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Association of the endobiont doubled-stranded RNA virus LRV1 with treatment failure of human leishmaniasis caused by *Leishmania braziliensis* in Peru and Bolivia

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ABSTRACT

Cutaneous and mucosal leishmaniasis caused by *Leishmania braziliensis* in South America, is difficult to cure by chemotherapy (primarily pentavalent antimonials; Sb(V)). Treatment failure does not correlate well with resistance *in vitro*, and the factors responsible in patients are not well understood. Many isolates of *L. braziliensis* (>25%) contain a double-stranded RNA virus termed LRV1, also reported in *L. guyanensis* where association with increased pathology, metastasis, and parasite replication was found in murine models. Here we probed the relationship of LRV1 to drug treatment success and disease in *L. braziliensis*, in a group of 97 patients from Peru and Bolivia. *In vitro* cultures were established, parasites were typed as *L. braziliensis*, and the presence of LRV1 determined by RT-PCR followed by sequence analysis. LRV1 was associated significantly with increased risk of treatment failure (odds ratio = 3.99; $P = 0.04$). There was no significant association with intrinsic parasite Sb(V) resistance, suggesting that failures arise from LRV1-mediated effects on host metabolism and/or parasite survival. The association of LRV1 with clinical drug treatment failure could serve to guide more effective treatment of tegumentary disease caused by *L. braziliensis.*

Footnote page.

Potential conflicts of interest. All authors have submitted the ICMJE form for disclosure of potential conflicts of interest; no conflicts were disclosed.

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Introduction

Leishmania are widespread protozoan parasites transmitted by phlebotomine sand flies, afflicting more than 12 million people world-wide, with 1.2 million new cases/year [1] . The true incidence is likely far higher, as most infections are persistent and asymptomatic, only emerging as disease following immune compromise [2-3]. Leishmaniasis can be viewed as a 'spectral' disease, with a range of manifestations including tegumentary (cutaneous (CL) or mucosal (ML)) or visceral disease (VL); these manifestations are typically associated with different parasite species [4-5]. Amongst the different species, *L. braziliensis* is considered one of the most important in the New World, because of its prevalence, difficulty to cure, and public health importance. Notably, *L. braziliensis* is the most frequent cause of ML, which typically manifests first as CL and progresses to ML in up to 10% of the cases [6]. The factors responsible for the progression from CL to ML are not well understood, and likely involve both host and parasite factors [5].

As yet there is no effective vaccination against *L. braziliensis*, and treatment depends on diagnosis and chemotherapy. Pentavalent antimonials (Sb(V)), typically sodium stibogluconate/Pentostam or meglumine antimoniate/Glucantime) are presently the primary treatment. However, in Latin America, Sb(V)-treatment is characterised by a variable outcome, with treatment failure rates reaching 39% [7-8]. While in some *Leishmania* species Sb(V) resistance has been linked to intrinsic changes in parasite susceptibility, this does not appear to be the case in *L. braziliensis* in Peru [9-10]. Thus far identified risk factors include the presence of concomitant distant lesions, and immunological responses [11-12]. For example, the persistence of high levels of IL-10 in the lesions is associated with a poor response to treatment and it is well known that the efficacy of antimonials is

strongly influenced by immune responses [5, 10, 13-14]. Other factors contributing to the relative insensitivity of *L. braziliensis* to Sb(V) chemotherapy are likely.

Several species of *Leishmania* show the presence of a persistent monosegmented double-stranded RNA (dsRNA) virus named *Leishmaniavirus* (LRV), a member of the family Totiviridae [15-17]. LRV1 is most often seen in the *Leishmania* (*Viannia*) species *L. braziliensis* and *L. guyanensis*, where the overall occurrence is about 20-30%, with some populations showing more than 50% prevalence ([this work; 18, 19-22]. While the biological relevance of LRV1 had been elusive since its discovery more than 20 years ago, in murine models LRV1 is now known to be associated with increased parasite replication, pathology, and metastasis following infection with either *L. guyanensis* [23] or *L. braziliensis* (in preparation). There LRV1 can act as an immunomodulator, through the interactions of its dsRNA genome with the host Toll-like receptor 3 (TLR3), leading to a hyper-inflammatory response [24]. Similar responses in humans likewise would be expected to result in increased disease severity as well, but currently the clinical impact of LRV1 is uncertain. Two studies reported little association of LRV1 with cutaneous vs. mucocutaneous presentation [20, 25].

