MAJOR ARTICLE

Antigen Genes for Molecular Epidemiology of Leishmaniasis: Polymorphism of Cysteine Proteinase B and Surface Metalloprotease Glycoprotein 63 in the *Leishmania donovani* Complex

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Background. Efficient monitoring of endemic and resurgent visceral leishmaniasis (VL) requires discriminatory molecular tools that allow direct characterization of etiological agents (i.e., the *Leishmania donovani* complex) in host tissues. This characterization is possible through restriction fragment–length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)–amplified sequences (PCR-RFLP).

Methods. We present 2 new PCR-RFLP assays that target the gene locus of cysteine proteinase B *(cpb)*, an important *Leishmania* antigen. The assays were applied to the characterization of 15 reference strains of the *L. donovani* complex, and their discriminatory power was compared with that of PCR-RFLP analysis of the *gp63* gene, another *Leishmania* antigen, and with that of multilocus enzyme electrophoresis (MLEE), which is the reference standard for parasite typing.

Results. Restriction patterns of the *cpb* locus were polymorphic, but less so than *gp63* patterns. When data for both loci were combined, differences between PCR-RFLP and MLEE results were encountered. Antigen gene analysis was more discriminatory and supported a different classification of parasites, one that fitted with their geographic origin. PCR-RFLP analysis of *cpb* also allowed direct genotyping of parasites in bone marrow aspirate and venous blood samples obtained from patients with VL.

Conclusion. Antigen genes constitute valid targets for PCR-based *Leishmania* typing without the need for isolation of parasites.

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis and is lethal if left untreated. The disease is encountered in endemic and epidemic conditions in 47 countries worldwide, with an estimated 500,000 new cases/year [1]. Control of the disease is threatened by 3 major risk factors: human-made and environmental changes to the epidemiology, immunodeficiency (mainly caused by HIV coinfection), and resistance against antimonials (the first-line drugs) [2]. Monitoring the emergence and spread of the disease requires, among other things, effective and efficient molecular epidemiology tools.

Thus far, the reference standard for genetic characterization of *Leishmania* species has been multilocus enzyme electrophoresis (MLEE) [3]. On the one hand, this method, together with epidemiological criteria, supports a classification that groups the etiological agents of VL within the *L. donovani* complex of species [4]: *L. infantum* and synonym *L. chagasi* (which cause zoonotic VL in Europe/Africa and Latin America, respectively), *L. donovani* (which causes anthroponotic VL in Asia and

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Table 1. Stocks used in the present study for identification of *Leishmania* species.

Species, study code	International code	Country of origin	Zymodeme
L. infantum			
LG1	MHOM/FR/1978/LEM75	France	MON1
LG2	MHOM/FR/1995/LPN114(LEM3001)	France	MON1
LG3	MHOM/ES/1993/PM1(LEM2608)	Spain	MON1
LG4	MHOM/FR/1997/LSL29(LEM3420)	France	MON1
LG5	MHOM/ES/1986/BCN16(LEM1078)	Spain	MON1
LG6	MHOM/PT/2000/IMT260(LEM3975)	Portugal	MON1
LG7	MHOM/FR/1996/LEM3249	France	MON29
LG8	MHOM/ES/1991/LEM2298	Spain	MON183
LG12	MHOM/SD/1982/GILANI[a]	Sudan	MON30
LG14	MHOM/FR/1980/LEM189	France	MON11
LG15	MHOM/MT/1985/BUCK	Malta	MON78
L. donovani			
LG9	MHOM/IN/00/DEVI(LEM138)	India	MON2
LG10	MHOM/IN/1996/THAK35(LEM3178)	India	MON2
LG13	MHOM/ET/00/HUSSEN	Ethiopia	LON42
L. archibaldi, LG11	MHOM/ET/1972/GEBRE1	Ethiopia	MON82
L. major	MHOM/SU/73/5-ASKH	USSR	MON4
L. aethiopica	MHOM/ET/72/L100	Ethiopia	MON14

NOTE. Species identification was based on multilocus enzyme electrophoresis typing.

[a] The zymodeme is disputedly attributed to *L. infantum* or *L. donovani.*

East Africa), and *L. archibaldi* (which causes zoonotic VL in East Africa) [3, 5]. On the other hand, MLEE distinguishes >50 different genotypes (called zymodemes) within the *L. donovani* complex and, as such, constitutes a molecular typing tool that is helpful for molecular tracking. Thus, MLEE can be considered to be a very effective characterization tool. However, its efficiency is hampered by several problems, including the need to isolate parasites, the selection of parasite populations during cultivation, and the underestimation of genetic variability. Accordingly, MLEE should be complemented by polymerase chain reaction (PCR)–based molecular methods, because they are discriminatory but also allow direct typing in host tissues.

