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

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Research

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Abstract

Glucantime® (Sb^{V}) is the first-line treatment against leishmaniasis in South America. Its effectiveness has been associated with modulation of the parasite detoxification system that, in turn, is related to serine proteases such as subtilisins. In this study, 12 *Leishmania (Viannia) braziliensis* isolates from patients that presented clinical cure (Responders - R) and relapse or therapeutic failure (Non-responders - NR) were used. The parasites were assessed by *in vitro* susceptibility to Sb^{III} and Sb^{V}, serine proteases activity – measured with z-FR-AMC as substrate and specific inhibitors – and expression of subtilisins and tryparedoxin-peroxidase (TXNPx). *In vitro* susceptibility of axenic amastigotes to Sb^{III} showed a significant difference between R and NR groups. TLCK inhibited almost 100 % of activity in both axenic amastigotes and promastigotes while AEBSF inhibited around 70 %, and PMSF showed lower inhibition of specific isolates. Principal component and clustering analysis yielded one homogeneous cluster with only NR isolates and three heterogeneous clusters with R and NR isolates. Additionally, transcripts of subtilisins (LbrM.13.0860 and LbrM.28.2570) and TXNPx (LbrM.15.1080) were detected in promastigotes and axenic amastigotes from both groups. The data presented here show a phenotypic heterogeneity among the parasites, suggesting that exploration of *in vitro* phenotypes based on Sb^{III} and serine proteases profiles can aid in the characterization of *L. (V.) braziliensis* clinical isolates.

Introduction

Leishmaniasis is a neglected tropical disease that affects around 1 million people worldwide, causing 26 000 to 65 000 deaths per year (1). *Leishmania* spp. are eukaryotic protozoan parasites responsible for a broad range of clinical manifestations, such as cutaneous, mucocutaneous and visceral forms, which depend on the parasite species and immune state of the mammalian host (2). In the American continent, *Leishmania mexicana* complex and subgenus *Viannia* species cause cutaneous and mucocutaneous forms that can develop into localized, disseminated or diffuse lesions, which is known as American Tegumentary leishmaniasis (ATL) (1).

A pentavalent antimony 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 (low and high dose) vary according to therapeutic response patterns seen in specific geographical areas (3–5). Its mechanism of action is still not completely understood, but, it has been well accepted the hypothesis that Sb^{V} needs to be reduced to its trivalent form (Sb^{III}), either directly by the parasite or within the host macrophages, to excel leishmanicidal activity (6–8).

Moreover, the most abundant *Leishmania* spp. thiol, trypanothione reductase [$T(SH)_2$], is part of the parasite trypanothione reductase system which is a unique detoxification defence mechanism that relies on four key enzymes: trypanothione synthetase (TryS), trypanothione reductase (TR) and tryparedoxin (TXN) and tryparedoxin peroxidase (TXNPx) (9). This system is involved in the detoxification of metal ions, including reduction of Sb^{V} to Sb^{III}, within the macrophage phagolysosome (6,7). It is also

hypothesised that Sb^V reduction might happen through the oxidation of $T(SH)_2$ forming a stable complex – $Sb^{III}(TS)_2$ – that could be pumped out of the cells through parasite membrane transporters (6,9). Interestingly, parasite's resistance towards Sb^V has been associated with increased trypanothione levels and a decreased capacity to reduce Sb^V (10–12). Indeed, increased abundance of TXNPx has been correlated with an enhanced thiol redox potential in Sb^V resistant parasites, not only in lab-generated strains (13,14) but also in clinical isolates (15).

Furthermore, some studies have shown that parasite enzymes are involved in the reduction of Sb^V to Sb^{III} and *Leishmania* spp. resistance towards Sb^V (10,16,17). Particularly, serine proteases have been extensively studied in American *Leishmania* spp. due to their functional interrelationship in parasite physiology and their potential as therapeutic targets (18–24). Experimental evidence has demonstrated that these enzymes can act as signal peptidases, maturases of other proteins, and can have a metacaspase-like activity (20,25). Among them, subtilisins are proposed to have a functional interrelationship with other *Leishmania* spp. proteins, suggesting key functions of these enzymes in parasite physiology such as modulation of the trypanothione reductase system through direct action over TXNPx (18,25).

In this context, subtilisins are correlated to the balance of cytosolic and mitochondrial TXNPx levels in *L. (L.) donovani* clinical isolates. Subtilisin knockout promastigotes of these parasites failed to differentiate into viable amastigotes and their TXNPx peptide abundance was decreased, which suggested that subtilisins can act as maturases of specific proteins or pathways (25). Although, in *L. (V.) braziliensis,* subtilisins have distinct subcellular distributions and expression (26) it has not been assessed yet if these enzymes excel a similar role during parasite differentiation; and if impairment of trypanothione reductase system has any relation with Sb^V resistant phenotypes.

