v. 1, n. 1, 2021

International Journal of Health Science

In vitro insights of *Leishmania (Viannia)* species related to their outcomes and virulence in American tegumentary leishmaniasis

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Abstract: Different *Leishmania* species cause cutaneous leishmaniasis. *Leishmania (Viannia) braziliensis* [L(V)b] is responsible for the cutaneous and mucosal form, while *L. (V.) lainsoni* [L(V)l] and *L. (V.) naiffi* [L(V)n] are associated with the cutaneous one only, and the latter is responsible for self-healing. Since these mechanisms are not fully understood, we investigated *in vitro* aspects of these species that could be related to their different leishmaniasis outcomes. Three strains of L(V)b, one strain of L(V)n and one from L(V)l were selected for this study. The morphologies of the *Leishmania* species were observed. The activity of peptidases present into the membrane of promastigote forms of *Leishmania* spp. and in the supernatant of macrophages infected by *Leishmania* spp. were studied by the zymographic and inhibition assay. The *in vitro* infectiveness of each *Leishmania* spp. was evaluated in macrophages J774A.1 at 0h and 24h post-infection. The results showed that these parasites have different body lengths, forms, activity of promastigote cell-related metalloproteases and metalloproteases secreted by macrophages in response to the infection. Low expression of *Leishmania* peptidases may be associated with the cure after the treatment in L(V)b strains. Pro-metalloproteinase 2 seems to be related with more complicated cases of cutaneous leishmaniasis. L(V)n has the lowest infection index in macrophages. These species have differences in parasite constitution and host interaction that may explain their clinical manifestation in humans and further studies can contribute to the elucidation of new drug targets and biomarkers.

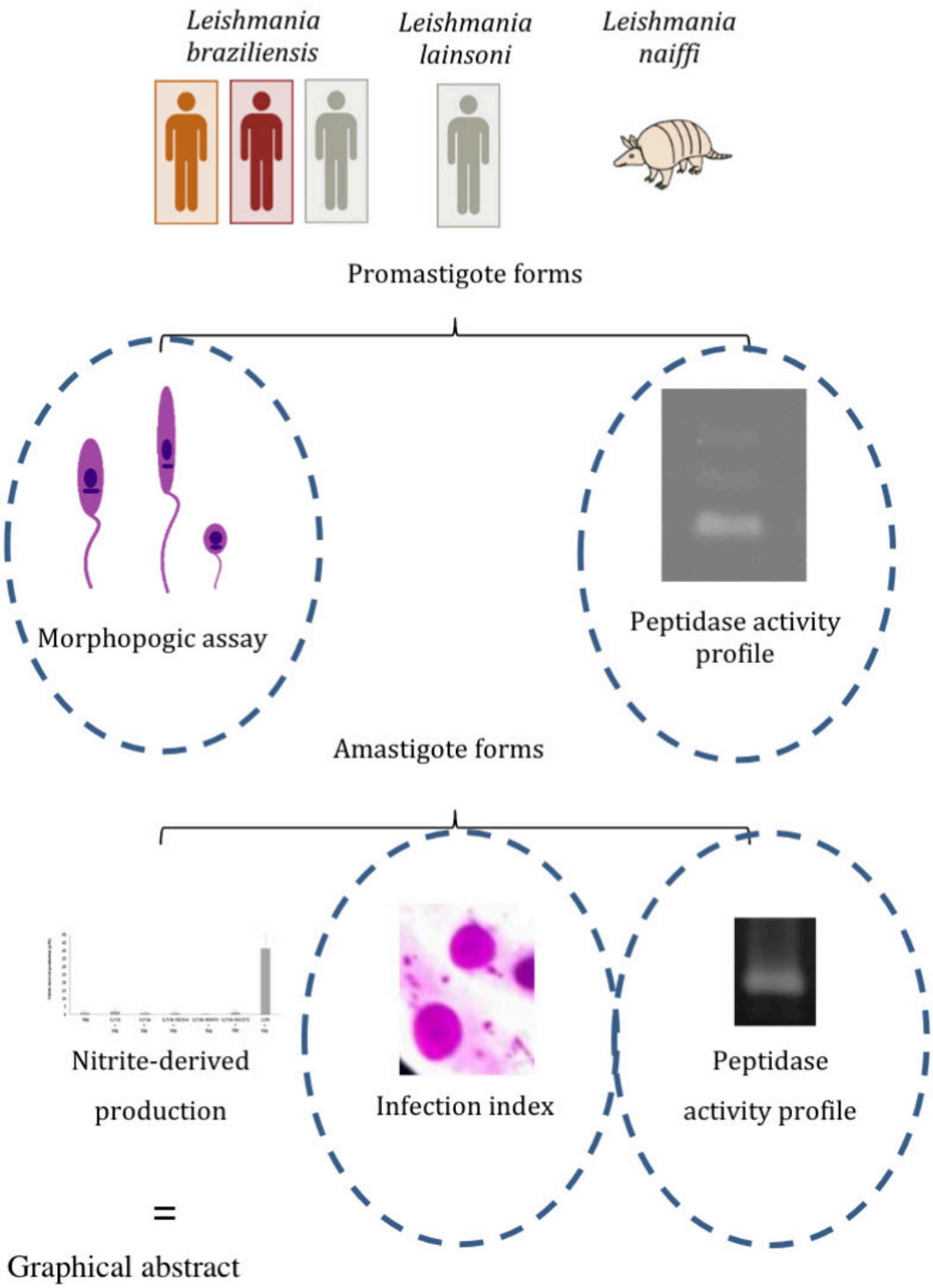
Keywords: Cutaneous leishmaniasis, new world leishmaniasis, virulence, *Leishmania*, host-parasite interactions, metalloproteases.

