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ORIGINAL PAPER

Organization of H locus conserved repeats in *Leishmania (Viannia) braziliensis* correlates with lack of gene amplification and drug resistance

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Abstract Resistance to antimonials is a major problem when treating visceral leishmaniasis in India and has already been described for New World parasites. Clinical response to meglumine antimoniate in patients infected with parasites of the *Viannia* sub-genus can be widely variable, suggesting the presence of mechanisms of drug resistance. In this work, we have compared *L. major* and *L. braziliensis* mutants selected in different drugs. The cross-resistance profiles of some cell lines resembled those of mutants bearing H locus amplicons. However, amplified episomal molecules were exclusively detected in *L. major* mutants. The analysis of the *L. braziliensis* H region revealed a strong conservation of gene synteny. The typical intergenic repeats that are believed to mediate the amplification of the H locus in species of the *Leishmania* sub-genus are partially conserved in the *Viannia* species. The conservation of these non-coding elements in equivalent positions in both species is indicative of their relevance within this locus. The absence of amplicons in *L. braziliensis* suggests that this species may not favour extra-chromosomal gene amplification as a source of phenotypic heterogeneity and fitness maintenance in changing environments.

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Introduction

Several species of the protozoan parasite *Leishmania* are human pathogens that cause a broad range of pathologies collectively called leishmaniasis, a complex disease with an estimated prevalence of 12 million cases worldwide. The different forms of this disease are generally associated with particular parasite species and may range from cutaneous or mucosal lesions to the fatal visceral form. American tegumentary leishmaniasis presents clinical manifestations that are highly variable and depend not only on the parasite species involved but also on its interaction with the host (Romero et al. 2001; Yardley et al. 2006).

The therapeutic arsenal against *Leishmania* infection is limited. Antimony-containing compounds were first used in leishmaniasis treatment almost a century ago and are still the mainstream form of anti-leishmanial therapy. Resistance to antimonials is a major problem for the treatment of visceral leishmaniasis in India and has already been described for New World parasites (Romero et al. 2001; Sundar 2001). Clinical response to meglumine antimoniate in patients infected with parasites of the *Viannia* sub-genus can be widely variable (Rojas et al. 2006; Romero et al. 2001; Yardley et al. 2006). In one of these studies, a higher level of therapeutic failure was reported by *Leishmania guyanensis* infections when compared to *L. braziliensis*, suggesting that the poorer response in the former species might be associated to mechanisms of drug resistance (Romero et al. 2001).

In the species of the *Leishmania* sub-genus, the proposed mechanisms of resistance to antimonials include the increase in intracellular levels of thiols, which leads to the formation of drug-thiol conjugates that can be either extruded out of the cell or transported into vesicles (Dey et al. 1996; Legare et

al. 2001; Mukhopadhyay et al. 1996). Studies of laboratory strains have demonstrated that the amplification of the H locus in different species belonging to the *Leishmania* sub-genus may mediate resistance to several unrelated drugs, including antimonials. P-glycoprotein A (*MRPA*), Pteridine reductase 1 (*PTR1*) and the H region-associated terbinafine resistance gene (*HTBF*) are the three loci related to drug resistance that have been identified within the H region (Callahan and Beverley 1991, 1992; Grondin et al. 1993; Haimeur et al. 2000; Marchini et al. 2003).

On the other hand, mechanisms of drug resistance in species of the *Viannia* sub-genus are not clearly established. Despite the report of a glucantime-resistant *L. guyanensis* strain bearing an amplified MRPA-like gene (Anacleto et al. 2003), the correlation between drug resistance and gene amplification is much less clear in this sub-genus. In this paper, we report the study of the H locus of *L. braziliensis*, a *Viannia* sub-genus species, and investigate its possible amplification under drug pressure. The comparison of *L. major* and *L. braziliensis* resistant mutants indicated that the elicited resistance profile of some mutants was comparable to that of cell lines bearing H locus amplicons. Nevertheless, amplified episomes were detected exclusively in *L. major* mutants. The analysis of the *L. braziliensis* H region sequence revealed a strong conservation of gene order and the presence of the typical repeated elements throughout the locus when compared to *L. major*. Despite the low sequence identity, the selective maintenance of repeats in equivalent positions in both species is indicative of their functional relevance within this locus.

Material and methods

Cells lines and culture conditions Promastigote forms of *Leishmania* were grown in M199 (Sigma) media supplemented with the appropriate antibiotic as described elsewhere (Kapler et al. 1990). We used *L. major* LT252 (MHOM/IR/84/LT252) and *L. braziliensis* LB2904 (MHOM/BR/75/M2904). The drug-resistant cell lines were derived from both clones by stepwise selection in an increasing concentration of terbinafine (mutants $Lm[Tbf]^{9}$, $Lm[Tbf]^{10}$ and $Lb[Tbf]^{10}$); antimony tartrate ($Lm[Sb(III)]^{70}$, $Lb[Sb(III)]^{10}$ and $Lb[Sb(III)]^{20}$); meglumine antimoniate ($Lm[Sb(V)]^{40}$ and $Lb[Sb(V)]^{10}$) and methotrexate ($Lm[Mtx]^{1000}$). The effect of the different drugs on wild-type and mutant cell lines was determined by monitoring the rate of cell growth in liquid culture containing varying amounts of drug concentration. In these experiments, log-phase cells were inoculated at a cell density of 10^5 cells ml^{-1} in media containing 2–12 mg ml^{-1} of terbinafine (TCI); 0.1–400 μg ml^{-1} of antimony tartrate (Sigma); 0.01–150 mg ml^{-1} of meglumine antimoniate (Aventis Pharma) or 0.5–1,000 μM of methotrexate (Orion). Mutant parasites were named according to Clayton et al. (1998).

