MAJOR ARTICLE

American Tegumentary Leishmaniasis: Is Antimonial Treatment Outcome Related to Parasite Drug Susceptibility?

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Background. Antimonials are the firs drug of choice for the treatment of American tegumentary leishmaniasis (ATL); however, their efficac is not predictable, and this may be linked to parasite drug resistance. We aimed to characterize the in vitro antimony susceptibility of clinical isolates of Peruvian patients with ATL who were treated with sodium stibogluconate and to correlate this in vitro phenotype with different treatment outcomes.

Methods. Thirty-seven clinical isolates were obtained from patients with known disease and treatment histories. These isolates were typed, and the susceptibility of intracellular amastigotes to pentavalent (SbV) and trivalent (SbIII) antimonials was determined.

Results. We observed 29 SbV-resistant isolates among 4 species of subgenus *Viannia,* most of which exhibited primary resistance; isolates resistant only to SbIII; and 3 combinations of in vitro phenotypes: (1) parasites sensitive to both drugs, (2) parasites resistant to both drugs, and (3) parasites resistant to SbV only (the majority of isolates fell into this category). There was no correlation between in vitro susceptibility to both antimonials and the clinical outcome of therapy.

Conclusion. Antimony insensitivity might occur in a stepwise fashion (firs to SbV and then to SbIII). Our data question the definitio of true parasite resistance to antimonials. Further studies of treatment efficac should apply standardized protocols and definition and should also consider host factors.

At present, leishmaniasis is reemerging and spreading worldwide, because of environmental changes, host immunity, and treatment failure [1, 2]. The latter phenomenon has been well described in the Indian subcontinent, where, in Bihar, >60% of patients do not respond to antimonials [3]. In other regions where the parasite is endemic, antimonials remain the first-lin drug. In Latin America, reports of the efficac of this class of drug have revealed contrasting figu es: 7% treatment failure in American tegumentary leishmaniasis (ATL) in Bolivia [4], 16% in Brazil [5], 23.9% in Peru (G. Tulliano, F.C., and A.L.-C., data not shown), and up to 39% in Colombia [6].

Treatment failure is a complex phenomenon with a potentially multifactorial origin. This may involve (1) host factors, such as genetics, immunological response [7], characteristics of the patients [6], and clinical presentation [8]; (2) treatment features, such as drug quality [9], duration of therapy, and compliance; and (3) parasite characteristics, such as intrinsic insensitivity (species) [10] and drug resistance [11]. With respect to the specifi contribution of parasite drug resistance, reports have been inconsistent. A correlation between resistance to pentavalent antimony (SbV) and treatment outcome has been demonstrated in *Leishmania (Leishmania) donovani* in India [11] but not in Sudan [12]. In neotropical *Leishmania* species, a correlation

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between in vitro susceptibility and clinical phenotypes was observed in patients infected with *L. (Viannia) braziliensis* or *L. (V.) panamensis* [13]. However, in that study, parasite susceptibility was determined using promastigotes (vector and culture stage), which are known to be intrinsically insensitive to pharmacological concentrations of SbV [14]. Very recently, a study that focused on *L. (V.) panamensis* suggested that SbV resistance define using intracellular amastigotes (the vertebrate stage) could contribute to 40% of treatment failure [15]. However, in that study, isolates from patients who responded to treatment were not included; hence, the predictive value of intrinsic parasite drug resistance could not be completely assessed.

