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Lack of relationship on *in vitro* susceptibility of *Leishmania* (*Viannia*) *braziliensis* promastigotes and *Leishmania RNA virus* 1 to the trivalent antimonial

Ausência de relação na susceptibilidade *in vitro* de promastigotas de *Leishmania (Viannia) braziliensis* e de *Leishmania RNA virus* 1 ao antimonial trivalente

Ausencia de relación entre la susceptibilidad *in vitro* de promastigotes de *Leishmania (Viannia) braziliensis* y el *Leishmania RNA virus* 1 al antimonial trivalente

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ABSTRACT

Objective: To evaluate the relationship between the presence/viral load of *Leishmania RNA virus* 1 (*LRV*1) and the *in vitro* susceptibility of *Leishmania* (*Viannia*) *braziliensis* promastigotes to trivalent antimony (SbIII). **Methods:** Experimental study with 20 strains of *L. (V.) braziliensis*, 12 positives for *LRV*1. Susceptibility to SbIII was assessed using smoothing curves and the activity index (AI). *LRV*1 was quantified by real-time PCR (qPCR). Statistical tests were applied to evaluate associations and correlations between the analyzed parameters. **Results:** The strains were classified as susceptible (N=8) and less susceptible (N=12) based on smoothing curves compared to the reference strain. According to the AI, all strains showed lower susceptibility compared to the reference strain. Among the 12 *LRV*1-positive strains, 75% had quantifiable viral loads, while 25% were below the quantification limit. *LRV*1-positive strains exhibited higher parasite density compared to *LRV*1-negative strains. **Conclusion:** Although *LRV*1-positive strains presented higher parasite density, no significant correlation was observed between the presence/viral load of *LRV*1 and *in vitro* susceptibility to SbIII.

Keywords: Susceptibility, *Leishmania*, viral load.

RESUMO

Objetivo: Avaliar a relação entre a presença/carga viral de *Leishmania RNA virus* 1 (*LRV*1) e a susceptibilidade *in vitro* de promastigotas de *Leishmania* (*Viannia*) *braziliensis* ao antimônio trivalente (SbIII). **Métodos:** Estudo experimental com 20 cepas de *L.* (*V.*) *braziliensis*, 12 positivas para *LRV*1. A susceptibilidade ao SbIII foi avaliada por curvas de suavização e índice de atividade (IA). O *LRV*1 foi quantificado por PCR em tempo real (qPCR). Testes estatísticos foram aplicados para avaliar as associações

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e correlações entre os parâmetros analisados. **Resultados:** As cepas foram classificadas como susceptíveis (N=8) e menos susceptíveis (N=12) com base nas curvas de suavização em relação à cepa de referência. No IA, todas as cepas apresentaram menor susceptibilidade em comparação à referência. Das 12 cepas positivas para *LRV*1, 75% tiveram carga viral quantificada, enquanto 25% ficaram abaixo do limite de quantificação. As cepas *LRV*1 positivas exibiram maior densidade parasitária em relação as negativas. **Conclusão:** Embora as cepas *LRV*1 positivas apresentem maior densidade parasitária, não houve correlação significativa entre a presença/carga viral de *LRV*1 e a susceptibilidade *in vitro* ao SbIII.

Palavras-chaves: Susceptibilidade, *Leishmania*, carga viral.

RESUMEN

Objetivo: Evaluar la relación entre la presencia/carga viral del *Leishmania RNA virus* 1 (*LRV*1) y la susceptibilidad *in vitro* de promastigotes de *Leishmania (Viannia) braziliensis* al antimonio trivalente (SbIII). **Métodos:** Estudio experimental con 20 cepas de *L. (V.) braziliensis*, 12 positivas para *LRV*1. La susceptibilidad al SbIII se evaluó mediante curvas de suavización e índice de actividad (IA). El *LRV*1 fue cuantificado mediante PCR en tiempo real (qPCR). Se aplicaron pruebas estadísticas para evaluar las asociaciones y correlaciones entre los parámetros analizados. **Resultados:** Las cepas fueron clasificadas como susceptibles (N=8) y menos susceptibles (N=12) basándose en las curvas de suavización en comparación con la cepa de referencia. Según el IA, todas las cepas mostraron menor susceptibilidad en comparación con la referencia. De las 12 cepas positivas para *LRV*1, el 75% tuvo carga viral cuantificada, mientras que el 25% estuvo por debajo del límite de cuantificación. Las cepas positivas para *LRV*1 presentaron una mayor densidad parasitaria en comparación con las negativas. **Conclusión:** Aunque las cepas positivas para *LRV*1 mostraron mayor densidad parasitaria, no se encontró una correlación significativa entre la presencia/carga viral del *LRV*1 y la susceptibilidad *in vitro* al SbIII.