The success of such PCR typing assays relies mainly on 2 features of the chosen DNA target: repetition for the detection threshold and sequence polymorphism for the discriminatory power. At present, most assays target intergenic sequences of repeated genes (e.g., rDNA, mini-exon, and *gp63*) [6, 7] that are known to be polymorphic as a result of their noncoding nature. However, antigen genes may also be interesting targets for characterization by PCR, especially genes repeated in tandem (such as the 63-kDa surface metalloprotease gene of *Leishmania* species, *gp63*), because they are prone to frequent rearrangements [8], which leads to polymorphisms that may constitute a selective advantage under immune pressure [9]. Analysis of the polymorphisms of antigen genes might give a unique perspective on the population structure of pathogens, which is shaped by the host's immune response [10]. A first attempt at using antigen genes for typing of *Leishmania* species was performed by use of restriction fragment–length polymorphism (RFLP) analysis of PCR-amplified sequences of *gp63* genes (PCR-RFLP) [11, 12]. In the present study, we focused on the gene locus of cysteine proteinase B *(cpb),* another important factor in the host-parasite relationship [13]. PCR-RFLP assays targeting those genes known to be repeated [14, 15] and their noncoding intergenic sequences were developed and applied to the characterization of 15 references strains of the *L. donovani* complex. Results were compared with data obtained by analysis of *gp63* and with data obtained by use of MLEE [16] and were discussed in terms of taxonomic and molecular typing applications, respectively. Direct applicability of the developed tools was tested on bone marrow aspirate (BMA) and venous blood samples obtained from patients with VL.

SUBJECTS, MATERIALS, AND METHODS

Subjects and samples. The *Leishmania* strains studied here belong to the *L. donovani* complex (*L. infantum, L. donovani,* and *L. archibaldi* species identified and typed by use of MLEE; DNA was provided by J.-P.D.). They were selected on the basis of their geographic origin and correspond to different zymodemes (table 1): 6 MON1 strains were included for analysis of genetic heterogeneity within this most widespread zymodeme.

L. major and *L. aethiopica,* which do not belong to the *L. donovani* complex, were used as species-complex controls. Patients with confirmed VL from the Dharan area in eastern Nepal, where VL is endemic, were recruited at the B. P. Koirala Institute of Health Sciences (BPKIHS; Dharan, Nepal). Patients were offered free treatment, in accordance with current policy of the BPKIHS and with guidelines of the World Health Organization. Informed consent was obtained from patients or their parents or guardians. Human-experimentation guidelines of the "Prins Leopold" Instituut voor Tropische Geneeskunde (PLITG) were followed, and ethical clearance was obtained from the review boards of the PLITG and the BPKIHS. After informed consent was obtained, 7 BMA samples (placed in EDTA) and 1 venous blood sample (180 μL, drawn into EDTA and mixed with an equal volume of AS1-buffer [Qiagen]) were obtained. DNA was extracted by use of the QIAamp DNA Mini Kit, for BMA samples, or the QIAamp DNA Blood Mini Kit, for venous blood samples, according to the manufacturer's instructions (Qiagen).

***PCR for amplification of intragenic and intergenic sequences of* cpb.** By use of sequences reported in GenBank, primers were designed by use of the software Primer Premier (version 5.0; Premier Biosoft International; available at: http://www.premierbiosoft.com), to amplify the intragenic and intergenic regions of the *cpb* genes from conserved sequences, for primer hybridization (figure 1). The primers designed for amplification of the intragenic region were *CPBFOR* (5′-CGA ACT TCG AGC GCA ACC T-3′) and *CPBREV* (5′-CAG CCC AGG ACC AAA GCA A-3′), at nucleotide positions 179–197 and 1239–1257, respectively, on the *L. donovani* reference sequence AF309626. The corresponding amplicon should contain 80% of the coding region and should include a major part of the carboxyl region, which has been shown to be the most divergent across species [15]. The primers designed for amplification of the intergenic region were *PIGS1A* (5′-CCT CAT TGC TTT GGT CCT GG-3′) and *PIGS2B* (5′-GGC GTG CCC ACG TAT ATC GC-3′), at nucleotide positions 1234–1253 and 79–98, respectively, on the *L. donovani* reference sequence AF309626. In both cases, the PCR mix (50 μL) contained 20–50 ng of DNA, 1× buffer, $MgCl_2$ (final concentration, 0.5 mmol/L), 40 nmol of dNTPs mix, 20 pmol of each primer, and 1.5 U of *Taq* DNA polymerase (Eurogentec). For intragenic PCR, thermal cycling parameters were initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. For intergenic PCR, thermal cycling parameters were initial denaturation at 95°C for 5 min; 35 cycles denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 2.5 min; and a final extension at 72°C for 10 min. PCR products and negative controls were analyzed in 1% agarose gel, and remaining amplification products were stored at 4°C until further analysis.

