MAJOR ARTICLE

Parasite Susceptibility to Amphotericin B in Failures of Treatment for Visceral Leishmaniasis in Patients Coinfected with HIV Type 1 and *Leishmania infantum*

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Background. Visceral leishmaniasis (VL) is an opportunistic infection that can occur among patients infected with human immunodeficieny virus type 1 (HIV-1) in areas where both infections are endemic. Highly active antiretroviral therapy has decreased the incidence of VL in southern Europe among HIV-1–infected patients, but VL is still observed among patients with low CD4 cell counts, and most coinfected patients receiving highly active antiretroviral therapy experienced relapse, despite initial treatment with liposomal amphotericin B.

Methods. Through long-term monitoring of VL in 10 patients with HIV-1 infection and/or AIDS, we compared parasite strains derived from primary and secondary episodes of VL. All the patients have received many courses of amphotericin B treatment and/or prophylaxis.

Results. Through molecular techniques, we have shown that secondary episodes of VL can be attributable to relapse (7 of 10 episodes) or reinfection (3 of 10). We developed an assay to measure amphotericin B susceptibility and found no evidence of decreased susceptibility among strains isolated from patients, some of whom were infected with the same isolate for up to 10 years.

Conclusions. This apparent absence of resistance, as determined by in vitro susceptibility testing, has important consequences and suggests that amphotericin B will remain a useful drug of choice against VL, even after repetitive treatments or prophylactic use.

Leishmaniasis is a protozoan disease distributed in many countries and gives rise to diverse clinical manifestations. The visceral form is potentially deadly and requires treatment [1–3]. Leishmaniasis is endemic in the Mediterranean region, and the agent of visceral leishmaniasis (VL) is *Leishmania infantum*. In southwestern Europe, most cases of VL reported occur in patients with HIV infection and/or AIDS (reviewed by Cruz et al. [4]). In these patients, infection shows the classic signs of VL, but unusual anatomical locations and atypical clinical signs are also frequent [5]. The introduction of HAART has considerably reduced the prevalence of all opportunistic infections among patients with HIV-1 infection and/or AIDS, including VL in southwestern Europe [6–8].

The first-line treatment for all forms of leishmaniasis is pentavalent antimony, but toxicity is frequent, and parasite resistance leading to treatment failures has been described in several parts of the world [9–11]. Over the past decade, amphotericin B (AmB) has been increasingly used to treat VL. The primary target of AmB in *Leishmania* species is membrane sterols, and *Leishmania* species, similar to fungal pathogens, have ergosterol as their main membrane sterol [12]. AmB use is nonetheless limited by its toxicity and adverse reactions experienced by recipients, but lipid formula-

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tions of AmB have improved the safety profile of the drug, and liposomal AmB has the highest therapeutic index of current anti-*Leishmania* drugs [13]. The classic anti-*Leishmania* drugs, antimonials and AmB, have also shown their usefulness in treating VL in patients with AIDS, but relapses are often observed [14, 15]. In southern Europe, liposomal AmB is the mainstay treatment given to patients coinfected with HIV and *Leishmania* species, either after primary diagnosis of VL or for secondary prophylaxis [13, 16].

Although HAART has reduced the incidence of VL, it does not prevent relapses [4], and a high proportion of coinfected patients receiving HAART experienced relapse within 24 months after initial effective treatment for VL [17, 18]. Most of these secondary episodes of VL involved patients with low CD4 cell counts (<200 cells/mm^3) [19, 20], which suggests that the immune status of the host is key for secondary episodes of VL. These secondary episodes of VL may be attributable either to a relapse from parasites living in sanctuaries or to reinfection. Distinguishing between relapses and reinfections may be important for secondary prophylaxis. Indeed, repetitive treatment of a relapse may select for parasites that are resistant to drugs. In one study using restriction fragment–length polymorphism PCR, it was shown that the majority of VL cases were attributable to relapses, although 7.5% of secondary episodes of VL were attributed to reinfection [21].