Introduction

Leishmaniasis is a set of zoonotic diseases with highly complex clinical manifestations and epidemiology with worldwide distribution, affecting mainly developing countries with a considerable impact on their health economy [1]. The most frequent clinical manifestation is the cutaneous leishmaniasis (CL) [2], with more than one million cases reported in the last five years, with 90 % of the cases worldwide in Bolivia, Brazil and Peru (WHO, 2018c). The main species for CL in the New World (the Western Hemisphere) is *Leishmania (Viannia) braziliensis* that is involved in the mucosal form as well [4]. Other species from the subgenus L. (*Viannia*) also develop CL with better prognosis and lower incidence, such as *L. (V.) lainsoni* (Jennings et al, 2014; WHO, 2010) and *L. (V.) naiffi* (Figueira et al, 2014; WHO, 2010) causing unique ulcers, and the latter species is also related to self-limiting infections [8].

These differences in prevalence may be a result of the vector species responsible for their transmission and demographic distribution, since these species are transmitted by different species of sandflies, and are endemic in different regions. The *L. (V.) lainson*i and *L. (V) naiffi* are restricted to the North of the South American continent, being transmitted by a limited number of low anthropophilic species. On the other hand, *L (V.) braziliensis* is distributed all over Western Hemisphere and it is transmitted by several species of sandflies with high affinity for humans [9,10]. However, these cannot fully explain those differences and some intrinsic factors of parasites that interfere with host-parasite interaction and immune response modulation, must be associated with less or more virulence against a host [11].

Leishmania are intracellular parasites, infecting especially mononuclear phagocytes. Unrestricted replication of these parasites cau-



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Graphical abstract

ses the eventual burst of the host cell, thereby releasing the infectious parasites [4]. Based on this, the abilities of species to infect and to multiply inside mononuclear phagocytes are important virulence factors. Additionally, the host-parasite interaction depends on parasite membrane enzymes and host-immune modulation, crucial in shaping parasite virulence [12,13]. Although this host-parasite interaction knowledge contributes to the elucidation of new drug targets and biomarkers [12,14], these are unclear and understudied especially for *L. (V.) lainsoni* and *L. (V) naiffi*.

Considering the different leishmaniasis outcomes related to parasite intrinsic characteristics, we investigated the different aspects *in vitro* of three species of *Leishmania (Viannia)* spp., as well as three different strains of *L. (V.) braziliensis* with distinct CL outcomes, targeting supporting facts for different clinical manifestations due to infecting species/strains.

Methods

Parasites and culture growth conditions

This study used five strains of *Leishmania* (*Viannia)* subgenus, with three strains of *L. (V.) braziliensis* [L(V)b]: one isolated from a patient with complete regression of the lesion after the Glucantime® treatment-MHOM/2003/2314 [L(V)b-M2314], other from a patient that did not recovered after the treatment-MHOM/BR/2009/3476 [L(V) b-M3476] and the last one was MHOM/BR/1987/M11272 [L(V)b-M11272] [15], that is a well-known strain of L(V)b in the Laboratory of Leishmaniasis at the Universidade Estadual de Maringá (UEM) that was isolated from a case of CL; one strain of *L. (V.) lainsoni* MHOM/BR/8l/M6424 [L(V)l] isolated from a patient with CL and other of *L. (V.) naiffi* MDAS/BR/79/M5533 [L(V)n] that was isolated from an armadillo. All strains ($5x10^6$ parasites/mL) were kept by weekly seed at 25ºC in 199 medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% (V/V) inactivated fetal bovine serum (FBS, Invitrogen, Grand Island, NY, USA) and antibiotics (penicillin G 63ng/mL, streptomycin 100 ng/mL, Sigma-Aldrich, Saint Louis, MO, USA).

Culture and growth conditions of J774A.1 macrophages

The macrophage-like cell line J774A.1 (ATCC: TIB67, *Banco de Células do Rio de Janeiro*, RJ, Brazil) were cultivated in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 20% (V/V) FBS and antibiotics (penicillin G 0.1 UI/mL, streptomycin 100 ng/mL) at 37 ºC with 5% of carbon dioxide (CO_2).

Morphologic assay

Promastigote forms in the stationary phase were centrifuged at 2500 rpm for 10 min and washed twice with phosphate-buffered saline buffer pH 7.2. Afterwards, they were fixed with 2% formalin, and a drop of each strain was put in a slide and stained with Giemsa (Sigma-Aldrich, Saint Louis, MO, USA) and observe at EVOS FL Imaging System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 100x magnification. The body length was estimated by the measure of two promastigotes per field of at least ten fields based on the scale provided by the microscopy [16, modified].

Infectiveness <u>in vitro</u>

A suspension of $1x10^6$ J774A.1 macrophages per mL was distributed in a plate of 24 wells with glass coverslips, followed by 2 h of incubation at 37 ºC with 5% of CO_2. Following, seven parasites in the stationary phase were added per macrophage and the plate was reincubated for 4 h. In the end, all coverslips were washed and part of the coverslips was stained with Panótic kit (Laborclin,

Vargem Grande, Pinhais, Brazil) (time zero "0 h") and the others part were reincubated with RPMI-1640 medium for more 24 h to analyse the behaviour of macrophage-*Leishmania* interaction. The coverslips were stained with Panótic kit (time twenty-four hours "24 h") and the supernatants were stored for further analysis (nitrite-derived production, and zymographic and inhibition assay). In microscopy (Olympus, Shinjuku, Tokyo, Japan), 200 macrophages were counted, quantifying the infected (I) and uninfected ones, and the number of parasites per macrophage was also assessed (P). The infection index was estimated by the expression [P x (I x 100/ 200)] [17, modified]. The experiments were performed in triplicate.