PCR-RFLP. After PCR amplification of intragenic or intergenic sequences of *cpb,* PCR products were ethanol-precipitated, dried, and resuspended in 20 μL of water. To determine the concentration of the PCR products after precipitation, 1 μL was analyzed in 1% agarose gel along with a ladder designed for DNA quantification (MBI-Fermentas). PCR products (~0.35 μg) were digested overnight in a total volume of 10 μL, with 10 U of restriction enzyme, as recommended by the manufacturer (MBI-Fermentas). Restriction enzymes were selected among those found to cleave >3 times in *cpb* sequences reported in the GenBank. The following enzymes were used for digesting *cpb:* for intragenic PCR-RFLP, *Hinf*I, *Taa*I, *Hae*III, *Cfr*I, *Hpa*II, and *Sdu*I; and, for intergenic PCR-RFLP, *Eam*1104I, *Nsp*I, *Hae*III,

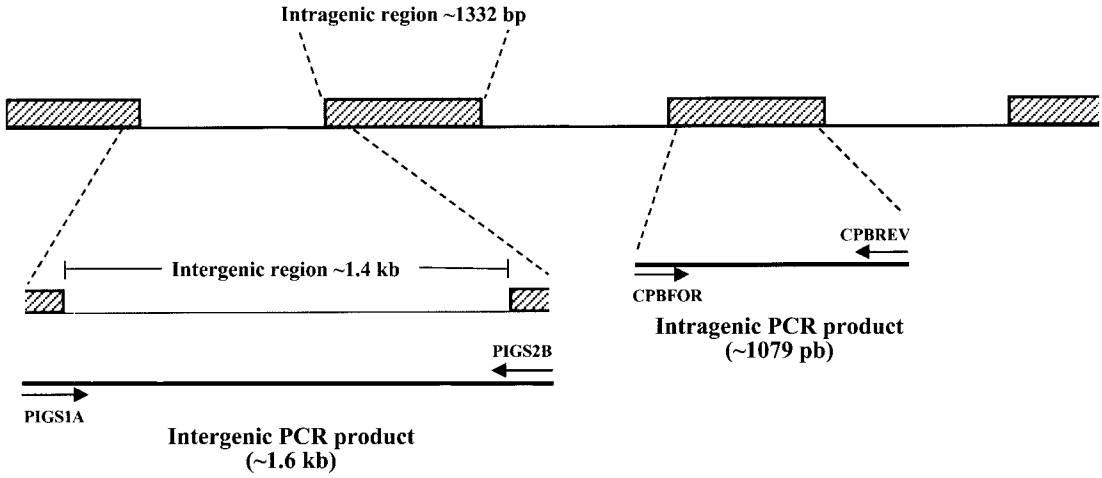

Figure 1. Localization of polymerase chain reaction (PCR) primers in the *cpb* coding sequences *(boxes)* for the amplification of the intragenic (CPBFOR and CPBREV) and intergenic (PIGS1A and PIGS2B) regions. The sizes of the coding and untranslated region were estimated from data of Brooks et al. [17].

*Acy*I, and *Hae*II. Products and reactions were stopped with EDTA (0.1 mol/L [pH 8.0]). PCR-RFLP products were analyzed by use of capillary electrophoresis (Agilent 2100 Bioanalyzer system; Agilent Technologies) in a microchip device (DNA 1000 LabChip; Caliper Technologies). This system [18] was selected not only for its high sensitivity and discriminatory power, but also because it requires only 1 μL of restriction product for loading the electrophoretic chip, versus the 18 μL required for an agarose gel.

All the samples were also analyzed by use of PCR-RFLP of the intragenic sequence of *gp63*, according to the method of Guerbouj et al. [12]. The following restriction enzymes were used: *Hinc*II, *Msc*I, and *Taq*I. Electrophoresis was performed by use of the Agilent 2100 Bioanalyzer system, as described above. All PCR-RFLP assays were performed in duplicate and were redigested, to avoid misleading results caused by incomplete restriction cleavage.

Phenetic analysis. A character-matrix was created by reporting all possible PCR-RFLP fragments in the sample studied. Next, for each strain, the presence or absence of bands was scored (a score of 1 for presence, and a score of 0 for absence). These matrices were processed for phenetic analyses, with the following programs of the PHYLIP package (version 3.6; available at: http://evolution.genetics.washington.edu/phylip.html): RESTDIST (restriction fragments distance, modification of Nei and Li restriction fragments distance method [19]), UPGMA (unweighted pair group method with arithmetic averages), CONSENSE (majority rule consensus), and SEQBOOT (bootstrap analysis). The bootstrap analyses were performed for 1000 replications, to estimate the robustness of the nodes. Dendrograms were drawn by use of the DRAWGRAM (tree-plotting) program. Analyses were done on individual matrices built from the intragenic sequence of *cpb,* the intergenic sequence of *cpb,* and the intragenic sequence of *gp63;* on global matrices gathering the 3 sets of PCR-RFLP data; and on data on MLEE typing (15 loci) obtained from Pratlong et al. [16].

RESULTS

Intragenic and intergenic amplifications of the* cpb *gene. DNA from 15 strains belonging to the *L. donovani* complex and 2 strains of *L. major* and *L. aethiopica* (table 1) was used for intragenic and intergenic PCR amplification, respectively, of *cpb* genes. Amplification products corresponded to the length expected for primers annealing on reported sequences from *L. chagasi* and *L. donovani* [15, 20]: 1080 bp and 1600 bp for intragenic and intergenic amplicons, respectively (data not shown).