Patients with low CD4 cell counts have a high rate of relapse (or reinfection) and poor response to treatment, which imply systematic secondary prophylaxis and the possibility of selection for resistant parasites. The link between in vitro susceptibility to antimonials and treatment outcomes of leishmaniasis has been established in some parts of the world [9, 10], although, in other parts, this is less clear [22]. Few studies have dealt with AmB, but, in one study, a decrease in AmB susceptibility of *L. infantum* was observed in a patient who experienced 6 relapses [23]. Through long-term monitoring of VL in patients with AIDS, we gathered parasite isolates derived either from the initial onset of VL or from secondary episodes of VL. We made use of molecular techniques for discriminating between reinfection and relapse and performed in vitro susceptibility assays to look at the possible role of AmB resistance in contributing to the reappearance of VL.

Table 1. Characterization of *Leishmania infantum* strains isolated from HIV-1–infected patients with multiple episodes of visceral leishmaniasis.

Patient, strain	Date of isolation	PFGE profile	IC_{50}, mean μg/mL ± SD	
			Promastigotes	Amastigotes[a]
Patient 1				
3049	May 1995	A	0.39 ± 0.01	0.46 ± 0.05
3110	Nov 1995	A	0.48 ± 0.01	…
4054	Sep 2000	A	0.40 ± 0.03	…
5159	Dec 2005	A	0.42 ± 0.01	0.34 ± 0.14
Patient 2				
3476	Sep 1997	B	0.39 ± 0.01	0.56 ± 0.12
4147	Feb 2001	B	0.40 ± 0.03	0.69 ± 0.08
Patient 3				
3320	Jan 1997	C	0.39 ± 0.02	…
3842	Jul 1999	C	0.40 ± 0.02	…
Patient 4				
3135	Dec 1995	D	0.62 ± 0.06	…
3256	Jun 1996	D	0.29 ± 0.02	…
Patient 5				
4038	Jun 2000	E	0.40 ± 0.03	…
4340	Jan 2002	E	0.60 ± 0.02	…
Patient 6				
3323	Dec 1996	F	…	…
3416	Apr 1997	G	…	…
3831	Jun 1999	H	0.42 ± 0.02	…
4237	Aug 2001	I	0.40 ± 0.01	…
Patient 7[b]				
4155	Mar 2001	J	0.35 ± 0.05	…
4866	Sep 2004	K	0.40 ± 0.01	…
Patient 8[b]				
4023	Jun 2000	L	0.39 ± 0.01	…
4253	Sep 2001	M	0.60 ± 0.05	…
4473	Aug 2002	N	0.44 ± 0.05	…
4885	Oct 2004	O	0.60 ± 0.01	…
Patient 9[b]				
2990	Jan 1997	P	0.50 ± 0.05	…
3239	Jul 1999	P′	0.40 ± 0.02	…
Patient 10[b]				
3800	May 1999	Q	0.26 ± 0.04	…
3851	Aug 1999	Q	0.20 ± 0.02	…

[a] For intracellular parasites expressing luciferase in U937 cell lines.
[b] Patient is an injection drug user.

MATERIALS AND METHODS

Patients. Ten adult patients coinfected with HIV and *L. infantum* (1 woman and 9 men) were followed up in the Centre Hospitalier Universitaire of Montpellier and Nimes, France, for a mean duration of 35.6 months (range, 3–137 months). Four of those 10 patients were injection drug users (IDUs) (table 1). The mean age of patients at the first diagnosis was 34 years (range, 30–51 years). During the follow-up period, all received HAART. However, despite HAART, the CD4$^+$ cell count was <200 cells/mm^3 for all the patients throughout the study period. The first episode of VL was treated with the AmB lipid formulation (1–3 mg/kg/day for 5–10 days), and patients received a specific secondary prophylaxis, also consisting of liposomal AmB, given to most patients once a month or once every 15–21 days (dose, 1–3 mg/kg). Diagnosis of relapse or reinfection was based on the presence of clinical signs and parasite isolation

by blood or bone marrow culture on Novy-MacNeal-Nicolle medium. Relapses and reinfections were treated similarly as the first episode of VL.