This study aims to contribute in the understanding of Glucantime® susceptibility based on *L. (V.) braziliensis* clinical isolates. Gathered data here incorporate new information on the heterogenic profile of clinical isolates, assessing their *in vitro* susceptibility towards Sb^V and Sb^{III} as a direct relation to serine proteases of this parasite. Additionally, transcripts of some enzymes of the parasite detoxification system as subtilisins (LbrM.13.0860 and LbrM.28.2570) and TXNPx (LbrM.15.1080) were accessed.

Results

In vitro susceptibility to antimony

Both, Sb^{III} and Sb^V, were unable to discriminate susceptibility profiles between promastigote forms of responders (R) and non-responders (NR) isolates. The mean IC_{50} value of R versus NR group was very close which did not allow discrimination among them (Fig. 1A and 1B). Meanwhile, 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 IC_{50} values are summarised in Supplementary file 1.

Sb^V was toxic for only two isolates, R1 (0.005 ± 0.27 mg/mL) and R5 (0.056 ± 0.14 mg/mL) while the IC_{50} values of all other isolates were ≥ 0.295 mg/mL (Supplementary file 1). 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).

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 (W/i) between promastigotes (76 ± 0.07 to 1025 ± 0.07 µmol . min^{-1} mg of $protein^{-1}$) and axenic amastigotes (133 ± 0.003 to 1063 ± 1.2 µmol . min^{-1} mg of $protein^{-1}$) (Fig. 2 and Supplementary file 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 and Supplementary file 2). 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 $protein^{-1}$, while 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 $protein^{-1}$, and in axenic amastigotes ranged from 14 ± 0.04 to 766 ± 0.1 µmol . min^{-1} mg of $protein^{-1}$. Conversely, PMSF residual activities were the highest among all isolates (Fig. 2).

Furthermore, inhibition assays using E-64, a cysteine protease inhibitor, suggests that, under our controlled conditions, Z-FR-AMC was not cleaved by cysteine proteases from the isolates' protein extracts (Fig. 2). The majority of residual activities were similar or even higher than those W/i. Exceptionally, only the axenic amastigote extracts from NR1 (147 ± 0.1 µmol . min^{-1} mg of $protein^{-1}$), NR5 (250 ± 0.5 µmol . min^{-1} mg of $protein^{-1}$) and NR7 (899 ± 0.2 µmol . min^{-1} mg of $protein^{-1}$) showed a significant difference when compared to their respective W/i controls.

The inhibition percentage of each serine inhibitor was calculated using each isolate W/i as a comparison (Supplementary file 3). 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 dispersed, 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 80 % of the data variance. Based on our analysis of the total sum of squares as a function of the number of clusters (Supplementary file 4) we opted for using 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 the 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 axenic amastigotes (0.9) (Supplementary file 5). Moreover, within Cluster 4, another heterogeneous cluster with two R and two NR isolates, TLCK inhibition between R and NR was the highest for both promastigotes and axenic amastigotes, while, PMSF and AEBSF values ranged from 0.06 to 0.85 without an established pattern (Supplementary file 6). Also, 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) (Supplementary file 7). 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 tryparedoxin 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, when compared to the profile of the transcripts of the 40S Ribosomal Protein S8 housekeeping gene LbrM.24.2160 which remained constant for all isolates (Fig. 4D). 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.

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) (12–15,27–29). 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 (30). Exposure and resistance towards several drugs could be a developed capacity to adapt and survive within hosts.

The IC_{50} values obtained in this study showed that *L. (V.) braziliensis* clinical isolates were better distinguished due to the Sb^{III} IC_{50} values over the axenic amastigote forms. Promastigotes showed a 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 observation, since they had almost the same IC_{50} value. Sb^{V} was toxic for promastigotes of each clinical isolate and showed a 2-fold difference between the R and NR group. These results are in concordance with another study using *L. (V.) braziliensis* promastigotes, isolated from clinical samples of the same endemic area, that showed Sb^{V} IC_{50} minimum and maximum values of 0.37 ± 0.09 and 5.75 ± 0.26 mg/mL (13). Similarly, another study showed great variance among Sb^{V} IC_{50} values, with a 3-fold difference between promastigotes from poor/bad and cured/good clinical response to antimonial therapy (31). It has been observed that *L. (V.) braziliensis* isolates, circulating in the state of Rio de Janeiro, share common genetic traits but have different responses to Glucantime® treatment (32). The varied *in vitro* susceptibility showed in this study reinforces the phenotypical heterogeneity reported for this species.

