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THE SENSITIVITY OF CLINICAL ISOLATES OF *LEISHMANIA* FROM PERU AND NEPAL TO MILTEFOSINE

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Abstract. Clinical isolates of *Leishmania*, from visceral leishmaniasis (VL) cases in Nepal and from cutaneous leishmaniasis (CL) cases in Peru, were cultured using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to type species and strain. Promastigotes from 38 isolates, within eight passages from isolation, were used to infect mouse peritoneal macrophage cultures *in vitro*, and the amastigote sensitivity to miltefosine was determined. The concentration required to kill 50% of intracellular amastigotes from Nepalese VL isolates, all typed as *Leishmania (L.) donovani* ($N = 24$) from both Sb^v responders and nonresponders, ranged from 8.7 to 0.04 $\mu\text{g}/\text{mL}$. In contrast, the concentration required to kill 50% intracellular amastigotes from isolates from Peru, typed as *L. (V.) braziliensis* ($N = 8$), was > 30 to 8.4 $\mu\text{g}/\text{mL}$, *L. (V.) guyanensis* ($N = 2$) > 30 to 1.9 $\mu\text{g}/\text{mL}$, *L. (L.) mexicana* ($N = 1$) > 30 $\mu\text{g}/\text{mL}$, and *L. (V.) lainsoni* ($N = 4$) was 3.4 to 1.9 $\mu\text{g}/\text{mL}$. This demonstrates a notable difference in the intrinsic sensitivity of *Leishmania* species to miltefosine *in vitro*. If this model can be correlated to therapeutic outcome, it may have implications for the interpretation of clinical trials.

INTRODUCTION

The current chemotherapy for leishmaniasis is limited. Pentavalent antimonials have been the recommended drugs for the treatment of both visceral (VL) and cutaneous leishmaniasis (CL) for more than 50 years, but long courses, toxicity, and resistance in India limit their use. New drugs have become available in recent years for the treatment of both VL, including highly efficacious but expensive lipid amphotericin B formulations, of which AmBisome is the only one widely available. Oral miltefosine has recently been licensed for use in India for the treatment of VL. Topical formulations of paromomycin and oral miltefosine have been used to treat CL.¹

Miltefosine is an alkylphosphocholine, initially developed as an anticancer agent, that also shows selective activity against *Leishmania*.² Miltefosine has undergone successful clinical trials for anthroponotic VL in Bihar state, India, with 94% cure rate, including antimony-resistant cases,³ and is currently in Phase IV trials in India and Nepal. This oral drug has demonstrated some activity in the treatment of zoonotic CL in South America^{4,5} where it is currently in Phase III clinical trials.⁶ There are more than 17 species of *Leishmania* that cause leishmaniasis in human populations; these species have been shown to vary in sensitivity to a range of anti-leishmanial drugs⁷ including miltefosine.⁸ The current study is the first to evaluate the *in vitro* anti-leishmanial activity of miltefosine against recently isolated, typed *Leishmania* spp. from patients in the Old and New Worlds.

MATERIALS AND METHODS

Ethical clearance was obtained from the ethical committees of the Health Research Council, Kathmandu, Nepal, and Cayetano Heredia University, Lima, Peru. Patients were recruited to the study from November 2002 until the beginning of 2004. Clinical cases of VL in Nepal were recruited at the B

P Koirala Institute of Health Sciences, Dharan, Nepal, from a catchment area covering the eastern Terai. Individuals less than 2 years old were excluded from the study. Suspected VL cases, fever for 14 days or longer with splenomegaly, were confirmed by bone marrow aspiration and visual identification of parasites. All patients received 20 mg Sb^v kg⁻¹ day⁻¹ intramuscularly for 30 days (sodium antimony gluconate; Albert David Ltd, Calcutta, India). Unresponsive patients subsequently received amphotericin B (amphotericin B deoxycholate) treatment.