Bacteria, growth conditions and molecular techniques The *Escherichia coli* strain DH10B (Gibco BRL) used in this study was grown in LB medium supplemented with 100 μg ml^{-1} of Hygromycin B (Invitrogen) when necessary. Plasmid DNA from bacteria was extracted using commercial kits (Qiagen), and DNA manipulation was carried out with restriction and modifying enzymes (Invitrogen; New England Biolabs) as previously described (Sambrook et al. 1989). DNA samples were resolved by electrophoresis in 0.8% agarose gels. Intact chromosomes were prepared in agarose blocks (Cruz and Beverley 1990) and used in Pulse Field Gel Electrophoresis (PFGE) with a Bio-Rad CHEF Mapper apparatus (10-s constant pulses for 18 h). After migration, DNA gels were stained with Ethidium bromide and blotted by alkaline transfer onto nylon membranes (Hybond N^+, Amersham). The LmHTBF probe of *L. major* is a 0.56-Kb fragment amplified by PCR using primers LT007 (5′-GCGCCCGGGCATATGCTCAACGAGGTGC) and LT008 (5′-CGCGGATCCTAAATACCAACCAGA) in 35 cycles consisting of 30 s at 94°C, 30 s at 55°C and 2 min at 72°C. The LmPTR1 and LmTTRS probes of *L. major* are 0.86-Kb *Sma*I/*Sfi*I and 1.2-Kb *Hin*dIII fragments, respectively, from pELHYGH2 (Tosi and Beverley 2000). The LmV-ATPase probe is a 0.21-Kb *Hin*dIII/*Sac*I fragment from pSNARH1 (Callahan and Beverley 1991). The LmRIME3 probe is a 1.9-Kb fragment amplified by PCR using primers LT041 (5′-GCAGATACCACACCGT CAACT) and LT042 (5′-TTCAGTGTTTCCGCTGA GACA) in 30 cycles consisting of 50 s at 94°C, 1 min at 58°C and 2 min at 72°C. The probes were labelled by random-priming synthesis and hybridized according to standard procedures (Feinberg and Vogelstein 1983). PCR amplification of the *L. braziliensis* 5-Kb fragment carrying the LbrM23.0310 gene was performed using primers LT032 (5′-CTTACGCTATGTGGCTTCT) and LT033 (5′-AACCGC AGAAACTCCCAG) in 40 cycles consisting of 30 s at 94°C, 30 s at 40°C and 5 min at 68°C. The amplification product was cloned into pELHYGII (Marchini et al. 2003).

L. braziliensis genomic libraries Two *L. braziliensis* partial genomic libraries were constructed into pELHYGII (Marchini et al. 2003) or pELHYG (Garraway et al. 1997). Both libraries were organized in high-density filters and used as described (Tosi et al. 1997). Clones cLbPTR1a, cLbPTR1b and cLbTTRS were rescued by hybridisation with the LmTTRS and LmPTR1 probes.

Contig assembly and sequence analysis Sequence data obtained from the different sub-clones used in this work were analysed with the Phred/Phrap/Consed package

(Ewing and Green 1998; Ewing et al. 1998; Gordon et al. 1998). The assembly of the locus also included sequences from the genome shotgun sequencing data freely available from http://www.sanger.ac.uk/Projects/L_braziliensis (version 1.0). The *L. braziliensis* H locus consensus was examined for putative protein-coding open reading frames (ORFs) using a combination of gene-prediction algorithms (Delcher et al. 1999; Tiwari et al. 1997; Tramontano et al. 1984). The *L. braziliensis* H locus sequence was annotated using the consensus sequence exported through MAGI (Aggarwal et al. 2003), the codon-usage algorithm built into the Artemis Software and the *L. major* annotation data. Sequence-similarity searches with BLAST (Altschul et al. 1990) and FASTA (Pearson and Lipman 1988) were carried out against public domain databases directly downloaded from the National Center for Biotechnology. The Artemis comparison tool (ACT; Carver et al. 2005), freely available from the Sanger Institute, permitted the visualisation of sequence comparisons.

