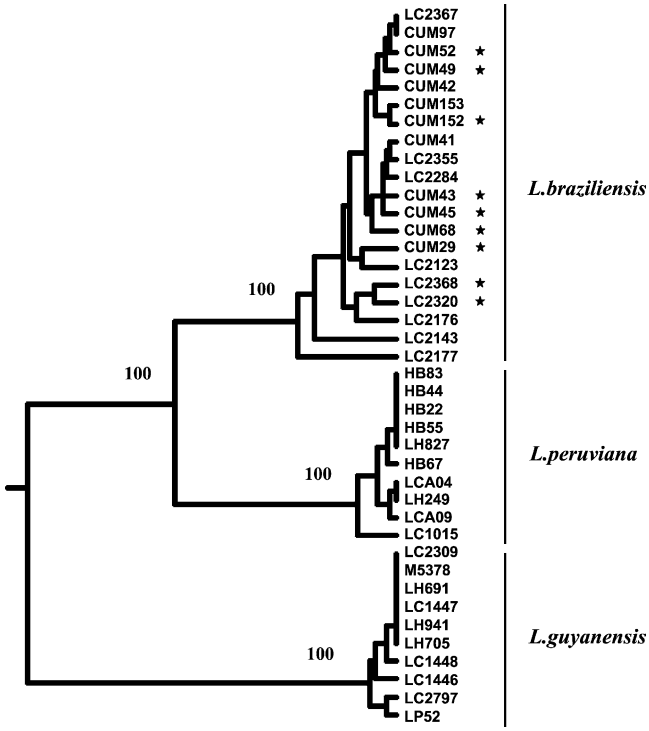

Fig. 2. Genetic polymorphism of *L.* (*V.*) *braziliensis* after PCR-RFLP analysis of *rDNA ITS*, *gp63*, *H2B*, *Cpb* and *Hsp70*. Bootstrap values (% from 1000 replicates) are shown above the branches. See Table 1 for origin of the isolates. Stars represent mucosal isolates.

91% of Bolivian isolates, while 67% of the Peruvian ones branched separately (Fig. 2).

4. Discussion

This study presents the analysis of the genetic polymorphism of five *Leishmania*-specific sequences in a broad sample of South American isolates: one non-coding (*rDNA ITS*) and four encoding major *Leishmania* antigens, i.e. *gp63* (Russo et al., 1993), *Hsp70* (Rico et al., 1999), *H2B* (Probst et al., 2001) and *Cpb* (Souza et al., 1992; Pollock et al., 2003). MLP analysis supported the current taxonomy in a highly robust way. Representatives of each of the five species considered, and currently defined by MLEE analysis, clustered together, with highly significant bootstrap values (>99%). Genetic relationships between the five species were similar to those evidenced by other methods (Cupolillo et al., 1994; Bañuls, 1998; Victoir et al., 1998; Eresh et al., 1995). Finally, MLP gave a high level of polymorphism among strains analysed (up to 95% within *L.* (*V.*) *braziliensis*), demonstrating a particular relevance for the detection of intra-species variation.

Four of the DNA targets here explored were previously analysed by PCR-RFLP directly in human tissues without the need of parasite isolation and cultivation, demonstrating a high sensitivity in detecting patterns (85% for *gp63*, Victoir et al., 2003; 97.3% for *rDNA ITS*, Schönian et al., 2003; 100% for *Hsp70*, Garcia et al., 2004; 100% for *Cpb* in Old World samples, Quispe Tintaya et al., 2004). Further work is necessary to compare precisely the sensitivity of all the methods used in this study and should be done on the same clinical samples using the same case definition. Altogether, these results demonstrate the unique and ideal combination by MLP analysis of four essential features for parasite typing: (i) direct applicability to clinical samples, (ii) easy, rapid and high throughput analyses, (iii) association with a robust taxonomy, and (iv) excellent fingerprinting properties. Accordingly, MLP analysis might become a new standard for genetic characterisation of *Leishmania* populations and allow numerous applications—for example, in large-scale epidemiological surveys for clinical risk assessment or parasite tracking in sand flies and animal reservoirs could be performed in a more rapid and cost-effective way than MLEE. This could be further improved by implementing protocols in which (i) compatible restriction enzymes would be used together, and (ii) digests of different amplicons would be run together in a same electrophoresis. Applications in travel medicine are also very promising—as a combination of a few markers, like *rDNA ITS* and *gp63* or *H2B* are enough to make *Leishmania* species identification possible within 24 h. This will help physicians in adapting their therapy and patient follow-up.