***PCR-RFLP of intragenic sequence of* cpb.** *cpb* intragenic amplicons were cleaved with 6 different restriction enzymes, revealing monomorphic (*Sdu*I) and polymorphic (*Hinf*I, *Taa*I, *Hae*III, *Cfr*I, and *Hpa*II) patterns among the 15 strains. Patterns consisted of several bands, and, in most cases, the sum of their size was ~2 kb (twice the size of undigested amplicons). This phenomenon was not the result of incomplete digestion, because the same patterns were obtained after second digestion of samples already digested. This result can be explained by the presence of at least 2 sequence variants (i.e., isogenes, which are characterized by different restriction patterns) within the *cpb* repeated array, a feature documented elsewhere [15, 21]. Among polymorphic patterns, those obtained with the *Hae*III enzyme were the most informative (figure 2*A*). Four restriction fragments were common to all 15 strains in the *L. donovani* complex (100, 152, 167, and 217 bp), and 1 of them (167 bp) was absent in *L. major* and *L. aethiopica.* Within the *L. donovani* complex, additional bands were specific for some strains only. The first group, formed by European *L. infantum* strains (LG1-8, -14, and -15), had a specific band of 263 bp and 2 more bands of 286 and 294 bp each that were absent in Maltese strain LG15. In the second group, which included *L. donovani, L. archibaldi,* and the Sudanese *L. infantum* strains, 2 specific bands of 71 and 186 bp each were observed. Within the latter group, Indian *L. donovani* (LG9 and LG10) were differentiated from the other strains by 2 bands of 260 and 296 bp each (figure 2*A*).

***PCR-RFLP of intergenic sequence of* cpb.** Restriction enzymes *Eam*1104I, *Nsp*I, *Acy*I, *Hae*II, and *Hae*III were selected for analysis of *cpb* intergenic amplicons. The patterns obtained with the first 2 enzymes were monomorphic. With the 3 latter enzymes, polymorphic patterns were encountered. Two *Hae*III fragments (190 and 390 bp) were common to all strains of the *L. donovani* complex and were not encountered in *L. major* and *L. aethiopica.* Within the *L. donovani* complex, *L. infantum* European/Maltese strains (2 specific *Hae*III fragments of 615 and 296 bp each; figure 2*B*) were distinguished from all other strains (specific *Hae*III fragment of 310 bp; figure 2*B*). The sum of the restriction fragments sizes in a single lane reached ~1600 or 3200 bp, which suggests that there is some degree of heterogeneity among intergenic sequences of the same strain.

Phenetic analysis. To obtain a comprehensive view of the genetic polymorphism within the *L. donovani* species complex, PCR-RFLP data were processed by phenetic analysis. Three types of analyses were performed.

First, to compare the polymorphism of coding and noncoding regions of the *cpb* locus, PCR-RFLP patterns obtained from intragenic and intergenic sequences of *cpb* were analyzed (figure 3*A*). Both dendrograms showed 2 separate clusters: European *L. infantum* (group I) and *L. donovani, L. archibaldi,* and the Sudanese *L. infantum* (group II). Within each cluster, relative positions of isolates were similar, except for LG8, LG15, and LG13. Within cluster II, *L. donovani* strains were grouped together (Indian and Sudanese) in the intergenic tree. In contrast, intragenic analysis separated Indian (LG9 and LG10) from all