The present article is a part of a multidisciplinary, prospective study aiming at a global understanding of antimony failure (see http://www.leishnatdrug.org). We have characterized the in vitro susceptibility to antimony of clinical isolates from Peruvian patients with ATL who were treated with sodium stibogluconate and who had different treatment outcomes. In total, 37 isolates belonging to 5 different species endemic in Peru were included, and particular attention was given to the most pathogenic species, *L. (V.) braziliensis*. We tested the susceptibility of intracellular amastigotes to SbV, which is considered to be a prodrug [14], and to trivalent antimony (SbIII), the reduced, active form of the drug (evidence for the role of SbIII in intracellular amastigote killing has been reviewed elsewhere [14]). We then compared these in vitro data to the treatment outcome of patients from whom parasites were isolated. We are aware that parasite tolerance to antimonies could originate from exposure to drug or could constitute an intrinsic unresponsiveness. However, because there is no clear answer to this question at this stage (for parasites of subgenus *Viannia*), and to be consistent with the recent literature on that subject, we have a priori used the term "resistance" throughout the present article.

PATIENTS, MATERIALS, AND METHODS

Patients and clinical protocol. Patients with clinical suspected cutaneous (CL), mucosal (ML), or mucocutaneous (MCL) leishmaniasis were investigated at the Instituto de Medicina Tropical "Alexander von Humboldt," Lima, Peru. Infection with *Leishmania* species was confi med by direct examination of punch-biopsy samples, parasite culture, or polymerase chain reaction (PCR) [16]. Between November 2001 and December 2004, patients with confi med leishmaniasis were enrolled if they provided written, informed consent. Pregnant women were excluded from the study. Patients were treated with intravenous meglumine antimoniate (Glucantime; Sanof Aventis) or generic sodium stibogluconate (Viteco), depending on drug availability, at dosages of 20 mg/kg/day for 20 (for CL) or 30 (for ML or MCL) days. Patients who failed a firs course of SbV were treated with a second course of SbV with or without topical imiquimod or with conventional amphotericin B. Daily treatment was administered, under medical supervision, in outpatient clinics. All patients were asked to attend follow-up visits 1, 2, 3, 6, and 12 months after treatment. At each visit, the clinical appearance of the lesion(s) was assessed by a physician for size and the presence of signs of inflammatio or scarring. The status of the lesion(s) was compared with digital pictures and drawings of the initial (pretreatment) lesion(s) and graded from M0 (no change or worsening of the lesion) to M4 (complete scarring of the lesion). Informed consent was obtained from patients or their parents or guardians. Human-experimentation guidelines of the Institute of Tropical Medicine were followed. Ethics clearance was obtained from the ethical committees of the Cayetano Heredia University, Lima, Peru, and Institute of Tropical Medicine, Antwerp, Belgium.

Definitio of clinical outcomes. Initial cure (⩽3 months after treatment) was define as follows: for ulcers, complete scarring of lesion(s) and disappearance of inflammato y signs; for nodular lesions, flattenin and the absence of infiltratio or other sign(s) of inflammation Unresponsiveness was define as the absence or incomplete scarring of lesion(s) and/or the persistence of inflammato y signs at 3 months after treatment or the worsening of existing lesion(s) or the appearance of new lesion(s) ⩽3 months after treatment. Relapse was define as the reappearance of an ulcer or nodule and/or local signs of inflammatio after initial cure. Treatment failure was define as unresponsiveness or relapse. Cure was define as initial cure without relapse ⩽12 months after treatment.

Parasites and in vitro culture. Parasites were isolated onto 3N blood slopes with a saline/antibiotic overlay [17], sent to the Institute of Tropical Medicine (Antwerp, Belgium) cryopreserved in aliquots, and typed within 8 passages of isolation. Frozen stocks were sent to the London School of Hygiene and Tropical Medicine, where the parasites were passaged initially onto 3N blood slopes with M199 with a 20% heat-inactivated fetal calf serum (HIFCS) overlay and then onto M199 with 20% HIFCS alone. It was necessary to introduce the use of M199 [18] to obtain a clean bulk culture of promastigotes that would be sufficien for further evaluation. The type of medium can affect the infectivity of the parasite [19]; however, in the present study, all isolates were exposed to exactly the same growth conditions, and the work was performed as close to the time of isolation as possible.