The studies above prompted us to consider the potential for a link between LRV1 and treatment success. Here we performed a cross-sectional analysis of collections of *L. braziliensis* isolates from patients in Peru and Bolivia exhibiting various forms of tegumentary leishmaniasis (CL, ML, or both (MCL)). Importantly, a significant association was seen between the presence of LRV1 and therapeutic failure with Sb(V) or Amphotericin B treatments.

Materials and Methods

Ethics statement

Research in this study was subject to ethical review by the European Commission and approved as part of contract negotiation for projects LeishBolPe (an epidemiological study in Bolivia and Peru), and LeishNatDrug-R (a multicenter study on Sb(V) treatment failure): the work conformed to all relevant European regulations. The research was also reviewed and approved by the Ethics Committees of the Universidad Peruana Cayetano Heredia and the Hospital Nacional Cayetano Heredia in Lima, Peru, and of the Universidad Mayor de San Simón in Cochabamba, Bolivia. All human strains of *Leishmania* had been isolated from patients as part of normal diagnosis and treatment with no unnecessary invasive procedures and with written and/or verbal informed consent recorded at the time of clinical examination. Data on human isolates were coded and anonymized.

Patients

Patients were recruited at the Instituto de Medicina Tropical "Alexander von Humboldt" in Lima, Peru, and at the Universidad Mayor de San Simón in Cochabamba, Bolivia as part of two prospective studies: i) the project LeishBolPe (Bolivia and Peru, 1994-1998), an epidemiological study aiming to discriminate factors underlying clinical variability in infection and disease; and ii) the project LeishNatDrug-R (Peru, 2001-2004), a case-control study of incident cases to understand risk factors of treatment failure; here we focused on *L. braziliensis* due to its prevalence and association with a higher risk of treatment failure [11]. From both prior studies, a total of 290 isolates were typed, and all 97 *L. braziliensis* isolates with adequate clinical and epidemiological documentation were considered (Table 1). Of these, 54 had been monitored for treatment outcomes for up to one year, and

included in our analysis (Table 1, Fig. 3). Patients were classified clinically as manifesting CL or ML lesions, or both (MCL). Patients underwent standard supervised treatment with intravenous or intramuscular meglumine antimoniate (Glucantime; Sanofi Aventis) or generic sodium stibogluconate (Viteco, Colombia or Albert-David, India), depending on availability; both drugs are considered equally effective [26]. We used dosages of 20 mg/kg/day for 20 (CL) or 30 (for ML or MCL) days, or conventional Amphotericin B (Fungizone, Bristol-Myers Squibb) at dosages of 0.6 mg/kg/day for 30 to 41 days [9]. Follow-up visits were scheduled at 1, 2, 3, 6, and 12 months after treatment ended. The clinical outcomes were defined as follows: 1) cure as complete re-epithelialization with a characteristic scar and no inflammation at the time point of follow-up assessment (3 to 12 months after treatment, which depended on patients returning for their medical evaluation); primary unresponsive as the absence or incomplete scarring of lesion(s) and/or the persistence of inflammatory signs at 3 months after treatment or the worsening of existing lesion(s) or the appearance of new lesion(s) ≤3 months after treatment; and relapse as the reappearance of an ulcer or nodule and/or local signs of inflammation after initial cure [9]. Cured patients were still observed until 12 months to detect possible relapses. Patients with treatment failure received either a repeat course of antimonials with or without topical imiquimod (Aldara; 3M Pharmaceuticals) or intravenous amphotericin B deoxycholate (Bristol-Myers Squibb) [9, 11]. Some patients had previously been treated for leishmaniasis; these were classified retrospectively as primary unresponsive if the second treatment led to cure, or secondary unresponsive if it did not. For statistical analysis, treatment failure was defined as either unresponsiveness or relapse (Table 1; Supplemental Figs. 1, 2).

Parasite isolates

Leishmania guyanensis LRV1+ strain Lg5313 (WHO code WHI/BR/78/M5313) and a LRV1-deficient line Lg17 [23] were obtained from Nicolas Fasel (U. Lausanne, Switzerland). Ninety seven isolates of *L. braziliensis* were available for analysis (62 from Peru, 35 from Bolivia); the designation, geographical origins, and clinical features of the isolates used in this study are summarized in Table 1, Fig. 1, and Supplemental Figs. 1 and 2. The Peruvian strains constitute an allopatric sample spanning the geographical range of *L. braziliensis*, mainly in the jungle. Conversely, the Bolivian strains comprise a sympatric sample, originating from the "Indigenous Territory and National Park Isiboro Sécure" at Cochabamba (Fig. 1). In this study, parasites were recovered from patients before treatment, cryopreserved, and later revived for culture for RNA and DNA isolation. The isolates studied were typed as *L. braziliensis* by PCR–RFLP targeting *gp63*, *Hsp70*, *cpb* and/or *H2b* genes as described elsewhere [27-28].

