Serine Proteases Profiles of *Leishmania* (Viannia) *Braziliensis* Clinical Isolates With Distinct Susceptibilities to Antimony

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Research

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Abstract

Background: Glucantime® (Sb^V) is considered the first-line treatment against American Tegumentary Leishmaniasis in South America, though increased parasite resistance towards it have been reported and hampered its effectiveness. In this context, subtilisins serine proteases have been related to parasites' susceptibility to drugs such as Sb^V and derivatives, through modulation of the parasite detoxification system. However, little is known about parasites causing ATL and their distinct responses towards this treatment.

Methods: The study was conducted using *Leishmania (Viannia) braziliensis* clinical isolates from patients that presented clinical cure or Responders (R) and relapse/therapeutic failure or Non-responders (NR). Twelve clinical isolates were used to assess their *in vitro* susceptibility to Sb^{III} and Sb^V, serine proteases activity, and expression of subtilisins-like and trypanothione-oxidoreductase (TXNPx) transcripts.

Results: Sb^{III} was able to better distinguish axenic amastigotes from each clinical group. These isolates were also assessed for serine protease activity, using z-FR-AMC as substrate and detecting distinct enzyme profiles with specific inhibitors. TLCK inhibited almost 100% of activity in both promastigotes and axenic amastigotes while AEBSF inhibited around 70%. PMSF showed low inhibition of specific isolates (35%). Gathering all the quantitative data, we performed principal component analysis and then used the K-means algorithm to cluster the isolates. This analysis yielded one cluster with only one isolate (R isolate), one homogeneous cluster (NR isolates), and three heterogeneous clusters (R and NR isolates). Additionally, gene transcripts of subtilisins and TXNPx were detected in promastigotes and axenic amastigotes from both groups.

Conclusions: Cluster analysis showed that there is a phenotypic heterogeneity among the isolates, however, exploration of *in vitro* phenotypes based on Sb^{III} and serine proteases profiles can aid in the characterization and better understanding of *L. (V.) braziliensis* clinical isolates.

Background

Leishmaniasis are neglected tropical diseases that affect around 1 million people worldwide, causing 26 000 to 65 000 deaths per year [1]. *Leishmania* spp. are eukaryotic protozoan parasites responsible for the broad range of clinical forms, such as cutaneous (localized and mucocutaneous manifestations) and the visceral form, which depends on the parasite specie and the immune state of the mammalian host [2]. In the American continent, *Leishmania mexicana* complex and subgenus *Viannia* species cause American Tegumentary Leishmaniasis (ATL), which varies from localized cutaneous to the diffuse and mucocutaneous form [1].

A pentavalent antimonial formulation – Glucantime® (Sb^V) – is the first-line treatment against ATL and has not changed in the last 70 years [1]. In South America this treatment doses varies according to the therapeutic response patterns [3, 4]. Its mechanism of action is still not completely understood, but, it has

been stated that Sb^{V} is a prodrug that is reduced to its trivalent form (Sb^{III}), either directly by the parasite or within the host macrophages [5–7].

Moreover, the most abundant *Leishmania* spp. thiol, trypanothione, $\text{T}(\text{SH})_2$, may be involved in the detoxification of metal ions, reducing Sb^{V} to Sb^{III} within the macrophage phagolysosome [5, 6]. $\text{T}(\text{SH})_2$ is part of the parasite trypanothione reductase system which is a unique detoxification defence system, and it relies on four key enzymes: trypanothione synthetase (TryS), trypanothione reductase (TR) and tryparedoxin (TXN) and tryparedoxin peroxidase (TXNPx) [8]. Another theory states that Sb^{V} reduction to Sb^{III} happens through the oxidation of $\text{T}(\text{SH})_2$ which forms a stable complex – $\text{Sb}^{\text{III}}(\text{TS})_2$ – that could be pumped out of the cells by parasite membrane transporters [6, 9].

The effectiveness of Sb^{V} as a treatment is hampered not only due to increased drug resistance but also by its highly toxic side effects [10, 11]. Moreover, drug resistance has been correlated to the action of some *Leishmania* spp. virulence factors [12–14]. For instance, proteases – enzymes that hydrolyse peptide bonds – are known to play important roles during life cycle transitions, manipulation of host immune response and development of infections in mammals [15, 16]. These enzymes are classified according to their residue in the catalytic domains: serine proteases, cysteine proteases, and aspartyl proteases. Exceptionally, metalloproteases carry this name because their catalytic mechanism involves a metal [17].

Specifically, serine proteases have been extensively studied in American *Leishmania* spp. due to their functional interrelationship in parasite physiology and their potential as therapeutic targets [18–25]. Experimental evidence has demonstrated that these enzymes can act as signal peptidases; maturases of other proteins; and have a metacaspase-like activity [21, 26]. Among them, oligopeptidases have important roles during infection of host macrophages [27], and subtilisins are established virulence factors in *Leishmania* spp. as well as in other intracellular parasites [28, 29].

In this context, subtilisins are correlated to the balance of cytosolic and mitochondrial TXNPx levels in *L. (L.) donovani* [26]. This study showed that subtilisin knockout promastigotes failed to differentiate into viable amastigotes and their TXNPx peptide abundance was decreased, which suggests that subtilisins can act as maturases of specific proteins or pathways [26]. Additionally, increased abundance of TXNPx has been correlated with an enhanced antioxidant defence seen in Sb^{V} resistant parasites; not only in lab-generated strains [30, 31] but also in clinical isolates [32].

In *L. (V.) braziliensis* clinical isolates, it is still not known if serine proteases excel a similar role in both axenic forms: promastigotes and axenic amastigotes, and if impairment of the trypanothione reductase system has any relation with Sb^{V} resistant phenotype. Therefore, this study aimed to characterize *L. (V.) braziliensis* clinical isolates to assess their *in vitro* susceptibility towards Sb^{V} and Sb^{III} , serine enzymatic activity and expression of subtilisin-like and TXNPx transcripts. We have evidence that different phenotypic profiles can be grouped based on *in vitro* susceptibility, serine protease activities profile and their specific inhibition.

Methods

Parasite samples

Twelve *L. (V.) braziliensis* clinical isolates were acquired from the biological collection of the Evandro Chagas National Institute of Infectious Diseases at Fundação Oswaldo Cruz (INI – Fiocruz). All parasites were isolated before Glucantime® treatment from five patients who were classified as responders (R) because they presented complete lesion healing and clinical cure; and seven non-responders (NR) who had therapeutic failure or relapse. The medical personal of the Leishmaniasis Clinical Research and Vigilance Laboratory (LaPClinVigiLeish) at INI – Fiocruz were responsible for this classification following the criteria reported for ATL patient's treatment in the state of Rio de Janeiro – Brazil [4,33,34].

For this study, each isolate was named after its patient response: Responder (R1, R2, R3, R4, R5) and Non-responder (NR1, NR2, NR3, NR4, NR5, NR6, NR7).