Nitrite-derived production

The supernatants from infectiveness *in vitro* assay were also used to determine the production of nitrite-derived by the method described by Fernandes et al (2015) modified. The supernatants were incubated with the Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylene diamine dihydrochloride and 2.5% orthophosphoric acid) in the proportion of 1:1 at room temperature for 15 min. The absorbance was read at 550/620 nm in a spectrophotometer (VersaMax™ ELISA Microplate Reader, Molecular Device, Sunnyvale, California, USA), and the concentration of nitrite-derived was based on a standard curve of sodium nitrite ($NaNO_2$). The experiments were performed in triplicate in ice. The supernatant of macrophages stimulated with lipopolysaccharide (Sigma-Aldrich, Saint Louis, MO, USA) was used as a positive control of nitrite-derived production [18].

Zymographic and inhibition assay

For the zymographic assay, peptidases present in promastigote forms of parasites (*Leishmania* peptidases) and peptidases secreted by macrophages in response to *Leishmania* infection (host peptidases) were isolated. Peptidases present in the stationary phase of parasites were obtained by disrupting $1x10^8$ promastigote forms in 100 µL of lysis buffer containing 4% of sodium dodecyl sulfate (SDS) in 0.1 M TRIS, pH 6.8, followed by three cycles of vortex for 1 min with breaks of 4 min into ice (Lima et al, 2009 modified). Host peptidases produced by infected and uninfected macrophages were obtained by concentrating 1.5 mL of the supernatant from the infectiveness *in vitro* assay with 0.75 mL of absolute ethanol (LabSinth, Diadema, SP, Brazil), followed by an incubation at -30 °C for 24 h. After that, this mixture was centrifuged at 4000 rpm for 15 min and the pellet was resuspended in 0.5 M of TRIS, pH 6.8. The same procedure was done with the RPMI-1640 medium as a control.

The proteins present in both types of extracts were measured by Qubit™ Protein Assay Kit (Invitrogen, Eugene, Oregon, USA). The extract of *Leishmania* peptidases (50µg) and host peptidases (1µg) were mixed with sample buffer and applied to 10 and 8% polyacrylamide SDS- PAGE, both with 0.1% of gelatin (Sigma-Aldrich, Saint Louis, MO, USA), respectively. Sample buffer alone was also added into the gels as an internal control. After electrophoresis, the resulting gels were washed twice for 30 min under agitation at room temperature with 2.5% triton X-100 (Sigma-Aldrich, Saint Louis, MO, USA). Protease activities were detected for both extracts by incubating the gels in the renaturation buffer (50 mM Tris, 75 mM NaCl and 2.5 mM $CaCl_2$) in pH 5.5 and 7.2, respectively, at 37^0C for 48 h. The gels were stained with 0.2% (w/v) Coomassie blue, 30% (v/v) methanol, 10% (v/v) acetic acid, and destained with 30% (v/v) methanol, 10% (v/v) acetic acid.

For inhibition assays, the metalloprotease inhibitor 1,10-phenanthroline (Sigma-Al-

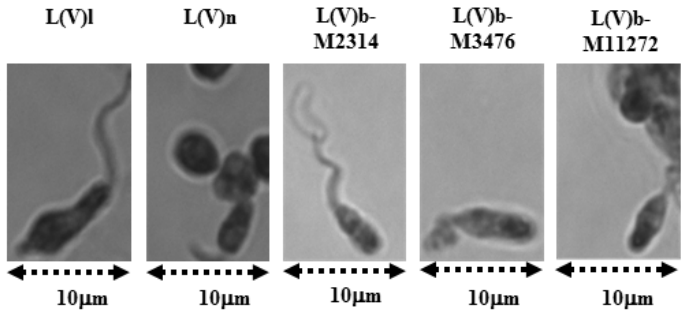
drich, Saint Louis, MO, USA) (10 mM for *Leishmania* peptidase and 5 mM for host peptidase) was added to the renaturation buffer. The weights of peptidases were measured based on a molecular weight standard (See Blue™Prestained Protein Ladder, Invitrogen, Eugene, Oregon, USA). The experiments were performed in triplicate under a low temperature.

The gels were digitized by a scanner for the densitometric analysis. The peptidase activities were estimated based on the intensity of light bands by the Image J 1.46 software (National Institutes of Health, Bethesda, Maryland, USA). Initially, the image was transformed to 8-bits, and a rectangle involving the band and the dark background was manually selected. The contrast of these elements was graphically demonstrated by inverted pics that were proportional to peptidase activities in the gel. For minimizing the error in the manual selection of area, this measure was made three times for each gel and at least two gels were used [20].

Statistical analysis

All results were plotted at Excel ⟶ (Microsoft Corporation, Albuquerque, New Mexico, USA) and the results were expressed as means ± standard deviations. For comparison between two groups with normal distribution by the Shapiro-Wilk test, the t-student test was used. When the data did not follow normal distribution the Mann-Whitney test was applied. In the analysis that included more than two groups, the analysis of variance by ANOVA followed by the Tukey post hoc test was used since the data followed the criteria of normality, independence and equality of residue variance in residual analyses. In the cases that data did not meet these criteria, Kruskal-Wallis followed by the Mann-Whitney pairwise test was used. All tests were per-

A



B

Leishmania (V.) spp.	Form	Body length (µm)± SD (µm)
L(V)l	Fusiform	11.71± 1.95[a]
L(V)n	Oval	4.82± 0.62[b]
L(V)b-M2314	Fusiform	8.32± 1.64[c]
L(V)b-M3476	Fusiform	8.50± 1.33[c]
L(V)b-M11272	Fusiform	7.55± 1.27[c]

