MAJOR ARTICLE

Influence of *Leishmania (Viannia)* Species on the Response to Antimonial Treatment in Patients with American Tegumentary Leishmaniasis

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Background. Pentavalent antimonials (Sb^V) are the first-line chemotherapy for American tegumentary leishmaniasis (ATL). There are, however, reports of the occurrence of treatment failure with these drugs. Few studies in Latin America have compared the response to Sb^V treatment in ATL caused by different *Leishmania* species.

Methods. Clinical parameters and response to Sb^V chemotherapy were studied in 103 patients with cutaneous leishmaniasis (CL) in Peru. *Leishmania* isolates were collected before treatment and typed by multilocus polymerase-chain-reaction restriction fragment–length polymorphism analysis.

Results. The 103 isolates were identified as *L. (Viannia) peruviana* (47.6%), *L. (V.) guyanensis* (23.3%), *L. (V.) braziliensis* (22.3%), *L. (V.) lainsoni* (4.9%), *L. (Leishmania) mexicana* (1%), and a putative hybrid, *L. (V.) braziliensis/L. (V.) peruviana* (1%). *L. (V.) guyanensis* was most abundant in central Peru. Of patients infected with the 3 former species, 21 (21.9%) did not respond to Sb^V chemotherapy. The proportions of treatment failure (after 12 months of follow-up) were 30.4%, 24.5%, and 8.3% in patients infected with *L. (V.) braziliensis, L. (V.) peruviana,* and *L. (V.) guyanensis,* respectively. Infection with *L. (V.) guyanensis* was associated with significantly less treatment failure than *L. (V.) braziliensis,* as determined by multiple logistic regression analysis (odds ratio, 0.07 [95% confidence interval, 0.007–0.8]; $P = .03$).

Conclusions. *Leishmania* species can influence Sb^V treatment outcome in patients with CL. Therefore, parasite identification is of utmost clinical importance, because it should lead to a species-oriented treatment.

American tegumentary leishmaniasis (ATL) is a parasitic protozoan disease that is endemic in most countries of Latin America. It is characterized by a significant clinical pleomorphism, which has been related to both the infecting *Leishmania* species and the host immune response [1, 2]. This complicates epidemiological monitoring and, eventually, clinical management.

This is well illustrated by the situation in Peru. In this country, 3 species are predominant and are of epidemiological importance: *L. (Viannia) braziliensis, L. (V.) peruviana,* and *L. (V.) guyanensis* [3, 4]. The 3 species cause cutaneous lesions, but the respective clinical evolution is characterized by a gradient of severity: (1) in *L. (V.) peruviana* infection, lesions generally remain small and are self-healing [5, 6]; (2) lesions caused by *L. (V.) guyanensis* may develop into diffuse cutaneous leishmaniasis (CL), which is characterized by disseminated nodular lesions that fail to heal spontaneously and that reappear after the cessation of treatment [1, 2]; and (3) *L. (V.) braziliensis* metastasize in up to 10% of cases [7], resulting in mutilating mucosal lesions

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(known as "espundia"). *L. (V.) peruviana* is mostly encountered in the rural Andean and inter-Andean valleys (between 1000 and 3000 m above sea level), whereas the 2 other species are both endemic in sylvatic regions. However, this epidemiological picture can be affected by environmental changes and human migrations [8].

Chemotherapy is the main control strategy for leishmaniases, and pentavalent antimonials (Sb^V) are the first-line drugs. Unfortunately, the increasing trend of treatment failure documented in several field sites is now challenging the clinical value of Sb^V therapy [9]. Studies conducted in Latin America have reported a range of efficacies of this drug against CL: 7% treatment failure in Bolivia [10], 16% in Brazil [11], and up to 39% in Colombia [12]. The identification of the factors associated with chemotherapy failure would allow better clinical management of patients.

Concerning the role played by different *Leishmania* species in the chemotherapeutic response of ATL, reports are scanty. Here, we demonstrate that Peruvian patients infected with *L. (V.) braziliensis* and *L. (V.) peruviana* respond similarly to Sb^V treatment, presenting a comparably high prevalence of treatment failure, whereas patients infected with *L. (V.) guyanensis* are much more responsive to Sb^V therapy. The epidemiological and clinical implications of our result are discussed.