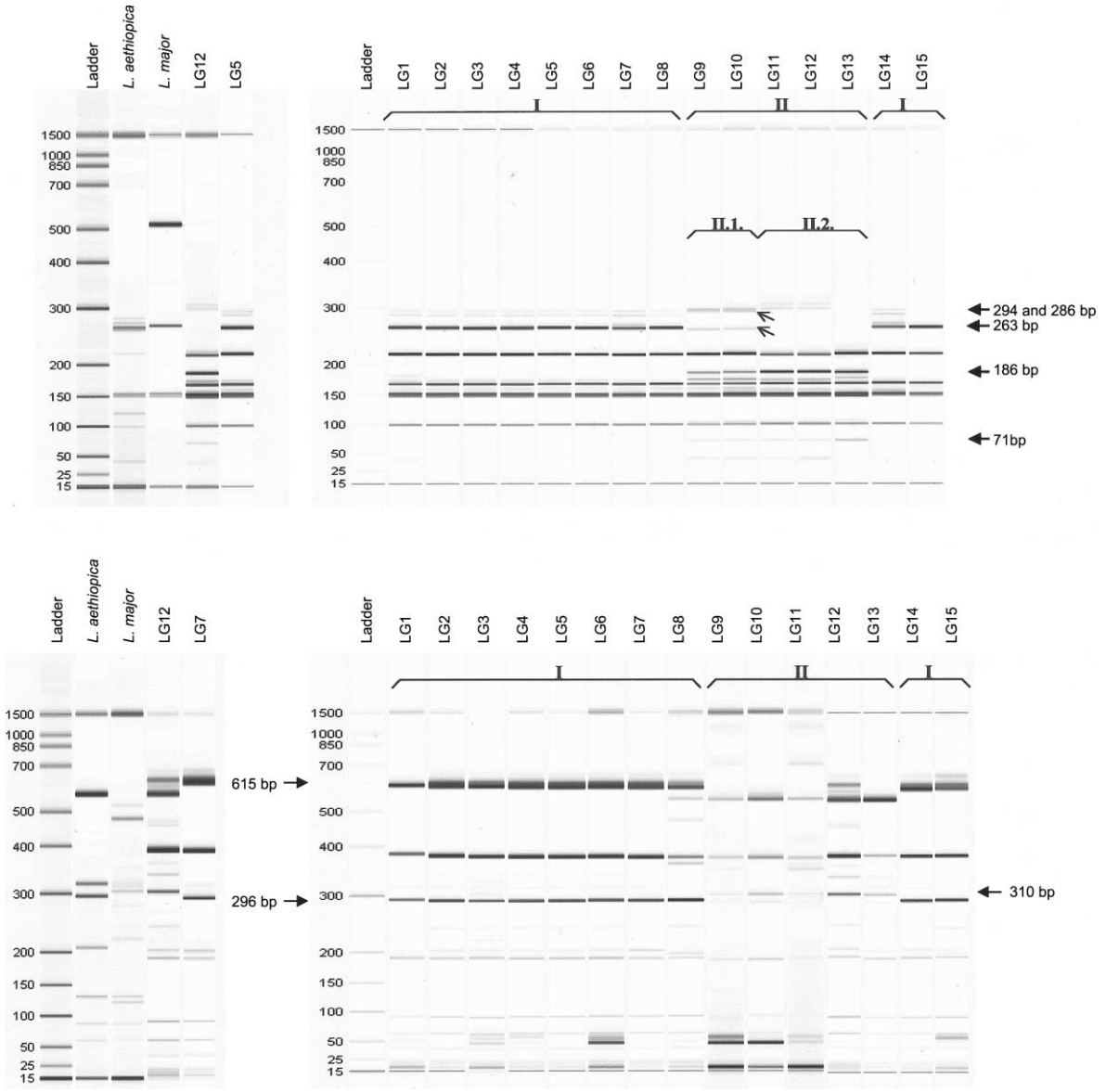

Figure 2. Polymerase chain reaction restriction fragment–length polymorphism analysis of intragenic *(A)* and intergenic *(B)* regions of *cpb* in *Leishmania donovani* complex, *L. major,* and *L. aethiopica.* The *Hae*III profiles were resolved by use of the Agilent 2100 Bioanalyzer system (Agilent Technologies). Group I corresponds to European isolates, including the one from Malta (LG15). Group II corresponds to isolates from India (II.1) and Africa (II.2). See table 1 for origin of the isolates. *Closed arrows,* fragments allowing discrimination between strains from groups I and II; *open arrows,* fragments specific to Indian strains.

East African (LG11, LG12, and LG13) strains. The degree of polymorphism, as estimated by counting the number of different genotypes in the tree, was higher with PCR-RFLP of the intergenic sequence of *cpb* than with PCR-RFLP of the intragenic sequence of *cpb* (12 vs. 7 genotypes). This trend was also observed for the 6 isolates of zymodeme MON1, which clustered together but could not be differentiated by PCR of the intragenic sequence of *cpb*, whereas 3 genotypes were encountered with PCR-RFLP of the intergenic sequence of *cpb*.

Second, to compare the levels of polymorphism in 2 proteases involved in host-parasite relationship, we analyzed PCR-RFLP patterns of intragenic sequences from both *cpb* and *gp63* (figure 3*B*). The same 2 major clusters mentioned above were evident. Within each cluster, most isolates occupied the same position, except LG13 and LG8. The degree of polymorphism was lower in *cpb* than in *gp63* (7 vs. 13 genotypes). Within MON1 strains, a single *cpb* genotype was encountered, versus 4 different genotypes for *gp63*.

Third, multilocus (ML) PCR-RFLP, which combines intragenic *cpb*, intergenic *cpb*, and intragenic *gp63* results, was compared with polymorphisms revealed by MLEE [16], which is considered to be the reference standard for *Leishmania* typing.