Palabras clave: Susceptibilidad, *Leishmania*, carga viral.

INTRODUCTION

Tegumentary leishmaniasis (TL) is a global public health problem, not only due to its wide geographic distribution in tropical and subtropical areas but also because of its various clinical manifestations that damage the epidermis and mucous membranes (ALVAR J, et al., 2012; WHO, 2023). After the healing of cutaneous leishmaniasis (CL), some individuals may develop mucosal leishmaniasis (ML) years after specific treatment or spontaneous healing. ML is a severe clinical manifestation, characterized by tissue destruction in the mucous membranes, primarily affecting the nasal or oral regions, and resulting in social stigma for the individual (BURZA S, et al., 2018; MEIRA CS and GEDAMU L, 2019).

The clinical progression from CL to ML is not fully understood, however associations with genetic and immunological factors in individuals (RAMASAWMY R, et al., 2010; CASTELLUCCI LC, et al., 2014; BARCELLAR O, et al., 2002; MEIRA CS and GEDAMU, 2019) and the presence of an endosymbiotic virus called *LRV*1 have been explored in the literature (IVES A, et al., 2011; CANTANHÊDE LM, et al., 2015; CARVALHO RVH, et al., 2019). *LRV*1 belongs to the Totiviridae family and the *Leishmaniavirus* genus, often present in some species of *Leishmania* in the *Viannia* subgenus (TARR PI, et al., 1988; SCHEFFTER SM, et al., 1995; CARRION JR, et al., 2008; ZANGGER H, et al., 2014; CANTANHÊDE LM, et al., 2015). One species from the *Leishmania* (*Leishmania*) subgenus and five species from the *Leishmania* (*Viannia*) subgenus have been recorded as hosts of *LRV*1, with particular concern that *L.* (*V.*) *braziliensis*, *L.* (*V.*) *guyanensis* and *L.* (*V.*) *naiffi*, the most common species in northern Brazil, frequently harbor this endosymbiont (TARR PI, et al., 1988; GUILBRIDE L, et al., 1992; CANTANHÊDE LM, et al., 2015; SANTANA MCO, et al., 2023). Furthermore, since ML is frequently associated with these species, the presence of *LRV*1 constitutes an important prognostic factor (CANTANHÊDE LM, et al., 2015; CARVALHO RVH, et al., 2019).

Studies have proposed that the presence of *LRV*1 may influence the therapeutic failure process in the Americas (ADAUI V, et al., 2016; BOURREAU E, et al., 2016). The treatment of TL is based on the administration of pentavalent antimony (SbV), a first-line drug in several countries, associated with various side effects and, in certain cases, with low therapeutic success (CROFT SL, et al., 2006; BERBERT TRN, et al., 2018; SANTOS GA, et al., 2023), including in South America, where patients infected with *L.* (*V.*) *braziliensis* and *L.* (*V.*) *guyanensis* have shown therapeutic failure (ADAUI V, et al., 2016; BOURREAU E, et al., 2016). Moreover, evidence of resistance to SbV has been observed *in vitro* studies with *L.* (*V.*) *braziliensis* strains isolated from patients with therapeutic failure (YARDLEY V, et al., 2006; ROJAS R, et al., 2006; RUGANI JN, et al., 2019). In Brazil, in 2022, it was found that approximately 50% of treated TL cases progressed to healing (BRASIL, 2022). Other possible reasons for the low therapeutic efficacy include inadequate patient treatment, the individual's immune system, and *Leishmania* species, which may play a differential role in treatment response due to biological aspects of the parasite and its interaction with the host's immune system, leading to variations in drug susceptibility (ROMERO GAS, et al., 2001; AREVALO J, et al., 2007; PONTE-SUCRE A, et al., 2017; SANTOS GA, et al., 2023).

Intrinsic changes in the parasite's susceptibility to antimony have been verified in strains of the *Leishmania* and *Viannia* subgenera through protein expression analysis (BIYANI N, et al., 2011; BROTHERTON MC, et al., 2013; MATRANGOLO FSV, et al., 2013), as well as through the assessment of the expression of *Leishmania* genes involved in antimony metabolism pathways (MUKHERJEE A, et al., 2007; LEPROHON P, et al., 2009; ANDRADE JM, et al., 2020). However, it is important to note that there are few studies evaluating the *in vitro* susceptibility profile of *Viannia* subgenus strains circulating in the Amazon region, rarely describing the presence of the viral endosymbiont *LRV*1 and its response to antimony.