Figure 1: Morphologic assay of *Leishmania (Viannia)* spp. A- Promastigote forms stained by Panotico kit observed in EVOS FL Imaging System at 100x magnification. B - Body length and promastigote forms of *Leishmania (Viannia)* spp. The mean and standard deviation (SD) was calculated based on the measure of 2 promastigotes per field of at least 10 fields in the EVOS FL Imaging System. L(V)b-M2314: *L. (V.) braziliensis* (MHOM/2003/2314); L(V)b-M3476: *L. (V.) braziliensis*(MHOM/BR/2009/3476);L(V)b-M11272: *L. (V.) braziliensis*(MHOM/BR/1987/M11272); L(V)l: *L. (V.) lainsoni* (MHOM/BR/8l/M6424); L(V)n: *L. (V.) naiffi*(MDAS/BR/79/M5533). p=0.001 by ANOVA and Tukey post hoc tests.

formed at the Past 3.X software (University of Oslo, Oslo, Norway). Statistical differences were considered at $p < 0.05$.

Results

Morphologic assay

The three species of *Leishmania (Viannia*) spp. showed different promastigote forms. There was no difference related to morphology among the three strains of L(V)b, the body lengths of L(V)b strains ranged from 7.55 to 8.50 µm with body width of approximately 1µm and the flagellum was bigger than the body length, without significant statistical difference in their body length. The L(V)n was the smallest, with a body length of 4.82±0.62 µm, body width variable and the flagellum was smaller than the body length. The L(V)l was the largest species with a body length of 11.71±1.95 µm ($p=0.001$), body width variable and the flagellum size variable. Differences in the promastigote forms were also found, with the L(V)n oval and the others fusiform (Figure 1).

Infectiveness in vitro

The infection indexes on macrophages of all *Leishmania (Viannia)* spp. at 0 h were statically equal ($p>0.05$), being them 188±29, 204±31, 180±19, 185.42±31 and 200.67±26 for L(V)b-M2314, L(V)b-M3476, L(V) b-M11272, L(V)l and L(V)n, respectively. Otherwise, the infection index after 24 h was lowest in L(V)l with 124±24 ($p=0.001$). The other *Leishmania (Viannia)* spp. had infection indexes of 213±28, 208±35, 181±33 and 183±28 for L(V)b-M2314, L(V)b-M3476, L(V)b-M11272 and L(V)n, respectively. The comparison of the infection indexes at 0h and 24h showed that it was significantly reduced in the L(V)l ($p=0.001$) (Figure 2).

The nitrite-derived production in the positive control with LPS was 41.37±8.02 µM. In all studied strains and species, nitrite-derived production was below 2.07 µM, while the basal production of these compounds by macrophages alone was of 1.21±1.05 µM. These results indicate that nitrite-derived production was not stimulated in any infection by *Leishmania (Viannia)* spp. (Supplementary data 1).

Peptidase activity profile

The analysis of *Leishmania* peptidases showed differences among the species. The L(V)l presented the largest number of bands (seven), being at 141, 73, 67, 60, 57, 55 and 51 kDa and the band that had the highest activity was at 55 kDa. The L(V)n had four bands and two of those were at 57 and 55 kDa, which were also present in the peptidase activity profile of L(V)l, the other two bands were at 78 and 62 kDa, with highest activity of the lowest molecular weight band. There was no difference in *Leishmania* peptidase profiles among the three strains of L(V)b that had three bands at 92, 78 and 62 kDa, with the major activity detected on the lightest band. Highlighting that these last two were present in the peptidase activity profile of L(V)n and L(V)b (Figure 3A). Although there were no different bands expressed in the L(V)b strains, when the levels of activity of these bands were analysed differences were found. The lightest band was equally active by the strains and the other two (92 and 78 kDa) were diminished in L(V)b-M2314 ($p=0.004$ and $p=0.003$) (Figure 3B). In the presence of 1-10 phenanthroline, the majority of bands were completely inhibited, except the band at 62 kDa in L(V)b strains and the band at 55kDa in L(V)l that were partially inhibited (Supplementary data 2).

The host proteolytic activity was not different among uninfected macrophages and infected ones, presenting six bands at 130, 98, 72, 68, 37 and 34 kDa (Figure 3C). Comparing the activity of the band with highest activity (72 kDa), it was down-regulated in the infec-