Parasite species identification *Leishmania* species identificatio was performed by multilocus PCR–restriction fragment length polymorphism analysis of the *gp63, Hsp70, cpb,* and/or *H2b* genes [20–22]: restriction patterns were compared with those of reference strains of *L. (V.) braziliensis* (MHOM/BO/94/CUM43), *L. (V.) guyanensis* (MHOM/BR/75/M5378), *L. (V.) lainsoni* (MHOM/BO/94/CUM78), *L. (V.) peruviana* (MHOM/PE/90/HB22), and *L. (L.) amazonensis* (MHOM/BR/73/M2269).

In vitro drug-susceptibility testing. Promastigotes were

maintained in M199 medium supplemented with 20% HIFCS at 25°C. All strains were tested for their in vitro sensitivity to SbV within 8 passages of isolation. Late-stage promastigotes were used to infect starch-induced murine peritoneal macrophages at a ratio of 7 promastigotes to 1 macrophage in Labtek 16-well tissue culture-well slides (VWR), in quadruplicate, and kept at 34°C in a 5% CO_2/air mix. Twenty-four hours after infection, 1 slide was fixe in methanol and stained with Giemsa, for the determination of the initial level of infection. If the level of infection was >80%, the infected cultures were exposed to sodium stibogluconate (Sb[V]; GSK) over a dose range of 80, 26.6, 8.8, and 2.9 μg/mL. Stock solutions of both NaSbV and Triostam (SbIII) were formulated by dissolving the white powder in sterile PBS, followed by further dilution in complete medium. After 5 days, the percentage of infected macrophages in each well was determined by microscopic analysis [23]. The percentage of inhibition was calculated from a comparison of counts from treated and untreated cultures using sigmoidal regression analysis (*xl*fi version 3; Microsoft), and ED_{50} values were determined. The strain *L. (V.) braziliensis* MHOM/BR/75/M2903, a World Health Organization reference strain that is sensitive to sodium stibogluconate and meglumine antimoniate, was included in each assay as a reference. The ratio of the ED_{50} of a tested strain to the ED_{50} of the reference strain (range, 4–15 μg/mL, according to the experimental series), which we termed the "activity index" (AI), was used to express the in vitro susceptibility of that tested strain and to easily compare the results obtained from different series of experiments. Sensitivity to SbIII was evaluated using the same assay. Triostam (trivalent sodium antimonyl gluconate; donated by Burroughs Wellcome) was used over a dose range of 30–1.1 μg of SbIII/mL. Comparable concentrations to SbV could not be used because of toxicity in host cells at higher concentrations. Animal experiments complied with UK government (Home Office guidelines.

Data analysis. Demographic, clinical, laboratory, treatment, and outcome data were entered into an Excel database (Excel 2003; Microsoft). All data were cross-checked with individual patient file during several data-monitoring visits by F.C. Statistical analysis was done using SPSS (version 11.0. for Windows; SPSS). For the analysis of correlations between clinical outcome and parasite sensitivity, only patients treated with antimonials who had a clear clinical outcome (cure or treatment failure) were included. Categorical variables were compared using cross-tabulations and χ^2 tests, and numerical variables (parasite sensitivity) were compared using the nonparametric Mann-Whitney *U* test, at a critical α-level of .05.