Parasites culture and *in vitro* differentiation

Parasites were defrosted and initially cultured in biphasic Novy-MacNeal-Nicolle (NNN) medium, containing 10% of inactivated FBS. Then, they were expanded in Schneider's insect medium at pH 7.2 supplemented with 20% of inactivated FBS, 200 International Units (IU) penicillin and 200 mg/mL streptomycin and maintained at 26 °C. To obtain each isolate growth curve, 3×10^5 promastigotes were initially cultured and maintained in 25 cm² flasks containing the medium described above. Daily, for eight days, a 10 mL aliquot was taken to determine the number of viable parasites using a Neubauer chamber. Each parasite isolate did not have more than 5 passages since isolation. Differentiation from promastigotes to axenic amastigotes was performed as previously described by Cysne-Finkelstein et al. [20], with some modifications. Briefly, 5×10^5 per mL of log-phase promastigotes were cultured in Schneider medium (pH 5.5) supplemented with 20% of FBS, 60 IU penicillin, 60 mg/mL streptomycin and maintained at 26 °C for 2 days. Then, to complete differentiation, each culture was subjected to temperature shock (32 °C) for 2 days.

In vitro susceptibility assays

Log-phase promastigotes and axenic amastigotes forms, of each isolate, were tested against Glucantime® or Sb^V and antimony potassium tartrate or Sb^{III} (Sigma Aldrich Chemical Co., St. Louis, MO, USA) to measure the half-maximal inhibitory concentration (IC₅₀) induced by these drugs. The IC₅₀ was determined by AlamarBlue™ reduction assay as previously described (Shimony and Jaffe, 2008), with some modifications. Briefly, each parasite form was seeded in 96-well plates in triplicate at adequate conditions: log-phase promastigotes (4×10^6 parasites/mL) and one-day axenic amastigotes (5×10^5 parasites/mL) in 100 µL of Schneider medium (pH 7.2 or pH 5.5, respectively) supplemented with 20% of FBS and each drug in decreasing concentrations, leaving one column without any drug to serve as control. For Sb^V ranging from 20 mg/mL to 6×10^{-4} mg/mL; for Sb^{III} ranging from 0.196 mg/mL to 5×10^{-6}

mg/mL, with a 2:1 dilution factor between each concentration. Parasites cultures were incubated (26 °C, 48 h and 32 °C, 24 h), respectively. After each incubation time, 10 mL of AlamarBlue™ was added to each well, the plates were then re-incubated at their respective temperatures for 4 hours. Then, each plate was read on a Spectramax 190 microplate spectrophotometer (Molecular Devices Corporation) at 570 excitation and 590 nm emission wavelengths, and the percentage of reduction of AlamarBlue™ was determined.

Protein extraction

Log-phase promastigotes (10^8 to 10^9 parasites/mL) and one-day axenic amastigotes (10^8 parasites/mL) were separately used to obtain whole protein extracts as it follows. Parasites were washed by centrifugation ($3\,000 \times g$, 4 °C, 10 min) in sterile cold PBS pH 7.2. Then, the pellets were re-suspended in 1 mL of lysis buffer (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol and 0.6% Triton X-100) and subjected to a minimum of 5 freeze-thaw cycles. After complete parasites lysis, the soluble fraction was obtained by centrifugation ($25\,000 \times g$, 30 min, 4 °C) and the supernatant stored at -80 °C until further use. Whole-protein concentrations were determined by the Lowry method using BSA as a standard protein, as described elsewhere [36].

Enzymatic assays

The serine protease activity of the whole protein extract (5 µg of total protein) was assessed in activation buffer (10 mM Tris-HCl pH 7.5) using a specific fluorogenic peptide substrate (Z-FR-AMC at 1 mM) at a final volume of 60 µL. Samples were incubated (60 min, 37 °C), and the variance in the relative fluorescence was monitored on a Molecular Devices SpectraMax spectrophotometer (Gemini XPS). Inhibition assays were performed by incubation (5 min, 25 °C) of the samples with E-64 [10 mM], PMSF [1 mM], AEBSF [1 mM] and TLCK [100 mM] (Sigma Aldrich Chemical Co., St. Louis, MO, USA); which are the maximum concentrations recommended for each specific inhibitor. The substrate cleavage rate was defined as follows: $v = Ds/Dt$, where v = velocity, Ds = substrate concentration variation and Dt = total reaction time. The self-degradation of the fluorescent peptide substrate was controlled throughout the assay to avoid incorrect readings. The enzymatic activity is expressed as $\text{mmol min}^{-1}.\text{mg of protein}^{-1}$.

Primers design

The primers used in this study were previously designed and used to detect serine proteases and trypanothione peroxidase transcripts [38]. Briefly, the design is based on the *L. (V.) braziliensis* subtilisins (LbrM.13.0860: sense-5' ATCTGGCGATTTCTCCCTTT 3' and antisense-5' GAGCTAACACCAG 3'; LbrM.28.2570: sense-5' CACTGCGCTCCACATACACT 3' and antisense-5' GCCTTCATTTCGAGCTACAGG 3') and trypanothione peroxidase (LbrM.15.1080: sense-5' CTCTGTGGACAGCGAGTACG 3' and antisense-5' TGGGGTCGATGATAAAGAGG 3') sequences recorded in the Genedb database (<http://www.genedb.org>). All the primers were synthesized by Invitrogen Brazil at a concentration of 50 nM and purified by desalting.

RNA extraction and cDNA synthesis

Log-phase promastigotes (10^8 to 10^9 parasites/mL) and one-day axenic amastigotes (10^7 parasites/mL) were separately lysed in 1 mL TRIzol containing 200 μ L of chloroform. RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany). according to the manufacturer's instructions and some modifications. The samples were centrifuged ($10\,000 \times g$, 10 min, 4 °C). The supernatants containing RNA were dissolved with Isopropanol ($12\,000 \times g$, 20 min, 4 °C) and washed with 70% ethanol ($9\,000 \times g$, 5 min, 4 °C). Then, each pellet was re-suspended in 30 to 40 μ L of DEPC-treated water and incubated (10 min, 56 °C) to complete dissolution. The RNA concentrations were measured by spectrophotometry at 260/280 nm and 230/260 nm. cDNA synthesis was performed using the SuperScript® III Kit (Invitrogen Life Technologies, Carlsbad, USA) using maximum 4 μ g of total RNA.

RT-PCR

Primers complementary to the LbrM.13.0860, LbrM.28.2570, LbrM.15.1080 genes were used for Reverse Transcriptional-Polymerase Chain Reaction (RT-PCR) under the following reaction conditions: 10X PCR Buffer, 50 mM $MgCl_2$, 10 mM of each dNTP, 0.3 μ M of each primer and 1 U Platinum™ Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, USA), together with 20 ng of cDNA, adjusted to a final volume of 25 μ L. The amplification cycles were: initial denaturation (94 °C, 3 min), 40 cycles of denaturation, hybridization and extension (95 °C, 20 s; 56 °C, 30 s; 72 °C, 30 s) and a final extension step (72 °C, 5 min) using a PTC-100 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The amplified products were evaluated by 1.2% (w/v) agarose gel electrophoresis and stained with ethidium bromide.