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* (33) and *L. (L.) infantum* (34). 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 (15,35), 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 (36–40)

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 (41), was relatively the same among promastigotes and axenic amastigotes. Serine enzymatic activity is generally confirmed by using PMSF, AEBSF and TLCK inhibitors (26). Besides, 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 (20,42–44). 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 investigate 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). Cluster analysis shows the associations

between *in vitro* susceptibility and clinical response. However, additional studies with more isolates from other geographical areas are necessary to explore and reinforce the associations found here.

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 (37,45). Other studies have shown that genetic and phenotypic characteristics among different *L. (V.) braziliensis* strains are associated with different clinical manifestations and drug resistance (32,46). Additionally, the variation among serine proteases inhibition indicates specific traits among the conformation of their serine protease network since the specificity of PMSF, AEBSF and TLCK is different (42,47). 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.

Additionally, based on the previous role seen for subtilisins as maturases of TXNPx (18,26), 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. 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 (48). Additionally, another study using *L. (L.) donovani* clinical isolates correlated TXNPx higher amplification levels with antimony resistance phenotypes (49). However, is important to bear in mind that this study did conventional PCR which is a qualitative method, thus, qPCR and proteomics experiments, which are more sensitive quantitative methods, need to be performed to quantify these transcripts expression and explore their relationship with different *in vitro* phenotypes.

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 (15,50–52). The present study adds to this discussion by bringing proteases to focus, one of the most studied parasite virulence factors in the last 30 years (18,21,53). The data presented here show the possibility to use serine proteases to *in vitro* characterize *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.

Methods

Chemical, Molecular Biology and Culture Media reagents

Antibiotics (penicillin and streptomycin), Schneider's insect medium, bovine serum albumin (BSA), Fluorogenic peptide substrate [N-benzyloxycarbonyl-l-phenylalanyl-l-arginine 7-amino-4-methylcoumarin (Z-FR-AMC)], Proteases inhibitors [trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E-64), Phenylmethylsulfonyl fluoride (PMSF), 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), Tosyl-L-lysyl-chloromethane hydrochloride (TLCK)], Glucantime® (Sb^{V}) and antimony potassium tartrate (Sb^{III}) were purchased from Sigma Aldrich Chemical Co. (USA). TRIzol®RNA Isolation Reagent (TRIzol), RNase Henzyme, DEPC-treated water, deoxyribonucleotide phosphate solution (dNTPs), SuperScript III First-strand cDNA Synthesis Kit (SuperScript III kit), Platinum™ Taq DNA Polymerase (DNA Polymerase), Taq Platinum PCR buffer and AlamarBlue™ cell viability reagent were purchased from Life Technologies/Thermo Fisher Scientific (USA). Fetal Bovine Serum (FBS) was purchased from Gibco/Thermo Fisher Scientific (Brazil). All reagents were of analytical or superior grade.

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) since 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 (4,5,54). All the isolates included in this study were previously characterized as *L. (V.) braziliensis* by multilocus enzyme electrophoresis (MLEE), according to procedures described elsewhere (55). 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 cultured in biphasic Novy-MacNeal-Nicolle (NNN) medium with 10 % of inactivated FBS. Then, they were expanded in Schneider´s insect medium at pH 7.2 supplemented with 20 % of inactivated FBS, 200 IU penicillin and 200 mg/mL streptomycin and maintained at 26 °C. To obtain each isolate growth curve, $3x10^5$/mL promastigotes were initially cultured and maintained in 25 cm^2 flasks containing 5 mL of the medium described above. Daily, for eight days, a 10 µL aliquot was taken to determine the number of viable parasites using a Neubauer chamber (data not shown). Each parasite isolate did not have more than 7 passages since isolation. Differentiation from promastigotes to axenic amastigotes was performed as previously described elsewhere (38), with a few modifications. Briefly, $5x10^5$ 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 at 32 °C for 2 days. After this period, full differentiation was verified under an optical microscope (Labomed, Labo America, Inc.) and these parasites – named as one-day axenic amastigotes – were immediately used for all experimental assays (Supplementary file 8).