RESULTS

In vitro susceptibility to SbV and SbIII. A total of 37 isolates was selected for in vitro susceptibility assays, all but 1 of which originated from the Amazonian forest (lowlands and high jungle), because our aim was to focus on *L. (V.) braziliensis* isolates. Species identificatio revealed the following composition of our sample: *L. (V.) braziliensis* (26 isolates), *L. (V.) guyanensis* (5 isolates), *L. (V.) lainsoni* (4 isolates), *L. (V.) peruviana* (1; the only isolate from the Andean region), and *L. (L.) amazonensis* (1 isolate). The *L. (V.) braziliensis* isolates had a widespread origin, whereas *L. (V.) guyanensis* and *L. (V.) lainsoni* were essentially isolated from the central high jungle (figu e 1). All of these isolates were tested for their susceptibility to SbV. Strains with an AI of 1–2 (i.e., similar to that of the reference strain) were considered to be sensitive. Strains with an AI of $\geqslant$5–6 (corresponding to an ED_{50} of >80 μg of SbV/mL) were considered to be resistant. With 1 exception (strain PER008, with an AI of 3), all strains that we tested belonged to 1 of these 2 categories. Sensitive and resistant parasites were observed in *L. (V.) braziliensis, L. (V.) guyanensis,* and *L. (V.) lainsoni,* whereas the *L. (V.) peruviana* isolate and the *L. (L.) amazonensis* isolate were resistant and sensitive, respectively (table 1). Interestingly, the majority (22/26) of *L. (V.) braziliensis* isolates were resistant to SbV. This was not likely due to bias in this sample, because (1) the isolates came from different regions of the Amazonian jungle and (2) there was a similar proportion of cures (13) and treatment failures (11) among the patients from whom they were obtained (the 2 remaining patients were lost to follow-up).

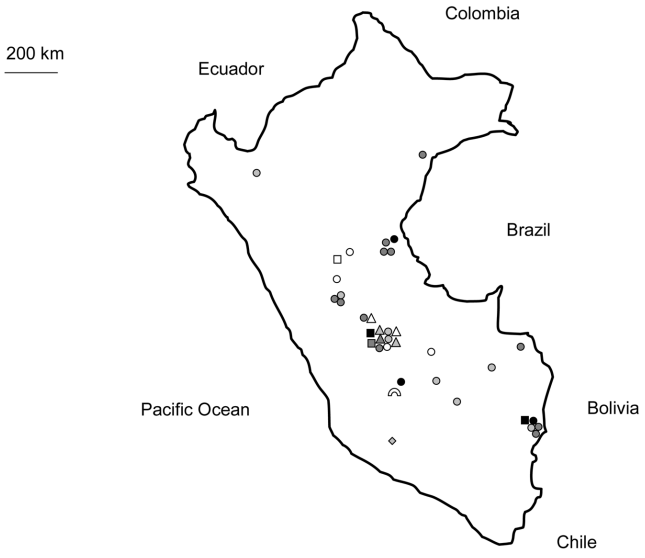

Figure 1. Distribution of clinical Peruvian isolates tested for their susceptibility to pentavalent antimony (SbV) and trivalent antimony (SbIII). *Leishmania (Viannia) braziliensis,* circles; *L. (V.) guyanensis,* triangles; *L. (V.) lainsoni,* squares; *L. (V.) peruviana,* diamond; and *L. (L.) amazonensis,* arc. The shading patterns represent the tested phenotype: sensitive to both drugs, white; resistant to SbV and not tested with SbIII, light gray; resistant to SbV and sensitive to SbIII, dark gray; and resistant to both drugs, black.

Table 1. Peruvian *Leishmania* isolates tested for their in vitro susceptibility to antimonials and link with clinical phenotype.