Statistical analysis

GraphPad Prism software (version 8.3.0) was used to calculate IC_{50} values and perform Student's T test, considering $p < 0.05$ as significant. To compare the inhibition regimens (W/i, E-64, PMSF, AEBSF, TLCK) in each isolate, we used 2way ANOVA followed by Dunnett's multiple comparison test.

Additionally, the quantitative variables obtained for each clinical isolate: i) promastigotes Sb^V IC_{50} , (ii) promastigotes Sb^{III} IC_{50} and (iii) axenic amastigotes, (iv) Z-FR-AMC protease activity substrate over promastigotes and (v) axenic amastigotes, (vi) PMSF inhibition over promastigotes and (vii) axenic amastigotes, (viii) AEBSF inhibition over promastigotes and (ix) axenic amastigotes, (x) TLCK inhibition over promastigotes and (xi) axenic amastigotes were normalized and subjected to principal component analysis (PCA). The first three PCs (PC1, PC2, PC3) were used to cluster the clinical isolates using the K-means algorithm clustering method. The total-within cluster sum of squares (twcss) (Equation 1), as a function of the number of clusters, was analysed to determine the optimal number of clusters; where the squared distances between each cluster centroid $(\bar{x}_{c_i})$ and each of its cluster members $(x, \text{ where } x \in c_i)$ are summed over each cluster c_i , N_c being the total number of clusters. Additionally, with the previously normalized data we explored the clusters characteristics by calculating the pairwise distance between

clusters using the normalized values of SbIII IC₅₀, PMSF, AEBSF and TLCK inhibition over promastigotes and axenic amastigotes. The statistical analysis was carried out using R (version 1.1.463).

$$WCSS = \sum_{i=1}^{N_c} \sum_{x \in c_i} d(x, \bar{x}_{c_i})^2 \quad (\text{Equation 1})$$

Results

In vitro susceptibility to antimony

Sb^{III} was able to discriminate the promastigote forms of all isolates but the *p* value between responders (R) and non-responders (NR) was not statistically significant (*p* = 0.07, Fig. 1A). Promastigotes exposed to Sb^V responded differently but the mean IC₅₀ value of the NR group was very close to the IC₅₀ value of the R group, which did not allow a discrimination among them (Fig. 1B). Moreover, the results showed a significant difference between the axenic amastigotes grouped as NR and R exposed to Sb^{III} (*p* < 0.05, Fig. 1C). Conversely, these forms did not respond well to Sb^V exposure and we were not able to discriminate among the groups (Fig. 1D). The IC₅₀ values are summarised in Additional file 1: Table S1.

Sb^V was highly toxic for promastigotes of the R group, with the lowest value being R1 (0.005 ± 0.27 mg/mL) and the highest R5 (0.951 ± 0.2 mg/mL); while for the NR group the lowest value was NR5 (0.295 ± 0.07 mg/mL) and the highest NR2 (3.996 ± 1.4 mg/mL). Regarding the axenic amastigotes, Sb^V did not differ among the isolates since the values were very similar, for example, R1 (0.007 ± 0.16 mg/mL) while NR3 (0.001 ± 0.4 mg/mL). However, Sb^{III} was selectively more toxic for axenic amastigotes from the R group: R3 (9x10⁻⁷ ± 0.39 mg/mL) and R5 (0.0003 ± 0.24 mg/mL), while for NR4 and NR5 (0.009 ± 0.04 mg/mL and 0.0003 ± 0.02 mg/mL).

Quantification of protease activity

All analysed samples presented protease activity, as they were able to hydrolyse the Z-FR-AMC substrate, a chromogenic synthetic peptide appropriate for serine proteases. Promastigotes had a fairly similar activity without inhibition (76 ± 0.07 to 1025 ± 0.07 µmol . min⁻¹ mg of protein⁻¹) as axenic amastigotes (133 ± 0.003 to 1063 ± 1.2 µmol . min⁻¹ mg of protein⁻¹) (Fig. 2). The specificity of serine proteases activity was assessed by measuring the hydrolysis of the Z-FR-AMC substrate in the presence of selective inhibitors (Fig. 2, Table 1). In general, enzymatic activities in presence of TLCK were significantly lower compared to the other serine proteases inhibitors. For promastigotes, the residual activities ranged from 9 ± 0.03 to 241 ± 0.1 µmol . min⁻¹ mg of protein⁻¹; and for axenic amastigotes from 8 ± 0.04 to 161 ± 0.09 µmol . min⁻¹ mg of protein⁻¹. Additionally, AEBSF in promastigotes ranged from 40 ± 0.03 to 937 ± 0.09 µmol . min⁻¹ mg of protein⁻¹, while in axenic amastigotes it ranged 14 ± 0.04 to 766 ± 0.1 µmol . min⁻¹ mg of protein⁻¹. Conversely, PMSF residual activities were the highest among all isolates (See Fig.2 and Table 1).

Furthermore, inhibition assays using E-64, a very well-known cysteine inhibitor, suggests that, under our controlled conditions, Z-FR-AMC did not cleave cysteine residues from the isolates' protein extracts (Fig. 2). The majority of residual activities were similar or even higher than those without inhibition. Exceptionally, only the axenic amastigote extracts from NR1 ($147 \pm 0.1 \mu\text{mol} \cdot \text{min}^{-1} \text{mg of protein}^{-1}$), NR5 ($250 \pm 0.5 \mu\text{mol} \cdot \text{min}^{-1} \text{mg of protein}^{-1}$) and NR7 ($899 \pm 0.2 \mu\text{mol} \cdot \text{min}^{-1} \text{mg of protein}^{-1}$) showed a significant difference when compared to their respective controls.

The inhibition percentage of each serine inhibitor was calculated using each isolate protease activity without inhibition as comparison (see Additional file 2: Figure S1). TLCK showed the strongest inhibition with almost 100% of inhibition for R1, R2, R3, R4, NR1 and NR2 both in promastigotes and axenic amastigotes extracts. Similarly, AEBSF strongly inhibited the same group of isolates (70%) but only for the promastigotes' extracts, while for the axenic amastigotes extracts the inhibition was disperse, only R1, R3 and NR6 were 70% inhibited. On the contrary, PMSF showed the lowest inhibition rates. In promastigotes extracts of R3, R4, NR6 and NR7, and axenic amastigotes extracts of R3, R5, NR3, NR6 and NR7 it inhibited less than 35%. Altogether these results indicated that these isolates have a distinct quantitative profile for serine protease activities in both assayed parasite forms.

Cluster analysis

The first three PCs explained approximately 90% of the data variance. Based on the optimization of the K-means algorithm analysis (see Additional file 3: Figure S2), we selected five clusters: Cluster 1, Cluster 2, Cluster 3, Cluster 4, and Cluster 5 (Fig. 3). There was one cluster that contained, exceptionally, only one clinical isolate (Cluster 3: NR3). One homogenous cluster containing two isolates from the same clinical group (Cluster 2: NR1, NR4) while the remaining three were heterogeneous (Cluster 1: NR6, R5; Cluster 4: R1, R3, NR5, NR7; and Cluster 5: NR2, R2, R4).

