Molecular Epidemiology of *Leishmania* (*Viannia*) *guyanensis* in French Guiana

Brice Rotureau,[1] Christophe Ravel,[2] Mathieu Nacher,[1] Pierre Couppié,[1,3] Isabelle Curtet,[4]
Jean-Pierre Dedet,[2] and Bernard Carme[1]*

*Laboratoire Hospitalo-universitaire de Parasitologie et Mycologie Médicale, Equipe EA 3593, UFR de Médecine de l'Université des
Antilles et de la Guyane, Cayenne, Guyane Française[1]; Laboratoire de Parasitologie Mycologie, Centre National de
Référence des Leishmania, UMR 5093 CNRS and Université de Montpellier 1, Montpellier, France[2];
Service de Dermatologie, Centre Hospitalier Andrée Rosemon, Cayenne, Guyane Française[3];
and Laboratoire d'Analyses Médicales de Montjoly, Remire-Montjoly, Guyane Française[4]*

Little information is available about the genetic variability of *Leishmania* populations and the possible correlations with ecoepidemiological features of leishmaniases. The present study was carried out in French Guiana, a country where cutaneous leishmaniases (CL) are endemic over the whole territory. The genetic polymorphism of a nuclear sequence encompassing the end of the ribosomal small subunit and the internal transcribed spacer 1 of 265 isolates from patients with CL was examined by restriction fragment length polymorphism analysis. Genotypes based on the fingerprinting phenetic integration were compared to epidemiological, clinical, and geographical data. In agreement with previous reports, five different *Leishmania* species were identified, but *Leishmania* (*Viannia*) *guyanensis* represented 95.8% of the samples. Two distinct *L.* (*V.*) *guyanensis* populations were found to originate in two ecologically characterized regions. Higher lesional parasite densities and the need for additional treatments were significantly linked to genotype group I. Parasites of genotype group II were more likely to cause chronic and disseminated cutaneous forms in patients. *L.* (*V.*) *guyanensis* was previously said not to be very polymorphic; however, the present analysis resulted in a significant degree of discrimination among *L.* (*V.*) *guyanensis* isolates from diverse ecological areas and with different clinical implications.

In the highly biodiversified region of the New World (NW) that encompasses the northern Amazon Basin and the Guiana Shield, at least seven named species belonging to the *Leishmania* genus coexist with often overlapping hosts and vectors (39). American visceral leishmaniases due to *Leishmania* (*Leishmania*) *infantum* are found there, but cases of cutaneous leishmaniases (CL) are far more numerous. Human cutaneous infections may be unapparent; however, many of the *Leishmania* parasites are able to produce a spectrum of diseases rather than a single clinical form. Cases reported in that region range from localized and diffuse cutaneous forms to mucocutaneous leishmaniases. They are now being reported in areas where leishmaniases had previously not been endemic (2, 17): increasing risk factors related to natural and especially manmade environmental changes are making leishmaniases a growing public health concern in this particular region. However, little information is available about the genetic variability of parasite populations and the possible correlation with ecoepidemiological features of the diseases. The present study was carried out in French Guiana, a country where CL are endemic over the whole territory (incidence of 0.2% between 1979 and 2000 [6, 14]). The genetic polymorphism of a nuclear sequence encompassing the end of the ribosomal small subunit (SSU) and the internal transcribed spacer 1 (ITS1) of 265 samples

from patients with CL was examined by restriction fragment length polymorphism (RFLP) analysis. Genotypes based on the fingerprinting phenetic integration were then compared to epidemiological, clinical, and geographical data.

MATERIALS AND METHODS

Patients. A total of 265 patients positive for CL that have consulted the Dermatology Department of Cayenne General Hospital (Cayenne, French Guiana) and/or the health centers of French Guiana in 2003 and 2004 were included in this study. The inclusion of a confirmed case of CL was based on the following: (i) the presence of cutaneous lesions with 2 or more weeks of evolution and a compatible epidemiological history, (ii) no specific treatment before consultation, and (iii) a positive result by at least one of the two methods used for routine diagnosis, microscopic examination of dermal scraping smears and/or in vitro cultivation. All individuals enrolled in this study provided informed consent. The supposed geographical origin of infection was assessed for each patient both by the health center where the sample was taken and by the patient interview. Strict biopsy protocol and treatment schemes were applied for all patients included in this study. Data were centralized, and cases were monitored in the Parasitology Department of Cayenne General Hospital (Cayenne, French Guiana). Table 1 summarizes the clinical data of the 265 patients enrolled in this study.

