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Low frequency of LRV1 in *Leishmania braziliensis* strains isolated from typical and atypical lesions in the State of Minas Gerais, Brazil

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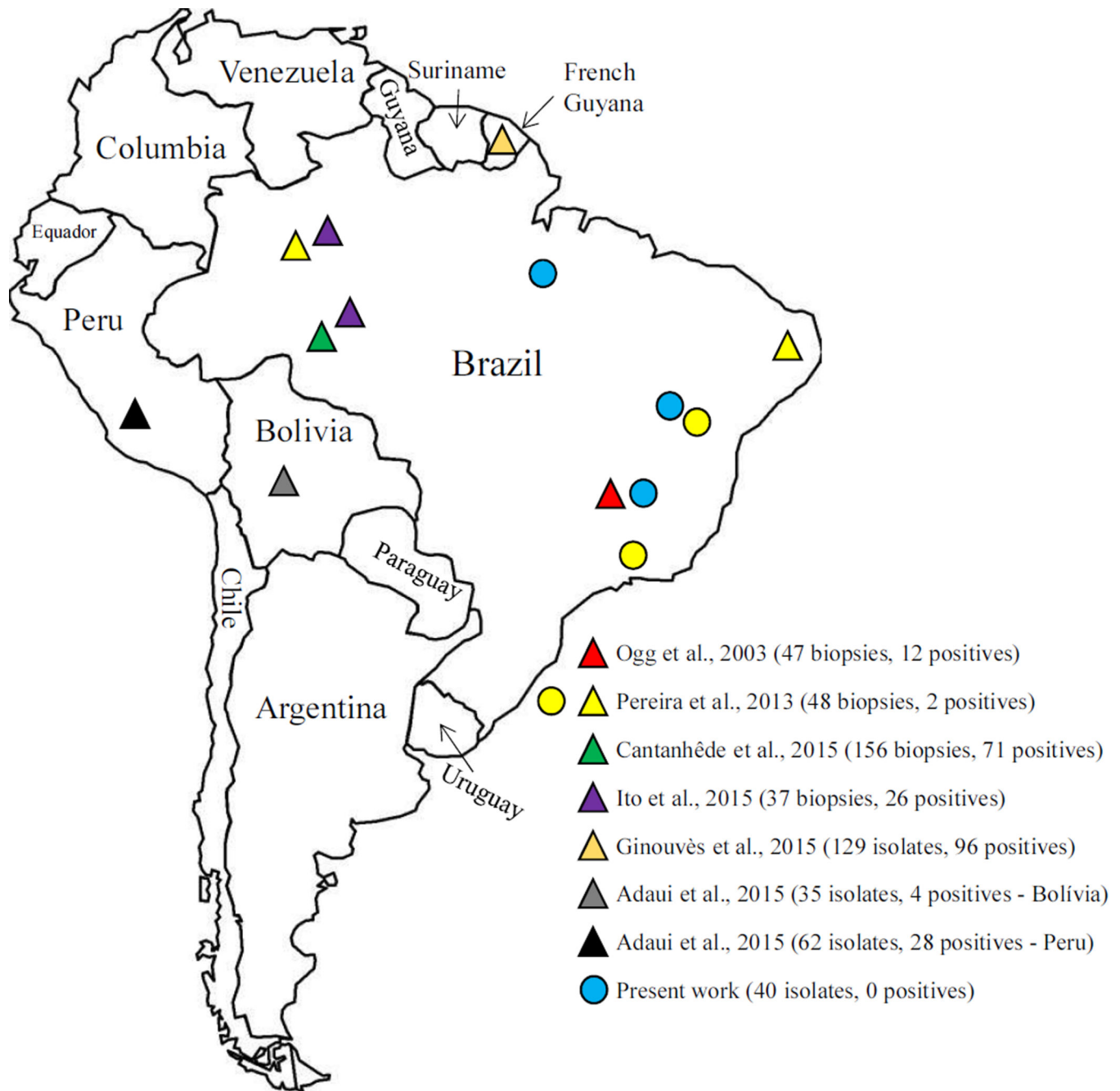
Abstract

The double stranded RNA (dsRNA) virus *Leishmanivirus* (*Totiviridae*) was first described in *Leishmania guyanensis* and *L. braziliensis* (LRV1), and more recently from *L. major* and *L. aethiopica* (LRV2). Parasites bearing LRV1 elicit a higher pro-inflammatory profile, arising through activation of Toll like receptor 3 (TLR3) interacting with the viral dsRNA. LRV1 is most common in *Leishmania* from the Amazon region; however data for other regions of Brazil are more limited. Here we applied PCR tests with validated ‘universal’ LRV1 primers to search for LRV1 in 40 strains of cultured *L. braziliensis* from several locales within Minas Gerais State, including patients presenting with atypical lesion pathology. All strains were negative however. These data are in agreement with results from other areas of Southeastern Brazil that LRV1 is relatively uncommon.