Interestingly, in heterogeneous Cluster 1 the distances between both members for all variables ranged from 0.2 to 0.7, except for TLCK inhibition over promastigotes (0.04) and over axenic amastigotes (0.9) (see Additional file 4: Figure S3). Moreover, within Cluster 4, a heterogeneous cluster with two R and two NR isolates, the TLCK inhibition between R and NR were the highest for both promastigotes and axenic amastigotes, while, PMSF and AEBSF values ranged from 0.06 to 0.85 without an established pattern (see Additional file 5: Figure S4). In addition, in Cluster 5, a cluster containing two R and one NR isolate, there was a pattern among variables with all distance values lower than 0.56, exceptionally, TLCK inhibition of NR3 versus R6 axenic amastigotes being the highest distance (0.72) (see Additional file 6: Figure S5). Regarding Sb^{III} distance within Cluster 4 and Cluster 5, there is no value higher than 0.43.

Gene transcripts

Subtilisins (LbrM.13.0860 and LbrM.28.2570) and trypanothione peroxidase (LbrM.15.1080) genes were investigated in both parasite biological forms of the R and NR group, by detection of RNA-transcripts of 200bp, 162bp, and 166bp, respectively (Fig. 4). In general, these RNA-transcripts were detected in all the cDNA samples, but with distinct bands intensity according to the biological form of the parasite. Our data

showed that LbrM.13.0860 gene transcripts (Fig. 4A) were not as well detected as LbrM.28.2570 and LbrM.15.1080 gene transcripts (Fig. 4B and 4C), both in promastigotes and axenic amastigotes of all isolates

Additionally, we performed densitometry analyses to assess the relative abundance of the PCR products (see Additional file 7: Figure S6). The arbitrary unit values (AU) corroborated the absence of amplification of LbrM.13.0860 gene from NR4 (both promastigote and axenic amastigote form), R1 and NR2 (axenic amastigote form). The amplification profile of LbrM.28.2570 gene was similar for all isolates, in both parasite forms, with all values higher than 50 ± 1.9 AU. Similarly, the amplification of LbrM.15.1080 gene was constant in all the assayed isolates with values higher than 60 ± 0.2 AU.

Table 1. Quantification of serine proteases activity in each protein extract

Clinical isolates	Enzymatic activity [mmol min ⁻¹ .mg of protein-1]*							
	<i>Promastigotes</i> **				<i>Axenic amastigotes</i> **			
	W/i ¹	PMSF ²	AEBSF ³	TLCK ⁴	W/i ¹	PMSF ²	AEBSF ³	TLCK ⁴
<i>R1</i>	495 ± 1.2	644 ± 1.2	133 ± 0.8	14 ± 0.05	576 ± 1.5	673 ± 0.02	146 ± 1.6	13 ± 0.01
<i>R2</i>	519 ± 1.7	548 ± 1.7	123 ± 0.05	44 ± 0.1	211 ± 1.2	301 ± 0.8	280 ± 0.9	75 ± 0.002
<i>R3</i>	690 ± 0.8	554 ± 1.6	128 ± 0.06	35 ± 0.09	391 ± 0.9	335 ± 0.9	44 ± 0.09	8 ± 0.04
<i>R4</i>	640 ± 0.03	592 ± 0.7	140 ± 0.03	14 ± 0.29	637 ± 1.3	764 ± 0.2	256 ± 0.9	131 ± 0.7
<i>R5</i>	396 ± 1.5	429 ± 0.03	547 ± 0.05	140 ± 0.03	516 ± 1.5	346 ± 0.01	542 ± 0.8	160 ± 0.9
<i>NR1</i>	595 ± 1.5	890 ± 0.05	60 ± 0.09	9 ± 0.03	350 ± 0.9	421 ± 0.09	158 ± 1.8	35 ± 0.02
<i>NR2</i>	566 ± 1.2	576 ± 1.2	64 ± 0.08	9 ± 0.02	516 ± 1.9	547 ± 0.5	258 ± 1.5	13 ± 0.004
<i>NR3</i>	452 ± 1.7	570 ± 0.8	457 ± 0.9	124 ± 0.1	366 ± 1.1	278 ± 0.1	264 ± 1.2	133 ± 1.1
<i>NR4</i>	76 ± 0.07	215 ± 0.1	40 ± 0.03	15 ± 0.9	167 ± 1.8	224 ± 0.3	152 ± 0.8	151 ± 0.8
<i>NR5</i>	353 ± 0.9	571 ± 0.2	165 ± 0.02	225 ± 0.09	597 ± 1.6	825 ± 0.2	261 ± 1.2	161 ± 0.8
<i>NR6</i>	318 ± 1.2	230 ± 0.2	340 ± 0.03	150 ± 0.02	133 ± 0.03	104 ± 0.2	14 ± 0.04	14 ± 0.002
<i>NR7</i>	1025 ± 0.07	888 ± 0.09	937 ± 0.09	241 ± 0.9	1063 ± 1.2	730 ± 0.4	766 ± 2.1	161 ± 0.9

(*) Serine protease activity of total protein extract [5µg] assessed in activation buffer (10 mM Tris-HCl pH 7.5) using Z-FR-AMC [1 mM] as a specific fluorogenic substrate. (**) Each sample was incubated (60 min, 37 °C) and the variance in the relative fluorescence was monitored. Inhibition assays were performed by incubation (5 min, 25 °C) with (1) no inhibitor, (2) PMSF [1 mM], (3) AEBSF [1 mM] and (4) TLCK [100 mM]. R: Responder; NR: Non-responder.

Discussion

The hypothesis that *L. (V.) braziliensis* clinical isolates have, indeed, different response profiles towards antimony was corroborated in this study by *in vitro* susceptibility experiments using Sb^V and Sb^{III}. It is

known that antimonial therapy failure and resistance do not only depend on host characteristics (nutrition, immune status, comorbidities, inadequate drug doses and treatment follow-up) but also on parasite factors (strains innate susceptibility, virulence factors, biologic profile) [10,11,39–43]. In this context, *Leishmania* spp. capacity to alternate between clonal and sexual reproduction increases their diversity, plasticity and biological capacity to expand under different stress conditions [44]. In fact, exposure and resistance towards several drugs could be a developed capacity to adapt and survive within the final host.