Clinical samples and standard diagnostic procedure. Tissue scrapings for microscopic smear examination were collected by slitting the internal border of skin lesions with a surgical blade, and the tissue scraping was smeared onto a clean glass slide, fixed with methanol, and stained in Giemsa. For each patient, two slides were read by trained parasitologists; if the slides were positive, they were given a parasite density score from 1 (rare [less than one parasite every 20 fields]) to 5 (very numerous [more than five parasites/field]). Punch skin biopsy specimens of 4 mm were taken in the internal border of lesions under sterile conditions and local anesthesia (lidocaine). In vitro cultivations were performed by inoculation of sterilely crushed biopsy specimens in 3 ml of RPMI 1640 (Sigma) supplemented with 20% fetal calf serum, 1% nonessential amino acids,

* Corresponding author. Mailing address: Laboratoire Hospitalo-universitaire de Parasitologie et Mycologie Médicale, Equipe EA 3593, UFR de Médecine de l'Université des Antilles et de la Guyane, Campus Saint-Denis, BP 718, 97336 Cayenne, Guyane Française. Phone: 33 594 28 72 60. Fax: 33 594 28 72 63. E-mail: ufrmedag2@wanadoo.fr.

TABLE 1. Clinical characteristics of 265 patients with
cutaneous leishmaniasis

Characteristic	Value
Patients	
Sex (% male)	79.5
Mean age (yr) (SD)	32.9 (13.5)
Median duration of the disease (mo) (range)................	1 (0.25–96)
Atypical clinical forms (%)[a]	3
Lesions	
Median no. (range)	1 (1–61)
Median parasite density score (range)[b]	4 (1–5)
Ulcerated lesion (%)	92.9
Nodular lesion (%)	15.8
Satellite papules (%)	5.6
Lesion location	
Arms (%)	49.3
Legs (%)	45.4
Trunk (%)	18
Head (%)	18.9
Treatment	
1 pentamidine injection (%)............................	66.3
2 pentamidine injections (%)	30.3
3 pentamidine injections (%)	2.2
4 pentamidine injections (%)	0
5 pentamidine injections (%)	1.1
Additional pentavalent antimonial courses (%)	16.3
Additional fluconazole oral cures (%)	2.2

[a] Atypical clinical forms are disseminated cutaneous leishmaniases and chronic presentations.

[b] Parasite density scores were estimated by direct microscopic examination and scored as follows. Parasite density scores were 1 (rare [≤1 parasite every 20 fields]), 2 (few [≥1 parasite every 20 fields]), 3 (quite numerous [≥1 parasite every 5 fields]), 4 (numerous [1 to 5 parasites/field]), and 5 (very numerous [>5 parasites/field]).

and 50 IU/ml penicillin. Culture flasks were incubated at 24°C in the dark and microscopically observed every 3 days for 4 weeks.

DNA extraction and PCR amplification. Only a single round of cultivation separated isolation from DNA extraction. For each isolate, DNA extraction was conducted from cultured biopsy medium by a simple salting-out procedure as described elsewhere (40). PCR control DNAs were extracted from *Leishmania* (*Viannia*) *guyanensis* MHOM/GF/2003/LBC40, *L.* (*V.*) *braziliensis* MHOM/BR/75/M2903, *L.* (*V.*) *naiffi* MHOM/GF/97/CRE88, *L.* (*V.*) *lainsoni* IUBI/BR/00/M12025, and *Leishmania* (*Leishmania*) *amazonensis* MHOM/BR/73/M2269. Negative controls were also used in each run, and all samples were tested at least twice. The 1,200-bp sequences located between the end of the ribosomal SSU and the 5.8S region were amplified as described elsewhere (41). The primers SSU-12103-D (5′-GGGAATATCCTCAGCACGT-3′) and 5.8S-13333-R (5′-CGA CACTGAGAATATGGCATG-3′) were used.