Understanding the relationship between *LRV*1 and *Leishmania* may contribute to monitoring the response to SbV and to the development of new therapeutic strategies in CL. Therefore, this study aims to determine whether there is an association between the presence and viral load of the *LRV*1 endosymbiont in the *in vitro* susceptibility of *L. (V.) braziliensis* strains to the active form of SbV and to propose an alternative approach for evaluating *in vitro* drug susceptibility.

METHODS

Ethical Aspect

The samples were previously collected by the Laboratory of Genetic Epidemiology (LabEpiGen) at the Fundação Oswaldo Cruz - Rondônia (FIOCRUZ/RO), with ethical approval under the Certificate of Presentation for Ethical Consideration - CAAE No. 54386716.1.0000.0011 and parecer No. 3445506.

Cultures of *Leishmania*

This study included 20 strains of *L.* (*V.*) *braziliensis* freshly isolated from patients diagnosed with CL in Porto Velho, Rondônia. Species identification and *LRV*1 detection were performed at LabEpiGen, and the strains were deposited in the Fiocruz *Leishmania* Collection (CLIOC). Some recent isolates have not yet been deposited in CLIOC (**Supplementary File 1**).

Standardization of parasites to *in vitro* experiments

The *L.* (*V.*) *braziliensis* strains were cultured in Schneider's medium (Gibco, Paisley, Scotland, UK), supplemented with 20% fetal bovine serum (Vitrocell, Campinas, SP, BR), 2% filtered human urine, and 50 µg/mL gentamicin, and incubated at 25°C. Parasite growth curves were determined by counting every 24 hours for 10 days, using Erythrosin B dye (Merck, Darmstadt, Alemanha) (0.04%), with an initial parasite concentration of 1×10^6/mL.

Trivalent antimony susceptibility *in vitro* assay

The parasites in the logarithmic phase were adjusted to a concentration of 1×10^6/mL and subjected to serial dilutions of SbIII (Sigma, St. Louis, MO, USA) at a 1:3 ratio in 96-well plates (Nunc, Roskilde, Denmark). The

serial dilutions of SbIII ranged from 1000 µM to 0.005 µM, with a final volume of 200 µL per well, and then incubated at 25°C for 72 hours. After incubation, Alamar Blue® solution (Sigma, St. Louis, MO, USA), at a concentration of 2 mM diluted in 1X PBS (pH 7.4), was added to assess the metabolic viability of the promastigotes. The plates were reincubated in a BOD incubator at 24°C ± 1 for 5 hours in the dark. Fluorescence readings were performed using a spectrofluorometer (Biotek, Synergy) with the parameters 530 nm/25 and 590 nm/35. The IOCL566 strain (MHOM/BR/75/M2903) was included in the *in vitro* experiments as a reference for SbIII sensitivity, as described in the literature (YARDLEY V, et al., 2006).

Viral load experiments

Total RNA was extracted from 200 µL of *L. (V.) braziliensis* positive for *LRV*1, in the logarithmic growth phase (1×10^6 parasites/mL), using the RNeasy Mini kit (Qiagen, Germany). RNA concentration was quantified by fluorescence using the Qubit 4 fluorometer, and purity was assessed by spectrophotometry on the NanoDrop ND-2000 (both from Thermo Fisher Scientific). The total RNA was normalized to 1 ng/µL, treated with DNase I (Promega, USA), and subjected to reverse transcription with the M-MLV Reverse Transcriptase enzyme (Invitrogen, USA), following the manufacturer's instructions.

To quantify the viral load of *LRV*1-containing strains, primers were designed using the Primer-BLAST tool, based on the sequence of the *LRV*1 1 Brazil/2013/308 strain (GenBank accession code KT347140.1) (CANTANHÊDE LM, et al., 2015). The primer pair *LRV*1_*ORF*1_76F - GACTGATTGGACGGAGGGCA and *LRV*1_*ORF*1_76R - TGCTGTGGAACGTGAGGAACT generates amplicons of 76 base pairs from the open reading frame 1 (*ORF*1) region of the *LRV*1 viral genome. This primer pair was tested in silico against the nr database of GenBank to check for non-specific amplification. Viral load standardization by qPCR was performed using the cloned *LRV*1 fragment in the pGEM®-T Easy Vector Systems plasmid (Promega - Wisconsin, USA), by LabEpiGen.