Cutaneous leishmaniasis patients were recruited at the Instituto Alexander von Humbolt, Lima, Peru, with geographical location noted in the patient data (Table 1). Pregnant women were excluded from the study. Patients presenting with an ulcerated or nodular lesion were considered. Infection by *Leishmania* was confirmed by direct examination of punch biopsy material with confirmation by parasite culture or PCR. Patients received 20 mg Sb^v kg⁻¹ day⁻¹ intravenously for 20 days, except for PER069 who received treatment of 30 days. Unresponsive patients received a repeat course of antimonials. If this was again unsuccessful, patients received intravenous amphotericin B (amphotericin B deoxycholate).

Patient material was isolated directly onto 3N⁹ blood slopes with a saline/antibiotic overlay and sent to ITG, Antwerp. Parasites were cryopreserved in aliquots and typed within eight passages from isolation. Frozen stocks were sent to LSHTM, London, where the parasites were passaged initially onto 3N slopes with M199 + 20% HIFCS overlay, then into M199 + 20% HIFCS alone. It was necessary to introduce the use of M199¹⁰ to obtain a clean, bulk culture of promastigotes that would be sufficient for further evaluation. There is some evidence that the type of medium can affect the infectivity of the parasite,¹¹ however, in this study all isolates were exposed to exactly the same growth conditions, and the work was carried out as close to the isolation as possible. Standard operating procedures were established between partners, which would minimize the effect of this variable between the strains. The parasites were tested for their *in vitro* sensitivity to miltefosine within eight passages from isolation.

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TABLE 1
Sensitivity of Peruvian cutaneous leishmaniasis isolates to miltefosine

Species	ID	ED ₅₀ ± SEM µg miltefosine/mL (ED ₉₀ ± SEM)	Patient details (Age; M/F)	Region (Department)	Clinical response to antimonial treatment¶
<i>L. (V.) braziliensis</i>	PER010	> 30 (> 30)			
<i>L. (V.) braziliensis</i>	PER012*	> 30 (> 30)	32; M	Cusco	Nonresponder
<i>L. (V.) braziliensis</i>	PER014	> 30 (> 30)	14; M	Junin	Nonresponder
<i>L. (V.) braziliensis</i>	PER067	28.3 (> 30)	23; M	Cusco	Nonresponder
<i>L. (V.) braziliensis</i>	PER122	21.3 ± 2.5 (19.7 ± 2.4)	35; M	Madre de Dios	Responder
<i>L. (V.) braziliensis</i>	PER016	> 30 (> 30)	22; M	Huanaco	Initial cure
<i>L. (V.) braziliensis</i>	PER069†	22.2 (> 30)	47; M	Madre de Dios	Nonresponder/relapse
<i>L. (V.) braziliensis</i>	PER010	> 30 (> 30)	22; F	Cajamarca	Initial cure
<i>L. (V.) guyanensis</i>	PER054	> 30 (> 30)	29; F	Junin	Unknown‡
<i>L. (V.) guyanensis</i>	PER132	8.4 ± 2.22 (14.42 ± 4.3)	65; M	Junin	Definite cure
<i>L. (V.) lainsoni</i>	PER092	3.37 ± 0.1 (4.02 ± 0.25)	33; M	Junin	Initial cure
<i>L. (V.) lainsoni</i>	PER105	1.89 ± 0.17 (3.51 ± 0.22)	13; M	Madre de Dios	Responder
<i>L. (V.) lainsoni</i>	PER 127	3.1 ± 0.94 (> 30)	9; M	San Martin	Responder
<i>L. (V.) lainsoni</i>	PER131	2.4 ± 0.22 (4.42 ± 0.43)	20; M	Junin	Initial cure
	PER068	> 30 (> 30)			
<i>L. (L.) mexicana</i>	PER068	> 30	10; M	Ayacucho	Unknown‡
<i>L. (V.) braziliensis</i>	M2903§	–	–		–

* Isolate taken after completion of antimonial treatment.

† Mucosal leishmaniasis.

‡ Patients lost to follow-up.

§ M2903 is WHO reference strain *L. (V.) braziliensis* MHOM/BR/75/M2903.