Graphical Abstract

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Keywords

Leishmania braziliensis, *Totiviridae*, American cutaneous leishmaniasis

Endemic in 98 countries, leishmaniasis comprises a spectrum of diseases caused by protozoa parasites from the *Leishmania* genus [1]. They have a complex digenetic cycle and in the vertebrate host, they are obligatory intracellular parasites. The *Leishmania* genus is divided in two subgenus according to their behavior in the vector's gut: *Leishmania*, where parasites

colonize both the foregut and midgut, and *Viannia*, where they colonize the hindgut [2]. In addition to these infecting mammals, a third subgenus is exclusive to reptiles (*Sauroleishmania*). In human leishmaniasis, a wide variation in symptoms and clinical manifestations are observed. In most cases, an ulcerated lesion with elevated borders is characterized as cutaneous leishmaniasis (CL). Depending on the species and parasite tropism, other forms may occur including: mucocutaneous leishmaniasis (MCL), anergic cutaneous diffuse leishmaniasis (ACDL), disseminated leishmaniasis (DL), or visceral leishmaniasis (VL) [3]. However, other presentations are known; in patients from the Xakriabá indigenous reserve, in São João das Missões, State of Minas Gerais, some cutaneous disease presentations differed from typical CL lesions, instead being described as lupoid, verrucous or vegetative. These were classified as 'atypical' CL and were associated with *Leishmania braziliensis* [4–5].

Some strains of the *Viannia* subgenus are infected with an endobiont virus of the family *Totiviridae*, formally termed *Leishmaniavirus* [6–9]. The most widely studied species LRV1 arises from the Amazonian region, and of these the virus present with *L. guyanensis* WHO reference strain M4147 is best known (formerly known as LRV1-4, now termed LRV1-LgyM4147 [10]. The virus is spherical or icosahedral with 30–40 nm of diameter and a dsRNA genome of 5,284 nucleotides [6,8,9]. A related species LRV2 is found in smaller fraction of isolates of *L. aethiopica* and *L. major* [11–12]. A significant percentage of *L. braziliensis* and *L. guyanensis* bear LRV1 45% up to 74.4 % [13–15]. Phylogenetic studies suggest that *Leishmania*/virus association is likely to be very ancient, since the phylogeny of LRVs closely parallels that of their host *Leishmania* [7,16]. This is also consistent with the behavior of most Totiviruses, which are typically neither shed nor infections [17].

In the past years, several research groups have focused on the relationship between the presence of LRV1 and virulence and disease presentation in different Latin America regions (Figure 1). Several studies have shown a strong association between LRV1 and metastatic leishmaniasis [13,18,19], while in other studies little association has been detected. Thus one important question is understanding the experimental and/or clinical factors which may cause LRV1-dependent pathology to manifest, or not. Two recent studies showed that the presence of LRV1 was associated with increased frequency of clinical drug treatment failures [19,20]. In contrast, in animal models the role of LRV1 is firmly established, where the use of isogenic LRV1+ or negative lines has established a clear effect on parasite burden and pathology in animals, and a clear TLR3-dependent hyperinflammatory response [21]. Together these data provide strong support for the hypothesis that LRV1 may play a significant role in human leishmaniasis.

Currently, there is strong evidence that a higher chance for developing MCL correlates with LRV1-infected parasites. Many of the mechanisms involved in the MCL occurrence prior the initial CL lesion are still unknown. In the case of LRV1, this virus could influence immunopathology by chemokine and cytokine production induced by TLR3 activation [21]. Interestingly, the incidence of LRV1 is higher using strains from the Amazon River basin than in other regions [13–15]. For example, in the southeast State of Rio de Janeiro, recent studies did not detect LRV1 in 40 *L. braziliensis* isolates [22]. In contrast, another study in

strains from the city of Caratinga, State of Minas Gerais, reported that 25% of the biopsies were positive for LRV1 [23].

As part of a wider survey on LRV1 prospection, here we aimed to detect its presence in parasites isolated from typical and atypical lesions from other regions of Minas Gerais State, Brazil. Our samples included typical lesion isolates from many locations of the state, including the previously reported region of Caratinga, and atypical lesion isolates from the Xakriabá indigenous reserve in São João das Missões (Table 1 and Figure 2).