For the linearity assays, standard curves were generated from serial dilutions of the recombinant plasmid, ranging from $2x10^1$ to $2x10^8$ viral copies/µL. The reaction was performed with primer concentrations of 0.3 µM, 10 µL of Mix Master Power Sybr Green® 1X (Applied Biosystems), 2 µL of dilution points, and ultrapure water to a final volume of 20µL. The experiments were conducted using the QuantStudio3 equipment (Applied Biosystems) on the RPT09F platform at FIOCRUZ/RO, under the following temperature conditions: activation of the AmpliTaq® Gold DNA Polymerase enzyme (95 °C for 10 min), followed by 40 cycles of 15 sec at 95 °C (denaturation), 1 min at 64 °C (annealing/extension), and 1 min at 62 °C (dissociation curve). The quantification of cultures positive for *LRV*1 was performed in technical duplicate under the same standardized conditions described above, with negative controls included in all experiments.

Data analysis

The fluorescence values generated by the spectrofluorometer were entered into an Excel spreadsheet (Microsoft Office). Smoothing curve graphs were generated using the LOESS method (Cleveland; Devlin, 1988) on the R platform v.4.4.1, as well as IC_{50} values, using the formula: $y = vmin – (vmax - vmin) \div [1 + (X \div EC_{50})]$ Hill (HYDE, 2018). The smoothing curve analysis was based on parasitic density in response to SbIII dilutions, with the curves subsequently compared to that of the standard strain IOCL566. The Activity Index (AI), defined as the ratio between the IC_{50} of the test strain and the IC_{50} of the reference strain IOCL566, was calculated as described by Yardley V, et al. (2006), with strains considered less susceptible to antimony when presenting AI values greater than 3. The parasitic density of *L.* (*V.*) *braziliensis* strains was analyzed from fluorescence data, based on the average of the initial point of viable parasites and the final point of non-viable parasites. With the obtained averages, the coefficient of variation (CV) formula was applied in the free R platform: $CV = (S \div \dot{x}) \times 100$, where S represents the standard deviation (REED et al., 2002).

The association between parasite susceptibility to SbIII, based on the results of the smoothing curve, and the presence or absence of *LRV*1 were analyzed using a 2x2 contingency table, with the application of Fisher's exact test for statistical evaluation. The Mann-Whitney test was applied to compare parasitic density and IC_{50} values between the conditions of presence/absence of *LRV*1. The viral load quantification of *LRV*1 was performed using the threshold cycle (Ct) values obtained from qPCR. The data were plotted in an Excel

spreadsheet (Microsoft Office) and analyzed using the formula: y = ax + b, relating the Ct value to the number of copies per reaction in log. To obtain the absolute viral load, the log value was converted from the linear scale using the calculation: $10^{\text{Log Quant } LRV1}$ = viral copies per reaction. The correlation between viral load and parasitic density was assessed using the Spearman correlation test. The association and correlation graphs, as well as the statistical analyses, were generated using free alternatives of GraphPad Prism v.10.4.1.

RESULTS

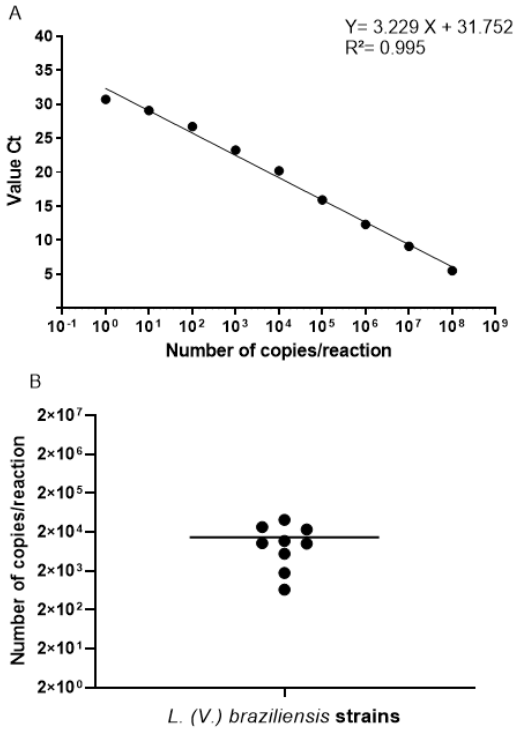
Growth curve and synchronization of cultures

Growth parameters are presented in **Supplementary File 2**. The growth phase ranged from 72 to 96 hours. The strains IOCL3564, IOCL3621, RO1113, and RO1160 reached the logarithmic phase within 72 hours, whereas the strains IOCL3545, IOCL3547, IOCL3549, IOCL3562, IOCL3567, IOCL3622, IOCL3626, IOCL3627, IOCL3642, IOCL3637, IOCL3639, IOCL3714, IOCL3817, IOCL3833, IOCL3851, and RO1022 reached this phase only after 96 hours. Following exponential growth, the parasite proliferation rate decreased, initiating the transition into the stationery and decline phases.