RNA purification

Parasites were thawed and grown in Schneider's medium containing fetal bovine serum until reaching late log/early stationary phase. 3×10^8 promastigotes were washed with ice cold phosphate-buffered saline, collected by centrifugation, and resuspended and lysed in 1ml TRIZOL (Invitrogen). Cell lysates were stored at -80°C prior to shipment or processing. Total RNA was isolated according to the manufacturer's instructions (Life Technologies, CA). Residual DNA was removed by treatment with DNase I (Life Technologies, CA) at 37º C for 45 min, and RNA was further purified with the Zymo RCC-25 kit following the manufacturer's instructions (Zymo Research, Irvine, CA). The integrity

of the purified RNA was verified by electrophoresis in 0.8% agarose gels in TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA, pH 8) at 4º C.

LRV1 detection and sequencing

These methods have been reported previously [17, 29-30]. cDNA was prepared from total RNA by priming with random hexamers, and then subjected to 30 cycles of PCR using 'universal LRV' degenerate primers amplifying a 488 nucleotide segment within the LRV1 capsid gene. Controls included buffer only, mock cDNA lacking reverse transcriptase, and both LRV1-positive and negative strains of *L. guyanensis*. RT-PCR products were analyzed on 1.5% native agarose gel in TAE buffer, and LRV1 amplicons were purified and subjected to automated sequencing. Sequence was obtained from both strands, assembled and trimmed to remove low quality bases and primer sequences, edited and aligned using DNASTAR Lasergene software. Molecular phylogenies were constructed on a 299 nucleotide segment using the MEGA 6 analysis software [31]. The final LRV1 dataset has been deposited in GenBank (KP682453-KP682484).

The presence of LRV1 was confirmed independently by the presence of an appropriately sized dsRNA following digestion with single-stranded nucleases [not shown; 30, 32]. Following a recent proposal to the International Committee on the Taxonomy of Viruses, LRVs are referred to as LRV1 or LRV2, followed by a species and then strain designation [33]. Thus, LRV1-Lguy-M4147 is the preferred name for M4147 LRV1-4, and LRV1-Lbr-CUM24 for the LRV occurring within strain MHOM/BO/94/CUM24.

Statistical analysis

The type of lesion was treated as a non-ordered three-level categorical variable. A simple exact logistic regression was used to independently model the total (unadjusted) effect of

the presence of LRV1 and lesion type on the probability of treatment failure, in scenarios of a small sample size. A multiple exact logistic regression was used to evaluate the direct effect on the probability of treatment failure of the presence of LRV1 after adjusting for the type of lesion. Statistical tests were performed under a 5% significance level, using the statistical software Stata 13.

Results

We analyzed a collection of 97 isolates of *L. braziliensis* taken from patients exhibiting CL, ML, or MCL in Peru and Bolivia (Table 1; Supplemental Figs. 1, 2). Axenic promastigote cultures were established *in vitro* following biopsy. Subsequently, patients were treated with Sb(V) (93) or Amphotericin B (4), and for 54 we were able to monitor the treatment outcome for up to 1 year. The patient response was classified as described previously [9], and for statistical analysis further classified as 'cure' or 'fail', the latter including both instances of unresponsiveness and relapses (Table 1; Supplemental Figs. 1, 2). Parasites were confirmed as *L. braziliensis* by molecular typing, and RT-PCR using 'universal' LRV primers was used to detect LRV1 (Fig. 2; Table 1). When present, the levels of individual LRV1 amplicons were similar (Fig. 2), and sequence of each was determined.

Thirty-two isolates clearly evidenced LRV1 (33%), with the proportion significantly higher in Peru (28/62; 45%) than Bolivia (4/35; 11%). LRV1+ parasites were found widely across Peru, with some regions showing higher prevalence than others (Fig. 1). Similar variation amongst localities was reported previously [19-22].

LRV1 is associated with a significant increase in the risk of treatment failure. We next examined the association of LRV1 with treatment outcome for all patients. Extensive

clinical data were available for 54 patients, including treatment history and outcome; all but 4 patients had been treated with Sb(V). The association of LRV1 and treatment outcome is shown in Fig. 3 for the entire dataset or subdivided by country in Supplemental Fig. 3. Overall, 33% (18/54) were classified as 'failures'; importantly, the percentage of failure was less in the LRV- than LRV+ isolates (24% (9/37), vs. 53% (9/17)). Exact logistic regression showed this difference to be significant (P = 0·043), and with a notably high odds ratio (OR) of 3.5, associating the risk of failure with LRV1. This finding was seen within both the Peruvian and Bolivian isolates, although the number of treatment failures in the latter was too few for statistical significance. Excluding the 3 patients treated with Amphotericin B (all LRV1-) caused the overall significance to decrease (OR = 3.12, P = 0·067).