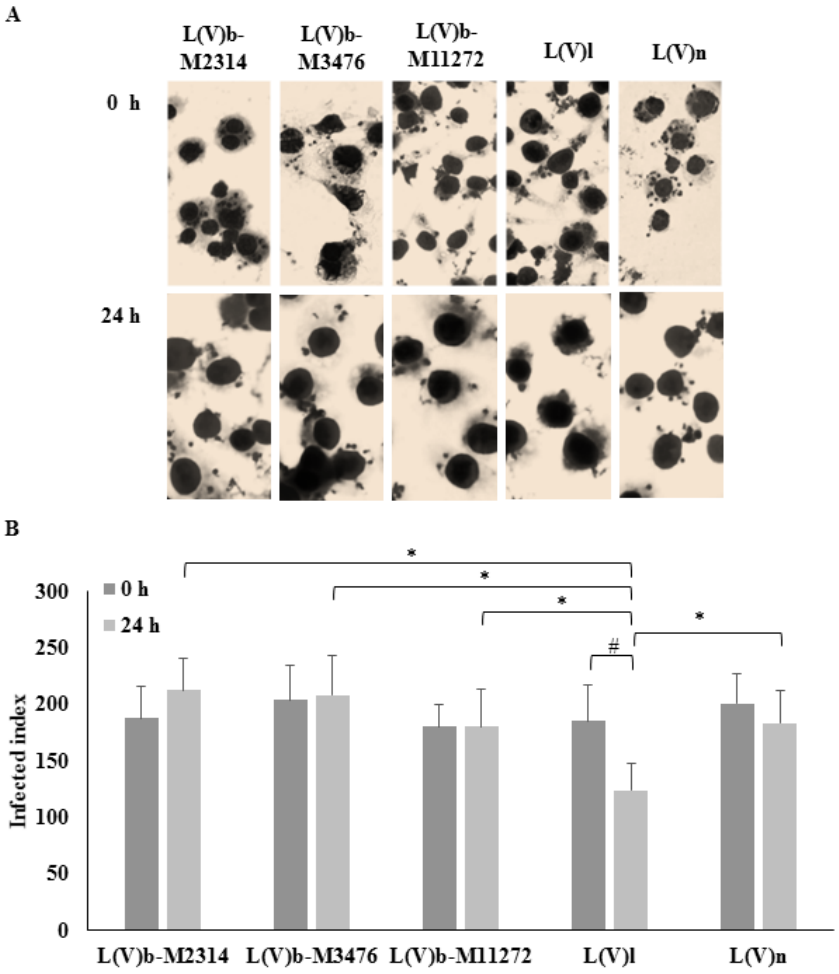


Figure 2: Infectiveness *in vitro*. A- Macrophages infected with *Leishmania (Viannia)* spp. at time zero (0 h) and macrophages infected with *Leishmania (Viannia)* spp. after 24 hours of infection (24 h) observed at a microscope at 100x magnification. C - Infection indexes at 0 h and after 24 h of infection. L(V)b-M11272: *L. (V.) braziliensis*(MHOM/BR/1987/M11272); L(V)b-M2314: *L. (V.) braziliensis* (MHOM/2003/2314); L(V)b-M3476: *L. (V.) braziliensis*(MHOM/BR/2009/3476); L(V)l: *L. (V.) lainsoni* (MHOM/BR/8l/M6424); L(V)n: *L. (V.) naiffi* (MDAS/BR/79/M5533). * p =0.001 by Kruskal-Wallis and Mann-Whitney pairwise tests. # p =0.001 by Mann-Whitney test. The results are expressed in mean and standard deviation of three independent experiments.