Viral load of *LRV*1

The quantification of *LRV*1 viral load was performed using a standard curve with an efficiency of 103% and an R^2 of 0.995 (**Figure 1A**). Absolute quantification was established by setting the fluorescence detection threshold at 0.04, resulting in specific Ct values for *LRV*1. Among the twelve *LRV*1-positive strains, viral load was quantified for IOCL3545, IOCL3562, IOCL3567, IOCL3621, IOCL3622, IOCL3637, IOCL3639, IOCL3642, and RO1022. For IOCL3564, IOCL3714, and IOCL3833 viral load could not be determined due to lack of reproducibility in the qPCR replicates. The viral load of the nine *L. (V.) braziliensis* strains had an average of 14,736.69 viral copies per reaction, ranging from $2x10^2$ to $2x10^4$ viral copies per reaction (**Figure 1B**, **Table 1**).

Figure 1 - *LRV*1 standard curve and viral load quantification.

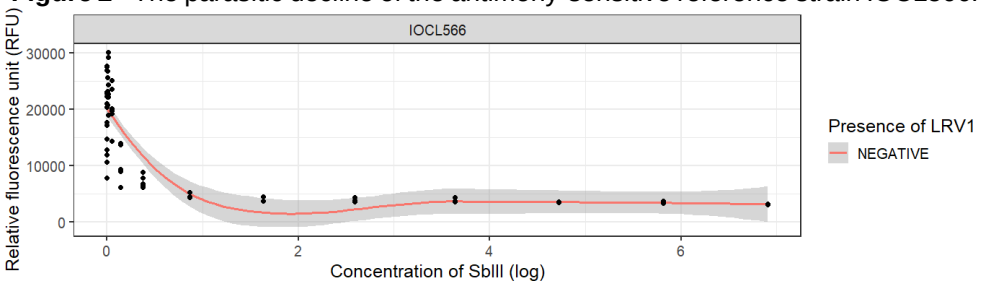


Legend: Figure A shows the standard curve for *LRV*1 quantification, with Ct values on the Y-axis and known *LRV*1 concentrations on the X-axis. Figure B displays the viral load of *LRV*1-positive *L. (V.) braziliensis* strains, with viral copies per reaction on the Y-axis and the corresponding strains on the X-axis. Figure generated using GraphPad Prism software, license: de63676e-59a3-469d-8489-b4d05151919c. **Source:** Medeiros EHRT, et al., 2025.

Smoothing curves of *L. (V.) braziliensis* strains to assess parasite density parasites in relation to the reference strain

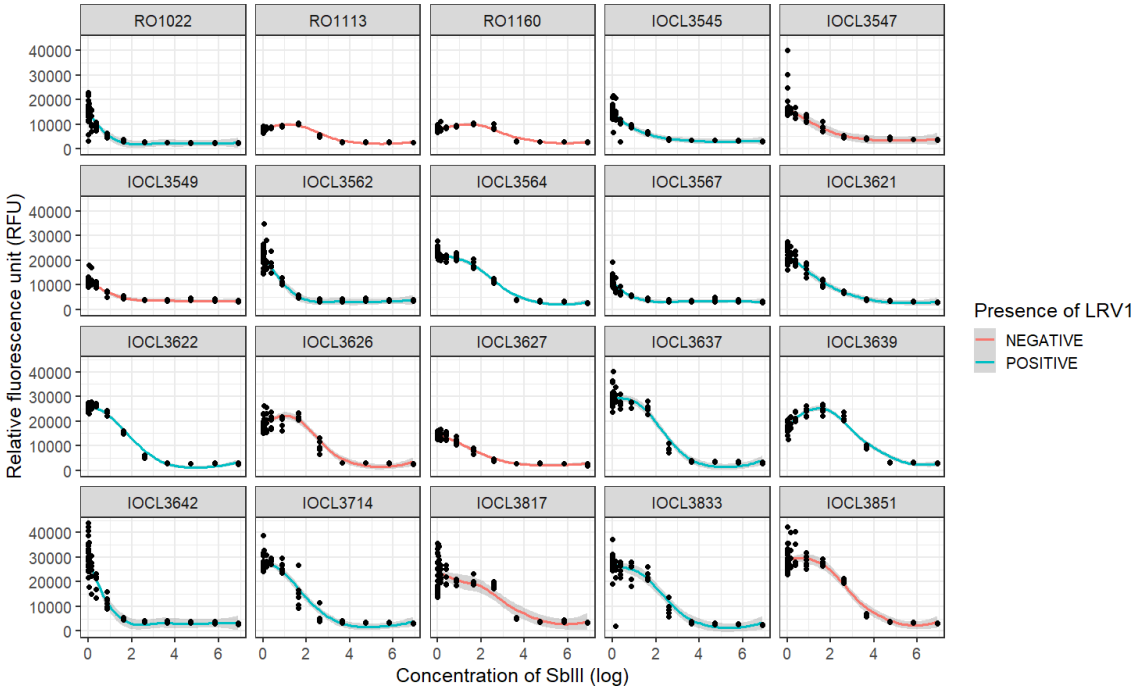
The positive *LRV*1 strains IOCL3545, IOCL3562, IOCL3567, IOCL3621, IOCL3642, and RO1022, as well as the negative *LRV*1 strains IOCL3547 and IOCL3549, had a parasitic decline similar to the smoothing curve of the reference strain IOCL566, while the positive strains IOCL3564, IOCL3622, IOCL3637, IOCL3639, IOCL3714 and IOCL3833 the negative strains IOCL3626, IOCL3627, IOCL3817, IOCL3851, RO1113, and RO1160 a point of parasitic decline at higher concentrations of the evaluated drug when compared to the reference strain (**Figure 2**, **3**). The Fisher's Exact Test showed that the difference in the proportion of *LRV*1-positive strains susceptible and less susceptible to the SbIII drug is not significantly different from the *LRV*1-negative strains (p=0.3729).