The IC₅₀ values obtained in this study showed that *L. (V.) braziliensis* clinical isolates were better distinguished due to the Sb^{III} IC₅₀ values over the axenic amastigote forms. Promastigotes showed a non-significant 1.9-fold difference while axenic amastigotes presented a significant 3.1-fold difference between the R and NR group. However, R5 and NR2 promastigotes did not follow this premise, since they had almost the same IC₅₀ value. Sb^V was toxic for promastigotes of each clinical isolate but it showed a non-significant 2-fold difference between the R and NR group, meaning that it did not distinguish one group from the other. These results are in concordance with another study using *L. (V.) braziliensis* promastigotes, isolated from clinical samples of the same endemic area as this study, that showed similar Sb^V IC₅₀ values for R and NR promastigotes [46]. Similarly, another study showed minimum and maximum Sb^V IC₅₀ values of 0.37 ± 0.09 and 5.75 ± 0.26 mg/mL for *L. (V.) braziliensis* promastigotes [39]. In contrast, another study showed great variance among Sb^V IC₅₀ values, with a 3-fold difference between promastigotes from poor/bad and cured/good clinical response to antimonial therapy [45]. Therefore, *L. (V.) braziliensis* isolates, circulating in the state of Rio de Janeiro, share common genetic traits but have very different responses to Glucantime[®] treatment [39,45,46]. The varied *in vitro* susceptibility showed in this study reinforce the typical phenotypical heterogeneity reported for this specie.

Even though intracellular amastigote is the “gold standard” model to test susceptibility, axenic amastigotes have been previously used to evaluate response towards Sb^V, which was found to be stage and strain-specific in *L. (L.) donovani* [47] and *L. (L.) infantum* [48]. Furthermore, it is important to remember that assays with intracellular amastigotes are difficult to standardize and strongly depend on the type of host cell and the medium used [40,49], which might bias the final results. Therefore, axenic amastigotes are a feasible model to characterize *in vitro* phenotypes since they maintain similar morphology, metabolic and virulence genes expression profiles as intracellular amastigotes [49–52].

The fact that promastigotes and axenic amastigotes have different *in vitro* susceptibilities, led us to evaluate the possible correlation of these phenotypes with virulence factors – serine proteases – under a biochemical approach. The serine protease activity measured by Z-FR-AMC, a substrate for serine proteases such as cathepsins, kallikrein and plasmin [53], was relatively the same among promastigotes and axenic amastigotes. Serine enzymatic activity is generally confirmed by using PMSF, AEBSF and TLCK inhibitors [38]. In addition, PMSF and AEBSF are known to inhibit a broad range of serine proteases including subtilisins while TLCK has a greater preference for *Leishmania* spp. oligopeptidases [21,54–

56]. The inhibition profiles seen in this study were different depending on each inhibitor assayed, suggesting that there are different groups or isoforms of serine proteases as part of the protease-network of each clinical isolate, and its respective biological forms.

Furthermore, cluster analysis was performed to know if the *in vitro* phenotypes correlate with the clinical response of each clinical isolate. Cluster 3 contained only one isolate, NR3, which was closely similar to the isolates from homogeneous Cluster 2 (NR1, NR4). These clusters suggest that *in vitro* susceptibility responses could predict clinical response. On the other hand, the presence of heterogeneous clusters was better understood once we compared the pairwise distance between the members of each cluster. This analysis showed a similar pattern of PMSF and AEBSF inhibition over both parasite biological forms while TLCK inhibition was significantly more varied among the heterogeneous clusters. This may suggest that, independently of each isolate clinical response, they have common enzymatic and *in vitro* Sb^{III} susceptibility traits. This observation supports the hypothesis that parasites of the subgenus *Viannia* are a polyclonal population with high genetic variability and, consequently, phenotypic diversity [20,57]. Other studies have shown that genetic and phenotypic characteristics among different *L. (V.) braziliensis* strains are associated with different clinical manifestations and drug resistance [46,58]. Additionally, the variation among serine proteases inhibition indicate specific traits among the conformation of their serine protease network since the specificity of PMSF, AEBSF and TLCK is different [27,54,59]. Therefore, *L. (V.) braziliensis* isolates can be composed of distinct enzymatic profiles that influence host-parasite interactions and, consequently, the success or failure of specific drugs.

In addition, based on the previously role seen for subtilisins as maturases of TXNPx [18,38], our study examined the expression of subtilisin transcripts from SB clan and S8 family (LbrM.13.0860 and LbrM.28.2570) and TXNPx (LbrM.15.1080). In general, almost all isolates (promastigotes and axenic amastigotes) expressed the three mentioned genes, however, densitometry analysis revealed lower intensities of subtilisin LbrM.13.0860 for few NR isolates. This finding suggests that subtilisins and TXNPx are expressed by *L. (V.) braziliensis* clinical isolates, but its level of expression varies depending on the isolate phenotype. Interestingly, a proteomic study using lab-generated *L. (V.) braziliensis* NR/resistant strains showed that TXNPx abundance was significantly higher than R/susceptible strains [60]. Additionally, another study using *L. (L.) donovani* clinical isolates correlated TXNPx higher amplification levels with antimony resistance phenotypes [32]. However, is important to bear in mind that this study did RT-PCR, which is a qualitative method and densitometry is a semi-quantitative analysis to measure bands intensity as absence/presence of transcripts. Thus, qPCR, which is more sensitive quantitative method, needs to be performed in order to quantify these transcripts expression and explore their relationship with different *in vitro* phenotypes.

Conclusions

Leishmania spp. resistance towards antimony treatment is an increasing multifactorial phenomenon, and several virulence factors have been studied and related to resistant phenotypes in clinical isolates [39,61–63]. The present study aids to this discussion by bringing proteases to focus, one of the most

studied parasite virulence factors in the last 30 years [18,22,64]. The data presented here shows the possibility to use serine proteases to *in vitro* characterize and distinguish *L. (V.) braziliensis* clinical isolates with different responses towards antimony. There is an important heterogeneity among the assayed isolates suggesting that some of them have different innate abilities to adapt to different environments and biological filters. Moreover, serine proteases activity and differential transcripts expression suggested that each isolate may have independent means to adapt to their niches and that *in vitro* response to antimony needs further characterization, especially in clinical isolates causing ATL.

Abbreviations

ATL: American Tegumentary Leishmaniasis; R: Responder; NR: Non-responder; TryS: trypanothione synthetase; TR: trypanothione reductase; TXN: tryparedoxin; TXNPx: Tryparedoxin peroxidase; Sb^V: Glucantime[®]; Sb^{III}: antimony potassium tartrate; FBS: Fetal Bovine Serum; Z-FR-AMC: N-benzyloxycarbonyl-L-phenylalanyl-L-arginine 7-amino-4-methylcoumarin; E-64: trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane; PMSF: Phenylmethylsulfonyl fluoride; AEBSF: 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochlorid; TLCK: Tosyl-L-lysyl-chloromethane hydrochloride.

Declarations

Availability of data and materials

All data generated or analysed during this study are included in this published article, and its additional information files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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Author's contribution

AZP conducted all the experiments, data analysis and wrote the original manuscript. GDL, LCF and FSS helped in the experiments planification and execution. FCS and LFCM provided the frozen parasite isolates. AF, MIFP, AOS provided information about the clinical origin of the isolates. LAM conducted the statistical analysis. GDL and CRA critically reviewed the data analysis and final manuscript.