Restriction analysis and control sequencing. Constant amounts of amplified DNA (approximately 100 ng) were then loaded and digested for 2 h with restriction enzymes according to the manufacturer's recommendations. Seven restriction enzymes were tested: TaqI, HaeIII, DdeI, PstI, and HpaII (Sigma) and RsaI and BfaI (New England BioLabs). Digestion products were separated for 2 h by simple electrophoresis in 2% agarose gels stained with ethidium bromide. Sequencing of the end of the RNA polymerase II large-subunit gene (330 bp) (8) was also performed to confirm the PCR-RFLP identification of 42 samples characteristic of each restriction pattern.

Numerical analyses. PCR-RFLP gel scans were analyzed with the LabImage 2.7.1 software (Kapelan GmbH). A character matrix was then created by reporting all possible fragments obtained with each enzyme in the samples studied. Then, for each sample, the presence or absence of bands was given a score of 1 or 0, respectively. In a given RFLP profile, differences of intensity among fragments were not taken into account. These differences are common in tandemly repeated genes consisting of sequence variants present in different copy numbers (24, 53). The binary matrix was then processed for phenetic analyses, with the following programs of the PHYLIP version 3.63 package (Joseph Felsenstein,

University of Washington, Seattle, Wash.) (July 2004 version): RESTDIST (restriction fragment distance method modified by the Nei and Li distance method [32]) followed by UPGMA (unweighted pair group method with arithmetic averages). A condensed tree was drawn using the Molecular Evolutionary Genetics Analysis version 2 (MEGA2) software (27). Statistical analyses were performed by the Intercooled Stata 8.2 version software (StataCorp LP). Qualitative binary data (genotype, gender, atypical clinical form, presence of ulcer, presence of nodule, presence of scab, presence of papule, presence of lymphangitis, lesion on the inferior parts, lesion on the superior parts, lesion on the head, lesion on the trunk, additional fluconazole cure, additional pentavalent antimonial cure, treatment different from one pentamidine injection), qualitative ordinal data (parasite density score), qualitative categorical information (supposed origin of infection), and quantitative values (number of lesions, number of pentamidine injections, age, and lesion duration) were successively analyzed by Pearson chi-square tests.

RESULTS

PCR on cultured biopsy specimens gave negative results in 49 (18.5%) of the 265 samples tested, most of these samples were positive by direct microscopic examination but negative by cultivation. Moreover, 32 samples (12.1%) were identified but not genotyped due to their low DNA levels. Species identification was based on comparison to the control strain profiles by checking the presence or absence of specific bands (41). In total, five different *Leishmania* species were identified from 216 samples: *Leishmania* (*Viannia*) *guyanensis* represented 95.8% of the samples, four *L. Leishmania* (*Leishmania*) *amazonensis* isolates (1.9%) were also found, as well as three *L.* (*V.*) *braziliensis* isolates (1.4%), one *L.* (*V.*) *lainsoni* isolate (0.5%), and one *L.* (*V.*) *naiffi* isolate (0.5%). Control sequencing of the RNA polymerase II gene (8) confirmed the PCR-RFLP identification of 42 samples characteristic of each restriction pattern. Finally, 175 *L.* (*V.*) *guyanensis* samples were genotyped with seven restriction enzymes.

As currently observed, PCR-RFLP profiles were relatively complex and polymorphic. All amplification patterns were reproducible. Each of the seven enzymes tested showed different levels of variability depending on the sample tested. The unrooted UPGMA molecular tree presented in Fig. 1 depicts relationships between the 175 *L.* (*V.*) *guyanensis* samples. The Nei genetic distances between *L.* (*V.*) *guyanensis* samples ranged from 0.0005 to 1.2600. This molecular epidemiological tree distinguished two genotype groups forming more or less tight clusters. Genotype group I included 133 homogeneous samples (76.0%) that were closely related (with branch lengths no longer than 0.0074), whereas genotype group II, which was much more heterogeneous, was characterized by a smaller population of 42 polymorphic samples (24.0%) which were sometimes highly distant (branch length > 0.01).