Isolate	Origin, state, province	Disease	SbV	SbIII	Clinical response
L. braziliensis					
MHOM/PE/01/PER 005/0	Loreto, Ucayali	CL	1	ND	Unresponsive
MHOM/PE/03/PER 130/0[a]	Cusco, Echarate	CL	1	0	Unresponsive
MHOM/PE/03/PER 163/0	Huanuco, Leoncio Prado	CL	2	0	Definite cure
MHOM/PE/03/PER 186/0[a]	Junin, Satipo	CL	2	1	Definite cure
MHOM/PE/02/PER 086/0[b]	Pasco, Oxapampa	CL	6+	0	Unresponsive
MHOM/PE/02/PER 011/0[c]	Huanuco, Huanuco	MCL	6+	1	Treatment with amphotericin B
MHOM/PE/03/PER 201/0	Loreto, Requena	ML	6	1	Definite cure
MHOM/PE/03/PER 164/0	Ucayali, Coronel Portillo	CL	6+	1	Initial cure
MHOM/PE/03/PER 231/0	Junin, Satipo	ML	5	2	Definite cure
MHOM/PE/01/PER 002/0	Madre de Dios, Tambopata	CL	6	2	Unresponsive
MHOM/PE/03/PER 215/0	Ucayali, Coronel Portillo	ML	6	2	Definite cure
MHOM/PE/02/PER 094/0	Huanuco, Puerto Inca	CL	6	2	Definite cure
MHOM/PE/03/PER 260/0	Madre de Dios, Tahuamanu	ML	6	2	Definite cure
MHOM/PE/03/PER 157/0	Madre de Dios, Tambopata	CL	6+	2	Definite cure
MHOM/PE/02/PER 015/0	Ucayali, Coronel Portillo	CL	6+	2	Unresponsive
MHOM/PE/03/PER 136/0[c]	Ucayali, Coronel Portillo	ML	6	5	Treatment with amphotericin B
MHOM/PE/03/PER 182/0	Ayacucho, La Mar	CL	6	5	Definite cure
MHOM/PE/02/PER 104/0[a]	Madre de Dios, Tambopata	CL	6+	6+	Unresponsive
MHOM/PE/02/PER 010/0	Cajamarca, Jaen	CL	6	ND	Initial cure
MHOM/PE/02/PER 069/0[a,c]	Madre de Dios, Manu	ML	6	ND	No treatment
MHOM/PE/01/PER 006/1	Junin, Satipo	CL	6+	ND	Unresponsive
MHOM/PE/01/PER 014/0[a]	Junin, Satipo	CL	6+	ND	Unresponsive
MHOM/PE/01/PER 012/1	Cusco, Calca	CL	6+	ND	Unresponsive
MHOM/PE/02/PER 016/0	Huanuco, Puerto Inca	CL	6+	ND	Definite cure
MHOM/PE/02/PER 067/0[a]	Cusco, La Convencion	CL	6+	ND	Unresponsive
MHOM/PE/02/PER 122/0	Madre de Dios, Tambopata	CL	6+	ND	Definite cure
L. peruviana MHOM/PE/01/PER 001/1	Ayacucho, Lucanas	CL	6+	ND	Unresponsive
L. guyanensis					
MHOM/PE/01/PER 003/0	Junin, Satipo	CL	1	ND	Definite cure
MHOM/PE/02/PER 008/0	Pasco, Oxapampa	CL	3	ND	Definite cure
MHOM/PE/03/PER 132/0	Junin, Satipo	CL	6+	1	Definite cure
MHOM/PE/02/PER 054/0[c]	Junin, Satipo	CL	6+	ND	Unknown
MHOM/PE/02/PER 072/0	Junin, Chanchamayo	CL	6+	ND	Definite cure
L. lainsoni					
MHOM/PE/03/PER 127/0	San Martin, Tocache	CL	1	ND	Definite cure
MHOM/PE/03/PER 131/0	Junin, Chanchamayo	CL	6+	1	Definite cure
MHOM/PE/02/PER 092/0	Junin, Chanchamayo	CL	6	6	Definite cure
MHOM/PE/02/PER 105/0	Madre de Dios, Tambopata	CL	6+	6+	Definite cure
L. mexicana MHOM/PE/02/PER 068/0[a]	Ayacucho, Huanta	CL	0	ND	Unknown

NOTE. CL, cutaneous leishmaniasis; MCL, mucocutaneous leishmaniasis; ML, mucosal leishmaniasis; ND, not done; SbIII, trivalent antimony; SbV, pentavalent antimony.

[a] Patients with a history of previous treatment with antimony.

[b] Patients with a history of previous treatment, but the drug used was not known.

[c] Patients not integrated in comparison with in vitro susceptibility data.

