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Assessment of drug resistance related genes as candidate markers for treatment outcome prediction of cutaneous leishmaniasis in Brazil

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ABSTRACT

The great public health problem posed by leishmaniasis has substantially worsened in recent years by the emergence of clinical failure. In Brazil, the poor prognosis observed for patients infected by *Leishmania braziliensis* (Lb) or *L. guyanensis* (Lg) may be related to parasite drug resistance. In the present study, 19 Lb and 29 Lg isolates were obtained from infected patients with different treatment outcomes. Translated amino acid sequence polymorphisms from four described antimony resistance related genes (*AQP1*, *hsp70*, *MRPA* and *TRYR*) were tested as candidate markers for antimonial treatment failure prediction. Possibly due to the low intraspecific variability observed in Lg samples, none of the prediction models had good prognosis values. Most strikingly, one mutation (T579A) found in hsp70 of Lb samples could predict 75% of the antimonial treatment failure clinical cases. Moreover, a multiple logistic regression model showed that the change from adenine to guanine at position 1735 of the *hsp70* gene, which is responsible for the T579A mutation, significantly increased the chance of Lb clinical isolates to be associated with treatment failure (OR = 7.29; CI 95% = [1.17, 45.25]; $p = 0.0331$). The use of molecular markers to predict treatment outcome presents practical and economic advantages as it allows the development of rapid assays to monitor the emergence of drug resistant parasites that can be clinically applied to aid the prognosis of cutaneous leishmaniasis in Brazil.

1. Introduction

Leishmania spp. is an intracellular protozoan parasite that infects humans and causes a wide spectrum of diseases known as leishmaniasis. In the Americas, leishmaniasis presents distinct clinical manifestations caused by several species, and there are reports of more than 60,000 cases of cutaneous leishmaniasis (CL) per year (Alvar et al., 2012). Control of the disease relies primarily on chemotherapy, in which the first-line choice of treatment against all forms of leishmaniasis involves drugs containing pentavalent antimonials [Sb(V)], such as sodium stibogluconate, Pentostam® (GlaxoSmithKline), and meglumine antimoniate, Glucantime® (Rhone Poulec). Unfortunately, increasing reports of treatment failure for this class of drug in several sites of the world have challenged its clinical value (Croft et al., 2006; Llanos-Cuentas et al., 2008).

Treatment failure should be carefully distinguished from parasite drug resistance. The former might have a multifactorial origin involving a parasite–host drug interaction, while the latter is a feature of parasite selection through contact with the drug alone. In leishmaniasis, a correlation between both phenomena has been demonstrated in India (Lira et al., 1999) and Iran (Hadighi et al., 2006), but not in Nepal (Rijal et al., 2007) or Sudan (Abdo et al., 2003).

Different treatment outcome scenarios have been described in Brazil, as reported in the states of Rio de Janeiro (Oliveira-Neto et al., 1997), Bahia (Romero et al., 2001; Machado et al., 2010) and Amazonas (Neves et al., 2011; Teixeira et al., 2008). However, an *in vitro* drug susceptibility test for the parasites was conducted only in isolates from the city of Rio de Janeiro, demonstrating that the observed good clinical response of CL might be related to the high sensitivity of the local *L. braziliensis* to meglumine antimoniate *in vitro* (Azeredo-Coutinho et al., 2007). Currently, the most reliable method for monitoring parasite drug resistance in *Leishmania* isolates is the slow and technically demanding *in vitro*

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amastigote-macrophage model (Carter et al., 2001; Lira et al., 1999; Vermeersch et al., 2009). For this reason, there is an urgent need to identify cellular and molecular markers that are easy and cost-effective to use, in order to evaluate resistance development in relation to treatment outcome.