When examining possible associations of the genotypic groups with epidemiological, clinical, and geographical data, several links could be observed (Table 2). All the *L.* (*V.*) *braziliensis*, *L.* (*V.*) *lainsoni*, and *L.* (*V.*) *naiffi* samples originated from the northern coastal part of French Guiana, whereas the four *L.* (*L.*) *amazonensis* isolates came from the southern part of the territory. Overall, *L.* (*V.*) *guyanensis* samples of genotype group I seemed to have originated in the northern part of French Guiana, whereas the samples of genotype group II seemed to have originated in the southern part of the country, from Suriname, and from the neighboring Brazilian states (Fig. 1). This link was supported by a Pearson
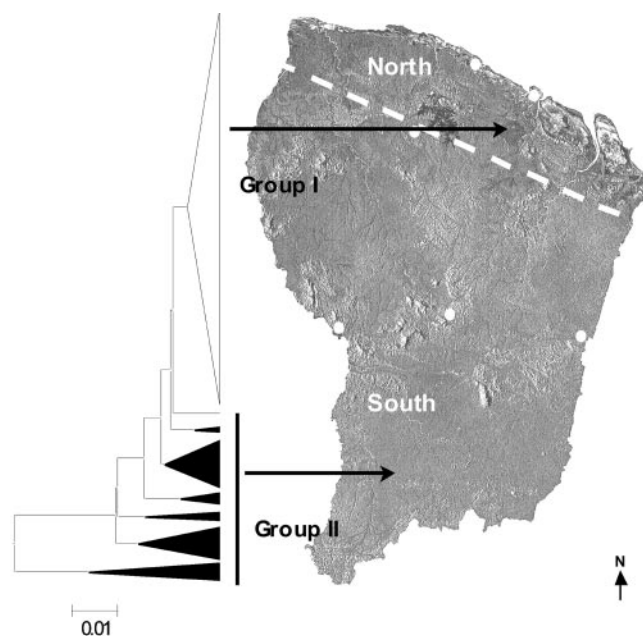
FIG. 1. Correlation between the UPGMA tree based on the PCR-RFLP genotype analyses of 175 *Leishmania* (*Viannia*) *guyanensis* samples and the supposed geographical origin of patient infection. A condensed unrooted UPGMA tree built with the PCR-RFLP data (seven restriction enzymes tested) of 175 samples is presented in the left part of the figure. The triangle width and height are commensurate with the divergence of the isolates and the number of samples, respectively. Samples were grouped according to their genotype: 133 closely related strains (branch length < 0.01) were included in genotype group I (white triangle), and 42 samples showing polymorphic heterogeneous patterns (branch length > 0.01) were included in genotype group II (black triangles). Sample origins are indicated by arrows pointing to the map of French Guiana. These links were supported by a Pearson chi-square test ($P < 0.01$).

relation was observed between the genotype and number of lesions, duration of the disease, gender, age, localization and aspect of lesions, and associated clinical features, such as satellite papules or lymphangitis.

DISCUSSION

The different *Leishmania* species prevalence were in agreement with previous studies (4, 14, 18; reviewed in reference 39), showing a relative stability of the different pathogenic complex in French Guiana during the last 20 years. Nevertheless, considering the important man-made environmental changes and associated vector population adaptations (B. Rotureau et al., submitted), the incidence of the most clinically relevant species, *Leishmania* (*Leishmania*) *amazonensis* and *Leishmania* (*Viannia*) *braziliensis*, should be checked regularly in the future.