Parasites. Throughout the course of the study, 26 *L. infantum* isolates were collected (table 1). Ten isolates were obtained during the first onset of VL, when patients had not yet received AmB. Sixteen strains were isolated at the time of relapse or reinfection. Strain typing was made by isoenzyme electrophoresis [24] and pteridine reductase 1 (*PTR1*) sequencing, which allows species discrimination [10]. All isolates corresponded to *L. infantum*. Parasites were stored in the international cryobank of *Leishmania* (LeishCryoBank WDCM879 in Montpellier).

The promastigotes of *L. infantum* field isolates were maintained in SDM-79 medium supplemented with 10% heat-inactivated fetal calf serum, 5 μg/mL hemin, and biopterin at 25°C. PFGE karyotyping was done as described elsewhere [10, 25]. The *L. infantum* reference strain (MHOM/MA/67/ITMAP-263) was selected for resistance to AmB by stepwise exposure to the drug as described elsewhere [26]. This in vitro–resistant isolate served as a standard for establishing susceptibility assays. AmB was purchased from Sigma chemical company, and Ambisome was purchased from Astellas Pharma Canada.

In vitro assay for AmB susceptibility. The inhibitory concentration of 50% (IC_{50}) of AmB for *L. infantum* promastigotes was determined by measuring absorbance at 600 nm by use of an automated microplate reader (Organon Teknica microwell) system as described elsewhere [27]. *Leishmania* promastigotes were transfected with pGEMαNEOαLUC1.2, which encodes the firefly luciferase gene, and were used to infect the human histiocytic lymphoma cell line U937, as described and validated previously with the human cell line THP-1 [28]. We found that the U937 line gave more reproducible results in IC_{50} determinations when dealing with AmB (M.P., unpublished observations). In brief, the human cell line U937 was propagated in suspension in complete RPMI 1640 medium (Sigma) containing 10% heat-inactivated fetal calf serum, 2 mmol/L glutamine, penicillin, and streptomycin at 37°C in the presence of 5% carbon dioxide. U937 cells were differentiated by incubation for 3 days in medium containing phorbol myristate acetate (20 ng/mL) and were infected with *L. infantum* promastigotes at a ratio of 2:1 for 3 h. Luciferase activity of the recombinant parasites was measured after 4 days of drug exposure, essentially as described elsewhere [29]. Values were expressed as relative light units.

Statistical analysis. The paired Wilcoxon test was used to compare AmB susceptibilities before and after treatment.

RESULTS

We succeeded in isolating *Leishmania* species from the first VL episode for 10 HIV-1–infected patients at the Centre Hospitalier Universitaire Montpellier and Nimes (table 1). These patients had 31 clinical secondary episodes of VL (see Materials and Methods), and 16 isolates of these secondary episodes were studied. The 26 isolates (10 primary and 16 secondary) listed in table 1 were identified as *L. infantum* by using the classic isoenzyme analysis [30] and by sequencing the *PTR1* gene as described elsewhere [10].