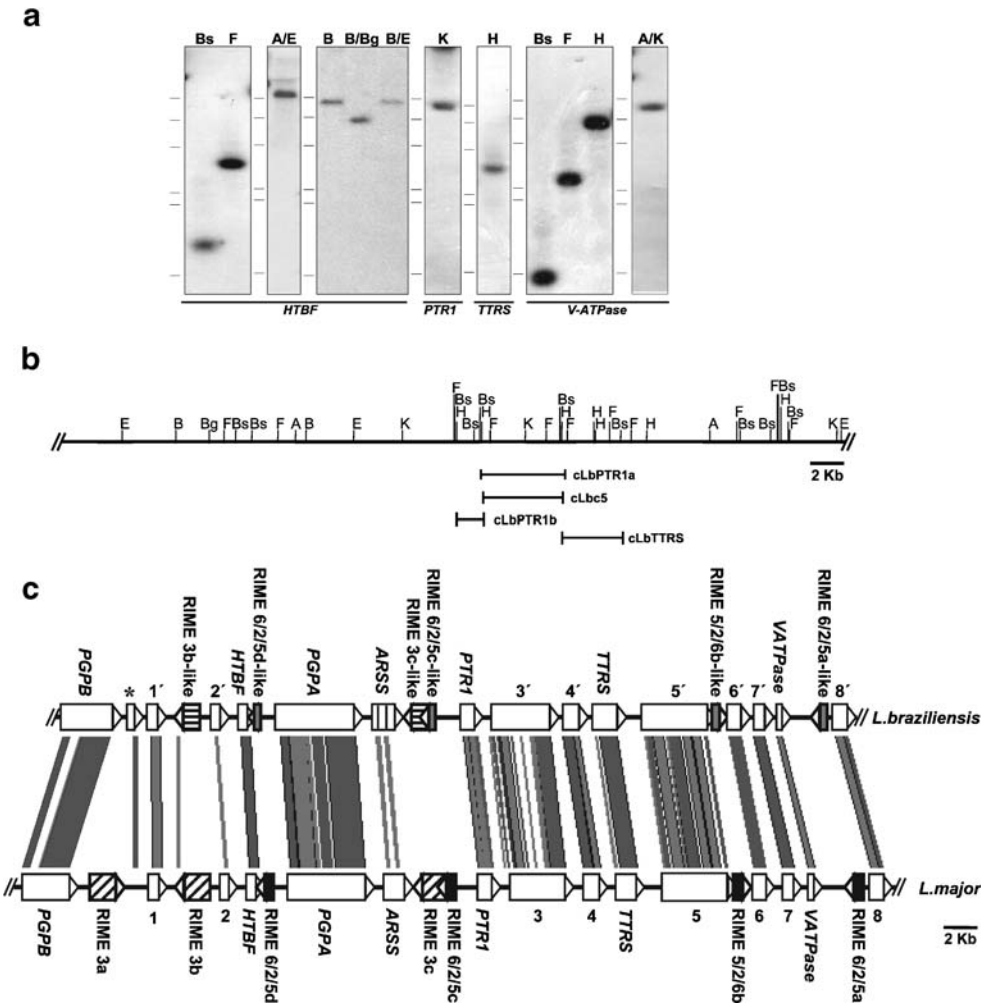


Fig. 1 Mapping of the *L. braziliensis* H locus. **a** Southern blots of *L. braziliensis* genomic DNA digested with BsaAI (*Bs*), FspI (*F*), AflIII (*A*), EcoRI (*E*), BglII (*Bg*), KpnI (*K*) and HindIII (*H*); the probes *HTBF*, *PTR1*, *TTRS* and *VATPase* were generated as described in "Material and methods." **b** The restriction map of the H locus was built from the Southern analysis and the *L. braziliensis* genome shotgun sequencing data; *cLbPTR1a*, *cLbPTR1b*, *cLbc5* and *cLbTTRS* indicate sub-cloned fragments. **c** Visualisation of the comparison between the *L. braziliensis* and *L. major* H loci using the ACT; sequences with a minimum identity of 79% are connected by *gray bands*, the intensity of the colour is proportional to the percent identity of the match; the *RIME 6/2/5* repeated elements are depicted as *grey* and *black boxes* in *L. braziliensis* and *L. major*, respectively; the *Viannia* species *RIME 3* elements are represented as *horizontal-dashed boxes*, and *L. major* RIME 3 sequences are shown as *diagonal-dashed boxes*; the *asterisk* represents ORF LbrM23.0240, exclusively found in *L. braziliensis*. Annotated ORFs numbered 1′–8′ are LbrM23.0250, LbrM23.0260, LbrM23.0310, LbrM23.0320, LbrM23.0340, LbrM23.0350, LbrM23.0360 and LbrM23.0380, respectively; the *L. major* annotated ORFs numbered 1–8 are LmjF22.0225, LmjF23.0230, LmjF23.0280, LmjF23.0290, LmjF23.0310, LmjF23.0320, LmjF23.0330 and LmjF23.0250, respectively