Linking the polymorphism of *Leishmania* samples with clinical outcomes has not always been evident in the past (19, 37, 44, 47, 52). As highlighted by Schriefer et al. (48), the wide geographic distribution and multiple sources (e.g., vectors, reservoirs, and human immunodeficiency virus-positive human hosts) of the isolates tested may be among the reasons for this lack of association. However, several studies using different methods have demonstrated at least a geographic structuring of *Leishmania* populations. Geographic structuring was demonstrated in *L.* (*V.*) *braziliensis* by randomly amplified polymorphic DNA (RAPD) analysis (48), RFLP analysis (21), multilocus enzyme electrophoresis (MLEE) (42), and serology (43); in *L.* (*L.*) *infantum* by MLEE (35), RAPD analysis (52, 54), and analysis with other molecular markers (22); in *L.* (*V.*) *peruviana* by RAPD analysis (3); in *L.* (*L.*) *aethiopica* by RFLP (44); and in *L.* (*L.*) *donovani* by sequencing (26, 28) and RFLP (51). Among *Viannia* parasites, reports have indicated that genetic variations were extensive, with some clones widely distributed and others localized to a particular endemic focus (13). Other results have suggested that their distribution was related to the origin of the gene pool as well as to present vector and reservoir movements (25). The present RFLP analysis resulted in a significant degree of discrimination among isolates from diverse regions, confirming the ability of this technique to discriminate between closely related parasites. Two main *L.* (*V.*) *guyanensis* genotype pools were distinguished by phenetic analysis and associated with two distinct geographical regions. *L.* (*V.*) *guyanensis* genotype group II originated in

chi-square test [$P (>\chi^2) = 0.007$]. Patients belonging to genotype group I were likely to present higher parasite density [$P (>\chi^2) = 0.008$] than patients belonging to genotype group II. Moreover, there were more patients from genotype group I who needed additional treatments (i.e., more than one intramuscular pentamidine injection) before they were cured (36.7%) compared to patients from genotype group II (8.3%) [$P (>\chi^2) = 0.055$]. Atypical clinical forms, such as disseminated presentations and chronic diseases, were mostly found in genotype group II [$P (>\chi^2) = 0.056$]. However, no clear cor-

TABLE 2. *L.* (*V.*) *guyanensis* genotype correlations with geographical, clinical, and epidemiological data[a]

Genotype	Population (%)	Supposed geographical zone of infection (%)	Parasite density score of >2 (%)[b]	Need for additional treatments (%)[c]	Atypical clinical forms (%)[d]
Group I	76.0	North (62.6)	74.7	36.7	1.5
Group II	24.0	South (60)	48.1	8.3	7.1
χ^2 test probability		$P = 0.007$	$P = 0.008$	$P = 0.055$	$P = 0.056$

[a] The genotype groups of the 175 *L.* (*V.*) *guyanensis* samples studied and the geographical zones are defined in Results and in Fig. 1.
[b] Parasite density scores were estimated by direct microscopic examination and scored as follows. Parasite density scores were 1 (rare [<1 parasite every 20 fields]), 2 (few [≥1 parasite every 20 fields]), 3 (quite numerous [≥1 parasite every 5 fields]), 4 (numerous [1 to 5 parasites/field]), and 5 (very numerous [>5 parasites/field]).
[c] In patients with nonchronic typical localized forms, treatments used in addition to one pentamidine intramuscular injection are other pentamidine intramuscular injections, intralesional pentavalent antimonial courses, and/or fluconazole oral cures.
[d] Atypical clinical forms are disseminated cutaneous leishmaniases and chronic presentations.