L. infantum is endemic in the South of France, with a number of zymodemes, MON-1 being the most prevalent [30]. However, within this specific zymodeme, there is considerable heterogeneity [31], and the use of PFGE has allowed additional characterization of these strains. None of the 10 isolates from the first episodes of VL had the same PFGE profile (figure 1 and table 1), further illustrating the genetic heterogeneity of *L. infantum,* even when from the same geographical region. This extensive parasite heterogeneity and the availability of isolates from secondary episodes of VL for each of the 10 patients has allowed the testing of whether patients experienced relapse or were reinfected with a novel parasite isolate. Patient 1 was followed up for 137 months and experienced 8 relapses, and a subset of the available isolates, separated by 5-year intervals, was characterized by PFGE. Under this criterion (along with *PTR1* sequencing), the different isolates were found to be identical (figure 1 and table 1). This strongly suggested that the secondary episode of VL in patient 1 was the result of the reactivation of latent parasites escaping AmB treatment. Among the non-IDUs, this scenario of relapse for explaining secondary episodes of VL was also observed for patients 2, 3, 4, and 5 (duration of follow-up, 6–42 months) (figure 1 and table 1). However, the situation was different for patient 6, a non-IDU. Indeed, patient 6 was followed up for 56 months and experienced 4 relapses; each of the 4 isolates studied from this patient had distinct PFGE profiles (table 1), which suggested reinfection by different isolates between AmB treatments. This reinfection scenario was also observed in patients 7 and 8, both IDUs, because the PFGE profiles of all isolates were different (figure 1 and table 1). The PFGE profile of the 2 isolates from patient 9 differed by only 1 band, suggesting that the secondary episode of VL in this patient was attributable to a relapse. Patient 10 had a short follow-up period and died 4 months after what appeared to be a relapse (table 1).

The majority of secondary episodes of VL (7 of 10) in the patients with HIV-1 infection and/or AIDS were attributable to a relapsing infection, as determined by identical PFGE profiles of strains isolated from the same patient at different time intervals. Patient 1 experienced relapsed 8 times and received, during a 10-year period, >76 months of AmB treatment and prophylaxis, but, remarkably, the same strain persisted throughout the years. It is possible that the susceptibility to AmB in this strain may have decreased. In vitro susceptibility testing is not yet standard for leishmaniasis, but it has been used to

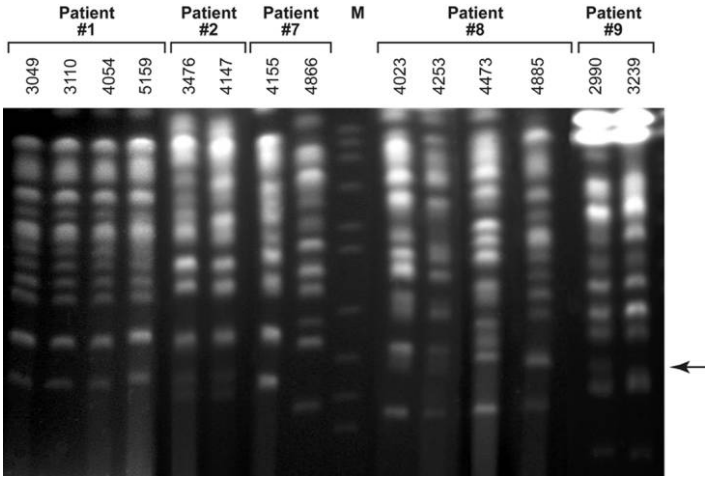

Figure 1. *Leishmania infantum* characterization by PFGE. *Leishmania* cells were embedded and lysed in agarose, and the chromosomes of various *L. infantum* isolates were electrophoresed and were stained with ethidium bromide. Representative isolates (see also table 1) from different patients were included. The arrow identifies the 1-band difference between 2 isolates derived from patient 9. *Lane M,* yeast chromosomes molecular-weight marker.

analyze pentavalent antimonial susceptibility [9, 10, 22]. To set up an assay for AmB and to measure susceptibility in the clinical isolates, we first established susceptibility values with an *L. infantum* reference isolate, in which we have selected for AmB resistance in vitro. As shown in figure 2*A*, the IC_{50} of the *L. infantum* reference strain for AmB was determined in its promastigote stage to be 0.4 μg/mL, which is in line with published data [28]. The in vitro–resistant promastigote mutant was ~10-fold more resistant to AmB, compared with the wild-type cell (figure 2*A*). An in vitro susceptibility assay was also set up with intracellular parasites. The AmB-susceptible and AmB-resistant *L. infantum* isolates were transfected with the firefly luciferase gene, and these recombinant parasites were used to infect the U937 macrophage cell lines. In this intracellular assay, we also observed that the IC_{50} values were higher for the in vitro–resistant line, compared with the wild-type line (figure 2*B*). This has validated our assay, which was used to look for AmB susceptibility in the isolates derived from the patients with HIV-1 infection and/or AIDS.