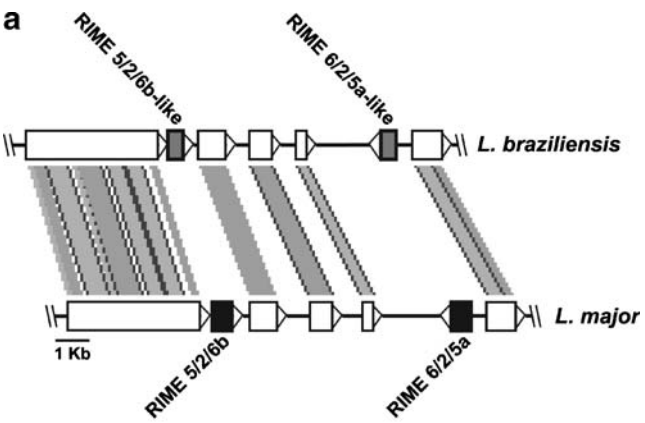
Results

H region conservation between Leishmania sub-genera The level of conservation across the H locus between *L. braziliensis* and the reference strain of *L. major* was first examined by Southern analysis of PFGE-separated chromosomes, which confirmed that the loci are located in a ~780-Kb band corresponding to chromosome 23 in both species (data not shown). The preservation of gene order across the locus was confirmed by mapping four H region genes (*HTBF*, *PTR1*, *TTRS* and *VATPase*) using Southern analysis of *L. braziliensis* genomic DNA digested with various enzymes. The restriction mapping of the locus presented in Fig. 1a,b directed the construction of genomic libraries that were used to rescue *L. braziliensis* genes. Figure 1b shows the sub-clones carrying *PTR1*, *TTRS* and the unknown genes LbrM23.0310 and LbrM23.0320, which were used as a starting point for contig assembly and the reconstruction of the entire H region of *L. braziliensis*.

The data sources for the assembly of the 42.4-Kb locus included not only the restriction mapping and sequences from the sub-clones but also the *L. braziliensis* genome shotgun sequencing data freely available from http://www.sanger.ac.uk/Projects/L_braziliensis/index.shtml (version 1.0). The database was subjected to successive rounds of clusterisation and used in the assembly of the contigs. The consensus sequences were examined for putative protein-coding ORFs with a combination of gene-prediction algorithms, which resulted in the graphical output presented in Fig. 1c. The *L. braziliensis* locus was compared to the *L. major* H region with the ACT, in which sequences with an identity above 79% are connected by gray bands. As seen in Fig. 1c, there is an extensive level of gene synteny and sequence conservation between the two species. However, annotation of the *L. braziliensis* locus revealed a 459-bp-long putative gene that was not originally reported in the *L. major*. Gene LbrM23.0240 is marked as an asterisk in Fig. 1c. Despite the high degree of synteny, the locus is 3 Kb shorter in *L. braziliensis*, which is mainly due to differences within intergenic sequences. In fact, the comparison of non-coding regions revealed a low nucleotide identity between the two species. This finding is supported by previously reported data showing that sequence conservation between *L. major* and *L. braziliensis* H loci was higher within coding regions when compared to non-coding sequences (Laurentino et al. 2004).

Repeated elements within the L. braziliensis H locus The annotation process identified repeated elements within non-coding sequences across the *L. braziliensis* H region. As shown in Fig. 1c, the position of these repeats is equivalent to the location of the repetitive sequences annotated as RIME 3 and RIME 6/2/5 elements in the *L. major* genome. In the *L. braziliensis* locus, the latter repeat is 443 bp long, and comparison between the two inverted copies, RIME 6/2/5a-like and RIME 5/2/6b-like located at the right end of the locus, revealed a sequence identity of 95.3% (Fig. 2b). As in the *L. major* locus, approximately 6 Kb separate these nearly identical inverted repeats. Despite the equivalent size and location, the repeated element from both species presented a low nucleotide identity of 23.9%, as shown in Fig. 2a,b.

Unlike the RIME 6/2/5 repeats, the distribution of repeated elements found across the first half of the H locus of *L. major* and *L. tarentolae* is not fully conserved in *L. braziliensis*. Figure 3a shows that the *Viannia* species bears sequence elements that are equivalent to two of the three RIME 3 repeats annotated in the *L. major* H locus. As shown above, the RIME 3 sequence was found in the amplicons generated in *L. major* resistant mutants (Fig. 5a). The RIME 3-like elements of *L. braziliensis* are 1,002 bp



b

	L. major		L. braziliensis	
	RIME 6/2/5a	RIME 5/2/6b	RIME 6/2/5a-like	RIME 5/2/6b-like
RIME 6/2/5a	—	96.7	24.3	23.5
RIME 5/2/6b		—	24.3	23.3
RIME 6/2/5a-like			—	95.3
RIME 5/2/6b-like				—

Fig. 2 Conservation of *RIME 6/2/5* repeats in *L. braziliensis*. **a** Visualisation of the comparison between the right end of *L. braziliensis* and *L. major* H loci using the ACT. **b** Percent identity among the different copies of RIME 6/2/5 repeats within the H locus of *L. major* and *L. braziliensis* by Clustal W (Chenna et al. 2003) alignment software of the MegAlign program (DNAStar, Madison, WI)