PATIENTS, MATERIALS, AND METHODS

Treatment and follow-up. Peruvian patients with a clinical diagnosis of leishmaniasis were recruited into the Leishnatdrug-R study between November 2001 and December 2004 at the Instituto de Medicina Tropical "Alexander von Humboldt," a national reference center for patients with leishmaniasis. The Leishnatdrug-R study was conducted to assess the occurrence of natural resistance among *Leishmania* isolates obtained from patients who either responded to treatment or experienced treatment failure with Sb^V (20 mg/kg/day intravenous Sb^V for 20 days [13]). Patients were interviewed to identify the most probable geographic origin of infection. They were treated with generic sodium stibogluconate (SSG) from Colombia (Viteco SA; $n = 80$) or India (Albert David Ltd.; $n = 23$), depending on drug availability. All used batches contained the recommended Sb^V concentration (quality control was performed by the International Dispensary Association). Patients received antimonial treatment under supervision and attended follow-up visits 1, 3, and 6 to 12 months after treatment. Patients with treatment failure received either (1) a repeat course of antimonials with or without topical imiquimod (Aldara; 3M Pharmaceuticals) or (2) intravenous amphotericin B (amphotericin B deoxycholate; Bristol-Myers Squibb).

Only patients with a first diagnosis of CL without concomitant mucosal involvement, who received $\geq$20 doses of antimonials, and who were followed for $\geq$6 months were included in the present study. Written, informed consent was obtained from all patients or their parents or guardians. Research protocols complied with national and international ethics policies. The human experimentation guidelines of the Institute of Tropical Medicine Antwerp were followed. Ethics clearance was obtained from the ethical committees of the Universidad Peruana Cayetano Heredia and the Institute of Tropical Medicine Antwerp.

Definition of clinical outcomes. Initial cure ($\leq$3 months after treatment) was defined as follows: for ulcers, complete scarring of lesion(s) and disappearance of inflammatory signs; for nodular lesions, flattening and the absence of infiltration or other sign(s) of inflammation. Unresponsiveness was defined as the absence or incomplete scarring of lesion(s) and/or the persistence of inflammatory signs 3 months after treatment or the worsening of existing lesion(s) or the appearance of new lesion(s) $\leq$3 months after treatment. Relapse was defined as the reappearance of an ulcer or nodule and/or local signs of inflammation after initial cure. Treatment failure was defined as unresponsiveness or relapse. Cure was defined as initial cure without relapse $\leq$12 months after treatment.

Parasite isolation and biopsy handling. Parasite samples (lesion aspirate samples and/or biopsy samples) were collected. Aspirate samples were cultured in Tobie blood agar medium [14] at 23°C. Mass cultures were harvested and cryopreserved for species identification. DNA was extracted by use of the QIAmp DNA Mini Kit (Qiagen) or by the classic phenol/chloroform method. For biopsy samples, frozen specimens were lysed at 65°C for 3 h in 50 μL of TNE buffer (25 mmol/L Tris, 100 mmol/L NaCl, and 5 mmol/L EDTA [pH 8]) containing 5% sodium dodecyl sulfate and 200 μg/μL proteinase K. After ethanol precipitation, DNA pellets were resuspended in 15 μL of buffer TE (10 mmol/L Tris and 1 mmol/L EDTA [pH 7.4]) [15].

Parasite species identification. *Leishmania* species typing was performed by multilocus polymerase-chain-reaction (PCR) restriction fragment–length polymorphism (RFLP) analysis. The target genes *rDNA ITS, gp63, hsp70, H2B,* and *cpb* were amplified and digested as reported elsewhere [15–17]. Restriction patterns were resolved by capillary electrophoresis (2100 Bioanalyzer system; Agilent Technologies) in a microchip device (DNA 1000 LabChip; Caliper Technologies) or by use of 12% polyacrilamide at 29:1, with silver stain. Obtained patterns were compared with those of reference strains of *L. (V.) braziliensis* (MHOM/PE/02/PER002), *L. (V.) guyanensis* (MHOM/BR/78/M5378), *L. (V.) lainsoni* (MHOM/PE/03/PER167), *L. (V.) peruviana* (MHOM/PE/90/HB22), and *L. (Leishmania) amazonensis* (MHOM/BR/73/M2269).