We first looked at strains 3049 and 5159, derived from patient 1, and strains 3476 and 4147, derived from patient 2. All these strains were transfected with pGEMαNEOαLUC1.2, encoding the firefly luciferase gene. We tested for AmB susceptibility in both the promastigote and intracellular forms of the parasite. Strains isolated during primary episodes of VL (3049 and 3476) and isolates derived from secondary episodes (5159 and 4147) had similar IC_{50} values, both as promastigotes and amastigotes (figure 3*A* and 3*B*). The IC_{50} values were similar for promastigotes and intracellular amastigotes, and, because the assay for promastigotes is simpler, the AmB IC_{50} values of the remaining isolates were measured in the promastigote stage. No significant differences were observed between strains isolated during primary episodes and the strains isolated during secondary episodes of VL ($P = .90$) (table 1).

DISCUSSION

Long-term monitoring of VL in patients with HIV-1 infection and/or AIDS has allowed testing of whether the secondary episode of VL is attributable to relapse or reinfection and also testing of whether long-term AmB treatment resulted in resistant parasites. This study has revealed that both reinfection and relapses can be the cause of a secondary episode of VL in patients with HIV-1 infection and/or AIDS, with relapses being more frequent. It is salient to point out that, in this study, all patients, despite HAART, had $CD4^+$ cell counts <200 cells/mm^3, a strong predictive factor for recurrence [19]. For 2 of the 4 IDUs with HIV-1 infection and/or AIDS (patients 7 and 8) and for 1 of the 6 non-IDUs (patient 6), the secondary episode of VL was associated with an isolate that differed from the isolate from the initial episode, suggesting that these 3 patients had reinfection instead of a relapse. A number of reasons for this reinfection can be invoked. The patients may have been infected with a heterogeneous population of parasites, and different clones were isolated during parasite isolation. In southwestern Europe, syringes have often replaced the sandfly as *Leishmania* species transmitters, and a study looking at syringes of IDUs in Spain found that they are indeed contaminated with *Leishmania* species [32]. One possible route for reinfection may thus also include syringe-mediated infections. For example, in patient 8, a different isolate was present almost every year (table 1). For patient 6, (a self-declared non-IDU), a different isolate was also found each time parasites were collected (table 1). This patient lived in regions of higher endemicity. We also noticed