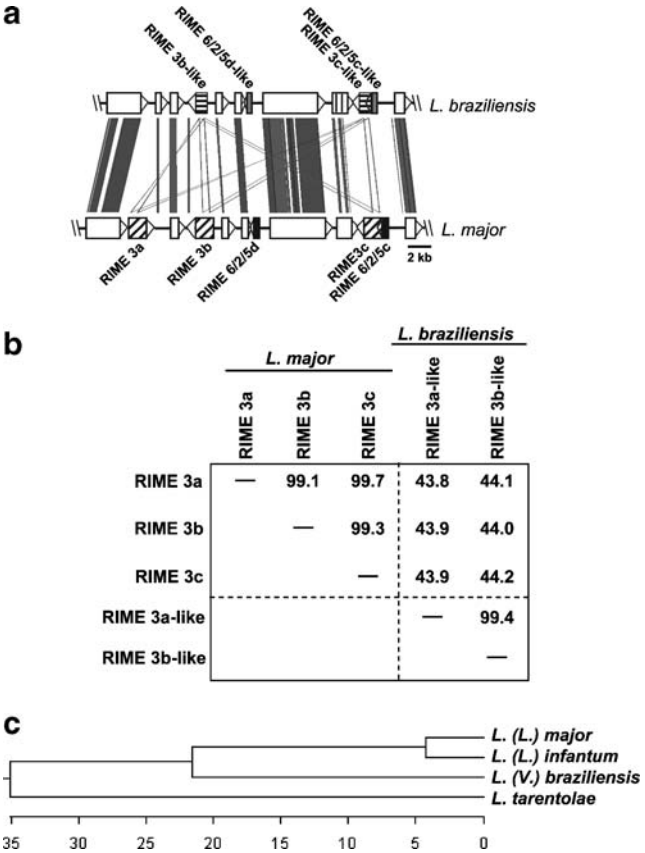


Fig. 3 Conservation of *RIME 3* repeats in *L. braziliensis*. **a** Visualisation of the comparison between the left end of *L. braziliensis* and *L. major* H loci using the ACT. **b** Percent identity among the different copies of *RIME 3a* repeats within the H locus of *L. major* and *L. braziliensis* by Clustal W alignment software of the MegAlign program (DNAStar). **c** Phylogenetic relationship of the RIME 3 repeats. An un-rooted dendogram was prepared by comparing the consensus nucleotide sequences of RIME 3 elements from *L. major*, *L. infantum*, *L. braziliensis* and *L. tarentolae* using Clustal W; the scale at the *bottom* measures the distance between sequences

long, and the average nucleotide identity with the *L. major* and *L. tarentolae* RIME 3 repeats was low. As expected, *L. tarentolae* has the most divergent RIME 3 sequence when compared to species of the *Leishmania* and *Viannia* subgenera (Fig. 3c). As seen in Fig. 3b, the sequence identity between *L. major* and *L. braziliensis* RIME 3 elements was only 43.9%. However, short stretches of 109 and 68 bp located within the repeats were highly conserved (Fig. 3a), with a nucleotide identity comparable to that observed in coding regions of the locus (~80%). It is noteworthy that the two *L. braziliensis* RIME 3-like copies have the same orientation and present a sequence identity of 99.4% (Fig. 3b). As shown in Fig. 1a, the inverted copy RIME 3a, which is located between the *PGPB* and LmjF22.0225 genes and defines the left end of the H locus in *L. major*, is absent in *L. braziliensis*. Therefore, the organization of the H region repeats in *L. braziliensis* is different from that described in *L. major* and *L. tarentolae*.

Selection of drug-resistant L. braziliensis and L. major mutants We have investigated the generation of amplicons in *L. braziliensis* cell lines selected for resistance to various drugs. The induction of resistance to meglumine antimoniate [Sb(V)], antimony tartrate [Sb(III)], terbinafine and methotrexate (MTX) was attempted in species of the parasite from both *Leishmania* and *Viannia* subgenera in a stepwise manner. The resistance of selected cell lines is summarised in Table 1. The *Viannia* species was consistently more sensitive when compared to *L. major*. The EC_{50} values determined for the various drugs were two to fivefold lower in *L. braziliensis*, and the values determined for *L. major* were somewhat higher than previously reported (Callahan and Beverley 1991; Ellenberger and

Table 1 Resistance of *L. major* and *L. braziliensis* mutants to different drugs

Cell line	EC_{50}[a]			
	Terbinafine (μg ml^{-1})	Sb(III) (μg ml^{-1})	Sb(V) (mg ml^{-1})	MTX (μM)
LB2904	2.7±0.35	5.2±0.04	3.4±1.56	0.3±0.03
Lb[Tbf]^{10}	10.0±0.47**	13.1±1.18*	6.3±2.01	0.2±0.001
Lb[Sb(V)]^{10}	8.3±0.04**	17.0±2.83*	54.6±2.15**	0.3±0.01
Lb[Sb(III)]^{10}	5.5±0.10*	15.0±0.11**	37.8±6.62*	0.3±0.003
Lb[Sb(III)]^{20}	5.4±0.08*	67.9±1.71**	47.4±5.64*	0.3±0.02
LT252	4.0±0.04	24.5±1.85	6.0±0.35	1.4±0.6
Lm[Tbf]^{9}	8.8±0.61**	150.5±8.98**	46.1±7.54*	2.3±0.54
Lm[Tbf]^{10}	11.0±0.16**	146.2±35.07*	43.9±1.83**	5.6±0.56*
Lm[Sb(V)]^{40}	4.2±0.04*	131.9±4.69**	127.5±13.39**	3.5±1.03
Lm[Sb(III)]^{70}	3.8±0.24	185.1±2.84**	82.0±4.54**	2.0±0.03
Lm[Mtx]^{1000}	2.4±0.69	33.2±3.15	25.8±15.24	1139±197*

Values significantly different from the wild-type values by Student's *t* test are marked with asterisks.
*$P>0.05$
**$P>0.01$
[a] The drug EC_{50} is the concentration that decreases the rate of cell growth by 50%.

Beverley 1989). Despite the differences found, resistant lines from both species had growth patterns similar to those observed in wild-type cells in the absence of drug.