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References

1. WHO. Leishmaniasis [Internet]. 2019 [cited 2019 Nov 26]. Available from: <https://www.who.int/news-room/fact-sheets/detail/leishmaniasis>
2. Akhoundi M, Kuhls K, Cannet A, Votýpka J, Marty P, Delaunay P, et al. A Historical Overview of the Classification, Evolution, and Dispersion of Leishmania Parasites and Sandflies. *PLOS Neglected Tropical Diseases*. 2016 Mar 3;10(3):e0004349.
3. Lima EB de, Porto C, Motta JOC da, Sampaio RNR. Tratamento da Leishmaniose Tegumentar Americana. *Anais Brasileiros de Dermatologia*. 2007 Apr;82(2):111–24.
4. Saheki MN, Lyra MR, Bedoya-Pacheco SJ, Antônio L de F, Pimentel MIF, Salgueiro M de M, et al. Low versus high dose of antimony for American cutaneous leishmaniasis: A randomized controlled blind non-inferiority trial in Rio de Janeiro, Brazil. *PLoS One* [Internet]. 2017 May 30 [cited 2020 Jun 16];12(5). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5448803/>
5. Frézard F, Demicheli C, Ferreira CS, Costa MAP. Glutathione-Induced Conversion of Pentavalent Antimony to Trivalent Antimony in Meglumine Antimoniate. *Antimicrob Agents Chemother*. 2001 Mar;45(3):913–6.
6. Frézard F, Demicheli C, Kato KC, Reis PG, Lizarazo-Jaimes EH. Chemistry of antimony-based drugs in biological systems and studies of their mechanism of action. *Reviews in Inorganic Chemistry*. 2013 May 1;33(1):1–12.
7. Wyllie S, Cunningham ML, Fairlamb AH. Dual Action of Antimonial Drugs on Thiol Redox Metabolism in the Human Pathogen *Leishmania donovani*. *J Biol Chem*. 2004 Sep 17;279(38):39925–32.
8. Flohé L, Steinert P, Hecht H-J, Hofmann B. Tryparedoxin and tryparedoxin peroxidase. *Meth Enzymol*. 2002;347:244–58.
9. Yan S, Li F, Ding K, Sun H. Reduction of pentavalent antimony by trypanothione and formation of a binary and ternary complex of antimony(III) and trypanothione. *J Biol Inorg Chem*. 2003 Jul;8(6):689–97.
10. Croft SL, Sundar S, Fairlamb AH. Drug Resistance in Leishmaniasis. *Clinical Microbiology Reviews*. 2006 Jan 1;19(1):111–26.

11. Ponte-Sucre A, Gamarro F, Dujardin J-C, Barrett MP, López-Vélez R, García-Hernández R, et al. Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. *PLoS Negl Trop Dis*. 2017 Dec;11(12):e0006052.
12. de Moura TR, Santos MLB, Braz JM, Santos LFVC, Aragão MT, de Oliveira FA, et al. Cross-resistance of *Leishmania infantum* isolates to nitric oxide from patients refractory to antimony treatment, and greater tolerance to antileishmanial responses by macrophages. *Parasitol Res*. 2016 Feb;115(2):713–21.
13. Ouakad M, Vanaerschot M, Rijal S, Sundar S, Speybroeck N, Kestens L, et al. Increased metacyclogenesis of antimony-resistant *Leishmania donovani* clinical lines. *Parasitology*. 2011 Sep;138(11):1392–9.
14. Vanaerschot M, De Doncker S, Rijal S, Maes L, Dujardin J-C, Decuypere S. Antimonial resistance in *Leishmania donovani* is associated with increased in vivo parasite burden. *PLoS ONE*. 2011;6(8):e23120.
15. McKerrow JH, Caffrey C, Kelly B, Loke P, Sajid M. PROTEASES IN PARASITIC DISEASES. *Annu Rev Pathol Mech Dis*. 2006 Feb;1(1):497–536.
16. Silva-Almeida M, Pereira BA, Ribeiro-Guimarães M, Alves C. Proteinases as virulence factors in *Leishmania* spp. infection in mammals. *Parasites & Vectors*. 2012;5(1):160.
17. Barrett AJ. Classification of peptidases. *Meth Enzymol*. 1994;244:1–15.
18. Alves CR, Souza RS de, Charret K dos S, Côrtes LM de C, Sá-Silva MP de, Barral-Veloso L, et al. Understanding serine proteases implications on *Leishmania* spp lifecycle. *Experimental Parasitology*. 2018 Jan;184:67–81.
19. Borsari C, Quotadamo A, Ferrari S, Venturelli A, Cordeiro-da-Silva A, Santarem N, et al. Chapter Two - Scaffolds and Biological Targets Avenue to Fight Against Drug Resistance in Leishmaniasis. In: Botta M, editor. *Annual Reports in Medicinal Chemistry* [Internet]. Academic Press; 2018 [cited 2019 Nov 27]. p. 39–95. (Neglected Diseases: Extensive Space for Modern Drug Discovery; vol. 51). Available from: <http://www.sciencedirect.com/science/article/pii/S0065774318300022>
20. Cysne-Finkelstein L, Silva-Almeida M, Pereira BAS, dos Santos Charret K, Bertho ÁL, Bastos LS, et al. Evidence of Subpopulations with Distinct Biological Features Within a *Leishmania* (*Viannia*) *braziliensis* Strain. *Protist*. 2018 Feb 1;169(1):107–21.
21. da Silva-Lopez RE, Giovanni-De-Simone S. *Leishmania* (*Leishmania*) *amazonensis*: purification and characterization of a promastigote serine protease. *Experimental Parasitology*. 2004 Jul 1;107(3):173–82.
22. Guedes HLM, Rezende NJM, Fonseca MA, Salles CMC, Rossi-Bergmann B, De-Simone G. Identification of Serine Proteases from *Leishmania braziliensis*. 2014;62(5–6):373–381.
23. Machado P de A, Carneiro MPD, Sousa-Batista A de J, Lopes FJP, Lima APC de A, Chaves SP, et al. Leishmanicidal therapy targeted to parasite proteases. *Life Sciences*. 2019 Feb 15;219:163–81.
24. Pereira B, Souza-Silva F, Silva-Almeida M, Santos-de-Souza R, Oliveira L, Ribeiro-Guimaraes M, et al. Proteinase Inhibitors: A Promising Drug Class for Treating Leishmaniasis. *Current Drug Targets*.