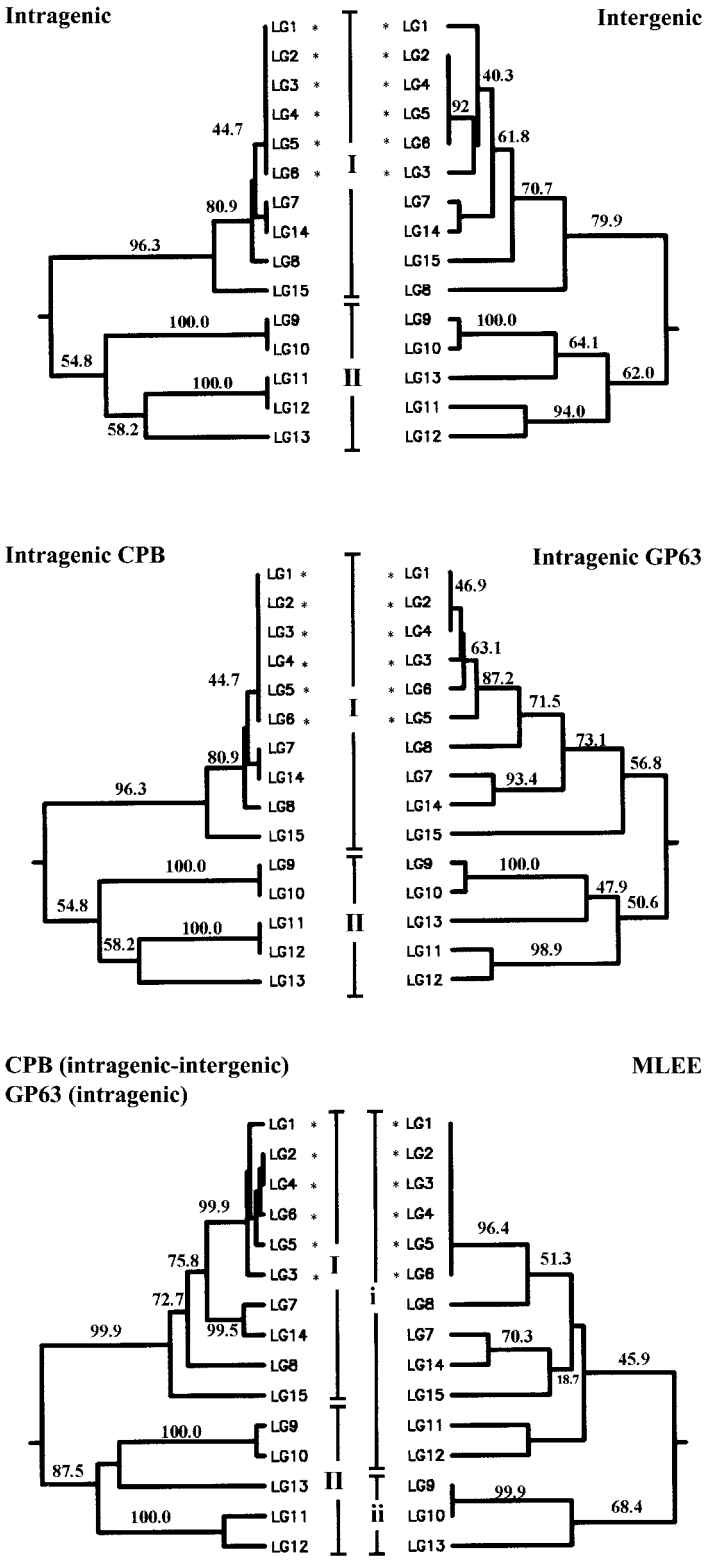

Figure 3. Genetic polymorphism within the *Leishmania donovani* complex. Shown are unweighted pair group method with arithmetic mean dendrograms built from data generated by polymerase chain reaction restriction fragment–length polymorphism (PCR-RFLP) analysis of intragenic and intergenic regions of *cpb (A),* data generated by PCR-RFLP analysis of intragenic regions of *cpb* and intragenic regions of *gp63 (B),* and data from combined PCR-RFLP and multilocus enzyme electrophoresis (MLEE) analyses *(C).* Bootstrap values (percentage from 1000 replicates) are shown above the branches. See table 1 for origin of the isolates. Asterisks represent strains of the MON1 zymodeme.

The 2 major clusters mentioned above were also individualized in the combined analysis of PCR-RFLP data and were associated with high bootstrap values (>85%). In contrast, MLEE analysis grouped isolates LG11 and LG12 *(L. archibaldi* and Sudanese *L. infantum)* together with the European/Maltese *L. infantum* isolates, whereas isolates LG9, LG10, and LG13 *(L. donovani)* clustered separately. However, bootstrap values of these 2 main MLEE clusters were not very high (<60%). With respect to the discriminatory power of both methods, ML PCR-RFLP indicated a higher degree of polymorphism than did MLEE (14 vs. 9 genotypes). Of interest, 5 different genotypes were observed within the 6 isolates of zymodeme MON1.

Direct application of PCR-RFLP assays of* cpb *in human tissues. To evaluate the possibility of performing genetic characterization of *Leishmania* without the need for isolating parasites, we directly applied the PCR-RFLP of intragenic sequence of *cpb* to DNA extracted from 7 BMA samples and 1 venous blood sample obtained from Nepalese patients with confirmed VL. Clear patterns were identified (figure 4) and were shown to be most similar to those presented by the reference strain, LG10 (Indian *L. donovani* isolate).