In vitro susceptibility assays

Log-phase promastigotes and one-day axenic amastigotes forms, of each isolate, were tested against Sb^{V} and Sb^{III} to measure the half-maximal inhibitory concentration (IC_{50}) induced by each drug. The IC_{50} was determined by AlamarBlue™ reduction assay as previously described (56), with some modifications. Briefly, each parasite form was seeded in 96-well plates in triplicate at adequate conditions: log-phase promastigotes ($4x10^{6}$ parasites/mL) and one-day axenic amastigotes ($5x10^{5}$ parasites/mL) in 0.1 mL of Schneider medium (pH 7.2 for promastigotes or pH 5.5 for axenic amastigotes) supplemented with 20 % of FBS and each drug in decreasing concentrations, leaving one column without any drug to serve as the control. Sb^{V} concentrations ranged from 20 mg/mL to $6x10^{-4}$ mg/mL; Sb^{III} concentrations ranged from 0.196 mg/mL to $5x10^{-6}$ mg/mL, with a 2:1 dilution factor between each one. After parasites incubation (Promastigotes: 26 °C, 48 h; Axenic amastigotes: 32 °C, 24 h), AlamarBlue™ reagent was added to each well (10 µL) followed by a new incubation at their respective temperatures for 4 hours. Then, each plate was read on a Spectramax 190 microplate spectrofluorometer (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) of each isolate were separately used to obtain whole protein extracts as it follows. Parasites were washed by centrifugation (3 000 × 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 parasites lysis, confirmed by optical microscopy, the soluble fraction was obtained by centrifugation (25 000 x g, 4 °C, 30 min) and the supernatant stored at -80 °C until further use. The parasites total protein concentrations were determined by the Lowry method using BSA as a standard protein (57).

Enzymatic assays

The serine protease activity of the whole protein extract, 5 µg of total protein, was assessed in activation buffer (Tris-HCl [10 mM], pH 7.5) using a specific fluorogenic peptide substrate, Z-FR-AMC [1 mM], at a final volume of 60 mL. Samples were incubated (37 °C, 60 min), and the variance in the relative fluorescence was monitored on a Molecular Devices SpectraMax spectrophotometer (Gemini XPS).

Inhibition assays were performed by incubation (25 °C, 5 min) of each sample with specific inhibitors of proteases: E-64 [10 mM] (for cysteine proteases), PMSF [1 mM] (for serine- and cysteine proteases), AEBSF [1 mM] (for serine proteases such as trypsin, chymotrypsin, plasmin, kallikrein and thrombin), and TLCK [100 mM] (for serine proteases such as trypsin and trypsin-like). All inhibitors were assessed at the maximum recommended concentrations (58).

The substrate cleavage rate was defined as follows: v = Ds/Dt, where v = velocity, Ds = substrate concentration variation and Dt = total reaction time, as determined elsewhere (36). The self-degradation of the fluorescent peptide substrate was controlled throughout the assay to avoid incorrect readings; the enzymatic activity is expressed as mmol min^{-1}.mg of $protein^{-1}$.

Primers design

The primers used in this study were previously designed and used to detect serine proteases and tryparedoxin peroxidase transcripts (26). Briefly, the design is based on the *L. (V.) braziliensis* subtilisins (LbrM.13.0860: sense-5' GAGCTAACACCAGTGGCACA 3' and antisense-5' ATCTGGCGATTTCTCCCTTT 3'; LbrM.28.2570: sense-5' CACTGCGCTCCACATACACT 3' and antisense-5' GCCTTCATTCGAGCTACAGG 3'), tryparedoxin peroxidase (LbrM.15.1080: sense-5' CTCTGTGGACAGCGAGTACG 3' and antisense-5' TGGGGTCGATGATAAAGAGG 3') and 40S Ribosomal Protein S8 housekeeping gene (LbrM.24.2160: sense-5' AGACGCTGGTGAAGAACTGC 3' and antisense-5' AAGTCGATGCCGTAATGCTT 3') sequences recorded in the GeneDB database (http://www.genedb.org). All 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 1mL TRIzol containing 200 mL of chloroform. For RNA extraction the samples were centrifuged (10 000 × g, 4 °C, 10 min) and the supernatants containing RNA were dissolved in Isopropanol (12 000 × g, 4 °C, 20 min) and washed with 70 % ethanol (9 000 × g, 4 °C, 5 min). Then, each pellet was re-suspended in 30 to 40 mL of DEPC-treated water and incubated (56 °C, 10 min) to complete dissolution. The RNA concentrations were measured by spectrophotometry at 260/280 nm and 230/260 nm. DNAse treatment and cDNA synthesis were performed using the SuperScript® III Kit with a maximum of 4 µg of total RNA. cDNA concentration of each sample was measured with Qubit™ ssDNA Assay Kit (Thermo Fisher Scientific), following the manufacturer's protocol.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Primers complementary to the LbrM.13.0860, LbrM.28.2570, LbrM.15.1080 and LbrM.24.2160 genes were used for conventional PCR under the following reaction conditions: 10X PCR Buffer, 50 mM $MgCl_2$, 10 mM of each dNTP, 0.3 µM of each primer and 1 U Platinum™ Taq DNA polymerase, together with 20 ng of cDNA, adjusted to a final volume of 25 µL. 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 establish significant differences by Student's T-test, considering p <0.05 as a significant difference. While, to compare 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). Here we have 11 variables per isolate and biological form which is why PCA analysis helped us reduce dimensionality and perform further cluster analysis (59). The first three PCs (PC1, PC2, PC3) were used to cluster the clinical isolates using the K-means algorithm clustering method. To determine the optimal number of clusters we used the total-within cluster sum of squares (twcss), as a function of the number of clusters, where the squared distances between each cluster centroid () and each of its cluster members (are summed over each cluster , Nc is the total number of clusters, equation (1). Additionally, with the previously normalized data, we explored the characteristics of the clusters by calculating the pairwise distance between clusters using the normalized values of Sb^{III} IC_{50}, PMSF, AEBSF and TLCK inhibition over promastigotes and axenic amastigotes. The statistical analysis was carried out using R (version 1.1.463).