2014 Nov 7;15(12):1121–31.

25. Silva-López RE, Santos TR dos, Morgado-Díaz JA, Tanaka MN, De Simone SG. Serine protease activities in *Leishmania (Leishmania) chagasi* promastigotes. 2010 [cited 2020 Mar 3]; Available from: <https://www.arca.fiocruz.br/handle/icict/21057>
26. Swenerton RK, Knudsen GM, Sajid M, Kelly BL, McKerrow JH. *Leishmania* Subtilisin Is a Maturase for the Trypanothione Reductase System and Contributes to Disease Pathology. *J Biol Chem*. 2010 Oct 8;285(41):31120–9.
27. Leite KM. Análise da participação da oligopeptidase B e triparedoxina peroxidase citoplasmática na virulência de *Leishmania (Leishmania) amazonensis*. [Internet] [text]. Universidade de São Paulo; 2015 [cited 2020 Mar 6]. Available from: <http://www.teses.usp.br/teses/disponiveis/42/42135/tde-14042016-100555/>
28. Blackman MJ. Malarial proteases and host cell egress: an “emerging” cascade. *Cell Microbiol*. 2008 Oct;10(10):1925–34.
29. Miller SA, Thathy V, Ajioka JW, Blackman MJ, Kim K. TgSUB2 is a *Toxoplasma gondii* rhoptry organelle processing proteinase. *Mol Microbiol*. 2003 Aug;49(4):883–94.
30. Matrangolo FSV, Liarte DB, Andrade LC, de Melo MF, Andrade JM, Ferreira RF, et al. Comparative proteomic analysis of antimony-resistant and -susceptible *Leishmania braziliensis* and *Leishmania infantum chagasi* lines. *Molecular and Biochemical Parasitology*. 2013 Agosto;190(2):63–75.
31. Wyllie S, Vickers TJ, Fairlamb AH. Roles of Trypanothione S-Transferase and Tryparedoxin Peroxidase in Resistance to Antimonials. *Antimicrobial Agents and Chemotherapy*. 2008 Apr 1;52(4):1359–65.
32. Wyllie S, Mandal G, Singh N, Sundar S, Fairlamb AH, Chatterjee M. Elevated levels of tryparedoxin peroxidase in antimony unresponsive *Leishmania donovani* field isolates. *Molecular and Biochemical Parasitology*. 2010 Oct 1;173(2):162–4.
33. Brahim LR, Valette-Rosalino CM, Antônio L de F, Pimentel MIF, Lyra MR, Paes LE de C, et al. Low dose systemic or intralesional meglumine antimoniate treatment for American tegumentary leishmaniasis results in low lethality, low incidence of relapse, and low late mucosal involvement in a referral centre in Rio de Janeiro, Brazil (2001-2013). *Memórias do Instituto Oswaldo Cruz*. 2017 Dec;112(12):838–43.
34. Cataldo JI, Conceição-Silva F, Antônio L de F, Schubach A de O, Marzochi MC de A, Valette-Rosalino CM, et al. Favorable responses to treatment with 5 mg SbV/kg/day meglumine antimoniate in patients with American tegumentary leishmaniasis acquired in different Brazilian regions. *Rev Soc Bras Med Trop*. 2018 Dec;51(6):769–80.
35. Shimony O, Jaffe CL. Rapid fluorescent assay for screening drugs on *Leishmania* amastigotes. *J Microbiol Methods*. 2008 Oct;75(2):196–200.
36. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*. 1951;193:265–75.

37. Santos-de-Souza R, Monteiro de Castro Côrtes L, dos Santos Charret K, Cysne-Finkelstein L, Alves CR, Souza-Silva F. Serine Proteinases in *Leishmania (Viannia) braziliensis* Promastigotes Have Distinct Subcellular Distributions and Expression. *International Journal of Molecular Sciences*. 2019;20(6):1315.
38. Baptista C, Miranda L de FC, Madeira M de F, Leon LLP, Conceição-Silva F, Schubach A de O. In Vitro Sensitivity of Paired *Leishmania (Viannia) braziliensis* Samples Isolated before Meglumine Antimoniate Treatment and after Treatment Failure or Reactivation of Cutaneous Leishmaniasis. *Dis Markers* [Internet]. 2015 [cited 2020 Aug 24];2015. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4329789/>
39. Jeddi F, Piarroux R, Mary C. Antimony Resistance in *Leishmania*, Focusing on Experimental Research [Internet]. *Journal of Tropical Medicine*. 2011 [cited 2020 Mar 3]. Available from: <https://www.hindawi.com/journals/jtm/2011/695382/>
40. Rodrigues AM, Hueb M, Santos TARR dos, Fontes CJF. Fatores associados ao insucesso do tratamento da leishmaniose cutânea com antimoniato de meglumina. *Revista da Sociedade Brasileira de Medicina Tropical*. 2006 Apr;39(2):139–45.
41. Rojas R, Valderrama L, Valderrama M, Varona MX, Ouellette M, Saravia NG. Resistance to Antimony and Treatment Failure in Human *Leishmania (Viannia)* Infection. *J Infect Dis*. 2006 May 15;193(10):1375–83.
42. Soto J, Soto P. Current situation and future of antileishmanial therapy in Colombia. *Biomédica*. 2006 Oct 1;26:194–206.
43. Heitman J. Sexual Reproduction and the Evolution of Microbial Pathogens. *Current Biology*. 2006 Sep;16(17):R711–25.
44. Gagini T, de Oliveira Schubach A, de Fatima Madeira M, Maria Valette-Rosalino C, Fernandes Pimentel MI, da Silva Pacheco R. Genotypic profiles of *Leishmania (Viannia) braziliensis* strains from cutaneous leishmaniasis patients and their relationship with the response to meglumine antimoniate treatment: a pilot study. *Parasite* [Internet]. [cited 2020 Jun 15];24. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5621350/>
45. Azeredo-Coutinho RBG, Mendonça SCF, Callahan H, Portal AC, Grögl M. Sensitivity of *leishmania braziliensis* promastigotes to meglumine antimoniate (glucantime) is higher than that of other *leishmania* species and correlates with response to therapy in american tegumentary leishmaniasis. *Journal of Parasitology*. 2007 Jun 1;93(3):688–93.
46. Ephros M, Bitnun A, Shaked P, Waldman E, Zilberstein D. Stage-Specific Activity of Pentavalent Antimony against *Leishmania donovani* Axenic Amastigotes. *Antimicrob Agents Chemother*. 1999 Feb;43(2):278–82.
47. Sereno D, Cavaleyra M, Zemzoumi K, Maquaire S, Ouaisi A, Lemesre JL. Axenically Grown Amastigotes of *Leishmania infantum* Used as an In Vitro Model To Investigate the Pentavalent Antimony Mode of Action. *Antimicrob Agents Chemother*. 1998 Dec;42(12):3097–102.