Of these 37 isolates, 21 could be tested in parallel for their in vitro sensitivity to SbIII, with only 5 isolates shown to be resistant to the drug (AI of 5–6; table 1). The remaining 16 isolates showed AIs of 0–2 (0, more sensitive than the reference strain M2903). When we considered the results of SbV and SbIII together, 3 phenotypes were observed: SbV sensitive/SbIII sensitive (5S3S), SbV resistant/SbIII sensitive (5R3S), and SbV resistant/SbIII resistant (5R3R). A possible fourth combination, SbV sensitive/SbIII resistant (5S3R), was not seen in this sample. The 5R3S and 5R3R phenotypes were both seen in *L. (V.) braziliensis* and *L. (V.) lainsoni:* 5R3S parasites were more abundant in the former species (11/14 isolates), whereas 2 of 3 *L. (V.) lainsoni* isolates were of type 5R3R.

Geographical clustering in terms of in vitro susceptibility was not observed. SbV-resistant isolates were seen in all regions, and even the less frequently occurring 5R3R parasites were found a great distance from each other. Within the same foci, it was possible to observe different phenotypes among isolates—for example, the coexistence of 5R3R and 5R3S *L. (V.) braziliensis* isolates in Tambopata (eastern lowlands, close to the Bolivian border) and of 5S3S and 5R3S parasites in Junin (central high jungle).

In vitro susceptibility and treatment outcome. We compared the results of the in vitro susceptibility of the clinical isolates of *L. (V.) braziliensis, L. (V.) guyanensis,* and *L. (V.) lainsoni* to the treatment outcome of the patients from whom they were obtained (table 2). After the exclusion of 2 patients treated with amphotericin B, 1 patient who defaulted before the start of treatment, and 2 patients with an unclear clinical outcome, 32 patients and isolates were included in the analysis. There was no statistical correlation between SbV and SbIII in vitro sensitivity of the parasite and clinical outcome when parasite species were analyzed together or separately. Moreover, when the analysis was restricted to the 25 patients without prior treatment with antimonials who had parasites isolated and tested in vitro, we found no statistically significan association between in vitro sensitivity and clinical outcome. Two of 4 sensitive *L. (V.) braziliensis* isolates came from patients with treatment failure, and 11 of 19 resistant isolates of that species came from cured patients. Of the 10 5R3S and 2 5R3R isolates, 7 and 1 came, respectively, from cured patients. For patients infected with *L. (V.) guyanensis* and *L. (V.) lainsoni,* the clinical picture was somewhat different—all of them were cured with antimony treatment, but this was not dependent on the in vitro susceptibility of the infecting parasites; 5 of the patients were infected with SbV-resistant parasites.

We also analyzed the data by distinguishing *L. (V.) braziliensis* isolates obtained before and after antimony treatment. "Post-treatment" was considered broadly, as recruited patients either (1) without a previous history of treatment with antimonials and samples obtained after the controlled therapy of present study or (2) with a previous history of treatment and samples obtained before the controlled therapy of present study (labeled with "a" and "b" table 1). Results were similar in both categories: we observed 7 SbV-resistant isolates among the 9 post-treatment samples and 15 SbV-resistant isolates among the 17 pretreatment isolates.

DISCUSSION

Reports on antimonial resistance among clinical isolates of define neotropical *Leishmania* species are scanty. Early reports described the existence of resistance to SbV in *L. (V.) panamensis* [13], *L. (V.) braziliensis* [13], and *L. (L.) amazonensis*

Table 2. Relationship between in vitro susceptibility of clinical isolates to antimonials and treatment outcome of respective patients.

Isolate, in vitro phenotype	Isolates, no.	Patients with treatment failure, no.	Patients cured, no.
Leishmania (Viannia) braziliensis			
5S	4	2	2
5R	19	8	11
5R3S	10	3	7
5R3R	2	1	1
L. (V.) guyanensis			
5S	2	0	2
5R	2	0	2
L. (V.) lainsoni			
5S	1	0	1
5R	3	0	3

NOTE. Only isolates for which in vitro and clinical phenotypes were available are included. 3R, resistant to trivalent antimony (SbIII); 3S, sensitive to SbIII; 5R, resistant to pentavalent antimony (SbV); 5S, sensitive to SbV.

