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Susceptibility to Miltefosine in Brazilian Clinical Isolates of *Leishmania (Viannia) braziliensis*

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Abstract. *Leishmania (Viannia) braziliensis* is the main causative species of tegumentary leishmaniasis in Brazil. In this study, we evaluated the susceptibility of 16 clinical isolates of *L. (V.) braziliensis* from different regions of Brazil to miltefosine in vitro. Half-maximal inhibitory concentrations of miltefosine varied from 22.9 to 144.2 μM against promastigotes and from 0.3 to 4.2 μM against intracellular amastigotes. No significant differences were found between isolates of different geographical origins. A clear correlation between the EC_{50} against promastigotes and amastigotes within each isolate was found. These findings contribute to the evaluation of miltefosine's potential and limitations for the treatment of tegumentary leishmaniasis in Brazil.

Tegumentary leishmaniasis is a disease of importance in Brazil, where it is mainly caused by *Leishmania (Viannia) braziliensis.* The efficacy of the first-line drug, meglumine antimoniate, for the treatment of cutaneous leishmaniasis in areas of *L. (V.) braziliensis* predominance in Brazil can be as low as 53%.[1] New therapeutic alternatives are highly desirable.

Miltefosine (MF) (hexadecylphosphatidylcholine) was approved for the treatment of visceral leishmaniasis in India in 2002, where pentavalent antimony was already considered as ineffective due to widespread parasite resistance.[2] This oral drug has also been approved for the treatment of tegumentary leishmaniasis in Colombia, after the demonstration of equivalent efficacy to antimony, and in other countries in South America.[3] However, the response is heterogeneous in areas of high prevalence of *L. (V.) braziliensis:* for example, a clinical trial showed 83% efficacy for MF in cutaneous leishmaniasis in Bolivia, whereas a 53% cure rate was observed in Guatemala.[3,4] In Brazil, 70% success rates were observed in two MF clinical trials of cutaneous leishmaniasis due to *L. (V.) braziliensis* and *Leishmania (V.) guyanensis*.[1,5]

The aim of this work was to characterize the MF susceptibility of *L. (V.) braziliensis* clinical isolates from Brazilian patients with tegumentary leishmaniasis from two geographically distinct regions.

Eight clinical isolates were obtained from lesion biopsies of patients with tegumentary leishmaniasis attending the Anuar Auad Tropical Diseases Hospital, Goiânia, Goiás, Brazil (*Leishbank*),[6] and eight isolates were obtained through needle aspiration of skin lesions from patients attending the health post of Corte de Pedra, Bahia, Brazil. After the isolation, cultures were frozen and recovered to perform this study. This study was approved by the Ethical Committee of the Hospital das Clínicas of the Goiás Federal University and by the Ethical Committee for Human Research of the Bahia Federal University (CEP/MCO/UFBA-Par/Res 034/2007). Consent was obtained from all the subjects enrolled in the study.

The clinical isolates and two *L. (V.) braziliensis* reference strains (MHOM/BR/75/M2903 and MHOM/BR/94/H3227) were grown in 199 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat inactivated fetal calf serum, 2% sterile male human urine, and 0.005% hemin.

The isolates were typed by polymerase chain reaction of internal transcribed spacer of ribosomal DNA and *hsp*70 gene followed by restriction analysis using *Hae* III, as described.[7,8] The M2903 reference strain and 16 clinical isolates produced the expected profile for *L. (V.) braziliensis* (data not shown).

The activity of MF against promastigotes was evaluated by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay as previously described.[9] Approximately 2×10^6 log-phase parasites were incubated in the presence of 25–200 μM MF diluted from a 10 mM stock solution in water. After 24 hours, cell viability was determined by incubation with MTT. Results were expressed as the mean percentage reduction of parasite numbers compared with untreated control wells. Half-maximal and 90% effective concentrations (EC_{50} and EC_{90}) were determined by sigmoidal regression curves using Graph Pad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA) and the activity index was obtained by the ratio between the clinical isolate's EC_{50} and the reference strain M2903.