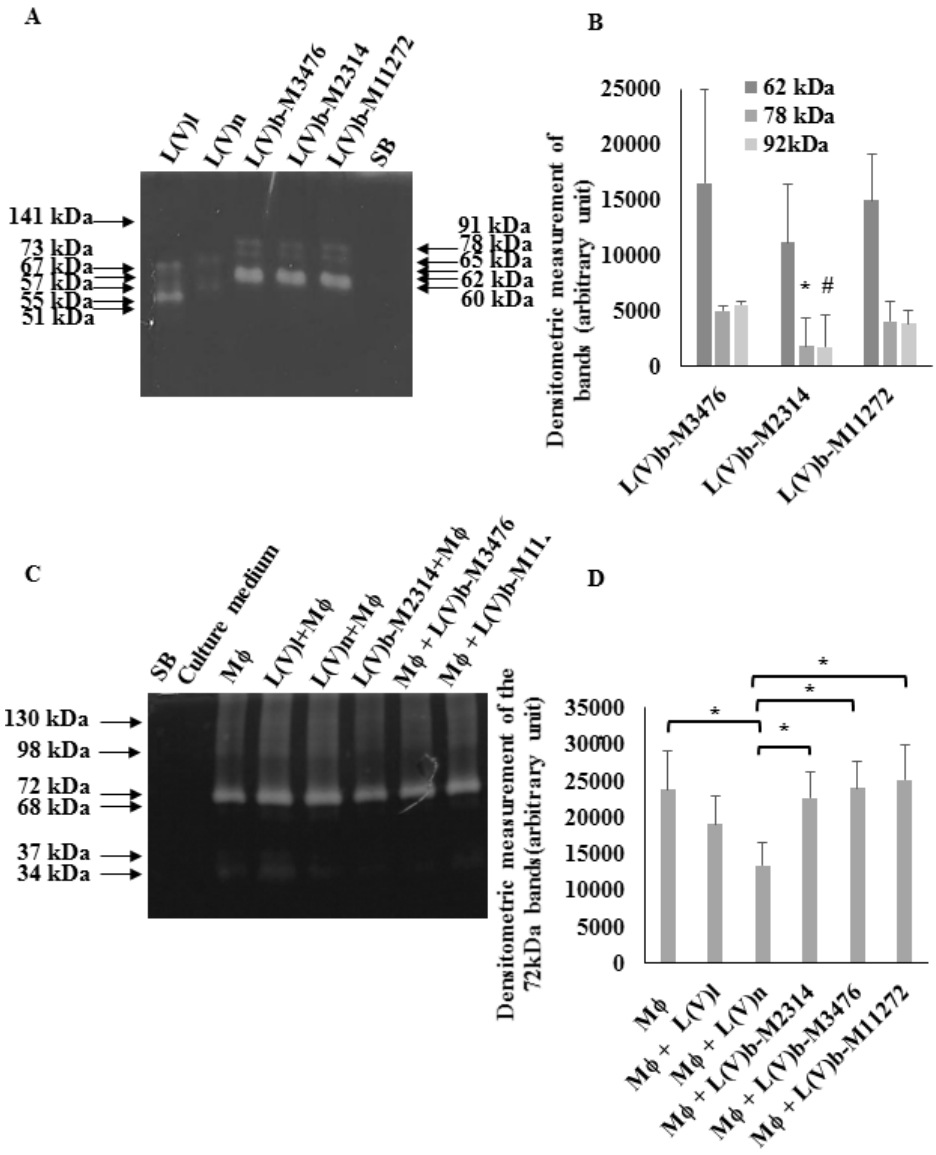


Figure 3- Peptidase proteolytic profile. A- Peptidase proteolytic profile of enzymes present in the membrane of promastigotes of *Leishmania (Viannia)* spp. in SDS-page. The *Leishmania (Viannia) lainsoni* (MHOM/BR/8l/M6424)[L(V)l] expressed seven bands (141, 73, 67, 60, 57, 54 and 51 kDa); the *Leishmania (Viannia) naiffi* (MDAS/BR/79/M5533) [L(V)n] expressed four bands (78, 62, 55 and 57 kDa); and the strains *L. (V.) braziliensis* MHOM/BR/1987/M11272 [L(V)b-M11272], *L. (V.) braziliensis* MHOM/2003/2314 [L(V)b-M2314] and *L. (V.) braziliensis* MHOM/BR/2009/3476 [L(V)b-M3476] expressed three bands (92, 78 and 62 KDa). B- Densitometric measurement (arbitrary unit) of bands expressed in the strains of *Leishmania (Viannia) braziliensis*; * p= 0.004 and # p= 0.003 by Kruskal-Wallis and Mann-Whitney pairwise tests. C- Peptidase proteolytic profiles of enzymes secreted by macrophages (Mϕ) alone and infected by *Leishmania (Viannia)* spp.; all samples presented the same peptidase proteolytic profile with six peptidases: 130, 98, 72, 68, 37 and 34 kDa. D- Densitometric measurement (arbitrary unit) of the band of 72 kDa expressed in the zymographic assay of secreted enzymes by macrophages in presence and absence of *Leishmania (Viannia)* spp; * p= 0.005 by Kruskal-Wallis and Mann-Whitney pairwise tests. Sample buffer (SB) is an internal control. The results are expressed in mean and standard deviation of two independent experiments.

tion caused by L(V)n in comparison to the uninfected macrophages and infected ones by the three strains of L(V)b (p=.0.005) (Figure 3D). All bands were completely inhibited by 1-10 phenanthroline (data not shown).

Discussion

In the *in vitro* cultures of the studied strains/species, remarkable differences in promastigote forms were observed and confirmed by the morphological assay (Figure 1). Comparing our results (Figure 1B) with [21] and [22], that researched the body length of L(V)l and L(V)n and, there is agreement that L(V)1 still has greater body length than L(V)n. The comparison of body length results for L(V)b was not possible because it was not found in the literature. We believe that the difference observed in body length cannot be discussed in terms of the species identification, but in terms of promastigote stages and parasite virulence.

Based on the body length, body width and the proportion of flagellum and body, it is possible to suggest that the majority of promastigotes in L(V)n culture were procyclic promastigotes. While in the L(V)l culture, they were mostly nectomonad promastigotes. Finally, in the three L(V)b cultures the main form was the metacyclic promastigote (Table 1). The promastigotes are flagellar forms present in vertebrate host (vector) and *in vitro* culture, while an aflagellar form called amastigote is present in the mammalian host [23]. In the vector, the promastigote forms have different evolutionary forms that are from procyclic to metacyclic promastigote, including more three different forms [16,24]. The infective potential of each form is not clearly understood, but there is a knowledge about metacyclic promastigotes being highly infective and that these different forms can also be observed in *in vitro* culture [16,24]. In the present study, the different results of promastigote forms (body length, body width and the proportion of flagellum and body) may reflect their differences in infectivity and differences in membrane constitution (peptidases), which were observed in the zymographic assay of *Leishmania* peptidases (Figure 3A). The forms and peptidases contribute to the virulence understanding of these species/strains, suggesting further studies.

Different patterns of *Leishmania* protease activity were observed among the species (Figure 3A). Peptidases are a group of enzymes produced by both parasites and hosts. They are related to different stages of leishmaniasis and can directly influence the course of the disease. The *Leishmania* peptidases are related to several developmental and proliferation processes, contributing to the interaction with both hosts (vertebrates and invertebrates) and evasion of immune response [14,25–28]. Similar to our results, Cuervo et. al (2005 and 2008) compared the peptidase active profile of different strains of L(V)b isolated from patients with different clinical manifestations and found different peptidases partners among them, ranging from 50-125 kDa, being the set of bands expressed in mucosal and cutaneous leishmaniasis distinct. Thus, the peptidases patterns appear to be related to different clinical manifestations, since L(V)b has been related to the mucosal and cutaneous leishmaniasis while L(V)l and L(V)n only with cutaneous form[4], and cases of self-healing are associated with L(V)n [8].

In the L(V)b strains complex, the patterns of *Leishmania* peptidase activity among L(V)b strains were equal, but the amount of peptidase activity in two bands out of the three expressed were decreased in the L(V)b-M2314 strain, that was isolated from a patient with good response to Glucantime® treatment [15]. Based on the study of Lima et. al (2009) that found a lower number of peptidase bands in a nonvirulent strain of L(V)b (one bands)

Leishmania (Viannia) spp.	Source	Predominant promastigote form in culture	Body length	Infection index ± SD		Metalloproteases profile presents in the membrane of promastigotes
			µm±SD	0h	24h	(kDa)
L. (V.) lainsoni MHOM/BR/8l/M6424	CL	Nectomonad	11.71± 1.95[a]	185.42±31[a]	123.93±23[b]	141, 73, 67, 60, 57, 54 and 51
L. (V.) naiffi MDAS/BR/79/M5533	Armadillo	Procyclic	4.82± 0.62[b]	200.67±26[a]	183.39±28[a]	78, 62, 55 and 57
L. (V.) braziliensis MHOM/2003/2314	CL (sensible to Glucantime®)	Metacylicl	8.32± 1.64[c]	187.79±29[a]	212.61±28[a]	92, 78 and 62
L. (V.) braziliensis MHOM/BR/2009/3476	CL (resistence to Glucantime®)	Metacylicl	8.50± 1.33[c]	204.00±31[a]	207.88±35[a]	92, 78 and 62
L. (V.) braziliensis MHOM/BR/1987/M11272	CL (standard)	Metacylicl	7.55± 1.27[c]	180.26±19[a]	180.54±28[a]	92, 78 and 62

Table 1- Main differences among studied *Leishmania (Viannia)* spp.

compared to a virulent one (four bands), it is possible to affirm that this decrease in L(V) b-M2314 peptidases is related to a less virulent parasite when compared with the other two strains.