We further assessed the impact of LRV1 after adjusting for lesion type (both variables showing no significant interaction), using multiple exact logistic regression analysis. Again the presence of LRV1 was associated with an increased risk of treatment failure (OR = 3.99, P = 0·05). Interestingly, patients with CL showed a higher risk of treatment failure compared to those showing mucosal involvement (ML+MCL; OR = 18.5, P = 0·009). This was unexpected, as prior studies had not revealed a consistent difference [34-39]. In our studies, ML or MCL patients received a longer course of Sb(V) treatment than CL patients (30 vs. 20 days [9]), perhaps accounting for this outcome. Given the implications for the success of Sb(V) treatments, this warrants further controlled studies in the future.

LRV1 does not confer intrinsic parasite antimony resistance in infected macrophages. We considered the hypothesis that, in some manner, the presence of LRV1 conferred intrinsic

drug resistance to the parasites. In a previous study, 26 of the Peruvian isolates had been examined for *in vitro* resistance to Sb(V) as intracellular amastigotes in macrophage infections [9]. Of the Sb(V)-resistant lines, 10 were LRV1+, while 12 were LRV1-; of the Sb(V)-sensitive lines, 2 were LRV1+, while 2 were LRV1-. Thus, LRV1 was not significantly associated with Sb(V) resistance directly ($P = 0.43$).

LRV1 subtypes are not associated with treatment outcome. We considered the possibility that the LRV1-treatment failure association arose not from the presence of LRV1, but from other parasite genetic factors. LRV1, like most other Totiviridae, are not shed or infectious and are transmitted only during cell division; thus by co-inheritance, isolates that bear closely related LRV1s are closely related at the nuclear DNA level [40-41]. If observed, clustering of treatment failures by LRV1 and presumably nuclear DNA relationship could signify that shared ancestry of other genetic factors was responsible, rather than LRV1. Differences in LRV1 sequence are unlikely to play a role, as it is the viral dsRNA itself (rather than any specific sequence motif) that serves to mediate virulence through interactions with TLR3 [23].

We constructed a dendrogram depicting LRV1 sequence relationships, onto which we displayed drug treatment outcomes where available (Fig. 4). It was clear that treatment failures (black boxes in Fig. 4) did not cluster preferentially by the degree of LRV1 relationship. Instead, failures were interspersed amongst cures in most LRV1 lineages, including two bearing identical LRV1s (PER012, PER010). Where known from microsatellite typing [42], the relationships of LRV1s were consistent with those of the underlying parasite genomes, including the close relationship of PER010 and PER012. While these data cannot rule out a direct contribution of other genetic factors, they suggest

that the LRV1 effect seen here is independent of these, if present. Future studies using high resolution methods to probe the relationships of the isolates studied here may further test and extend these findings.

We examined the LRV1 phylogeny for geographic associations; however, there was no clear cline of LRV1 across Peru or Bolivia. This is perhaps best illustrated by closer examination of two sympatric populations, one occurring in Pilcopata (Amazonian foothills, Cusco) in Peru and the other in Parque Isiboro (Amazonian lowlands), Bolivia. Both populations displayed considerable LRV1 diversity, spanning (or nearly so) the limits of the evolutionary tree (Fig. 5). These data further suggest considerable diversity of *L. braziliensis* parasite populations in these localities.

LRV1 is not preferentially associated with MCL or ML. For CL presentations, 28/67 (42%) were LRV1+, while for MCL and ML presentations the values were 1/13 (8%) and 7/17 (41%) respectively, or 8/30 (27%) for ML/MCL combined. These values did not differ significantly, analyzed separately or after combining ML/MCL (P = 0.29 and 0.57 respectively). These findings are consistent with studies of other *Leishmania* populations [19-20]; there was no significant association between disease status at time of biopsy and the presence of LRV1 in axenic cultured parasites.