The EC_{50} of MF against promastigotes of 16 isolates and two reference strains ranged from 22.9 ± 3.7 to 144.2 ± 16.1 μM (Figure 1 and Table 1) with a median of 47.8 μM. The EC_{50} for the reference strains M2903 and H3227 were 53.5 ± 6.6 and 40.7 ± 8.5 μM, respectively. The EC_{50} for the most and the least susceptible isolates (henceforth called polar isolates) varied 6.3-fold. The differences between the EC_{50} for polar isolates and the M2903 reference strain were statistically significant ($P < 0.0001$ analysis of variance (ANOVA) and Tukey's multiple comparison test). On the other hand, no significant differences were detected between isolates of different geographic origins (Figure 1A).

Based on the EC_{50} values against promastigotes, we selected the three most susceptible isolates, the two least susceptible and three isolates with intermediate EC_{50} to evaluate the in vitro susceptibility of intracellular amastigotes (Table 1).

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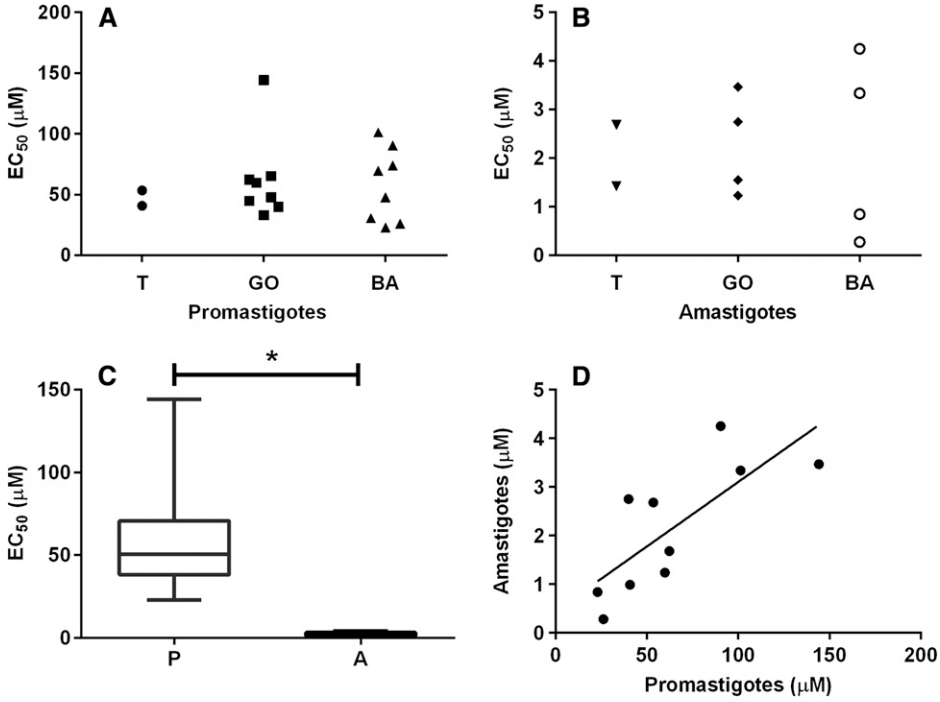

FIGURE 1. Susceptibility of promastigotes and intracellular amastigotes of *Leishmania (V.) braziliensis* isolates to miltefosine. (**A**) EC_{50} determined against promastigotes. (**B**) EC_{50} determined against intracellular amastigotes. "T" indicates the type strains M2903 and H3227, "GO" isolates from Goiás, and "BA" isolates from Bahia. (**C**) Comparison between the EC_{50} against promastigotes and amastigotes. The box indicates the 25th–75th percentiles. The line in the middle of the box indicates the median; * $P < 0.0001$ (Mann–Whitney test). (**D**) Correlation between the EC_{50} determined against promastigotes and amastigotes for each clinical isolate. Spearman coefficient $r = 0.793$; $P = 0.008$.