Statistical analysis. The response variable in the analysis was the clinical outcome, defined as cure or failure at the 6- to 12-month follow-up time point after completion of Sb^V therapy. By means of multiple logistic regression analysis, the

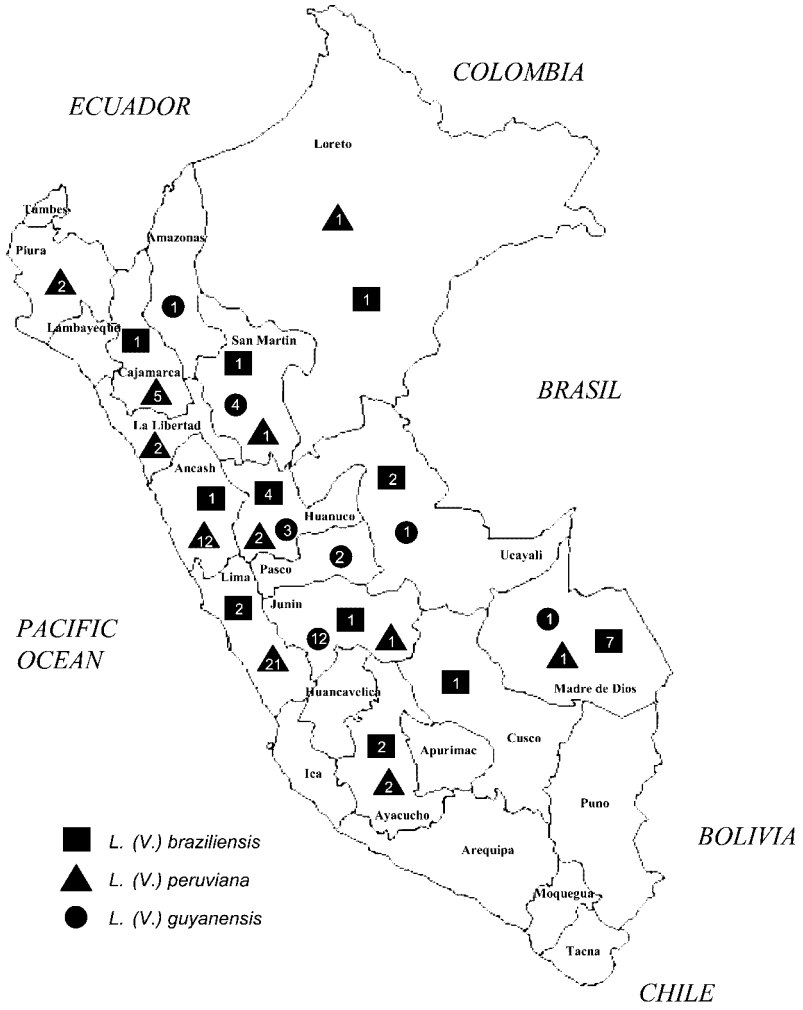

Figure 1. Geographic distribution of *Leishmania (Viannia) braziliensis, L. (V.) peruviana,* and *L. (V.) guyanensis* by department in Peru. The value inside each symbol corresponds to the no. of isolates of a given species encountered in each department.

odds of treatment failure were modeled considering the *Leishmania* species as the main predictor of interest. Other recorded variables that may act as confounding factors were included in the base model: (1) assumed species-independent variables—the patient's age, sex, occupation, and place of infection (Andes vs. Amazon); (2) assumed species-nonindependent variables—number of lesions, lesion type, duration of lesions, and total area of lesions; and (3) the source of SSG (Colombian vs. Indian). The best final model included age, number of lesions, duration of lesions, and the source of SSG as significant covariates. After adjustment for covariates, a separate comparison was performed for *L. (V.) guyanensis* versus *L. (V.) braziliensis* or *L. (V.) peruviana* infections. Absolute failure proportions and the 95% confidence intervals (CIs) were calculated on the basis of a binomial distribution. Statistical tests were performed under a 5% significance level, using STATA software (version 9.0; StataCorp).

RESULTS

A total of 171 patients received a diagnosis of leishmaniasis and had the infecting species of *Leishmania* typed during the study period. Of these patients, 56 did not meet eligibility criteria: 14 had previously received treatment for leishmaniasis, 18 presented concomitant mucosal involvement, 10 did not complete the first round of Sb^V treatment, and 14 presented an unclear clinical outcome (i.e., follow-up of <6 months). Twelve patients treated with meglumine antimoniate (Glucantime) or generic SSG from Peru (Marfan) were also excluded from the analysis, because they were too few in number for statistical comparison. Of the 103 patients enrolled in the study, 79 and 24 patients had *Leishmania* parasites isolated and typed from skin lesion aspirate and biopsy samples, respectively.