$$\mathrm{WCSS} = \sum_{i=1}^{Nc} \sum_{x \in c_i} d\left(x, \bar{x}_{c_i}\right)^2 \qquad (1)$$

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Declarations

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Author's contribution

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

Competing interests

The authors declare no competing interests.

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Figures

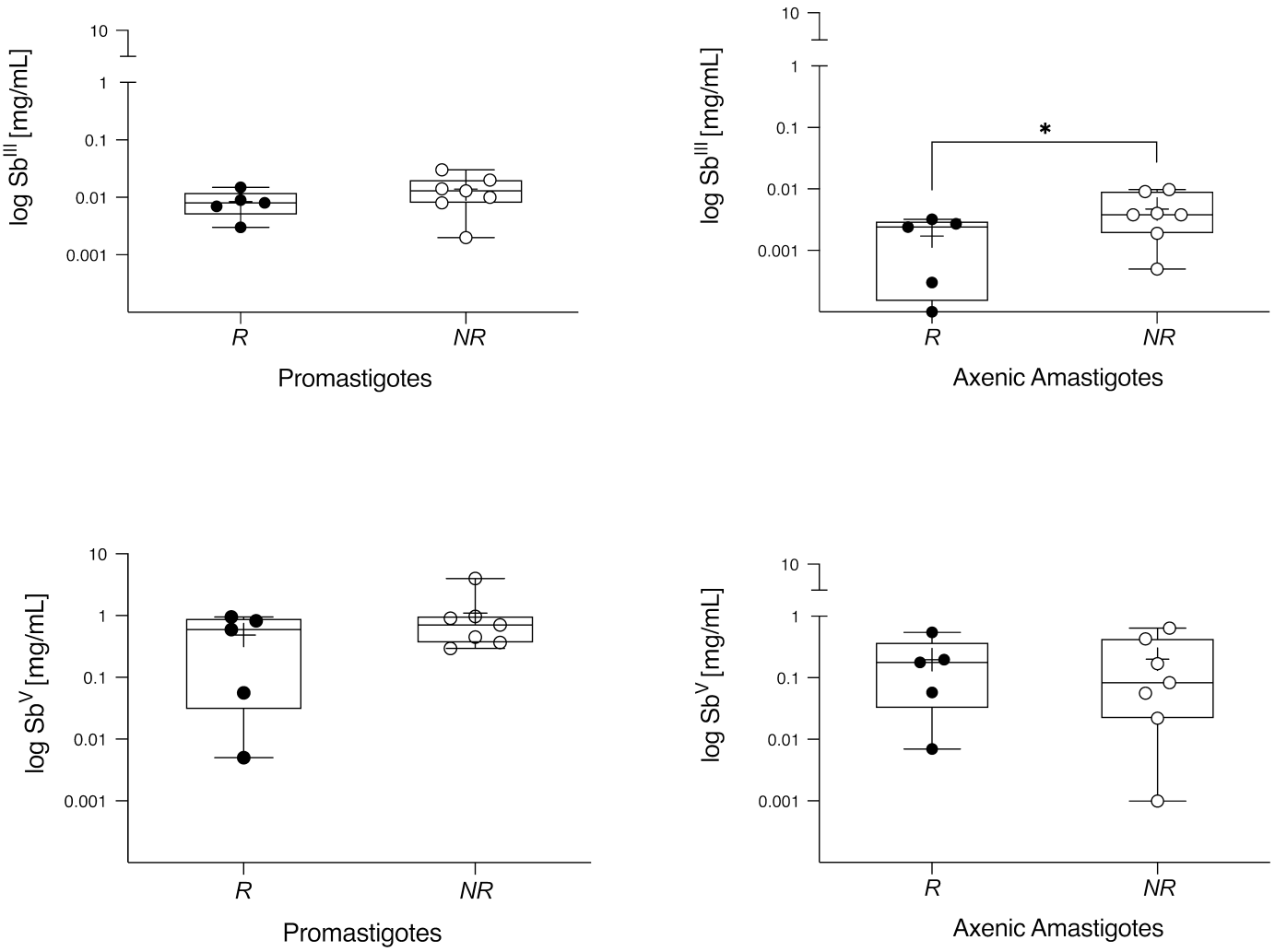

Figure 1

Promastigotes and axenic amastigotes in vitro susceptibility profile to antimony. Both parasite forms of each isolate (n=12), 4 × 106 promastigotes/well and 5 × 105 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 the mean of three biological replicates for each isolate. Asterisks indicate statistically significant differences: * $p < 0.05$