Discussion

Here we examined a large panel of isolates of *L. braziliensis*, and probed for associations between the presence of LRV1 and response to treatment or disease manifestations. Our data show a significant association between the presence of LRV1 and treatment failures (Fig. 3). We ruled out the possibility that this arose by 'intrinsic' LRV1-

mediated Sb(V) resistance, as there was no correlation between the presence of LRV1 and parasite Sb(V)-resistance manifested during infections of macrophages *in vitro*. Similarly, we ruled out a significant contribution from other parasite genetic factors, using LRV1 sequence relationships as a surrogate measure of parasite genetic relatedness (due to LRV1-parasite coevolution [41]) to assess whether the treatment failures were clustered preferentially into one or a few lineages, rather than the presence of LRV1 itself. This analysis provided no evidence for preferential genetic clustering of treatment failures, further pointing to the presence of LRV1 itself as key risk factor.

Importantly, a companion paper Bourreau *et al* reports a similar association of LRV1 with pentamidine treatment failures in *L. guyanensis*, in the absence of intrinsic parasite resistance [18]. Thus, current data suggest that LRV1 may act across species and drug classes to thwart efforts to treat leishmaniasis. These remarkable findings prompt us to consider potential mechanisms by which this occurs.

In the *L. guyanensis* murine model, LRV1-bearing parasites induce the expression of a distinctive set of macrophage inflammatory markers, constituting a 'hyper-inflammatory' response, resulting ultimately in a TLR3-dependent increase in parasite numbers and disease severity [23-24]. Correspondingly, many studies have shown a critical role for the host immune system in mediating Sb(V) activity [10, 13-14]. Thus, LRV1-mediated changes in the human host response could potentially serve to dampen the efficacy of Sb(V) action. A second and non-exclusive model suggests the elevated parasite burden associated with LRV1 would act to compromise the efficacy for any given drug treatment regimen, even in the absence of intrinsic parasite resistance or drug-specific host interactions. Indeed, this may be especially likely for most anti-leishmanial compounds, whose efficacy and selective index is far from optimal [43-44]. One key prediction of the

'higher parasite load' model is independence from the specific mode of drug action and/or drug-specific involvement of host metabolism, which differ considerably amongst Sb(V), Amphotericin B, and pentamidine. It also provides a potential framework to view the preferential association of treatment failures in CL (if this finding is confirmed in the future), as parasite numbers are generally much higher in this form of the disease than in chronic forms of ML.

The evidence presented here and the companion work of Bourreau *et al* [18] provide a strong rationale implicating LRV1 in important aspects of human – parasite biology. Current data do not permit a firm determination of the mechanism by which the presence of LRV1 leads to treatment failures, and further studies will be required to unravel this process. Regardless of the mechanism, these findings have important implications to anti-leishmanial therapy, as they suggest that knowledge of the LRV1 status in *L. braziliensis* and *L. guyanensis* could support prognostics and follow-up. Our findings should also guide further research on new options for combination therapy, including targeting LRV1.

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03 – project 95502) for parasite characterization and data analysis; and NIH AID R56 AI099364 for LRV1 analysis.

Figure Legends.

Figure 1. Geographical distribution of LRV1-containing *L. braziliensis* isolates from Peru and Bolivia.

The origins of *L. braziliensis* lines summarized in Table 1 are displayed on a map of Peru and Bolivia, displayed using the software package Quantum GIS version 2.0.1 (http://www.qgis.org/en/site/forusers/download.html) and the latitude and longitude coordinates of each locality. Both LRV+ (red star) and LRV- (blue circle) isolates occur in the same geographic areas, in Peru mostly along the jungle. Most Bolivian *L. braziliensis* isolates (33/35) originate from the "Indigenous Territory and National Park Isiboro Sécure" (Municipality of Villa Tunari) and two isolates (CUM67 and CUM68) originate from the town of Shinahota (Municipality of Tiraque), all located in the Department of Cochabamba.

Figure 2. RT-PCR detection of LRV1 in *L. braziliensis*.

Agarose gel electrophoresis of PCR products obtained using LRV 'universal' primers SMB4647 and SMB4648 with randomly primed cDNA derived from RNA from the species/strains is shown, as described in the methods. M, dsDNA molecular weight marker (1 kb plus; Life Technologies, CA); *L. braziliensis* (lanes 1-11), lane 1, LC2143; lane 2, LC2147; lane 3, LC2176; lane 4, LC2177; lane 5, LC2284, lane 6, LC2289; lane 7, LC2321; lane 8, LC2353; lane 9, LC2367; lane 10, LC2398; and lane 11, LC2318. *L. guyanensis:* lane 12, Lg17 (LRV1 negative); lane 13, Lg5313 (LRV1+).

Figure 3. Treatment failure vs. LRV1 prevalence.