DISCUSSION

In the present study, the genetic polymorphisms of intragenic and intergenic regions of *cpb* genes have been analyzed by use of PCR-RFLP in a sample of strains representative of the *L. donovani* complex, the etiological agents of VL, and have been compared with the results of PCR-RFLP analysis of *gp63* and MLEE. Our results demonstrate a series of technological and conceptual advances in the realm of *Leishmania* typing.

To our knowledge, this is the first report on the genetic typing of protozoan parasites by use of capillary electrophoresis in a microchip device (Agilent 2100 Bioanalyzer system). This electrophoresis system has already been evaluated for analysis of DNA polymorphisms in human chromosomes [22] and *Campylobacter jejuni* [23] and has demonstrated several advantages, including great precision in sizing of DNA and great repro-

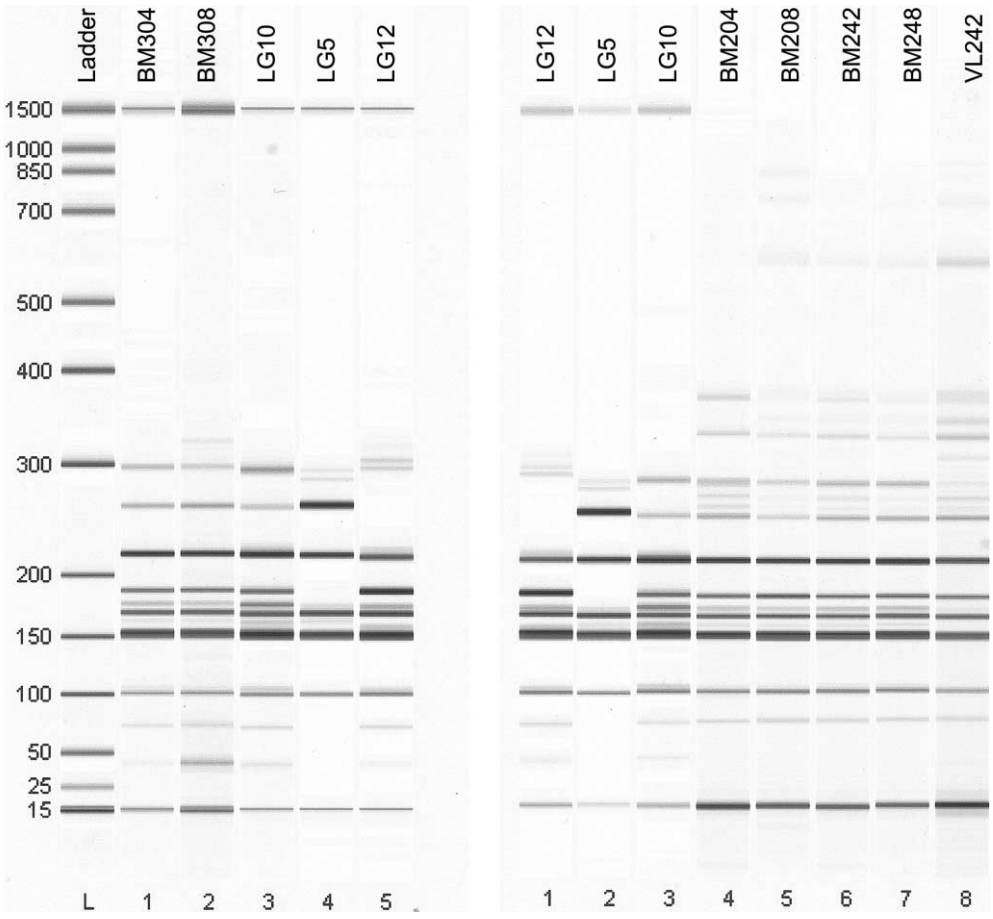

Figure 4. Direct characterization of parasites in human tissues by polymerase chain reaction restriction fragment–length polymorphism analysis of the intragenic region of *cpb. A: Lane L,* ladder; *lanes 1* and *2,* bone marrow aspirate (BMA) samples from Nepalese patients with visceral leishmaniasis (VL); and *lanes 3–5,* reference isolates (see table 1 for origin of the isolates). *B: Lanes 1–3,* reference isolates; *lanes 4–7,* BMA samples from Nepalese patients with VL; and *lane 8,* venous blood samples from Nepalese patients with VL.

ducibility. Here, we have demonstrated 2 additional qualities that are essential for the typing of pathogens. On the one hand, the high discriminatory power of the system allows for better identification of polymorphic markers during a screening phase of new potential targets. On the other hand, the high detection threshold allows work to be performed with minute amounts of DNA; for example, with a 1-μL loading volume of digested amplicons, restriction fragments corresponding to 0.2 ng of DNA could be detected (data not shown). This result does not exclude other methods, such as polyacrylamide electrophoresis or even conventional agarose electrophoresis, for the application of our assays. However, in the latter case, the inclusion of an additional hybridization step might be required to increase the level of detection.