The sensitivity of intracellular amastigotes to MF was evaluated as reported previously.[9] Bone marrow derived macrophages (BMDM) were plated on round glass coverslips in 24-well plates (3×10^5 cells per well). Infections were performed with *L. (V.) braziliensis* stationary-phase promastigotes (30 parasites per macrophage) for 3 hours at 33°C. Noninternalized parasites were removed by washing with warmed phosphate-buffered saline (PBS), followed by the addition of medium containing increasing MF concentrations, varying from 0.25 to a maximum of 40 μM, since 50% cytotoxicity determined against BMDM was calculated as 46.5 ± 3.9 μM. After 72 hours, the cells were washed with PBS, fixed in methanol, and stained with the panoptical Instant Prov kit (Newprov, Pinhais, Paraná, Brazil). The EC_{50} was determined based on the infection index (number of infected macrophages multiplied by the number of amastigotes per infected macrophage) in sigmoidal regression curves as described earlier.

The infection rates varied between 33% and 86% (Table 1). Compared with promastigotes, intracellular amastigotes were more susceptible to MF ($P < 0.0001$ ANOVA) (Figure 1B and C). The EC_{50} against intracellular amastigotes was also heterogeneous between the isolates varying between 0.3 ± 0.1 and 4.2 ± 0.2 μM (Table 1). The EC_{50} for the reference strain M2903 was 2.7 ± 0.2 μM and the median of EC_{50} values was 2.1 μM for these isolates. The EC_{50} for the less susceptible isolate was approximately 15-fold greater than the EC_{50} against amastigotes of the more susceptible isolate.

The data available for each isolate as well as EC_{50} and EC_{90} values determined for promastigotes and intracellular amastigotes are summarized in Table 1. Fourteen patients presented localized cutaneous lesions, one had simultaneous cutaneous and mucosal lesions and one was a mucosal leishmaniasis patient. Fifteen patients were treated with meglumine antimoniate. Of these, five failed to cure after the first course of treatment and one did not return for follow up. One patient was lost to follow up before treatment. The therapeutic failure after antimonial did not correlate with susceptibility to MF.

A clear correlation between the susceptibility of promastigotes and intracellular amastigotes was observed ($r = 0.793$; $P = 0.008$ Spearman's correlation test) (Figure 1D). Similar findings were also observed in *Leishmania donovani* isolates,[10] and indicate that in vitro susceptibility of promastigotes may be considered a surrogate of susceptibility of intracellular amastigotes. Therefore, in vitro assays using promastigotes are useful to evaluate the susceptibility of clinical isolates to MF.

The characterization of MF susceptibility of eight Peruvian *L. braziliensis* isolates found EC_{50} for intracellular amastigotes in the range of 52 to greater than 73 μm,[11] therefore markedly higher than EC_{50} values determined in this work for Brazilian isolates, emphasizing the existence of considerable diversity in susceptibility to MF within this species.

Since the isolation of parasites used in this study occurred before treatment, there was no previous exposition to MF or any other antileishmanial drug, indicating that this differential susceptibility is an intrinsic characteristic of these isolates.

The correlation between MF treatment failure or success and in vitro susceptibility to the drug is still unresolved, with some evidence pointing to selection of less tolerant parasites with drug exposure but without the accompanying relationship to cure rates.[12–16] One of the limitations of the

TABLE 1
Susceptibility of *Leishmanis (Viannia) braziliensis* clinical isolates to MF