¶ Initial cure—resolution of clinical signs and symptoms at 1 month. Currently in 6-month follow-up period. Definite cure—complete resolution of clinical signs and symptoms with no evidence of relapse at 6, 12, and 24 months after treatment.

Late stage promastigotes were used to infect primary isolated mouse peritoneal macrophages at a ratio of 7 promastigotes to 1 macrophage in Labtek 16-well tissue culture well slides (VWR Ltd, Poole, UK), in quadruplicate. Infected cultures were maintained in RPMI 1640 medium (Sigma, Gillingham, UK) with 10% heat-inactivated fetal calf serum (Harlan SeraLab, Loughborough, UK) at 37°C (VL strains) or 34°C (CL strains) in a 5% CO₂/95% air mixture. Twenty-four hours after infection, one slide was methanol-fixed and Giemsa-stained to determine the initial level of infection. Cultures were then exposed to miltefosine (hexadecylphosphocholine, HPC; A G Scientific, San Diego, CA) over a dose range of 30, 10, 3, and 1 µg/mL, in quadruplicate at each concentration. Higher concentrations were not tested due to toxicity to macrophage host cells: at concentrations above 30 µg/mL, macrophages infected are destroyed and the slide cannot be evaluated. After 5 days, all slides were methanol-fixed and Giemsa-stained. The percent of infected macrophages in each well was determined by microscopy. From a comparison of counts from treated with untreated cultures, the percent inhibition was calculated by sigmoidal regression analysis (MS *x*/fit) and ED₅₀ (ED₉₀) values determined. *Leishmania (V.) braziliensis* MHOM/BR/75/M2903 was the reference strain used for Peruvian isolates. *L. (L.) donovani* MHOM/ET/67/HU3 was used as the reference strains for Nepalese isolates. Both strains are known as WHO reference strains, as typed by isoenzyme analysis, and are sensitive to the standard antimonials, sodium stibogluconate and meglumine antimoniate.

Leishmania species typing. In Antwerp, *Leishmania* species typing was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of cysteine proteinase b (cpb) and gp63 genes for Nepalese strains and by multi-locus PCR-RFLP for Peruvian strains. Briefly, DNA was extracted from cultivated promastigotes with the QIAmp DNA mini Kit (Qiagen, Hilden, Germany), amplified and digested as reported elsewhere.^{12–14} Restriction patterns were resolved by capillary electrophore-

sis (2100 Bioanalyzer system, Agilent Technologies, Karlsruhe, Germany) in a micro-chip device (LabChip 1500, Caliper Technologies, Mountain View, CA) and compared with those of reference strains. Standard strains were also re-typed and confirmed to be *L. (L.) donovani* and *L. (V.) braziliensis*.

RESULTS

The range of activity of miltefosine against South American isolates of cutaneous leishmaniasis is shown in Table 1. *L. (V.) lainsoni* (PER105) was the most sensitive strain tested with an ED₅₀ value of 1.89 µg/mL. *L. (V.) braziliensis* and *L. (V.) guyanensis* isolates were insensitive to miltefosine over the range tested (30–0.3 µg/mL). The only isolates, for example PER127, which showed sensitivity to miltefosine were typed as *L. (V.) lainsoni*, with ED₅₀ values ranging from 3.37 to 1.89 µg/mL.

The activity of miltefosine against VL isolates from Nepal is shown in Table 2 with ED₅₀ values ranging from less than 0.04 µg/mL to 8 µg/mL. Results were similar in repeated experiments. Although the strains were identified as two *L. (L.) donovani* types (I and II, based on restriction pattern differences),^{12,14,15} there was no significant difference in sensitivity to miltefosine between them. The differences seen in the RFLP pattern are useful when tracing the genetic relationships among the strains. *L. (L.) donovani* HU3 is an Ethiopian strain of VL maintained in passage animals. An Indian reference strain would perhaps have been a more appropriate comparator but was not available to us at the time of this study.