[24], but they were based on parasite promastigotes, which are known to be intrinsically insensitive to SbV [11, 25]. This appeared clearly when we compared ED_{50}s of *L. (V.) braziliensis* promastigotes (220–4100 μg/mL) [13] with those of amastigotes: 4 to >80 μg/mL (present study) versus 2.6 to >128 μg/mL [15]. The higher ED_{50}s reported by Rojas et al. [15] are likely explained by differences in the respective protocols. For the present study, macrophages were infected at a ratio of 7 parasites to 1 macrophage. Previously, it has been shown that a higher amastigote:macrophage ratio can influenc the sodium stibogluconate ED_{50} [26]. A lower dose range over a longer exposure period (5 days) was also used in the present study, which allowed the compound to accumulate sufficientl within infected cells [26, 27]. This demonstrates the specificit of our study and highlights the extreme care needed in comparing our data with those of previous reports.

It is known that different *Leishmania* species may present a different intrinsic sensitivity to antimonials. Using the amastigote-macrophage model, reference strains of *L. (V.) braziliensis, L. (V.) guyanensis,* and *L. (V.) panamensis* were found to be 3–5-fold more sensitive to SbV (average ED_{50}, <5 μg/mL) than *L. (L.) major, L. (L.) tropica,* and *L. (L.) mexicana* [10]. Hence, we determined that it was important to use a reference strain of the same, or close, species—*L. (V.) braziliensis* M2903. By comparison with this reference strain, isolates presenting at least 6-fold lower sensitivity to SbV (considered to be resistant to SbV) were encountered in 4 species of subgenus *Viannia: L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) lainsoni,* and *L. (V.) peruviana*.

However, a more surprising result was the high frequency of SbV-resistant strains within the present sample: 28 of 35 isolates of all species and 22 of 26 *L. (V.) braziliensis* isolates. This contrasts with a previous report [15] in which 3 of 19 isolates were resistant before treatment and 7 of 19 were resistant after treatment failure. These differences could be due to experimental procedures, to species factors (*L. [V.] panamensis* was not included in our study), or to geographical variation, if hot spots of SbV resistance exist in Peru. The latter hypothesis was not supported by our results, given that all of the Peruvian SbV-resistant parasites appeared to be geographically spread over the territory covered by the present study. Interestingly, we saw a totally different picture of parasite drug susceptibility after exposure to SbIII, the reduced and active component of the drug. Indeed, 16 of 21 tested isolates of *Viannia* subgenus and 14 of 17 *L. (V.) braziliensis* isolates appeared to be sensitive to SbIII. If a strain is resistant to SbV but sensitive to SbIII, can it be classed as truly resistant to antimonials? This argument may be resolved as more becomes known about the mechanism of action of antimonials and the role of various host factors. In the meantime, it might be wise to clearly defin the terms of resistance at the outset of any future study or report and to make a clear distinction between real resistance caused by exposure to drug and intrinsic unresponsiveness.

A fin analysis of our results of SbV and SbIII susceptibility has already given some clues about the mechanisms leading to SbV/SbIII resistance. Indeed, the observation of 3 combinations of SbV and SbIII sensitivity phenotypes (5S3S, 5R3S, and 5R3R) and the absence in the present sample of the 5S3R combination suggests a cumulative process in which parasites would become resistant to SbV firs and resistant to SbIII second. A similar observation was made in *L. (L.) donovani* clinical isolates from Nepal (S. Rijal, V.Y., and J.-C.D., data not shown). It is generally accepted that all pentavalent antimonials are prodrugs that require biological reduction to the trivalent form (SbIII) for antileishmanial activity, although the site (amastigote or macrophage) and mechanism of reduction remain controversial [14]. Accordingly, resistance to SbV and not to SbIII would imply a lower activation in the parasite, whereas the cumulated resistance to SbIII could be due to an additional and broader spectrum of mechanisms, such as modifie influ or efflu and change of target [28].