Figure 2 - The parasitic decline of the antimony-sensitive reference strain IOCL566.



Legend: The Y-axis represents the RFU, and the X-axis represents the different dilutions of SbIII on a logarithmic scale (log). The dots represent the six replicates of each SbIII concentration. Controls were used to validate the assays. The analysis was performed using raw fluorescence data. Figure generated using R software, license: https://www.r-project.org/. **Source:** Medeiros EHRT, et al., 2025.

Figure 3 - Parasitic decline of *LRV*1 positive/*LRV*1 negative strains to SbIII.



Legend: The Y-axis represents the RFU, and the X-axis represents the different dilutions of SbIII on a logarithmic scale (log). The dots represent the six replicates of each SbIII concentration. Controls were used to validate the assays. The analysis was performed using raw fluorescence data. Figure generated using R software, license: https://www.r-project.org/. **Source:** Medeiros EHRT, et al., 2025.

In vitro susceptibility of the parasite to SbIII and its relation to *LRV*1

The estimated IC_{50} values for the strains under study ranged from 0.97 µM to 22.45 µM in the negative strains and from 0.42 µM to 33.37 µM in the positive strains when exposed to SbIII. When analyzing the AI, it was observed that the *LRV*1-negative strains required 9.50 to 220.09 times the dose of SbIII to inhibit 50% of the parasite population, while the *LRV*1-positive strains required 4.11 to 327.15 times the same drug to achieve the same biological effect, compared to the reference strain. When evaluating parasitic density, considering the initial and final points of the smoothing curve, a variation in the initial density among the studied strains was observed. Among the *LRV*1-negative strains, the CV ranged from 38.37% to 57.82%, while in the *LRV*1-positive strains, it ranged from 47.76% to 74.38% (**Table 1**).

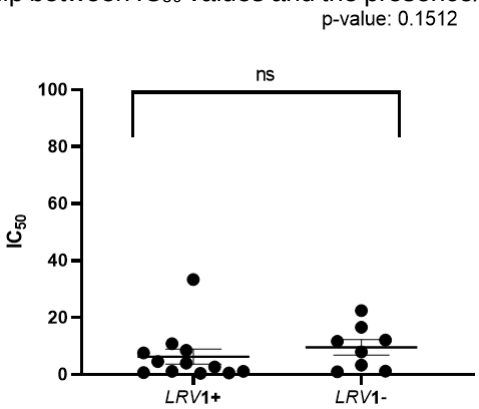
Table 1 - The overall result of positive and negative strains for *LRV*1.