Geographic distribution of characterized* Leishmania *species. The geographic distribution of the selected *Leishmania* isolates covered all of the Peruvian territory in which leishmaniasis has

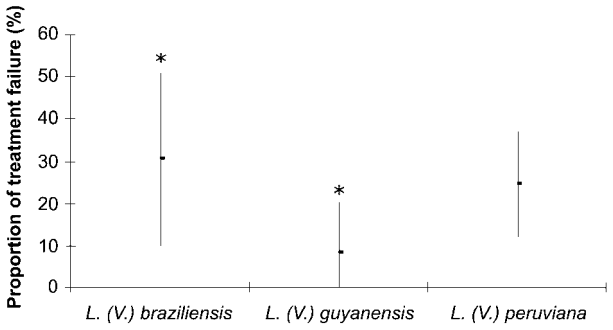

Figure 2. Proportion of pentavalent antimonial treatment failure by *Leishmania (Viannia)* species in Peru: for *L. (V.) braziliensis,* 30.4% (95% confidence interval [CI], 10%–50%; $n = 23$ isolates); for *L. (V.) peruviana,* 24.5% (95% CI, 10%–40%; $n = 49$ isolates); and for *L. (V.) guyanensis,* 8.3% (95% CI, 0%–20%; $n = 24$ isolates). CIs were calculated on the basis of a binomial distribution. **Leishmania* species that presented a significant difference in proportion of treatment failure ($P < .05$).

been reported. The 3 most prevalent *Leishmania* species were *L. (V.) peruviana* ($n = 49$ isolates [47.6%]), *L. (V.) guyanensis* ($n = 24$ [23.3%]), and *L. (V.) braziliensis* ($n = 23$ [22.3%]) (figure 1). Five isolates of *L. (V.) lainsoni* (4.9%), 1 of *L. (L.) mexicana* (1%), and 1 of a putative hybrid, *L. (V.) braziliensis/ L. (V.) peruviana* (1%), were also identified, but the corresponding patients were not considered in further analyses. The *L. (V.) peruviana* isolates originated mainly from the Andean region, whereas those of *L. (V.) braziliensis* presented a wide distribution with a major occurrence along the sylvatic part of the country. *L. (V.) guyanensis* was mainly isolated from patients infected in the central high jungle.

Treatment failure according to* Leishmania *species. Of the 96 patients infected with *L. (V.) braziliensis, L. (V.) peruviana,* or *L. (V.) guyanensis*, 21 (21.9%) did not respond to treatment. The proportions of treatment failure were 30.4% in patients infected with *L. (V.) braziliensis* (7/23), 24.5% in those infected with *L. (V.) peruviana* (12/49), and 8.3% in those infected with *L. (V.) guyanensis* (2/24) (figure 2).

On multiple logistic regression analysis, patients infected with *L. (V.) guyanensis* were significantly less likely to experience Sb^V treatment failure than were patients infected with *L. (V.) braziliensis* (odds ratio [OR], 0.07 [95% CI, 0.007–0.8]; $P = .03$). No significant difference was found regarding the odds of treatment failure when *L. (V.) guyanensis* infection was compared with *L. (V.) peruviana* infection (OR, 0.4 [95% CI, 0.04–4.7]; $P = .5$) or when *L. (V.) braziliensis* infection was compared with *L. (V.) peruviana* infection (OR, 0.3 [95% CI, 0.06–1.3]; $P = .1$).

Finally, we pooled the data on patients infected with either *L. (V.) braziliensis* or *L. (V.) peruviana* and compared them with the data on patients infected with *L. (V.) guyanensis*. Again, albeit not statistically significant, infection with *L. (V.) guyanensis* was associated with a lower proportion of treatment failure (OR, 0.2 [95% CI, 0.02–1.2]; $P = .07$).

DISCUSSION

In the present study, we assessed the influence that *Leishmania (V.)* species has on the outcome of Sb^V therapy in patients with CL in Peru. Our data support there being a significant association between the infecting *Leishmania* species and treatment outcome. The proportion of treatment failure in patients infected with *L. (V.) braziliensis* was significantly much higher than that in patients infected with *L. (V.) guyanensis*, after adjustment for potential confounding factors.

The influence of *Leishmania* species on treatment outcome has been documented with different drugs in different regions of endemicity (table 1). Our finding of a higher probability of therapeutic failure in patients infected with *L. (V.) braziliensis* than in those infected with *L. (V.) guyanensis* contrasts with

Table 1. Differential response by *Leishmania* species to chemotherapy in cutaneous leishmaniasis.