The number of 'cures' (open bars) or 'failures' (closed bars) following chemotherapy is shown for the complete dataset (n = 54). Within each grouping the number of isolates bearing or lacking LRV1 (LRV1+, LRV1- respectively) are shown. Data are taken from Table 1.

Figure 4. LRV1 molecular phylogeny and drug treatment outcomes. The figure shows a molecular tree based upon comparisons of a 299 nucleotide region of LRV1 (methods). When known for a given isolate, the clinical outcome of Sb(V) therapy is shown (no patients treated with Amphotericin B yielded strains bearing LRV1). Cure, open box; fail, dark box (see Table 1 for data and classification). The tree was constructed using the Neighbour-Joining algorithm based on the uncorrected number of nucleotide differences and uniform rate assumptions; the scale shows a branch length of 5 nt differences. Bootstrap values calculated from 5000 replicas are shown at each node.

Figure 5. LRV1 relationships for two sympatric populations of *L. braziliensis* in Peru and Bolivia.

The figure associates the LRV1 sequence relationships depicted in Fig. 4 with the geographical relationships shown in Fig. 1. The fine distribution of LRV1 genotypes is shown in the insets for the Pilcopata district at Paucartambo, Cusco, Peru; the Isiboro Sécure National Park; and Shinahota town at Cochabamba, Bolivia. From this and the data in Fig. 1, it can be seen that both LRV1+ and LRV1- lines occur within both populations (insets), including LRV1s whose sequences differ considerably.

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Table 1. Properties of *L. braziliensis* isolates from Peru and Bolivia including LRV1 typing

International code	Origin (Department, Province)[ϕ]	Lesion type	Treatment outcome[§]	Classification	LRV1 present	Reference
Pentavalent antimonial treatment						
MHOM/PE/03/PER260	Madre de Dios, Tahuamanu	ML	Cured (12m)	Cure	-	[9]
MHOM/PE/02/PER094	Huanuco, Puerto Inca	CL	Cured (12m)	Cure	-	[9]
MHOM/PE/02/PER122	Madre de Dios, Tambopata	CL	Cured (12m)	Cure	-	[9]
MHOM/PE/03/PER163	Huanuco, Leoncio Prado	CL	Cured (12m)	Cure	-	[9]
MHOM/PE/03/PER157	Madre de Dios, Tambopata	CL	Cured (6m)	Cure	-	[9]
MHOM/PE/03/PER182	Ayacucho, La Mar	CL	Cured (6m)	Cure	-	[9]
MHOM/PE/03/PER164	Ucayali, Coronel Portillo	CL	Cured (3m)	Cure	-	[9]
MHOM/PE/03/ PER215	Ucayali, Coronel Portillo	ML	Cured (6m)	Cure	-	[9]
MHOM/PE/84/LC03 cl6	Madre de Dios, Tambopata	CL	NA		-	[45]
MHOM/PE/91/LC1409	Huanuco, Huanuco	CL	NA		-	[46]
MHOM/PE/91/LC1412	Huanuco, Huanuco	CL	NA		-	[46]
MHOM/PE/91/LC1565	Cusco, Paucartambo	CL	NA		-	[47]
MHOM/PE/91/LC1580	Cusco, Paucartambo	MCL	NA		-	[27]
MHOM/PE/91/LC2123	Cusco, Paucartambo	CL	NA		-	[28]
MHOM/PE/91/LC2125	Cusco, Paucartambo	MCL	NA		-	[27]
MHOM/PE/91/LC2141	Cusco, Paucartambo	MCL	NA		-	[27]
MHOM/PE/93/LC2143	Cusco, Paucartambo	CL	NA		-	[28]
MHOM/PE/91/LC2147	Cusco, Paucartambo	CL	NA		-	[47]
MHOM/PE/91/LC2177	Cusco, Paucartambo	CL	NA		-	[28]
MHOM/PE/91/LC2289	Cusco, Paucartambo	CL	NA		-	[27]
MHOM/PE/91/LC2320	Cusco, Paucartambo	MCL	NA		-	[28]
MHOM/PE/94/LC2353	Cusco, Paucartambo	CL	NA		-	[27]
MHOM/PE/94/LC2355	Cusco, Paucartambo	CL	NA		-	[28]
MHOM/PE/94/LC2367	Cusco, Paucartambo	CL	NA		-	[28]
MHOM/PE/94/LC2368	Cusco, Paucartambo	MCL	NA		-	[28]
MHOM/PE/91/LC2398	Cusco, Paucartambo	CL	NA		-	[27]
MHOM/PE/00/LH699	Madre de Dios, Manu	CL	NA		-	[47]
MHOM/PE/00/LH800	Madre de Dios, Tambopata	CL	NA		-	[47]
MHOM/PE/01/PER005	Loreto, Ucayali	CL	Primary unresponsiveness (p)	Fail	-	[9]
MHOM/PE/01/PER006	Junin, Satipo	CL	Primary unresponsiveness (p)	Fail	-	[9]
MHOM/PE/02/PER015	Ucayali, Coronel Portillo	CL	Primary unresponsiveness (p)	Fail	-	[9]