Several methods, such as PCR-RFLP of kDNA minicircles and microsatellite analysis, have not shown a direct correlation with the drug resistance phenotype in *L. donovani* (Laurent et al., 2007). More recently, whole genome sequencing approaches have been successfully applied to identify single nucleotide polymorphisms (SNPs) and genome structural variation related to drug susceptibility in *L. major* (Coelho et al., 2012) and *L. donovani* (Downing et al., 2011) strains. Moreover, multilocus sequence typing on clinical *L. donovani* strains showed that 3 genomic locations suffice to specifically detect Sb(V) resistant parasites isolated from infected patients (Vanaerschot et al., 2012).

In the present study, we analyzed DNA sequence polymorphisms of drug resistance-related genes in *L. braziliensis* (Lb, $n = 19$) and *L. guyanensis* (Lg, $n = 29$) isolated from infected Brazilian patients with different antimonial treatment outcomes. The mechanisms of resistance to antimonials are still unclear, but it is generally believed they might involve reduction of the prodrug [Sb(V)] to its active form [Sb(III)] and the uptake, efflux, or sequestration of the drug in the parasite (Decuypere et al., 2012). The target genes were chosen because they are thought to be involved in antimony resistance functions, including transport (*AQP1*, aquaglyceroporin; *MRPA*, ABC-thiol transporter: multidrug resistance associated protein A), redox metabolism (*TRYR*, trypanothione reductase) and stress response (*hsp70*, heat-shock protein 70). Different degrees of polymorphism were observed among the genes and species studied, and several variations encoded non-synonymous mutations. Most strikingly, one polymorphism in the hsp70 amino acid translated sequence presented up to 75% prediction accuracy for treatment outcome in patients infected by *L. braziliensis*.

2. Materials and methods

2.1. Parasite isolates and genomic DNA isolation

Clinical isolates were obtained from patients presenting CL prior to treatment. All patients were treated intravenously with daily doses of 20 mg/kg meglumine antimoniate for 20 days, and the therapeutic endpoint was established three months after the end of the treatment. Patients were considered cured in case of complete healing, characterized by full epithelialization, absence of any inflammatory signs, no evidence of new lesions and complete regression of satellite adenomegaly and lymphangitis. Patients who did not progress in this way by the therapeutic endpoint were considered as having therapeutic failure.

All isolates are deposited at CLIOC (Coleção de *Leishmania* do Instituto Oswaldo Cruz) and were characterized by multilocus enzyme electrophoresis as *L. braziliensis* zymodeme 27 and *L. guyanensis* zymodeme 23, following the zymodeme classification from CLIOC (Cupolillo et al., 1994). The samples comprehend four distinct groups: *L. braziliensis* infected patients with treatment cure (LbC, $n = 14$), *L. braziliensis* infected patients with treatment failure (LbF, $n = 5$), *L. guyanensis* infected patients with treatment cure (LgC, $n = 15$), and *L. guyanensis* infected patients with treatment failure (LgF, $n = 14$). The geographical origin, isolate identity, therapeutic outcome and international code are listed in Table 1.

Promastigote forms of the parasite were grown at 25 °C in Schneider's medium supplemented with 20% (v/v) heat-inactivated fetal bovine serum. Parasites (10^7/ml) were harvested by centrifugation, and genomic DNA was purified using the Wizard® Genomic DNA Purification Kit (Promega).

Table 1
Details of *L. braziliensis* and *L. guyanensis* parasites isolated from patients with different treatment outcomes of cutaneous leishmaniasis.