48. Ait-Oudhia K, Gazanion E, Vergnes B, Oury B, Sereno D. Leishmania antimony resistance: what we know what we can learn from the field. *Parasitol Res.* 2011 Nov;109(5):1225–32.
49. Gomes CB, -Silva FS, Charret KDS, Pereira BAS, Finkelstein LC, Santos-de-Souza R, et al. Increasing in cysteine proteinase B expression and enzymatic activity during in vitro differentiation of *Leishmania (Viannia) braziliensis*: First evidence of modulation during morphological transition. *Biochimie.* 2017 Feb;133:28–36.
50. Nasereddin A, Schweynoch C, Schonian G, Jaffe CL. Characterization of *Leishmania (Leishmania) tropica* axenic amastigotes. *Acta Tropica.* 2010 Jan 1;113(1):72–9.
51. Saunders EC, Ng WW, Kloehn J, Chambers JM, Ng M, McConville MJ. Induction of a Stringent Metabolic Response in Intracellular Stages of *Leishmania mexicana* Leads to Increased Dependence on Mitochondrial Metabolism. *PLoS Pathog* [Internet]. 2014 Jan 23 [cited 2020 Aug 5];10(1). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3900632/>
52. Jara M, Berg M, Caljon G, de Muylder G, Cuypers B, Castillo D, et al. Macromolecular biosynthetic parameters and metabolic profile in different life stages of *Leishmania braziliensis*: Amastigotes as a functionally less active stage. *PLoS One* [Internet]. 2017 Jul 25 [cited 2020 Aug 5];12(7). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5526552/>
53. Morita T, Kato H, Iwanaga S, Takada K, Kimura T, Sakakibara S. New Fluorogenic Substrates for α -Thrombin, Factor Xa, Kallikreins, and Urokinase. *The Journal of Biochemistry.* 1977;82(5):1495–8.
54. Basak S, Stewart NA, Chrétien M, Basak A. Aminoethyl benzenesulfonyl fluoride and its hexapeptide (Ac-VFRSLK) conjugate are both in vitro inhibitors of subtilisin kexin isozyme-1. *FEBS Letters.* 2004 Aug 27;573(1):186–94.
55. Silva CL. Caracterização bioquímica da Prolil Oligopeptidase de *Leishmania chagasi*, um potencial alvo quimioterápico para as Leishmanioses. 2014 Jan 23 [cited 2020 Mar 6]; Available from: <https://repositorio.unb.br/handle/10482/16720>
56. Wang R, Liu S, Wang J, Dong Q, Xu L, Rui Q. Purification, characterization and identification of a senescence related serine protease in dark-induced senescent wheat leaves. *Phytochemistry.* 2013 Nov 1;95:118–26.
57. Cupolillo E, Brahim LR, Toaldo CB, Oliveira-Neto MP de, Brito MEF de, Falqueto A, et al. Genetic Polymorphism and Molecular Epidemiology of *Leishmania (Viannia) braziliensis* from Different Hosts and Geographic Areas in Brazil. *Journal of Clinical Microbiology.* 2003 Jul 1;41(7):3126–32.
58. Schriefer A, Schriefer ALF, Góes-Neto A, Guimarães LH, Carvalho LP, Almeida RP, et al. Multiclonal *Leishmania braziliensis* Population Structure and Its Clinical Implication in a Region of Endemicity for American Tegumentary Leishmaniasis. *Infection and Immunity.* 2004 Jan 1;72(1):508–14.
59. Chu N-M, Chao Y, Bi R-C. The 2 Å crystal structure of subtilisin E with PMSF inhibitor. *Protein Eng Des Sel.* 1995 Mar 1;8(3):211–5.
60. Moreira D de S, Pescher P, Laurent C, Lenormand P, Späth GF, Murta SMF. Phosphoproteomic analysis of wild-type and antimony-resistant *Leishmania braziliensis* lines by 2D-DIGE technology. *PROTEOMICS.* 2015;15(17):2999–3019.

61. Kumar D, Singh R, Bhandari V, Kulshrestha A, Negi NS, Salotra P. Biomarkers of antimony resistance: need for expression analysis of multiple genes to distinguish resistance phenotype in clinical isolates of *Leishmania donovani*. *Parasitology Research*. 2012 Jul;111(1):223–30.
62. Oliaee RT, Sharifi I, Afgar A, Kareshk AT, Asadi A, Heshmatkhah A, et al. Unresponsiveness to meglumine antimoniate in anthroponotic cutaneous leishmaniasis field isolates: analysis of resistance biomarkers by gene expression profiling. *Trop Med Int Health*. 2018 Apr 30;
63. Rugani JN, Gontijo CMF, Frézard F, Soares RP, do Monte-Neto RL. Antimony resistance in *Leishmania* (*Viannia*) *braziliensis* clinical isolates from atypical lesions associates with increased ARM56/ARM58 transcripts and reduced drug uptake. *Mem Inst Oswaldo Cruz* [Internet]. 2019 Aug 15 [cited 2020 Feb 20];114. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6697410/>
64. Gómez MA, Olivier M. Proteases and phosphatases during leishmania-macrophage interaction: Paving the road for pathogenesis. *Virulence*. 2010 Jul;1(4):314–8.

Figures

Figure 1

Promastigotes and axenic amastigotes in vitro susceptibility profile to antimony. Both parasites forms of each isolate (n=12), 4×10^6 promastigotes/well and 5×10^5 axenic amastigotes/well were exposed to serial dilutions of trivalent (SbIII) and pentavalent (Sbv) antimonial for 48 hours and 24 hours in 96-well plates, respectively. The half-maximal inhibitory concentration (IC₅₀ [mg/mL]) of parasites viability was measured using a fluorescence method with AlamarBlue reagent. Parasites isolated before treatment of patients with ATL cured after antimonial therapy (R: ●) or with poor clinical response to therapy, either therapeutic failure or relapse (NR: ○). The data is presented by boxplot diagrams as mean and standard deviation ($\pm$) of three biological replicates. Asterisks indicate statistically significant differences: * $p < 0.05$

Figure 2

Enzymatic residual activity of whole soluble proteins from the clinical isolates. In these assays, protein extract [5 μ g] of promastigotes and axenic amastigotes were measured using a specific fluorogenic peptide substrate of serine proteinases, z-FR-AMC [1mM]. The enzymatic activities (mmol min⁻¹.mg of protein⁻¹) were assessed without inhibitor and in the presence of inhibitors: E-64 [10 μ M], PMSF [1mM],

AEBSF [1 mM] and TLCK [100 μ M]. R: Responder; NR: Non-responder. The results are shown as mean and the standard deviation ($\pm$) of three independent experiments. Asterisks indicate statistically significant differences: * $p < 0.05$; ** $p < 0.0005$; # $p < 0.0001$.

Figure 3

Cluster analysis of *L. (V.) braziliensis* clinical isolates. Principal components analysis (PCA) was performed to group clinical isolates based on the normalized quantitative variables. Each point represents the first three principal components of a clinical isolate. The points are coloured according to the cluster they belong to, C1 (●): NR6 and R3; C2 (●): R1, R3, NR5 and NR7; C3 (●): NR3; C4 (●): NR2, R2 and R4; C5 (●): NR1 and NR4.

Figure 4

Detection of RNA transcripts in the isolates. The RT-PCR assays were performed using specific primers sets: (A) LbrM.13.0860, subtilisin gene (200 bp); (B) LbrM.28.2570, subtilisin gene (162 bp); (C) LbrM.15.1080, trypanothione peroxidase gene (166 bp). The amplified products of clinical isolates were assessed by agarose gels electrophoresis (2% agarose). Lanes (L) are 100 bp DNA ladder (Invitrogen), used as a molecular weight marker.

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