By use of capillary electrophoresis, we have demonstrated the potential of the *cpb* gene locus as a target for genetic characterization of *Leishmania* species at different levels of complexity. First, when applying PCR-RFLP analysis of *cpb*-coding regions, strains of the *L. donovani* complex could be distinguished from other species complexes *(L. major* and *L. aethiopica)* and showed different patterns according to their geographic origin. A similar feature was previously demonstrated by PCR-RFLP of *gp63* and was explained by a possible host (vector or vertebrate) selective pressure [9, 12]. During the current period of reemergence and spreading of VL, this geographic distinction of parasites would constitute excellent support for molecular tracking of *Leishmania* species characterized by different clinical and epidemiological profiles. Second, within these groups, strain molecular typing was possible, to a certain extent, even within the most widespread MON1 zymodeme. However, the degree of polymorphism was lower in the coding region than in the intergenic region of *cpb* genes (7 vs. 12 different genotypes). This is a classic feature of tandemly repeated genes, in which coding regions are submitted to a stronger selective pressure than noncoding intergenic sequences, as in rDNA genes [6, 24]. There may be some exceptions, such as the case of *gp63* genes analyzed here. Indeed, in the present samples, we encountered 13 different PCR-RFLP genotypes when targeting the coding region of the *gp63* genes. Previous work already showed that the level of polymorphism was very high in *gp63* genes and higher than in the corresponding intergenic sequence [12]. Localization of hot spots of sequence polymorphism in regions corresponding to major epitopes (T and B) led to the hypothesis that genetic polymorphisms in this important antigen may bring selective advantages by allowing immune escape [9, 25]. *cpb* is also a major antigen [13], but its genes were shown here by PCR-RFLP to be less polymorphic than *gp63* genes. Although our assay targeted *cpb* gene regions known to diverge across species [15], it is possible that the choice of restriction enzymes led to an underestimation of the degree of polymorphism. This possibility is supported by a comparison of sequences extracted from GenBank, which suggests that a similar degree of dissimilarity exists between *cpb* (AJ420286, Z49965, and U43706) and *gp63* (M80669, M80671, M80672, AF039721, and X64394) genes of *L. donovani, L. mexicana,* and *L. major.* Further work is necessary to confirm these data and to know whether genetic polymorphisms in *cpb* genes also affect regions coding for major epitopes.

At present, the reference standard for genetic characterization of *Leishmania* species is MLEE [3]. The main advantage of this method is that it allows characterization in different regions of the genome, which is important for population genetics (i.e., for undertaking recombination tests with different loci) and phylogenetics (i.e., hypotheses are stronger if they are based on several genes [26]). However, MLEE also has various disadvantages: it does not detect silent mutations and requires a large amount of cultivated material, along with all the associated operational problems, such as contamination and selection biases. Therefore, multigenic PCR assays that avoid the need for parasite isolation and that combine sensitivity and discriminatory power should constitute a new generation of characterization tools. A first step in this direction has been explored in the present study by combining PCR-RFLP data of 3 sequence targets (intragenic and intergenic sequences of *cpb* together with those of *gp63*). Phenetic analysis of these ML PCR-RFLP and MLEE data differed essentially in the classification of 2 African strains of *L. infantum* (LG12 and MON30) and *L. archibaldi* (LG11 and MON82) that clustered together (bootstrap values >85%) with *L. donovani* (Indian and Ethiopian) in ML PCR-RFLP. The same discrepancy with respect to the position of MON30 and MON82 was observed after analysis of the intergenic sequence of *gp63* [7]. Controversy with regard to classification of species and zymodemes within the *L. donovani* complex was also supported by other molecular methods [27]. Species delineation within the *L. donovani* complex is relatively difficult in East Africa, which is thought to be the original focus of VL [16] and where a broad continuum of genetic diversity can be observed among natural strains. This has even led some authors to propose to regroup all these taxa in a single group, *L. donovani sensu lato,* without referring to different species [28]. It would be premature to derive taxonomic conclusions from our results, but our results confirm the need for a careful reevaluation of MLEE-based classification of *Leishmania* species with several independent molecular methods.

Finally, we have demonstrated that PCR-RFLP assays of *cpb* also allow the performance of direct characterization of *Leishmania* parasites in human tissue samples. The BMA and venous blood samples from Nepalese patients analyzed here presented restriction patterns (PCR-RFLP of the intragenic sequence of *cpb*) identical to those observed in Indian *L. donovani* reference strains. Similar performances were recently demonstrated by

PCR-RFLP of *cpb* of blood samples obtained from Somalian patients with VL [29] and PCR-RFLP of *gp63* of skin biopsy samples obtained from Peruvian patients with tegumentary leishmaniasis [30]. Accordingly, antigen genes constitute valid targets for *Leishmania* typing without the need for isolation of parasites. This should significantly increase the accessibility of *Leishmania* genetic characterization, because any molecular laboratory could apply our assay. The next step should be to compare, on host tissues, the sensitivity and discriminatory power of the corresponding assays to those targeting intergenic sequence of rDNA and mini-exons.

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