IOC-L[a]	Geographic origin[b]	Therapeutic response	International code
L. braziliensis			
2463	BA	Failure	MHOM/BR/2001/JOLIVAL
2538	RJ	Cure	MHOM/BR/2002/EMM
2823	BA	Cure	MHOM/BR/2001/RGJ
2842	BA	Cure	MHOM/BR/2002/LTCP14438
2846	BA	Cure	MHOM/BR/2002/LTCP14432
2867	BA	Failure	MHOM/BR/2002/NMT-LTCP 14418-P
2869	BA	Cure	MHOM/BR/2002/NMT-LTCP 14440-P
2871	BA	Cure	MHOM/BR/2002/NMT-LTCP 14447-P
2872	BA	Cure	MHOM/BR/2002/NMT-LTCP 14451-P
2876	BA	Failure	MHOM/BR/2002/NMT-LTCP 14509-P
2878	BA	Cure	MHOM/BR/2002/NMT-LTCP 14515-P
2889	BA	Cure	MHOM/BR/2002/NMT-LTCP 14616-P
2891	BA	Cure	MHOM/BR/2002/NMT-LTCP 14621-P
2892	BA	Cure	MHOM/BR/2002/NMT-LTCP 14624-P
2893	BA	Cure	MHOM/BR/2002/NMT-LTCP 14627-P
2918	RJ	Cure	MHOM/BR/2006/ICA
2927	BA	Failure	MHOM/BR/2001/NMT-LTCP14369-P
2928	BA	Failure	MHOM/BR/2002/NMT-LTCP14563-P
2934	BA	Cure	MHOM/BR/2002/NMT-LTCP14566-P
L. guyanensis			
2334	AM	Failure	MHOM/BR/1997/NMT-MAO 202P
2335	AM	Cure	MHOM/BR/1997/NMT-MAO 203P
2336	AM	Cure	MHOM/BR/1997/NMT-MAO 203G
2337	AM	Cure	MHOM/BR/1997/NMT-MAO 210P
2341	AM	Failure	MHOM/BR/1997/NMT-MAO 223P
2346	AM	Failure	MHOM/BR/1997/NMT-MAO 233P
2354	AM	Failure	MHOM/BR/1997/NMT-MAO 243P
2356	AM	Failure	MHOM/BR/1997/NMT-MAO 246P
2357	AM	Failure	MHOM/BR/1997/NMT-MAO 247P
2358	AM	Failure	MHOM/BR/1997/NMT-MAO 248P
2362	AM	Failure	MHOM/BR/1997/NMT-MAO 253P
2366	AM	Failure	MHOM/BR/1997/NMT-MAO 258P
2370	AM	Cure	MHOM/BR/1997/NMT-MAO 264P
2371	AM	Failure	MHOM/BR/1997/NMT-MAO 292P
2372	AM	Failure	MHOM/BR/1997/NMT-MAO 292G
2378	AM	Failure	MHOM/BR/1997/NMT-MAO 299P
2389	AM	Cure	MHOM/BR/1997/NMT-MAO 307P
2391	AM	Cure	MHOM/BR/1997/NMT-MAO 308G
2397	AM	Failure	MHOM/BR/1997/NMT-MAO 316P
2398	AM	Failure	MHOM/BR/1997/NMT-MAO 317P
2404	AM	Cure	MHOM/BR/1997/NMT-MAO 324G
2405	AM	Cure	MHOM/BR/1997/NMT-MAO 325P
2407	AM	Cure	MHOM/BR/1997/NMT-MAO 328P
2414	AM	Cure	MHOM/BR/1997/NMT-MAO 342P
2956	AM	Cure	MHOM/BR/2007/011
2957	AM	Cure	MHOM/BR/2007/014-JIS
2958	AM	Cure	MHOM/BR/2007/018-MAS
2959	AM	Cure	MHOM/BR/2007/025-LFA
2960	AM	Cure	MHOM/BR/2007/029-ZAV

[a] Specimens were deposited in the Coleção de *Leishmania* do Instituto Oswaldo Cruz (CLIOC), Rio de Janeiro, Brazil. Further information may be obtained at http://clioc.ioc.fiocruz.br/.
[b] States from Brazil (BA: Bahia; AM: Amazonas; RJ: Rio de Janeiro).

2.2. PCR amplification and cloning

Four PCR assays were developed to target DNA segments of the following genes: *AQP1*, *hsp70*, *MRPA* and *TRYR*. The designed primers and amplification strategies are depicted in Fig. 1 and Table 2. Amplification reactions for the genes *hsp70*, *MRPA* and *TRYR* were performed in a final volume of 50 μL containing 10–100 ng of genomic DNA (template), 1× buffer, 2 mM $MgSO_4$, 200 μM of each dNTPs, 15 pmol of each primer and 1 U Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen). PCR reactions for *AQP1* were