DISCUSSION

This study supports previous work comparing species sensitivity to miltefosine, which found significant differences between New World and Old World CL and demonstrated sig-

TABLE 2
Sensitivity of Nepalese visceral leishmaniasis isolates to miltefosine

Species	ID	ED ₅₀ ± SEM µg miltefosine/mL (ED ₉₀ ± SEM)	Patient details (Age; M/F)	Region (District)§	Clinical response to antimonial treatment¶
<i>L. donovani</i> (I)					
<i>L. donovani</i> (I)	BPK025/0	0.5 ± 0.2 (0.9 ± 0.07)	32; M	Sunsari	Definite cure
<i>L. donovani</i> (II)	BPK026/0	1.9 ± 0.6 (6.9 ± 0.8)	48; M	Bhojpur	Definite cure
<i>L. donovani</i> (I)	BPK035/0	1.32 ± 0.09 (2.5 ± 0.21)	9; M	Saptari	Nonresponder
<i>L. donovani</i> (I)	BPK043/0	1.0 ± 0.04 (2.2 ± 0.1)	10; M	Sunsari	Definite cure
<i>L. donovani</i> (II)	BPK077/0†	5.6 ± 0.7 (13.5 ± 1.5)	40; M	Saptari	Nonresponder
<i>L. (L.) donovani</i> (I)	BPK080/0	1.2 ± 0.1 (3.3 ± 0.2)	36; F	Sunsari	Relapse
<i>L. (L.) donovani</i> (I)	BPK085/0	5.6 ± 0.6 (10.0 ± 2.2)	32; M	Saptari	Definite cure
<i>L. (L.) donovani</i> (I)	BPK087/0	5.6 ± 0.6 (10.1 ± 2.7)	40; M	Morang	Definite cure
<i>L. (L.) donovani</i> (I)	BPK090/0	4.9 ± 0.2 (7.4 ± 0.5)	46; M	Sunsari	Definite cure
<i>L. (L.) donovani</i> (I)	BPK091/0	1.9 ± 0.06 (6.9 ± 0.8)	40; M	Sunsari	Definite cure
<i>L. (L.) donovani</i> (I)	BPK164/1†	2.2 ± 0.1 (8.2 ± 1.3)	40; M	Dhanusa	Nonresponder
<i>L. (L.) donovani</i> (I)	BPK177/0†	8.1 ± 0.06 (0.9 ± 0.2)	40; M	Dhanusa	Relapse
<i>L. (L.) donovani</i> (I)	BPK178/0	5.7 ± 0.7 (12.6 ± 0.6)	45; M	Sunsari	Initial cure
<i>L. (L.) donovani</i> (I)	BPK190/0	0.9 ± 0.2 (7.3 ± 4.0)	10; M	Morang	Nonresponder
<i>L. (L.) donovani</i> (I)	BPK191/0	2.0 ± 0.1 (5.8 ± 0.8)	16; F	Morang	Initial cure
<i>L. (L.) donovani</i> (I)	BPK192/0	2.0 ± 0.2 (4.1 ± 0.5)	7; F	Morang	Initial cure
<i>L. (L.) donovani</i> (I)	BPK206/0	3.9 ± 1.6 (10.2 ± 3.2)	12; M	Morang	Initial cure
<i>L. (L.) donovani</i> (I)	BPK208/0‡	0.04 ± 0.02 (1.7 ± 0.9)	20; F	Morang	Initial cure
<i>L. (L.) donovani</i> (I)	BPK212/0	3.2 ± 0.3 (6.3 ± 1.1)	50; M	Sunsari	Initial cure
<i>L. (L.) donovani</i> (I)	BPK274/0	0.85 ± 0.14 (4.0 ± 0.5)	18; F	Sunsari	Initial cure
<i>L. (L.) donovani</i> (I)	BPK275/0	1.03 ± 0.04 (2.6 ± 0.2)	12; F	Morang	Nonresponder
<i>L. (L.) donovani</i> (I)	BPK276/0	0.2 ± 0.1 (2.4 ± 0.7)	10; F	Sunsari	Initial cure
<i>L. (L.) donovani</i> (I)	BKP278/0	2.2 ± 0.2 (8.8 ± 1.3)	7; F	Morang	Initial cure
<i>L. (L.) donovani</i> (I)	BPK279/0	0.09 ± 0.73 (1.18 ± 0.2)	10; M	Morang	Initial cure
<i>L. (L.) donovani</i>	HU3*	8.7 ± 0.6 (25.7 ± 2.6)	—	—	—

* HU3 is WHO reference strain *L. donovani* MHOM/ET/67/HU3.