The high frequency of SbV resistance raises a particular concern in the generally accepted zoonotic context of leishmaniasis in the *Viannia* subgenus. Indeed, in this situation, humans are generally considered to be a "dead end" for transmission, and most of the parasites are in animals in which drug pressure is nonexistent. One explanation for these results may be a shift from zoonotic to anthroponotic transmission, as has been hypothesized by other researchers [15]. Several reports have suggested that some transmission cycles of neotropical *Leishmania* tend toward domestication [29]. However, if this were the case, geographical clustering of isolates according to their phenotype would be expected, and this was not seen in the present study. Alternatively, in a real zoonotic context, drug resistance could be acquired by many patients (secondary resistance), as has been shown in Colombia by the isolation—from the same patient—of sensitive and resistant parasites before and after treatment failure, respectively [15]. This could be due to suboptimal therapy, poor quality of the drug, or other factors. This was controlled for as much as possible in the present study. (1) Commercial brands (Glucantime) or generic sodium stibogluconate was used, and, in the latter case, each batch was shown to contain the adequate content of SbV (IDA). (2) A directly observed therapy protocol was used to ensure the correct administration of the drug. Furthermore, we encountered in *L. (V.) braziliensis* a high proportion (15/17) of primary SbV-resistant parasites (isolated before supervised treatment from patients with no history of antimonial therapy). A last explanation compatible with zoonotic transmission could be that the observed SbV resistance does not result from previous contact with the drug but could be a secondary effect of another phenomenon. The recent demonstration of cross-resistance to

antimony and nitric oxide [30] supports this possibility and should be further explored.

Another major findin was the lack of correlation between in vitro susceptibility to antimony and the clinical outcome of therapy. In a recent report on resistance to SbV in *L. (V.) panamensis,* a correlation of 40% was observed with treatment failure [15], but the occurrence of resistant strains among patients responding to the treatment was not reported. Our data show that, of 13 cured patients, 11 were infected with SbV-resistant *L. (V.) braziliensis* parasites. Obviously, our sample size could be further increased to confi m this lack of correlation, bur other explanations should be considered. The possibility that SbV susceptibility is not the correct phenotype to correlate with treatment outcome should not be excluded: indeed, of 10 cured patients for whom we had isolates with known SbV and SbIII susceptibility phenotypes, it appeared that 9 isolates were sensitive to SbIII. Nevertheless, we did not fin any correlation between SbIII susceptibility and clinical outcome. The fact that many of our isolates were obtained before treatment could be a second explanation, if secondary resistance were the most frequent situation (in that case, the pretreatment sensitive isolates would obviously not correlate with treatment failure). However, as was mentioned above, most of the pretreatment isolates were already resistant in the present study. A third explanation could be that treatment failure is not due simply to the degree of parasite sensitivity to antimony but also to host factors. This is supported by previous reports that have shown that a poor response to antimonial therapy in patients infected with *L. (V.) braziliensis* was associated with a low lymphoproliferative response [31]. This possible explanation is further strengthened by recent trials that have demonstrated that the administration of antimony plus topical imiquimod (an innate immune-response modulator) to subjects for whom an initial course of antimony therapy failed accelerated the reepithelization of lesions and improved scar quality [32, 33].

The present results demonstrate the need for more standardization in studies of drug resistance and its link to treatment outcome. This concerns the in vitro susceptibility assays themselves (e.g., those based on intracellular amastigotes that measure SbV and SbIII) but also the clinical aspects themselves, protocols, and, most of all, definitions Further work should be performed in Peru, to confi m our data in a larger sample, and in other countries in the region. Ideally, they should be performed in a multicenter and multidisciplinary context, and they should certainly contain an immunological component, to consider the respective weight of parasite and human factors in the fina treatment outcome.

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