MHOM/PE/02/PER086¶	Pasco, Oxapampa	CL	Relapse (re) + secondary unresponsiveness	Fail	-	[9]
MHOM/PE/02/PER104‡	Madre de Dios, Tambopata	CL	Secondary unresponsiveness (re + p)	Fail	-	[9]
MHOM/PE/03/PER201	Loreto, Requena	ML	Cured (12m)	Cure	+	[9]
MHOM/PE/02/PER016	Huanuco, Puerto Inca	CL	Cured (12m)	Cure	+	[9]
MHOM/PE/02/PER096	Madre de Dios, Manu	CL	Cured (12m)	Cure	+	[42]
MHOM/PE/03/PER207	Madre de Dios, Tambopata	CL	Cured (12m)	Cure	+	[42]
MHOM/PE/03/PER231	Junin, Satipo	ML	Cured (12m)	Cure	+	[9]
MHOM/PE/02/PER069‡	Madre de Dios, Manu	ML	Incomplete treatment, lost		+	[9]
MHOM/PE/02/PER010	Cajamarca, Jaen	CL	Cured (3m)	Cure	+	[9]
MHOM/PE/91/LC2041	Cusco, Paucartambo	CL	NA		+	[47]
MHOM/PE/91/LC1568	Cusco, Paucartambo	CL	NA		+	[48]
MHOM/PE/91/LC1569	Cusco, Paucartambo	CL	NA		+	[47]
MHOM/PE/91/LC1578	Cusco, Paucartambo	CL	NA		+	[47]
MHOM/PE/91/LC1586	Cusco, Paucartambo	CL	NA		+	[27]
MHOM/PE/91/LC2043	Cusco, Paucartambo	MCL	NA		+	[27]
MHOM/PE/91/LC2176	Cusco, Paucartambo	CL	NA		+	[28]
MHOM/PE/94/LC2284	Cusco, Paucartambo	CL	NA		+	[28]
MHOM/PE/91/LC2318	Cusco, Paucartambo	CL	NA		+	[48]
MHOM/PE/91/LC2319	Cusco, Paucartambo	CL	NA		+	[48]
MHOM/PE/91/LC2321	Cusco, Paucartambo	CL	NA		+	[48]
MHOM/PE/90/LH825	Ucayali, Padre Abad	CL	NA		+	[47]
MHOM/PE/01/PER002	Madre de Dios, Tambopata	CL	Primary unresponsiveness (p)	Fail	+	[9]
MHOM/PE/01/PER012	Cusco, Calca	CL	Primary unresponsiveness (p)	Fail	+	[9]
MHOM/PE/01/PER014‡	Junin, Satipo	CL	Primary unresponsiveness (re)	Fail	+	[9]
MHOM/PE/03/PER130‡	Cusco, Echarate	CL	Primary unresponsiveness (re)	Fail	+	[9]
MHOM/PE/03/PER186‡	Junin, Satipo	CL	Primary unresponsiveness (re)	Fail	+	[9]
MHOM/PE/02/PER065	Cusco, La Convencion	CL	Relapse (p)	Fail	+	This work
MHOM/PE/03/PER212‡	Madre de Dios, Tambopata	CL	Secondary unresponsiveness (p)	Fail	+	This work
MHOM/PE/02/PER067‡	Cusco, La Convencion	CL	Secondary unresponsiveness (re + p)	Fail	+	[9]
MHOM/BO/94/CUM153	Parque Isiboro, Limoncitos	CL	Cure/Scar	Cure	-	[28]
MHOM/BO/94/CUM25	Parque Isiboro, Moleto	CL	Cure/Scar	Cure	-	[48]
MHOM/BO/94/CUM27	Parque Isiboro, Moleto	MCL	Cure/Scar	Cure	-	[48]
MHOM/BO/94/CUM29	Parque Isiboro, Moleto	MCL	Cure/Scar	Cure	-	[28]
MHOM/BO/94/CUM31	Parque Isiboro, Moleto	CL	Cure/Scar	Cure	-	[48]
MHOM/BO/94/CUM34	Parque Isiboro, Moleto	CL	Cure/Scar	Cure	-	[48]