† Isolates taken after completion of antimonial treatment.

‡ Pregnant patient treated with amphotericin B.

§ All districts are on the southeastern border of Nepal.

¶ Initial cure—resolution of clinical symptoms 6 months after treatment, negative bone marrow aspirate. Currently in follow-up period. Definite cure—complete resolution of clinical signs and symptoms with no evidence of relapse 12 months after treatment, negative bone marrow aspirate.

nificantly different sensitivities between New World species.⁸ This report, one of the few that includes both molecular typing and sensitivity studies of a large number of recent isolates from patients, reflecting results seen in a recent clinical trial of miltefosine against cutaneous leishmaniasis.⁵ That study concluded that miltefosine, at 2.5 mg kg⁻¹ day⁻¹, had comparable efficacy to antimonials when treating presumed *L. (V.) panamensis* in Colombia, less so against presumed *L. (V.) braziliensis* in Guatemala, but needed further evaluation against more endemic species. Earlier studies have described differences in the sensitivity of species and strains of *Leishmania* to antimonials, azoles, allopurinol, and paromomycin (see Ref. 7). Six laboratory-maintained isolates of *Leishmania* have also shown variation in sensitivity to miltefosine. In a 3-day amastigote-macrophage assay, ED₅₀ values ranged from 2.6 to 37.7 µM (1–15.4 µg/mL), *L. (L.) donovani* and *L. (L.) aethiopicum* being the most sensitive species and *L. (L.) major* the least sensitive.⁸ In that study, the ED₅₀ values of *L. (L.) mexicana* and *L. (V.) panamensis* were reported as 6.8–10.1 (2.8–4.1 µg/mL) and 10.6 µM (4.3 µg/mL), respectively. The results presented in this paper on 38 clinical isolates also indicate that *L. (L.) donovani* is intrinsically sensitive to miltefosine. In contrast, most of the isolates typed to the *L. (V.) braziliensis* complex were insensitive with the exception of those identified as *L. (V.) lainsoni*.

As with *in vitro* sensitivity to antimonials and other anti-leishmanial drugs, there appears to be intrinsic variation in species sensitivity to miltefosine. If this is reflected *in vivo* this has important implications for the evaluation of clinical trials of the drug in Latin America. A dosing regimen of miltefosine

at 2.5 mg kg⁻¹ day⁻¹ (150 mg of drug) for 28 days was required to have a significant effect against Colombian *L. (V.) panamensis* but was not as effective against Guatemalan *L. (V.) braziliensis*.⁵ It should be noted that the species were identified by historical data in these studies. The evidence suggests that miltefosine may be a useful drug for the treatment of South American CL, however, species identification should perhaps be a priority before treatment to give the best chance of efficacy and to reduce the possibility of either encouraging resistance to develop, a potential problem when considering anthroponotic transmission, or by re-enforcing intrinsic drug insensitivity, as may be the case in zoonotic foci. Resistance mechanisms to miltefosine been identified in laboratory-induced *Leishmania*.^{16,17} It would be interesting to see if these were present in unresponsive “wild-type” parasites.

The utility of the *in vitro* test when assessing isolate drug sensitivity could be improved by testing parasites pre- and post-treatment, but, to date, the number of samples where this is possible is not significant. Finally, the relevance of *in vitro* data to clinical outcome must also be considered as the two parameters may not necessarily correlate—confounding factors such as host immunity are absent from these tests.¹⁸ The data serve to emphasize the extremely complex clinical picture of leishmaniasis.

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