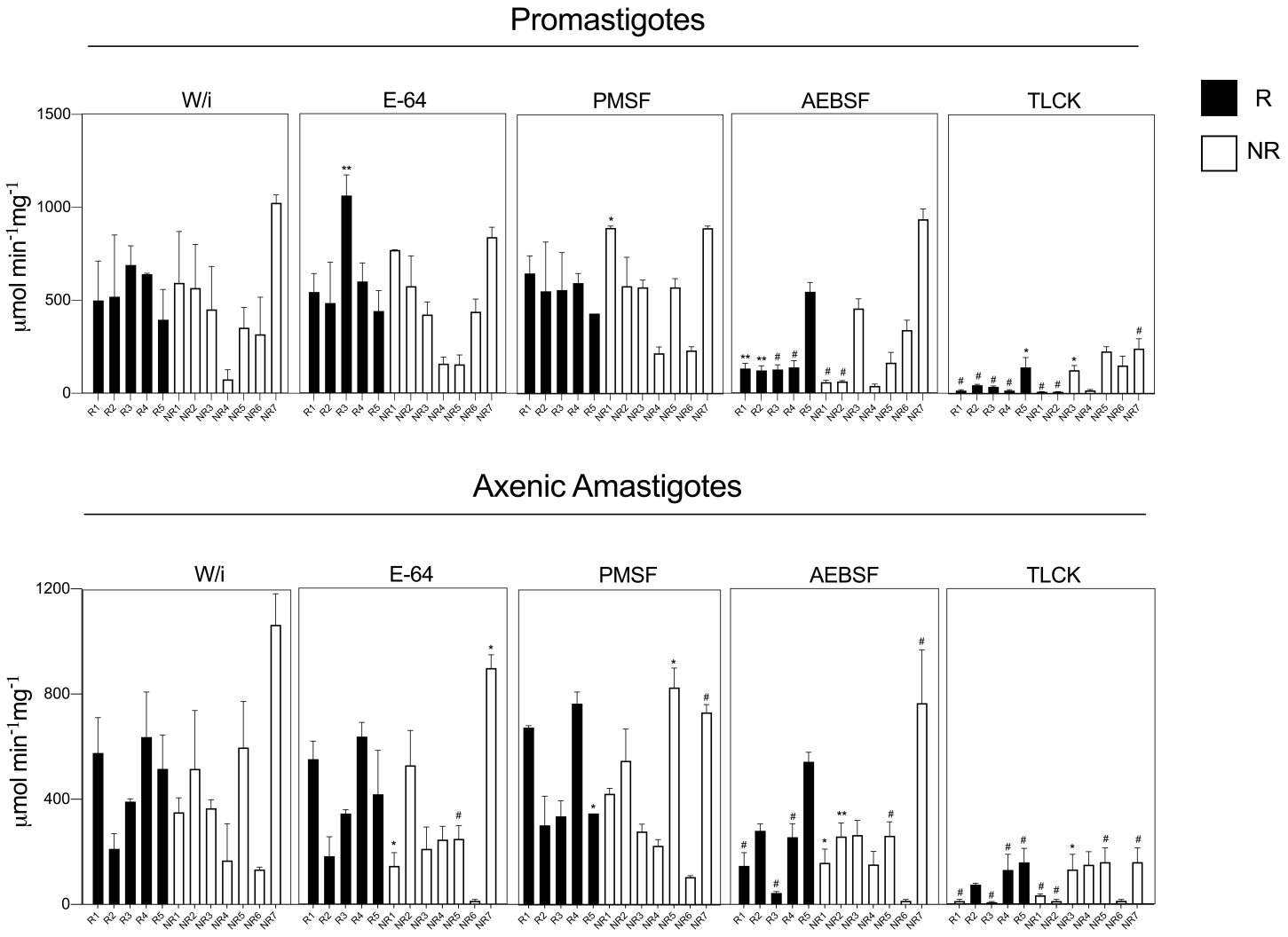

Figure 2

Enzymatic residual activity of whole soluble proteins from the clinical isolates. In these assays, protein extract [5 µg] 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 (w/i) and in the presence of inhibitors: E-64 [10 µM], PMSF [1mM], AEBSF [1 mM] and TLCK [100 µM]. R: Responder; NR: Non-responder. The results are shown as mean and the standard deviation (±) of three independent experiments. Asterisks indicate statistically significant differences of enzymatic activities in the absence (w/i) and presence (E-64, PMSF, AEBSF, TLCK) of inhibitors for each isolate: * p = 0.01; ** p = 0.005; *** p = 0.0005; **** p <0.0001.