southern French Guiana and neighboring areas in Brazil and Suriname. These regions are characterized by a continuous dense primary rain forest spotted with gold-digging sites. Most of the patients were infected during occupational activities as reported by Dedet et al. (15). *L. (V.) guyanensis* genotype group I originated in the northern part of the country. As along the whole northern part of the Guiana Shield, this costal region is typically divided in distinct ecotopes: beaches, mangroves, coastal swamps, and rain forests. This area is heavily settled by humans, and most of the sylvatic ecotopes are secondary. Infections in this geographic area likely occurred principally during occupational or leisure activities (15). These two distinct *L. (V.) guyanensis* populations observed by ribosomal fingerprinting may have originated in the two different ecologically characterized regions. Thus, they may represent at least two nonsympatric lineages of this parasite. As it was proposed by Cupolillo (9, 11, 13), the polymorphism observed could be related to the great number of sand fly vectors and animal reservoir hosts of the rain forests, especially in the southern part of the country. This particular *Leishmania* species is said not to be very polymorphic; however, to our knowledge, no large-scale study on *L. (V.) guyanensis* populations has been conducted before. Cupolillo et al. (12, 13) did not find much intraspecific polymorphism in *L. (V.) guyanensis* isolates compared to *L. (V.) braziliensis* samples. This was probably due to the smaller number of samples studied. As suggested by Cupolillo for *L. (V.) braziliensis* (10), the distinct genotypic groups described herein within 175 *L. (V.) guyanensis* samples could explain the plasticity of these parasites and their ability to adapt to changing ecological conditions, especially to the man-made environmental changes happening in French Guiana. Entomological and zoological data are required to confirm this hypothesis.

The possibility of mixed infections has been previously reported (23, 29, 31, 49). However, in the present study, we did not observe any hybrid profiles and control genotyping did not detect any multiple infections. Biopsy and treatment protocols were identical at all the health centers, and dermal smear parasite density scores were all estimated by the same trained parasitologists at the Cayenne General Hospital. A higher parasite density in lesions and the need for additional treatments were significantly linked to genotype group I. This suggests the presence of two distinct nonsympatric *L. (V.) guyanensis* populations with different clinical implications. Patients with atypical clinical presentations, i.e., chronic and disseminated cutaneous forms, were mostly found in genotype group II. The immunogenetic susceptibility of the host is likely to be partially responsible for these particular presentations, but atypical forms could also be related to the higher biological diversity of the virgin rain forest in the southern part of French Guiana, and thus, to a potentially higher parasitic cycle diversity. This result reinforces the existence of significant genetic diversity within *L. (V.) guyanensis* samples and the possible association of genotypes with specific transmission cycles. The identification of specific patterns for *L. (V.) guyanensis* subpopulations also enables detection of unfavorable outcomes and proper adjustment of therapy.

The degree of polymorphism within each species varied according to the genetic markers studied. Because of the high level of polymorphism in the composition of the ITS

locus, several molecular epidemiological studies have been conducted by RFLP analysis in New World taxa (5, 10, 21, 38), but most studies have been performed on Old World *Leishmania* species (7, 19, 34, 44–47). Analyses of the ribosomal ITS regions of different parasites have demonstrated the utility of this marker in detecting genetic diversity and any association of this diversity with several epidemiological aspects (16, 33). The mechanisms that control the expression of genes in the *Leishmania* genus remain unclear. In the absence of the characteristic eukaryotic modulation of primary transcription via individual promoter activity, *Leishmania* organisms rely on posttranscriptional (mostly *trans*-splicing and polyadenylation) or posttranslational mechanisms to generate the appropriate levels of gene products within the cell (reviewed in reference 50). Multicopy tandemly repeated genes are characterized by a high degree of conservation in peptide sequence, although there is considerable divergence in the 5′ and 3′ untranslated regions (UTRs) of each gene transcript, a feature perhaps related to differences in their regulation (1). Posttranscriptional regulation of transcript levels appeared to be dependent on 3′ UTR sequences (via their effects on mRNA processing or by binding of stability/degradation factors) (20, 30, 36). Although UTR and ITS regions present functional differences, such as transcription by distinct polymerases, one could hypothesize that the rRNA ITS region may also have a role in ribosomal gene expression itself. It is unlikely that the ITS sequences are directly genetically linked to the pathogenic mechanisms, due to the continuum of variation observed in this locus. These variations are undoubtedly linked to the divergence among the isolates. Nevertheless, the important genetic polymorphism, statistically linked to various geographical, epidemiological, and clinical parameters evokes a much more complex potential functional role of this particular locus. However, this is conjecture, and further studies will be required to clarify this point.

L. (V.) guyanensis was previously said not to be very polymorphic; however, the present RFLP analysis on a ribosomal sequence resulted in a significant degree of discrimination among *L. (V.) guyanensis* isolates from diverse ecological areas and with different clinical implications.

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