MHOM/BO/94/CUM43	Parque Isiboro, Moleto	MCL	Cure/Scar	Cure	-	[28]
MHOM/BO/94/CUM45	Parque Isiboro, NA	MCL	Cure/Scar	Cure	-	[28]
MHOM/BO/94/CUM49	Parque Isiboro, Isinuta	MCL	Cure/Scar	Cure	-	[28]
MHOM/BO/94/CUM50	Parque Isiboro, Primavera	MCL	Cure/Scar	Cure	-	[48]
MHOM/BO/94/CUM55	Parque Isiboro, Isinuta	MCL	Cure/Scar	Cure	-	[48]
MHOM/BO/94/CUM57	Parque Isiboro, NA	CL	Cure/Scar	Cure	-	[49]
MHOM/BO/94/CUM59	Parque Isiboro, Moleto	CL	Cure/Scar	Cure	-	[48]
MHOM/BO/94/CUM67	Shinahota	MCL	Cure/Scar	Cure	-	[48]
MHOM/BO/94/CUM96	Parque Isiboro, Moleto	CL	Cure/Scar	Cure	-	[48]
MHOM/BO/94/CUM152	Parque Isiboro, Limoncitos	MCL	Cure/Scar	Cure	-	[28]
MHOM/BO/96/CUM180	Parque Isiboro, Primavera	MCL	Cure/Scar	Cure	-	[50]
MHOM/BO/94/CUM42¶	Parque Isiboro, Primavera	CL	Secondary unresponsiveness	Fail	-	[28]
MHOM/BO/2002/CUM623	Parque Isiboro, Llallagua	CL	Primary unresponsiveness	Fail	-	[49]
MHOM/BO/2002/CUM700	Parque Isiboro, Llallagua	CL	Primary unresponsiveness	Fail	-	[49]
MHOM/BO/2002/CUM704	Parque Isiboro, Moleto	CL	Primary unresponsiveness	Fail	-	This work
MHOM/BO/84/CEN002	NA	CL	NA		-	[45]
MHOM/BO/85/CEN007	NA	CL	NA		-	[45]
MHOM/BO/94/CUM97	Parque Isiboro, Primavera	CL	NA		-	[28]
MHOM/BO/94/CUM138	Parque Isiboro, Isinuta	MCL	NA		-	[27]
MHOM/BO/96/CUM181	Parque Isiboro, NA	MCL	NA		-	This work
MHOM/BO/--/CUM363	Parque Isiboro, NA	CL	NA		-	This work
MHOM/BO/--/CUM505	NA	MCL	NA		-	[49]
MHOM/BO/94/CUM52	Parque Isiboro, Isinuta	MCL	NA		-	[48]
MHOM/BO/94/CUM65	Parque Isiboro, Moleto	MCL	Cure/Scar	Cure	+	[48]
MHOM/BO/94/CUM68	Shinahota	MCL	Cure/Scar	Cure	+	[28]
MHOM/BO/94/CUM24	Parque Isiboro, Isinuta	MCL	Primary unresponsiveness	Fail	+	[48]
MHOM/BO/94/CUM41	Parque Isiboro, Moleto	CL	NA		+	[28]
Amphotericin B treatment				Cure		This work
MHOM/BO/2002/CUM637	Parque Isiboro, Primavera	MCL	Cure/Scar		-	
MHOM/BO/2002/CUM639	Parque Isiboro, Primavera	CL	Cure/Scar	Cure	-	This work
MHOM/PE/02/PER011	Huanuco, Huanuco	MCL	NA		+	[9]
MHOM/PE/03/PER136	Ucayali, Coronel Portillo	ML	Cured (12m)	Cure	-	[9]

CL = cutaneous leishmaniasis; MCL = mucocutaneous leishmaniasis; ML = mucosal leishmaniasis; NA = not available. ϕRegion, town in Bolivia. §p = prospective (within the LeishNatDrug-R study); re = retrospective (previous leishmaniasis episode). ¶Patients with a history of previous treatment, but the drug used was not known. ‡Patients with a history of previous treatment with antimonials. LRV1 = *Leishmania* RNA virus 1. Unless otherwise indicated, 'Cure' signifies that the patient was monitored for 12 months. In some cases patients could only be monitored for 3 or 6 months. For analysis, these were classified as 'cured' since previous studies showed that the cure rate assessed at 3 months was very nearly that seen at 12 months [11].