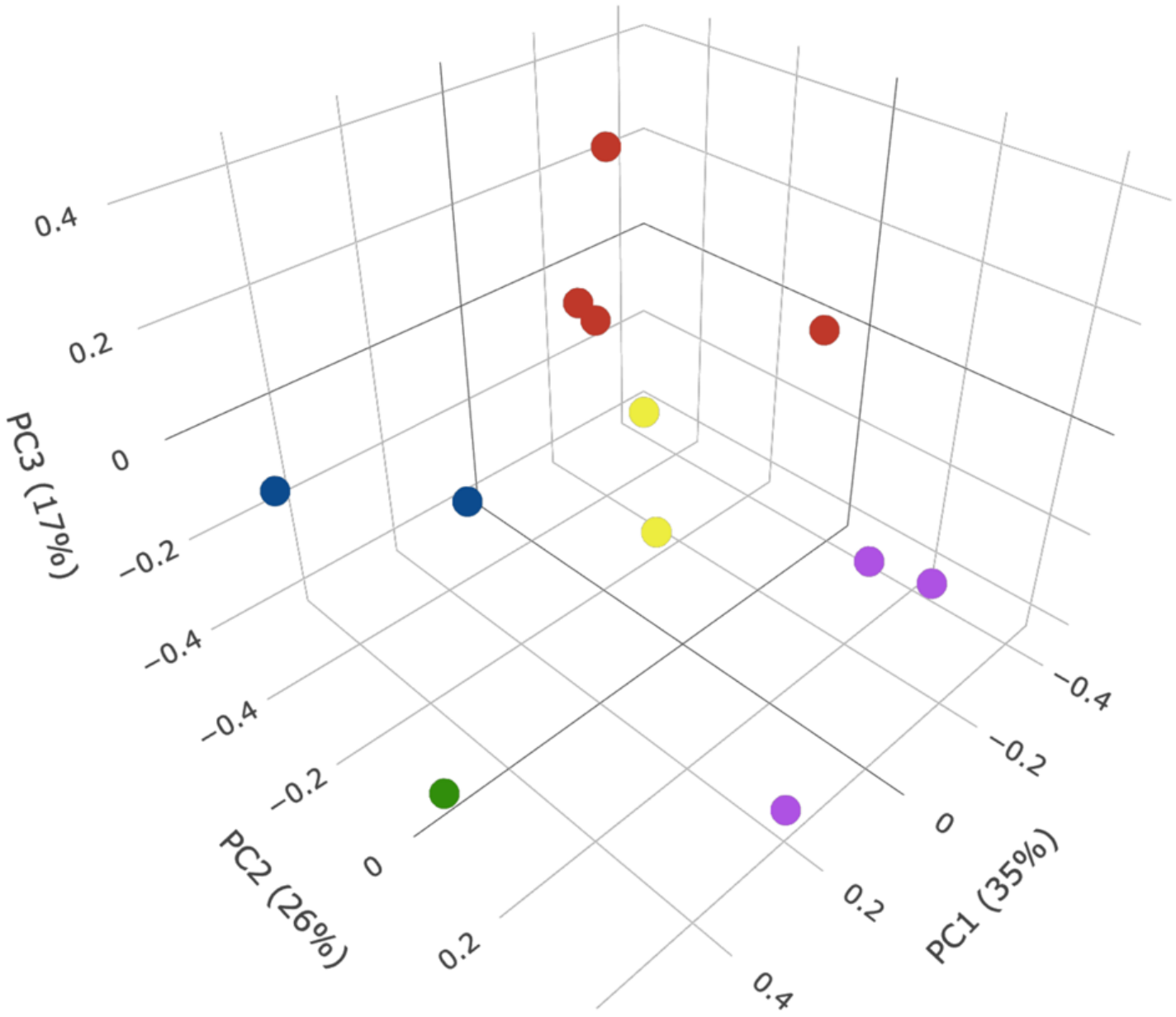

Figure 3

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 (blue): NR6 and R3; C2 (red): R1, R3, NR5 and NR7; C3 (green): NR3; C4 (purple): NR2, R2 and R4; C5 (yellow): NR1 and NR4.

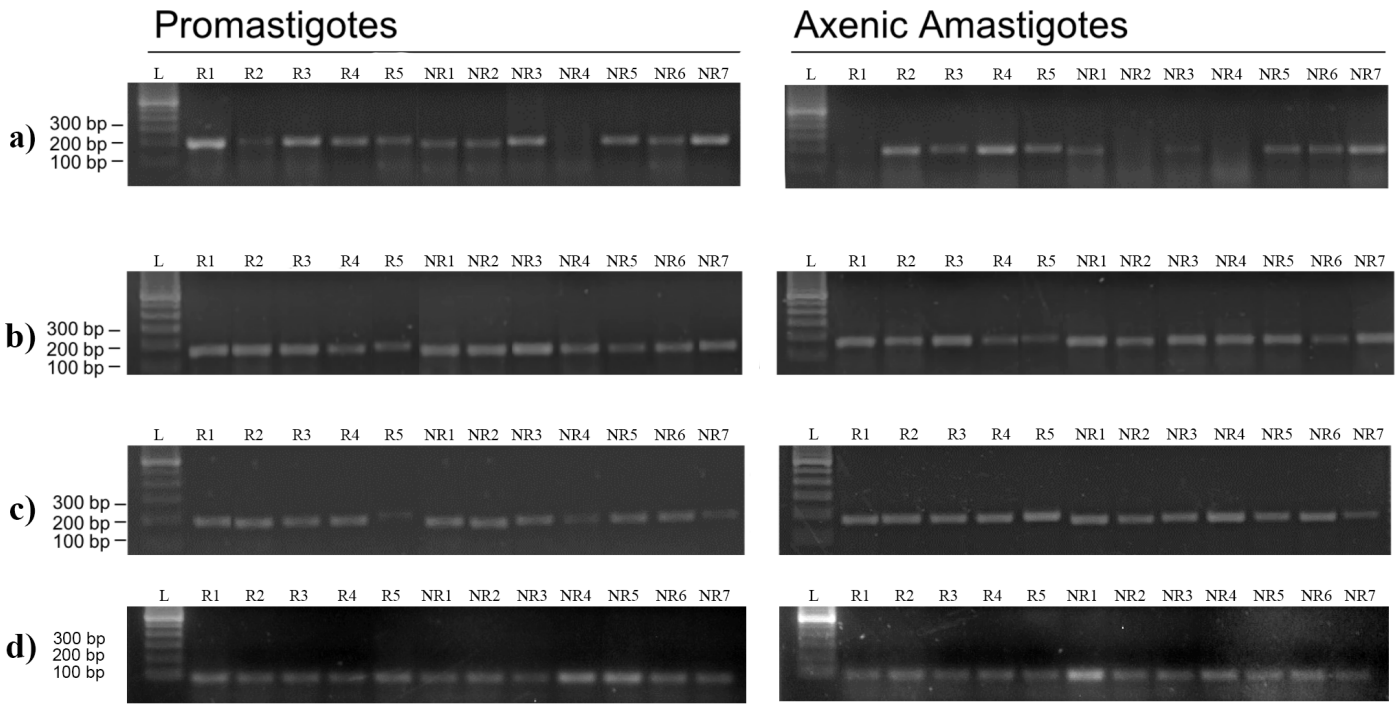

Figure 4

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, tryparedoxin peroxidase gene (166 bp); and d) LbrM.24.2160, 40S Ribosomal Protein S8 - the housekeeping gene used (98 bp). The amplified products of clinical isolates (1 to 12) were assessed by agarose gels electrophoresis (2% agarose). Lanes (L) are 100 bp DNA ladder (Invitrogen), used as a molecular weight marker.

Supplementary Files

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