Code	*LRV*1	IC_{50} (µM)	AI	Smoothing curve	CV (%)	Load viral
IOCL566	Negative	0.102	-	Susceptible	-	-
IOCL3547	Negative	0.97	9.50	Susceptible	57.82	-
IOCL3549		1.18	11.56	Susceptible	46.76	-
IOCL3626		11.67	114.41	Less susceptible	54.29	-
IOCL3627		7.94	77.84	Susceptible	54.45	-
IOCL3817		3.29	32.25	Less susceptible	54.83	-
IOCL3851		16.60	162.74	Less susceptible	52.62	-
RO1113		12.07	118.33	Less susceptible	40.40	-
RO1160		22.45	220.09	Less susceptible	38.37	-
IOCL3545	Positive	1.12	10.98	Susceptible	57.32	10.150.88
IOCL3562		1.12	10.98	Susceptible	65.60	1.788.13
IOCL3564		10.80	105.88	Less susceptible	52.61	-
IOCL3567		0.50	4.90	Susceptible	53.07	11.832.80
IOCL3621		2.64	25.88	Susceptible	57.53	41.657.82
IOCL3622		4.53	44.41	Less susceptible	60.60	23.547.34
IOCL3637		7.56	74.11	Less susceptible	58.25	10.370.37
IOCL3639		33.37	327.15	Less susceptible	47.76	666.00
IOCL3642		0.69	6.76	Susceptible	74.38	27.060.26
IOCL3714		4.00	39.21	Less susceptible	61.29	-
IOCL3833		8.46	82.94	Less susceptible	59.94	-
RO1022		0.42	4.11	Susceptible	73.13	5.556.64

Legend: Code IOCL, collection de *Leishmania* of Instituto Oswaldo Cruz and code RO, Rondônia; *LRV*1, *Leishmania RNA virus* 1; AI, activity index; IC_{50}, growth inhibition of 50%; CV, coefficient of variation; - Strains that was not possible to determine the *LRV*1 viral load; IOCL566, SbIII-sensitive reference strain. **Source:** Medeiros EHRT, et al., 2025.

When analyzing the IC_{50} values between the *LRV*1-positive and *LRV*1-negative groups, no statistically significant difference was identified (p = 0.1512, **Figure 4**), corroborating Fisher's test, which also showed no significant differences in susceptibility based on the smoothed curve data.

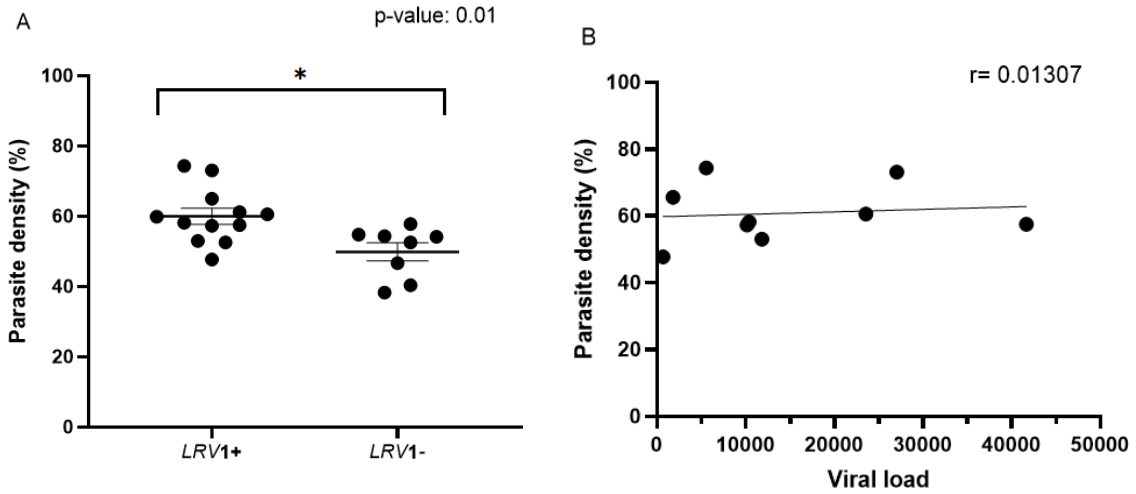
Figure 4 - Relationship between IC_{50} values and the presence/absence of *LRV*1.



Note: ns = no statistically significant difference. Figure generated using GraphPad Prism software, license: de63676e-59a3-469d-8489-b4d05151919c. **Source:** Medeiros EHRT, et al., 2025.

On the other hand, the statistical analysis of parasite density revealed a p-value of 0.01, indicating a statistically significant difference between *LRV*1-positive and *LRV*1-negative strains (**Figure 5A**). However, the correlation (r) between parasite density and *LRV*1 viral load was extremely weak (**Figure 5B**).

Figure 5 - Parasite density in relation to the absence/presence and viral load of *LRV*1.



Note: *= p-value 0.01. Figure generated using GraphPad Prism software, license: de63676e-59a3-469d-8489-b4d05151919c. **Source:** Medeiros EHRT, et al., 2025.