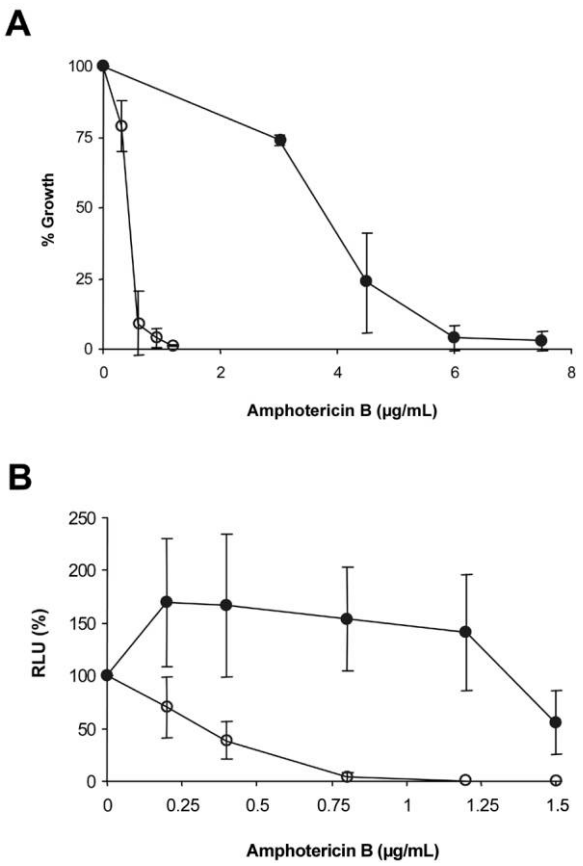

Figure 2. Amphotericin B susceptibility of *Leishmania infantum. A,* Growth of the promastigote parasites, monitored by measuring the optical density at 600 nm after 72 h of incubation at 25°C while varying the concentration of amphotericin B. The mean of 3 experiments is shown. *B,* Luciferase-expressing amastigotes of *Leishmania* species grown in U937 cells. Intracellular parasites were incubated for 4 days with the indicated concentrations of amphotericin B. The mean of 4 duplicate experiments is shown. *Unblackened circles, L. infantum* (MHOM/MA/67/ITMAP-263). *Blackened circles, L. infantum* AmB 1000.1, which is the reference strain selected for amphotericin B resistance in vitro. RLU, relative light units.

that only 4 months separated the first VL episode from the reinfection. This was during the winter season, when there are no sandfly-related transmissions. If contaminated syringes were not involved, alternatives to reinfection could be blood transfusion [33] or sexual transmission [34, 35], 2 rare but possible routes of *Leishmania* infection. The reinfection in 3 of 10 patients may have been the result of hig-risk behavior, which can facilitate parasite transmission. However, it is surprising that an existing parasite population is being replaced so easily by another population. This suggests that the latent parasites from the previous infections are less fit than the newly incoming parasites. The rate of reinfection reported here is higher than what was observed in an independent study [21]. Interestingly, when Morales et al. [21] used solely isoenzyme analysis, they had no evidence for reinfection, but, when they used a more discriminatory PCR method, they reported a rate of reinfection of 7.5%. If we had also used only isoenzyme analysis for strain characterization, we would have also concluded that all patients experienced relapse. Because PFGE analysis has proven to be the most discriminatory method to distinguish between *Leishmania* species isolates [36], it has allowed the detection of more cases of reinfection.

Relapse was observed for 5 of the 6 non-IDUs and for 2 of the 4 IDUs with HIV-1 infection and/or AIDS (table 1). Patient 1 has been infected with the same strain for >10 years, despite >76 months of AmB treatment, further supporting the notion that an immunocompromised patient can be chronically infected despite extensive chemotherapy. Parasite isolates derived from primary and secondary episodes of VL in patient 9 differed in PFGE profile by only 1 band (figure 1), suggesting that it was indeed the same strain and, thus, that this patient experienced relapse. Indeed, guidelines for PFGE analysis for bacterial epidemiology suggest that a 1-band difference is an indication that strains are closely related [37], and we reported previously small changes in karyotype after the culturing of parasites [38]. It is thus possible that this rearrangement occurred during in vivo growth. Indeed, in vivo evolution has been reported for some microbial pathogens [39]. It must be relatively rare for *Leishmania* species, however, because most isolates derived from the same patient were identical (figure 1 and table 1). Relapses thus appear to be more frequent than reinfections. Remarkably, the IC_{50} to AmB of all primary isolates varied very little between strains, with karyotypes having IC_{50} values ranging from 0.26 to 0.6 μg/mL. Similar IC_{50} values were observed between promastigotes and amastigotes, further supporting the idea that the stage of the parasite does not seem to influence AmB susceptibility [40, 41]. One of the most important findings of this study is that parasites that have been in contact with AmB for many episodes are not less susceptible to the drug than are primary isolates (table 1). This low level of clinical resistance to AmB among *Leishmania* species is also mirrored with fungal pathogens. Indeed, although resistance to azoles is becoming a problem, resistance to AmB is also infrequent in fungal pathogens in HIV-1–infected patients [42, 43]. The relatively nonspecific mode of action of AmB at the level of the membrane may be responsible for this lack of resistance to AmB.