As observed in Table 1 and Fig. 4a, the level of terbinafine resistance of mutants selected with this drug was three to four times higher than that of wild-type cells. The cell lines selected in terbinafine also presented significant cross-resistance to the other unrelated drugs tested. The *L. major* mutant *Lm[Tbf]*^{10} was up to sixfold more resistant to antimony tartrate (Fig. 4b) and meglumine antimoniate (Fig. 4c) when compared to unselected cells. On the other hand, the terbinafine-selected *L. braziliensis* mutant *Lb[Tbf]*^{10} had a much lower level of cross-resistance to antimonials (Figs. 4b,c) and was not resistant to MTX, an inhibitor of DHFR.

Sb(III)-resistant cell lines from *L. major* and *L. braziliensis* were, respectively, eight and 13 times more resistant than unselected cells (Fig. 4b). These mutants were cross resistant to Sb(V), but only the *Viannia* mutant, *Lb[Sb (III)]*^{20}, had some resistance to terbinafine (Fig. 4a). Cell lines selected in the chlorocresol-free preparation of Sb(V), meglumine antimoniate, were up to 20-fold more resistant than wild-type cells to the action of this form of the drug (Fig. 4c). As seen in Table 1, these mutants were also resistant to antimony tartrate, and only the *Viannia* mutant *Lb[Sb(V)]*^{10} showed a minor resistance to terbinafine. None of the mutants selected in Sb(III) or Sb(V) presented any resistance to MTX. The two species behaved differently in selection protocols that used methotrexate. While the *L. major* mutant *Lm[Mtx]*^{1000} showed considerable resistance to the drug, we were unable to select a *L. braziliensis* cell line in the medium containing MTX at concentration as low as 0.25 μM.

Another major difference between the two species was that the majority of the *L. major* resistant cell lines carried amplicons. Pulsed-field electrophoresis, which allows the separation of episomal DNA, detected extra-chromosomal elements in *L. major* mutants selected in terbinafine and Sb (III), as seen in Ethidium bromide-stained gels shown in Fig. 5. These elements were only stable under selective

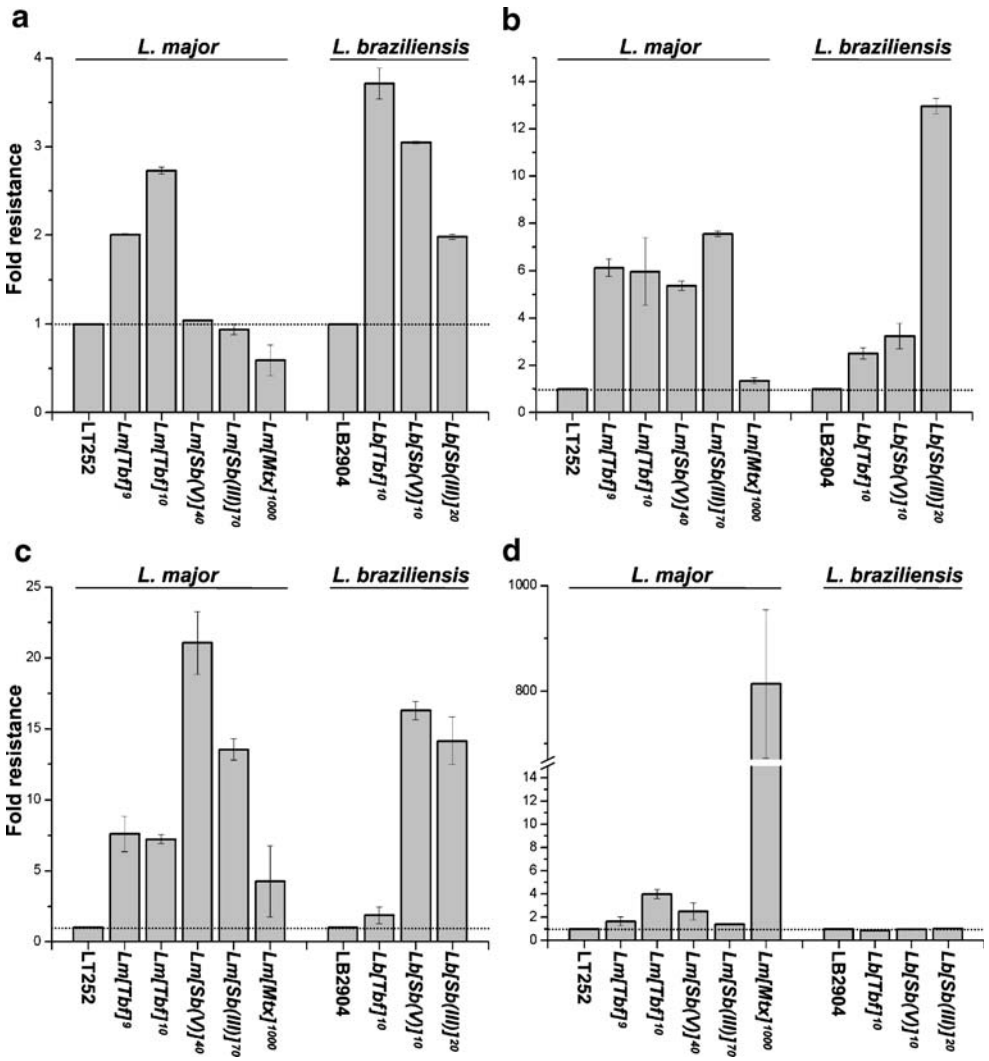


Fig. 4 Resistance profiles of *L. major* and *L. braziliensis* mutants selected in different drugs. Fold resistance is the ratio of drug EC_{50}s for experimental and wild-types cells measured in the same experiment in the drugs: terbinafine (**a**); antimony tartrate (**b**); meglumine antimoniate (**c**); and methotrexate (**d**). The EC_{50} values used for the different cell lines are presented in Table 1