DISCUSSON

The proposal to investigate the *in vitro* susceptibility of *L.* (*V.*) *braziliensis* strains to the SbIII compound and correlate it with the presence and viral load of *LRV*1 is based on studies showing an association between therapeutic failure with SbV compound and *Leishmania* infection of the *Viannia* subgenus containing the viral endosymbiont *LRV*1 (ADAUI V, et al., 2016; BOURREAU, et. al., 2016). In this study, we present an approach that combines smoothing curves, IA and CV to estimate different biological variables and advance susceptibility analyses. The strains were isolated from patients in Rondônia, a state in the Brazilian Amazon region, endemic for TL and with *LRV*1 circulation (CANTANHÊDE LM, et al., 2015; CANTANHÊDE LM, et al., 2018). It is important to highlight that we do not have access to clinical outcomes and treatments administered to the patients from whom the isolates were identified. It is worth noting that there are few studies on the susceptibility of *Viannia* subgenus strains, especially considering the presence of a viral endosymbiont. While other studies have assessed the *in vitro* susceptibility of *Leishmania* using AI (YARDLEY V, et al., 2006; ZAULI-NASCIMENTO R, et al., 2010), in this study, smoothing curves were considered a complementary parameter in describing susceptibility profiles due to the overview of parasitic density across drug dilutions.

Considering only the IA results with the reference strain, all the strains in this study showed less susceptible profiles. On the other hand, when adding the smoothing curve to the analysis, it was observed that eight strains exhibited results similar to the decline in parasitic density of the standard strain IOCL566, which is sensitive to SbIII. For the other 12 strains, the comparison of the decline points showed a less susceptible profile compared to the standard strain IOCL566. Indeed, these less susceptible strains presented higher IA estimates compared to the susceptible ones. The inconsistencies in determining *in vitro* susceptibility between the exploratory approach (smoothing curve) and the most commonly used determinants (IC_{50} and IA) reinforce the need for further evolution in dose-response analyses in *Leishmania*.

The contribution of smoothing curves in interpreting susceptibility minimizes the effects of variability between the strains. These data suggest that it may be important to evaluate the IC_{50} together with another parameter, such as the smoothing curve. Additionally, proliferation parameters and other characteristics should be standardized for *in vitro* susceptibility evaluation across different studies. The inclusion of other strains with

a well-defined response profile, in addition to the reference strain IOCL566, could provide a better understanding of the observed divergences.

The observed differences in susceptibility were not related to the presence of *LRV*1, as both *LRV*1-positive and *LRV*1-negative strains exhibited lower susceptibility to SbIII. The viral load of the endosymbiont also showed no relationship with the parasite's susceptibility to SbIII, as it was variable between the susceptible and less susceptible *LRV*1-positive strains to the drug tested. This variation in viral load may be associated with the parasitic population, in which there are differences in the number of viral particles per *Leishmania* cell (ZANGGER H, et al., 2014). Therefore, intrinsic factors of the parasite, such as variations in the expression of genes related to redox metabolism may explain the parasite's susceptibility (FRÉZARD F, et al., 2014).

Another important feature of the parasite's biology was observed in the exploratory results, performed during the logarithmic growth phase with the same initial parasite concentration. The exploratory analyses, using smoothing curves, allowed us to observe that, after 72 hours in culture, both *LRV*1-positive and *LRV*1-negative strains showed variation in parasitic density between the initial and final points. Heterogeneity was observed in the CV of parasitic density among the *LRV*1-positive strains. This variation in growth patterns between strains has been previously observed and linked to the presence of *LRV*1, which may be modulating the parasite's proliferation and contributing to parasitic persistence (IVES A, et al., 2011).

The presence of the endosymbiotic virus *LRV*1 in the promastigote forms of *L. (V.) braziliensis* did not influence susceptibility to SbIII, but it cannot be stated that *LRV*1 does not play an important role in human infection. Although the study used recently isolated strains from patients, the lack of association between the presence of *LRV*1 and susceptibility to SbIII may be questioned when considering the simplicity of the promastigote model. This reflection makes it essential to assess susceptibility *in vivo* or in interactions in an *in vitro* model, due to the complexity involved. As this is one of the first studies to explore the role of *LRV*1 in the Brazilian Amazon region in drug challenge experiments, further research in a multicenter and multidisciplinary context could validate our findings and expand the hypotheses using a larger sample.

CONCLUSION

This study demonstrated variability in the *in vitro* susceptibility of the analyzed strains, with no correlation to the presence and viral load of *LRV*1. This variation may be linked to the biological characteristics of the strains, but other factors, such as the inclusion of reference strains and analyses of behavior under infection with host cells *in vitro* and *in vivo*, should be considered for a more accurate assessment. Furthermore, correct normalization and detailed characterization of the strains are essential for dose-response experiments, and the approach used to determine susceptibility should not be simplified to a single parameter.

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