Interestingly, the results provided by each of the individual genetic characters considered here were very different, further emphasising the need of multi-locus approaches for comprehensive genetic characterisation of the parasites. Discrimination of the different species and their branching on dendrograms varied according to the genetic markers. This might be influenced by the choice of restriction enzymes used in this study, and homoplasy in some restriction sites due to random genetic drift may not be excluded. Alternatively, these inconsistencies and the consequent convergence of some characters in very different species might be explained by a variable selective pressure between *Leishmania* species on respective genes. This is to be expected when comparing non-coding sequences (*rDNA ITS*) with genes encoding major parasite antigens. Also, the degree of polymorphism within each species varied according to the genetic markers. Considering antigen-gene diversity as an adaptive strategy of the parasites (Guerbouj et al., 2001; Victoir and Dujardin, 2002), these results might indicate the existence of different molecular interactions between the respective *Leishmania* species and host's immune system. This hypothesis should be further explored by confirming that polymorphism indeed concerns regions encoding for T- or B-epitopes, as previously shown for *gp63* (Victoir and Dujardin, 2002). The diversity of the host itself should also be considered: as well as humans who are often accidental hosts, the principal reservoir of American tegumentary leishmaniasis is typically a wild animal, which can vary according to the species involved (Lainson and Shaw, 1979). Considering the potential immunological differences among mammalian hosts (White et al., 1989; Mestas and Hughes, 2004), heterogeneous selective pressure is to be expected.

Mucosal leishmaniasis (MCL, also called Espundia), like any other clinical form of the disease, has most probably a multi-factorial origin. Pathogenesis is explained by an exacerbated cellular response, usually with very few parasites in the lesions (Bacellar et al., 2002). This may be related to host factors, as suggested by the direct association encountered between susceptibility to MCL and regulatory polymorphism affecting TNF-α production (Cabrera et al., 1995). The importance of the host genetic background is reinforced by the observations that Bolivian patients of African origin were more susceptible to Espundia than others (Walton and Valverde, 1977). Similarly, the past experience of the host would also contribute to the disease pattern, migrants showing a higher risk of MCL than natives (Alcais et al., 1997). In parallel, parasite factors may also play a major role in MCL development. Specific antigens are detected by MCL sera (versus CL sera) and the corresponding IgG would recognise similar determinants in host mucosal tissues, producing an auto-immune phenomenon (Valli et al., 1999). Furthermore, a biased pro-inflammatory response might be induced by selective overexpansion of T-cell clones specific for certain *Leishmania* antigens (Bacellar et al., 2002). Despite the fact that four of the targeted genes were encoding major *Leishmania* antigens, this study could not demonstrate a subpopulation of *L.* (*V.*) *braziliensis* that would be associated with MCL; to the contrary, MLP-5 analysis revealed that all the mucosal strains were different from each other. Con-

sidering that the restrictions sites detected by our PCR-RFLP assays cover only a minor fraction of the genes here studied, our results do not exclude relevant polymorphisms in other regions of these genes or in other antigen-genes. This is supported by a recent report showing that different forms of American tegumentary leishmaniasis presented a statistically significant difference in their frequencies among RAPD-defined *L.* (*V.*) *braziliensis* clades (Schriefer et al., 2004).

So far, most studies aiming to understand the clinical pleomorphism of leishmaniasis and MCL in particular are fragmented. There is a need for major multi-disciplinary approaches combining studies on (i) immuno-genetics of human hosts, (ii) parasite antigen polymorphism, (iii) extrinsic immuno-modulator factors like sand fly saliva or the co-infection with other parasites, and (iv) environmental factors. The knowledge provided by these studies would allow a better evaluation of clinical and epidemiological risks as well as provide a sound theoretical base for future vaccine trials.

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