Biochemical characteristics of these peptidases showed that all of them belong to the metalloproteases class since they were abrogated by 1-10 phenanthroline, a specific inhibitor of this class of enzyme [29]. One of the most important metalloproteases present in the membrane of *Leishmania* is the glycoprotein 63 (GP-63) which is strongly related to the parasite virulence [11]. This is a multifunctional enzyme that is associated with the inactivation of complement compounds [28], degradation of extracellular matrix contributing to cell migration [27,28], inhibition of natural killing cells and activation of tyrosine phosphatase in the host [30,31]. Resting on these facts, we encourage further investigations in GP-63, which would give major contributions in the virulence understanding of *Leishmania (Viannia)* spp, highlighting that the role of *Leishmania* metalloproteases remains unclear particularly for *L. (V.)lainsoni*and *L.(V.)naiffi*.

The host metalloproteases also are involved in the leishmaniasis pathogenesis[12,32–36], but there are few studies on New World *Leishmania* species [12,32,33,35]. The host peptidase profiles of macrophages infected or not with *Leishmania (Viannia)* spp. were the same, with six bands belonging to the metalloproteases class based on their biochemical characteristics [29]. According to the molecular weight, bands found in this study could be a membrane-type matrix metalloprotease (MMP) probably involving MMP-9 (130kDa), pro-MMP-9 (98 KDa), pro-MMP-2 (72 kDa), MMP-2 (68 kDa), and two metalloprotease degraded products (37 and 34 kDa). Lee et al (2012) found that pro-MMP-9, MMP-9, pro--MMP-2 and MMP-2 are expressed constitutively in J774A.1 macrophages, which is in concordance with our results.

Nowadays the MMP-9 and MMP-2 have been related to the immunopathogenesis of leishmaniasis, and the high expression of MMP-9 is related to clinical manifestations of post kala-azar dermal leishmaniasis [36] and the dissemination of parasites in the mucosal form of diseases[32]. Both metalloproteases were related to therapeutic failure [32] and progression of tegumentary leishmaniasis [35]. In general, these facts seem to associate MMP-9 and MMP-2 with a severe and benign

form of diseases, respectively. These findings are in disagreement with ours because we found that the low activity of the band 72 kDa, supposedly pro-MMP-2, was diminished in the macrophages infected with L(V)n, the strain that presented to be less virulent to human (self-healing)[8]. One explanation for this is that other cell lines, despite the macrophages, can produce metalloproteases in leishmaniasis, such as monocyte lines [35] and activated neutrophils [37] and all the mentioned studies were made in patient's blood or biopsies, a much more complex physiological system than just macrophages. Another important point is that in literature only few studies that investigate these metalloproteases were found in cutaneous and mucosal leishmaniasis [13], suggesting that these results are preliminary and need more confirmations.

In addition to the promastigote and peptidase assays, we investigated the amastigote infection on macrophages. The absence of difference in the infection indexes at 0 h among the strains of L(V)b, L(V)l and L(V)n may reflect that the macrophage-*Leishmania* interactions mediated by ligand-receptor are not so different among them. However, there is evidence that molecules of parasites such as GP 63 and lipophosphoglycan involved in the interaction and other virulence processes are redundantly expressed in *Leishmania* sp.[27,38]. Then, they could operate in a compensatory manner depending on the abundance of them [39], which is a limitation of the used method. The infection indexes found in our study showed that L(V)l was less infective to macrophages than the others and, interestingly, it was the only tested species which the infected index at 0 h was significantly decreased 24 h later (Figure 2). This result showed that the macrophages are able to control better the infection caused by L(V)l.

Considering the importance of nitric oxide for parasite elimination [40], the nitrite-derived production was investigated. In the present study, no production of nitrite-derived by macrophages infected with *Leishmania (Viannia)* spp. was detected. Matta et al (2010) compared the L(V)n with L(V)b and *L. (V.) guyanensis in vitro* and did not report any difference in their infection indexes, similar to our results. In relation to the findings involving the strains of L(V)b, no significant difference was found neither in the infection indexes nor in the nitrite-derived production, which is similar to the results of Fernandes et. al (2015).

Conclusion

The results reported in this study suggest that low expression of *Leishmania* peptidases may be associated with the cure after the treatment in L(V)b strains. They also pointed out that L(V)b, L(V)l and L(V)n have differences in virulence and peptidase activity profile (Table 1) that could be related to their clinical manifestation in humans.

Acknowledgements

We gratefully acknowledge all members of the Laboratory of Leishmaniasis at the Universidade Estadual de Maringá (Brazil) for their support in this research. We also acknowledge Dr. Priscila Portugal dos Santos of Universidade Estadual Paulista (Brazil), Dr. Patrícia de Souza Bonfim de Mendonça of Universidade Estadual de Maringá (Brazil) and Dr. Sandra Mara Alessi Aristides of Universidade Estadual de Maringá (Brazil) for their valuable suggestions and careful analysis of the manuscript.