Isolate	Origin‡	Clinical form	Age	Treatment§	Clinical cure¶	Promastigotes*			Infection rate (%)**	Amastigotes†		
						EC_{50} (μm)	EC_{90} (μm)	AI‖		EC_{50} (μm)	EC_{90} (μm)	AI‖
MHOM/BR/1975/2903	Ceará	–	–	–	–	53.5 ± 6.6	70.21 ± 7.7	–	77 ± 7	2.7 ± 0.2	5.1 ± 1.1	–
MHOM/BR/1994/H3227	Pará	–	–	–	–	40.7 ± 8.5	69.5 ± 5.5	0.76	50 ± 6	1.0 ± 0.0††	8.7 ± 1.9	0.37
MHOM/BR/2006/GDL	Goiás	CL	60	Gluc	Yes	45.0 ± 7.8	57.1 ± 5.1	0.84				
MHOM/BR/2006/BES	Goiás	CL	41	Gluc	No	62.2 ± 13.5	92.9 ± 13.3	1.16	40 ± 6	1.7 ± 0.5	4.8 ± 1.3	0.62
MHOM/BR/2006/EFSF	Goiás	CL	44	Gluc	Yes	144.2 ± 16.1	277.0 ± 16.3	2.70	54 ± 8	3.5 ± 0.6	11.9 ± 1.1	1.29
MHOM/BR/2006/UAF	Goiás	CL	29	Gluc	Yes	47.8 ± 6.6	58.3 ± 6.9	0.89				
MHOM/BR/2005/WSS	Goiás	CL	22	Gluc	NR	39.9 ± 4.2	75.9 ± 3.0	0.75	71 ± 6	2.7 ± 0.4	6.9 ± 0.3	1.02
MHOM/BR/2006/HPV	Goiás	CL	46	Gluc	Yes	65.2 ± 3.7	78.4 ± 2.1	1.22				
MHOM/BR/2006/PPS	Goiás	MCL	69	NR	NR	59.8 ± 4.7	73.6 ± 4.8	1.12	58 ± 6	1.2 ± 0.4	8.2 ± 1.6	0.46
MHOM/BR/2006/TBM	Goiás	CL	15	Gluc	Yes	33.1 ± 4.7	56.9 ± 13.2	0.62				
MHOM/BR/2006/LTCP 16907	Bahia	CL	27	Gluc	Yes	101.2 ± 6.0	133.6 ± 12.0	1.89	33 ± 1	3.3 ± 0.4	11.5 ± 0. 4	1.24
MHOM/BR/2010/LTCP 20221	Bahia	CL	2	Gluc	No	69.8 ± 9.0	102.9 ± 14.6	1.30				
MHOM/BR/2009/LTCP 19512	Bahia	CL	58	Gluc	No	26.1 ± 1.6	41.5 ± 3.0	0.49	86 ± 8	0.3 ± 0.1	1.8 ± 0.6	0.10
MHOM/BR/2003/LTCP 15344	Bahia	MCL	23	Gluc	No	47.8 ± 7.9	67.4 ± 3.9	0.89				
MHOM/BR/2009/LTCP 19446	Bahia	CL	28	Gluc	Yes	90.4 ± 5.2	140.3 ± 16.4	1.69	70 ± 3	4.2 ± 0.2	8.4 ± 2.1	1.58
MHOM/BR/2006/LTCP 16596	Bahia	CL	16	Gluc	No	74.1 ± 4.0	106.4 ± 16.9	1.39				
MHOM/BR/2005/LTCP 16012	Bahia	CL	21	Gluc	Yes	22.9 ± 3.7	30.9 ± 6.4	0.42	45 ± 5	0.8 ± 0.1	2.0 ± 0.2	0.31
MHOM/BR/2010/LTCP 20190	Bahia	CL	30	Gluc	Yes	30.5 ± 2.3	61.8 ± 6.3	0.57				

CL = cutaneous leishmaniasis; MCL = mucocutaneous leishmaniasis; MF = miltefosine; SD = standard deviation.
*Inhibitory concentrations against promastigotes, EC_{50} ± SD and EC_{90} ± SD. Experiments were performed three times in triplicate.
†Inhibitory concentrations against intracellular amastigotes, EC_{50} ± SD and EC_{90} ± SD. Experiments were performed three times in duplicate.
‡State in Brazil where the infection was most likely acquired.
§NR = did not return for treatment; Gluc = Glucantime 20 mg/kg for 20 days.
¶Clinical cure at 180 days posttreatment; NR = patient did not return for follow up.
‖AI = activity index (ratio between the isolate's EC_{50} and M2903 reference strain's EC_{50}).
**Percentage of infected macrophages after 72h ± SD (%) (N = 6).
††EC_{50} values for intracellular amastigotes were reported previously.[18]

present study was to lack the evaluation of a relationship between in vitro susceptibility with in vivo response to MF. However, these isolates were not from patients who used MF.

Considering that MF's efficacy against cutaneous leishmaniasis due to *Leishmania (Viannia)* species varies from 53% to 91%,[3,4,17,18] and in Brazil, MF was effective against *L. (V.) braziliensis* in 75% of patients,[1] it would be interesting to investigate whether clinical isolates from unresponsive patients presented low susceptibility to MF in vitro.

MF is not yet approved for the treatment of tegumentary leishmaniasis in Brazil and this study may contribute to the evaluation of MF's treatment potential in the country.

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