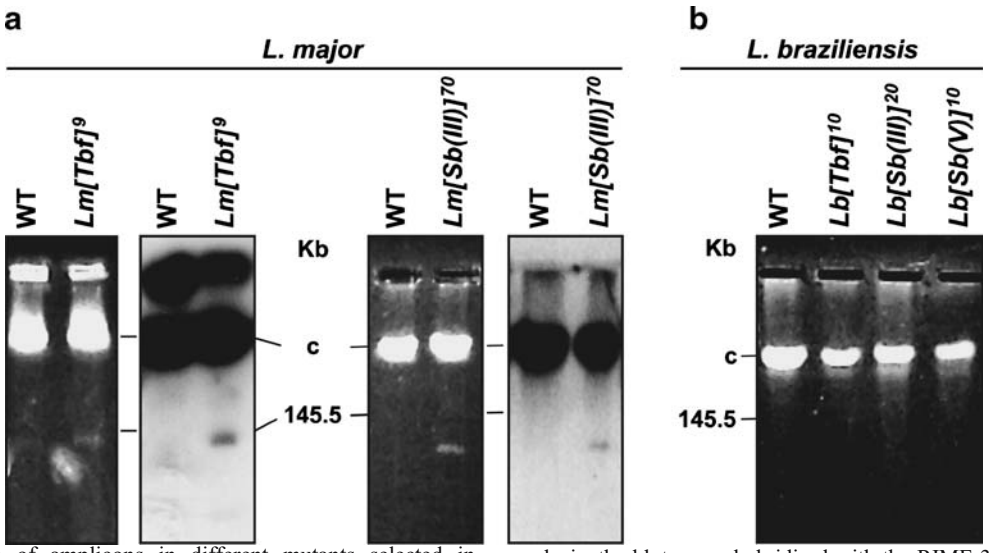


Fig. 5 Detection of amplicons in different mutants selected in terbinafine and antimonials. **a** Intact chromosomes of *L. major* wild-type (*WT*) and cell lines selected in terbinafine (*Lm[Tbf]*^{9}) and antimony tartrate (*Lm[Sb(III)]*^{70}) were subjected to short-run PFGE, which permits the separation on episomal molecules and Southern analysis; the blots were hybridised with the RIME 3 repeated element from *L. major*. **b** Short-run PFGE of intact chromosomes of *L. braziliensis* WT and cell lines selected in terbinafine (*Lb[Tbf]*^{10}), antimony tartrate (*Lb[Sb(III)]*^{20}) and meglumine antimoniate (*Lb[Sb(V)]*^{10}); c-compression zone

pressure, as they were diluted out when mutants were cultured in the absence of the drug (data not shown). On the other hand, amplicons were not observed in *L. braziliensis* mutants selected under the same conditions (Fig. 5b).

Species of *Leishmania* selected in antimonials, methotrexate, terbinafine or primaquine may derive resistance to these drugs through the amplification of a ~45-Kb locus, the H region (Ellenberger and Beverley 1989; Haimeur et al. 2000; Haimeur and Ouellette 1998; Ouellette et al. 1998). The cross-resistance observed in some of the cell lines selected in this work resembles the resistance profile of mutants presenting the H locus amplification. However, Southern analysis of the different mutants using the H region genes as probes suggested that amplicons generated in *L. major* mutants did not originate from this locus (data not shown). Surprisingly, the inverted repeated sequence present in the left end of the H locus, which is believed to mediate the formation of H circles in *L. tarentolae* (Grondin et al. 1993), was found in the amplicons generated in mutants selected in terbinafine or Sb(III) (*Lm[Tbf]*^{9} and *Lm[Sb(III)]*^{70}), as shown in Fig. 5a. These results led us to the investigation of the H locus of the *Viannia* subgenus species.

Discussion

In this study, we analysed the *L. braziliensis* H locus and compared *L. major* and *L. braziliensis* drug resistant mutants selected in unrelated drugs. The *L. major* cell line selected in terbinafine was significantly cross-resistant to antimony-containing drugs and also presented modest resistance to MTX, resembling mutants carrying the H region-derived amplicons (Ellenberger and Beverley 1989). On the other hand, *Viannia* sub-genus mutants were consistently less resistant to the different drugs tested, and the cross-resistance observed in these cell lines was not extended to the DHFR inhibitor. In fact, we were unable to select a MTX-resistant *L. braziliensis* mutant. As expected, the comparison between *L. major* and *L. braziliensis* H loci revealed a nearly complete conservation of gene order. A small break in synteny was revealed by the presence of a 459-bp-long gene of unknown function in *L. braziliensis*. Interestingly, this gene is found in the site of the *Viannia* locus lacking the inverted repeated element RIME 3a, which borders the *L. major* locus. The presence of an inverted copy of a repetitive sequence at this position has been associated with the amplification of the locus in species from different sub-genera (Beverley 1991; Grondin et al. 1996; Ouellette et al. 1991).