Financial support

This study was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES, Portuguese: *Coordenação de aperfeiçoamento de pessoal de nível superior*) and The Brazilian National Council

for Scientific and Technological Development (CNPq, Portuguese: *Conselho nacional de desenvolvimento científico e tecnológico*) (PROAP n^{0}817627/2015).

Conflict of interest

None.

Ethical standards

Not applicable.

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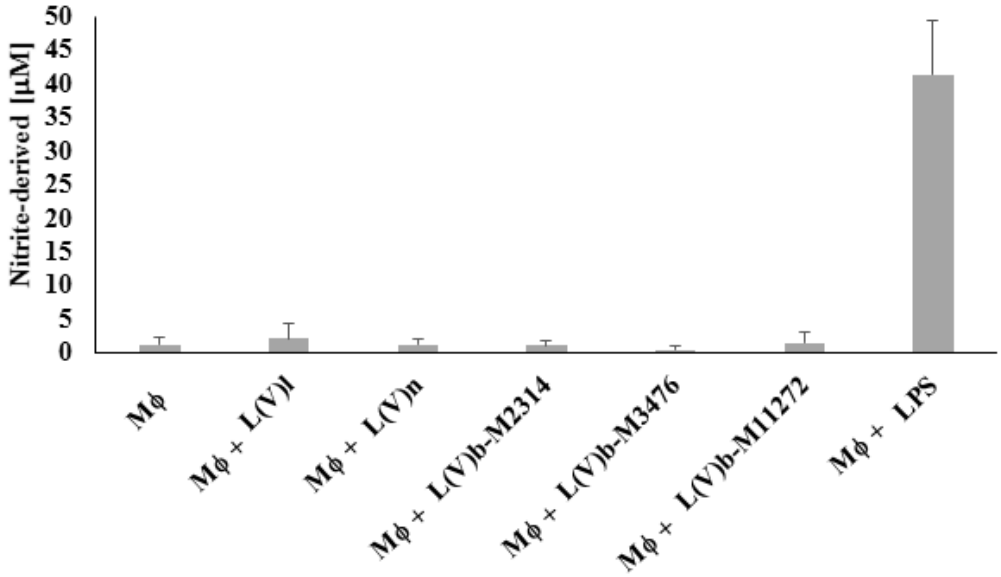
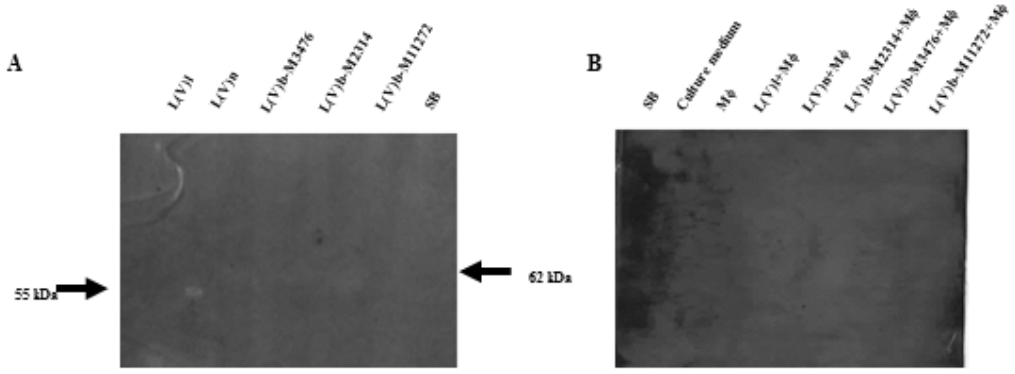


Supplementary data 1: Nitrite-derived production. Production of nitrite-derived in µM by J774A.1 macrophages alone and in response to *Leishmania (Viannia)* spp. infection. Mφ: J774A.1 macrophages; L(V) l: *L. (V.) lainsoni* (MHOM/BR/8l/M6424); L(V)n: *L. (V.) naiffi* (MDAS/BR/79/M5533); L(V)b-M2314: *L. (V.) braziliensis* (MHOM/2003/2314); L(V)b-M3476: *L. (V.) braziliensis* (MHOM/BR/2009/3476); L(V) b-M11272; *L. (V.) braziliensis* (MHOM/BR/1987/M11272); LPS: lipopolysaccharide (positive control of nitrite-derived production). The results are expressed in mean and standard deviation of three independent experiment.



Supplementary data 2: Inhibition peptidase proteolytic assay. A- Peptidase proteolytic profile of enzymes present in the membrane of promastigotes of *Leishmania (Viannia)* spp. in SDS-page treated with 10 mM of 1-10 phenanthroline. The *L. (V.) lainsoni* (MHOM/BR/8l/M6424) [L(V)l] had the activity of six peptidase bands completely inhibited (141, 73, 67, 60, 57 and 51 kDa) and the peptidase band at the 55 kDa was partially inhibited; the *L. (V.) naiffi* (MDAS/BR/79/M5533) [L(V)n] hab all peptidase bands completely inhibited (78, 62, 55, 57 kDa); and the strains of *L. (V.) braziliensis* MHOM/BR/1987/M11272 [L(V)b-M11272], *L. (V.) braziliensis* MHOM/2003/2314 [L(V)b-M2314] and *L. (V.) braziliensis* MHOM/BR/2009/3476 [L(V)b-M3476] hab two peptidases bands inhibited (92 and 78 kDa) and the peptidase band at 62 kDa was partially inhibited. C- Peptidase proteolytic profiles of enzymes secreted by macrophages (Mϕ) alone and infected by *Leishmania (Viannia)* spp.; all strains had their peptidases band inhibited by 5 mM of 1-10 phenanthroline; Sample buffer (SB) is an internal control.