Leishmania species	Region of endemicity	Chemotherapeutic response, % of cure		Reference
		Sb^V	Miltefosine	
L. (Viannia) braziliensis	Brazil	50.8	…	[18]
L. (V.) guyanensis	Brazil	26.3	…	[18]
L. (V.) braziliensis	Guatemala	96.0	…	[19]
L. (Leishmania) mexicana	Guatemala	57.0	…	[19]
L. (V.) braziliensis	Peru	69.6	…	Present study
L. (V.) peruviana	Peru	75.5	…	Present study
L. (V.) guyanensis	Peru	91.7	…	Present study
L. (V.) panamensis	Colombia	…	91.0	[20]
L. (V.) braziliensis	Guatemala	…	33.0	[20]
L. (L.) mexicana	Guatemala	…	60.0	[20]

NOTE. Sb^V, pentavalent antimonials.

the report of Romero et al. [18], in which the proportion of treatment failure after 6 months of antimonial therapy (Glucantime) was significantly higher in patients infected with *L. (V.) guyanensis* (73.7%) than in those infected with *L. (V.) braziliensis* (49.2%). Notably, the proportion of patients infected with *L. (V.) guyanensis* who experienced Sb^V treatment failure was ~9-fold higher in the Brazilian report than in ours (73.7% vs. 8.3%, respectively). This observation contrasts with the closer proportions of Sb^V treatment failure in patients infected with *L. (V.) braziliensis* in both studies (49.2% vs. 30.4%, respectively). Therefore, the discrepancy between these reports specifically concerns patients infected with *L. (V.) guyanensis*. Different factors associated with parasites, host responses, or treatments—or a combination of these factors [21]—could explain the contrasting findings.

Some *Leishmania* species are known to present differing intrinsic susceptibility to antimonials [22]. We recently found clinical isolates of *L. (V.) braziliensis* and *L. (V.) guyanensis* that were either susceptible or highly tolerant to Sb^V [23]. Accordingly, one might assume that the proportion of Sb^V-tolerant *L. (V.) guyanensis* isolates might vary geographically and explain the differences in treatment outcome encountered with this species between Brazil and Peru. However, in our previous report, we did not find any correlation between parasite Sb^V susceptibility (as measured in the in vitro amastigote-macrophage model) and treatment outcome [23]. Furthermore, 2 patients infected with *L. (V.) guyanensis* showed a definite cure (after follow-up during 12 months), despite being infected with parasites highly tolerant to Sb^V [23]. This highlights the need for comparative multicentric and multidisciplinary studies addressing both the host and the parasite, with standardized protocols and definitions.

Our results also provided an updated insight into the distribution of *Leishmania* species in Peru. A previous report from 1998 [3] was based on multilocus enzyme electrophoresis. Globally, this report and the present study are in agreement, such as the relevant finding of sympatric circulation of *L. (V.) braziliensis* and *L. (V.) guyanensis* in the east-central region of the Andes (Departments of Junin, Huanuco, San Martin, and Ucayali). Two specific differences, however, exist. First, the reported proportion of *L. (V.) guyanensis* isolates from the departments mentioned above increased from 25% (1986–1993) to 55% (2001–2004). Second, some species were encountered in unexpected regions, such as *L. (V.) peruviana* in the Amazonian jungle and *L. (V.) braziliensis* in the Andes. Differences in study design might explain this variation in frequency; in particular, part of our species identification was performed directly on patient biopsy samples, avoiding the selection biases introduced by isolation and in vitro maintenance. However, comparison of both reports might be indicative of a real change in epidemiological patterns, a phenomenon that is well described in Latin America [24]. Altogether, these results emphasize the need to conduct continuous prospective studies in Peru as well as in many other countries of the subcontinent.

In conclusion, our study demonstrates the complex and dynamic epidemiology of ATL in Peru, a situation that probably occurs in other countries of Latin America. Furthermore, the link between *Leishmania* species and treatment outcome highlights the relevance of incorporating species typing to improve the clinical management of patients. Moreover, species typing is needed for epidemiological surveys conducted within control programs and for clinical trials. Thus, rapid and very specific methods for parasite identification are urgently needed. PCR-based genotyping methods, such as multilocus PCR-RFLP analysis [15] and multilocus sequence typing [25], have been developed and can be applied directly to biopsy samples. Nevertheless, they are still too cumbersome to be applied in areas of endemicity that lack sophisticated equipment. Simplification of these characterization tools is needed for their clinical use in endemic regions in which several *Leishmania* species can coexist (as is the case in Central and South America) but also in travel medicine, in which a clinician can be confronted with different species and the geographic location cannot be used as the only criterion for species discrimination.

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