The amplification of the H locus is conserved during *Leishmania* evolution and has been described in the lizard parasite *L. tarentolae* and in species of the *Leishmania* subgenus (Borst and Ouellette 1995; Callahan and Beverley 1991; Callahan and Beverley 1992; Chiquero et al. 1994; Ellenberger and Beverley 1989; Grondin et al. 1993; Ouellette et al. 1998). The capacity to amplify DNA imparts a greater flexibility to a genome and may affect not only the maintenance and stability of chromosomes but also the pattern of gene expression in a wide range of organisms from bacteria to man (Beverley 1991; Genest et

al. 2005; Hastings et al. 2000; Stark and Wahl 1984; Yasui et al. 2004). Although extra-chromosomal gene amplification is extensively documented in drug resistant *Leishmania* strains (Beverley 1991; Borst and Ouellette 1995), this is not a common feature in field isolates that are resistant to drugs (Croft et al. 2006; Moreira et al. 1998). This is an indication that the strategies used by the protozoan to overcome drug pressure may differ considerably among species. In fact, a recent DNA microarray analysis of drug resistant parasites from different species has suggested that other mechanisms besides amplification can mediate the overexpression of genes implicated in resistance (El Fadili et al. 2005; Guimond et al. 2003). Moreover, resistance mechanisms may not always involve RNA overexpression. For instance, defective splicing of a C-24-Δ−Sterol methyltransferase (SCMT) from *L. donovani* is believed to interfere in the production of ergosterol, resulting in Amphotericin B resistant parasites (Pourshafie et al. 2004). The competence to amplify and re-arrange DNA may be pivotal to an organism that does not seem to undergo sexual crosses and faces the challenge of survival in diverse environments. Despite one report of gene amplification in *L. braziliensis* (Sampaio and Traub Cseko 2003), our results suggest that this species does not favour the generation of amplicons when submitted to drug pressure. Such inability to modulate resistance through the amplification of relevant loci could explain the higher susceptibility of *L. braziliensis* to the different drugs tested in this study and might denote a significant divergence between the *Viannia* and *Leishmania* sub-genera. A lower tolerance to DNA amplicons indicates that *L. braziliensis* derives phenotypic heterogeneity and maintains its fitness in changing environments using different mechanisms, which do not seem to include genome rearrangements and/or extra chromosomal gene amplification of relevant genes.

In this study, we have identified non-coding repeated elements that may be under a functional constraint within the *L. braziliensis* H locus. While protein-coding sequences are strongly constrained, functional or sequence conservation in non-coding DNA is much less strict. However, we observed a lower rate of nucleotide substitutions across H region repeats when these sequences were compared to other intergenic regions of the genome. The conservation of these elements in equivalent positions across the H region of different species, including the lizard parasite *L. tarentolae* (Grondin et al. 1993), suggests their presence in a common ancestor and is a strong indication of selective pressure. Despite the observed sequence divergence among species, the repeated copies are virtually identical within each of them. This finding demonstrates the relevance of these repetitive elements and underlines the occurrence of positive selection, which has guaranteed their preservation in the genome.

The presence of repetitive elements is one of the factors that can contribute to the plasticity of protozoan genomes (Wickstead et al. 2003). The number of copies, size and distribution of repeats are widely diverse in trypansomatids (El-Sayed et al. 2005; Wickstead et al. 2003). Despite the fact that repeated elements are important mediators of DNA rearrangements in *Leishmania*, this parasite's genome is relatively poorer in these sequences (El-Sayed et al. 2005; Ivens et al. 2005). The majority of repeated elements described are located within its sub-telomeric regions (Fu and Barker 1998; Myler et al. 1999; Pedrosa et al. 2006) and have been implicated in size variation between chromosome homologues (Sunkin et al. 2000). The possible participation of H region repeats in the amplification of this locus was brought forward in the study of drug-resistant *L. major* and *L. tarentolae* mutants (Beverley 1991; Grondin et al. 1996; Ouellette et al. 1991). Based on the proposed model for the H locus amplification (Grondin et al. 1996), the absence of RIME 3a repeat, demonstrated here, could constitute an obstacle to the amplification of the *L. braziliensis* locus. However, the analysis of the *L. major* Friedlin genome sequence revealed that these RIME elements are not limited to the H locus and are found in other *Leishmania* chromosomes (data not shown). In fact, we found the RIME 3 repeat in amplicons that are generated from other genomic loci apart from the H region. This finding suggests that these elements could have a more extensive participation in the dynamics of this parasite's genome. Therefore, the conservation of repeats within the H locus of the *Viannia* species may be related to different cellular processes or functions. It has been shown that non-coding sequences involved in the genome maintenance and/or expression are more sensitive to alterations during evolution (Halligan et al. 2004). Given the fact that these sequences could be co-transcribed with their neighbouring genes, the evidence of functional constraints of intergenic repeats in the *Leishmania* genome indicates the existence of sequence elements that may participate in the modulation of gene expression. The availability of whole-genome sequence information will allow the comparative and functional studies needed to understand the role of conserved intergenic sequences in the maintenance and expression of the *Leishmania* genome.

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