The failure of therapy for VL is therefore probably attributable to factors other than resistance. In a mouse experimental model of VL treated with AmB, T cells, CD40L, IFN-γ, and TNF were shown to be required for prevention of relapse after AmB treatment [44]. Consistent with these animal studies, $CD4^+$ cell counts are critical markers of VL relapse despite appropriate secondary AmB prophylaxis [19, 20]. This absence

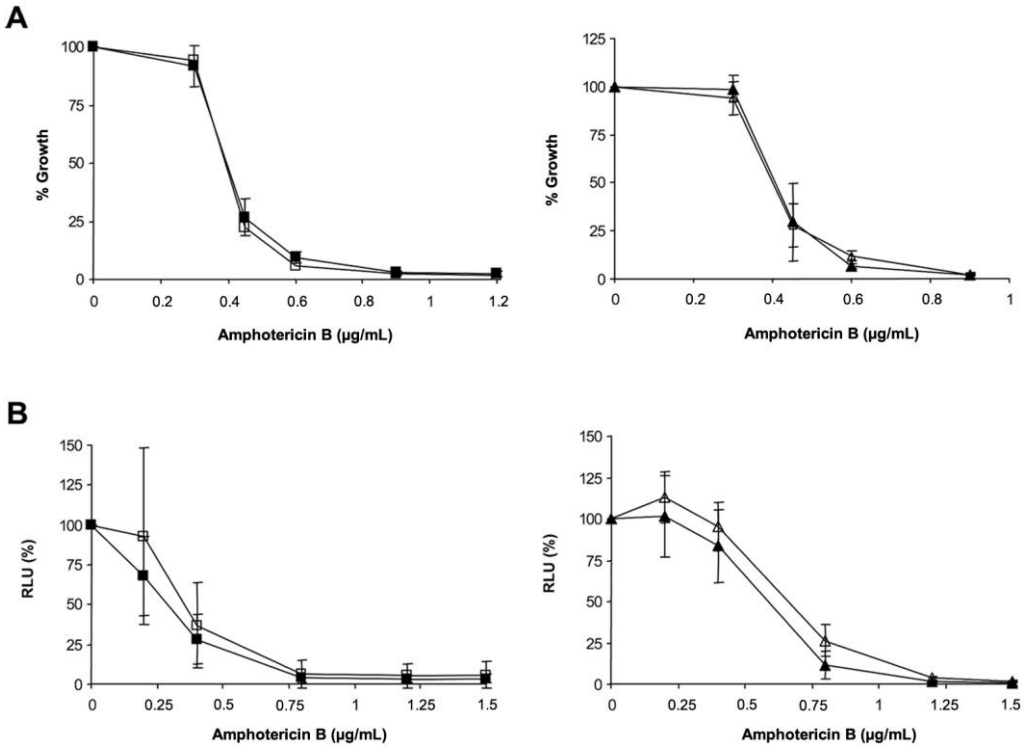

Figure 3. Amphotericin B susceptibility of *Leishmania infantum* strains isolated from HIV-1–infected patients. *A,* Growth of the promastigote parasites, monitored by measuring the optical density at 600 nm after 72 h of incubation at 25°C while varying the concentration of amphotericin B. The mean of 3 experiments is shown. *B,* Luciferase-expressing amastigotes of *Leishmania* species grown in U937 cells. Intracellular parasites were incubated for 4 days with the indicated concentrations of amphotericin B. The mean of 4 duplicate experiments is shown. *L. infantum* strains 3049 *(filled squares)* and 5159 *(open squares)* from patient 1 were isolated 10 years apart. *L. infantum* strains 3476 *(filled triangles)* and 4147 *(open triangles)* from patient 2 were isolated 4 years apart. RLU, relative light units.

of emergence of parasite resistance to AmB has important consequences, and this study suggests that AmB will remain the first line drug treatment even after repetitive cure or prophylaxis use.

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