Forty isolates of *L. braziliensis* from different clinical forms and regions from Minas Gerais and other States (Pará and Bahia) were used (Figure 2) (Table 1). All isolates were molecularly typed using genomic DNA, extracted from log-phase *Leishmania* using the phenol/chloroform method (1:1) (Thermo Scientific™, Waltham, MA). Amplification of the HSP70 fragment prior to digestion with HaeIII (Invitrogen, Carlsbad, CA) was performed [24]. For RNA, *L. braziliensis* promastigotes were grown in M199 media to a density of 1×10^7 cells/mL [25]. Parasites were collected by centrifugation at 3000g for 10 minutes, and RNA extraction was performed by the Trizol™ (Invitrogen) method following the manufacturer's guidelines (Life Technologies, Carlsbad, CA). Extracted RNA was treated with DNase I prior to complementary DNA (cDNA) synthesis using the Super Script III-First strand synthesis kit™ (Invitrogen) following the manufacture's specifications. LRV1 detection was performed by PCR of cDNA, using primers for the viral capsid SMB4647 (or UNIVf): 5'-TBRTWGCRCACAGTGAYGAAGG- and SMB4648 (or UNIVr): 5'-CWACCCARWACCABGGBGCCAT, yielding a 496-bp product. The primers were designed to conserved regions of the LRV1-LgyCUMC1-1A, LRV1-LgyM4147, LRV1-LbrLEM2700, LRV1-LbrLEM2780, LRV2-Lmj5ASKH and LRV2-LaeL494 genomes. These primers have been validated experimentally with many LRVs across diverse *Leishmania* species including the more distantly related LRV2s [23], and it should be noted that only LRV1 is expected in South American *Leishmania*. PCR amplification of the β -tubulin gene was used as quality control (QC) of cDNA using primers SMB2109 (5'-ACTGGATCCATGCGTGAGATCGTTTCCTGCC) and SMB2110 (5'-GACAGATCTCATCAAGCACGGAGTCGATCAGC), yielding a product of 396 bp [12]. Amplified sequences were resolved in 1% agarose gel and stained with GelRed (Biotium, Hayward, CA). LRV1 positive and negative controls were *L. guyanensis* (MHOM/BR/75/M4147) and *L. braziliensis* (MHOM/BR/75/M2903), respectively. Cycling conditions were as described elsewhere [12].

However, all 40 test strains were LRV-negative, while positive and negative controls behaved as expected (Fig. 3A). As a control for RNA quality, we performed PCR using primers specific for the β -tubulin gene, and as expected, a strong 396 bp amplicon was always observed (Fig. 3B). Figure 3 shows results from three "atypical pathology isolates" (MG24, 330 and MG20) and one typical (MG16), as well as a strain isolated from a MCL case from Bahia State, and one isolated from the sand fly vector *Psychodopygus wellcomei* in Pará State, all of which were negative for LRV1 (Table 1).

Our findings are consistent with previous surveys of LRV1 in Southeastern Brazil. Previous studies [22] found a low frequency of LRV1 in isolates from Rio de Janeiro while Ogg et al.

[23] found 25% in a region of Minas Gerais geographic closer to that studied by our group. One virtue of the latter study was that it was performed directly on biopsies rather than cultured organisms, however confirmatory sequence analysis of PCR amplicons was not performed to confirm the presence of unique LRV1 variants expected for a new geographic region. In contrast, the frequency of LRV1 in isolates in the Amazonian regions of South America is consistently higher, ranging upwards of 45% up to 74.4 % [13–15]. More recently, in the western parts of Latin America, LRV1 frequencies were 11% and 45.2% in Bolivia and Peru, respectively [20] (Fig. 1).

The results of our study differ from the first LRV1 survey in Caratinga, Minas Gerais, where ~25% of the samples were positive (12/47) [23], whereas in our study 0/9 Caratinga strains exhibited LRV1. This discrepancy could arise from differences in the methodologies used, or the use of biopsies vs. cultured, laboratory adapted strains. Future studies will be required to better assess the impact of these factors.

LRV1 surveys are relevant because the presence of this virus may be one of the determinants of infection pathology, potentially associated with MCL or other disseminated presentations [26]. As yet, surveys of LRV1 across South America, and its correlation with disease outcome are incomplete. Based on the studies presented in Figure 1 and together with this manuscript, there is strong evidence that the frequency of LRV1 in the Southeast of Brazil, and perhaps in the Southern regions of Latin America is very low. This is consistent with other studies of *L. braziliensis* from Rio de Janeiro and Bahia [22]. On the other hand, some samples from the North and Northeast of Brazil were LRV1 positive [22], and biopsies from patients and isolates from Northern regions of Brazil and French Guyana showed significant levels of LRV1 [13–15]. Significantly, Cantanhêde et al. [13] surveyed LRV1 in a large number of patients with different clinical forms and *Leishmania* species. The authors provided strong evidence that there is an association between LRV1 and the occurrence of mucosal leishmaniasis. However, the absence of LRV1 in other regions of Brasil despite the occurrence of MCL reinforces the idea that along with LRV1, other factors likely contribute independently or in combination to the severity of the infection [13].

This was the first work to prospect LRV1 in isolates from atypical lesions. Those isolates, from the Xakriabá indigenous reserve were all negative for LRV1. The majority of the above-mentioned data and ours indicated that the frequency of LRV1 in *L. braziliensis* samples from Southeastern Brazil is very low. In this region, a clear correlation between LRV1 presence and the clinical form of leishmaniasis could not be established. Consistent with the study of Cantanhêde et al., [13], those different clinical forms could also be a result of other factors (host and strain-specific), that remain to be elucidated. However, some unknown aspects still need to be elucidated in the LRV1 epidemiology such as its presence in other mammal hosts/reservoirs and sand fly vectors.

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Highlights

- LRV1 is an intracellular Leishmaniavirus.
- LRV1 presence is often correlated with an increase in leishmaniasis severity.
- LRV1 has high prevalence in the Northern and Western Regions of South America.
- LRV1 has low prevalence in the Southeast of Brazil.

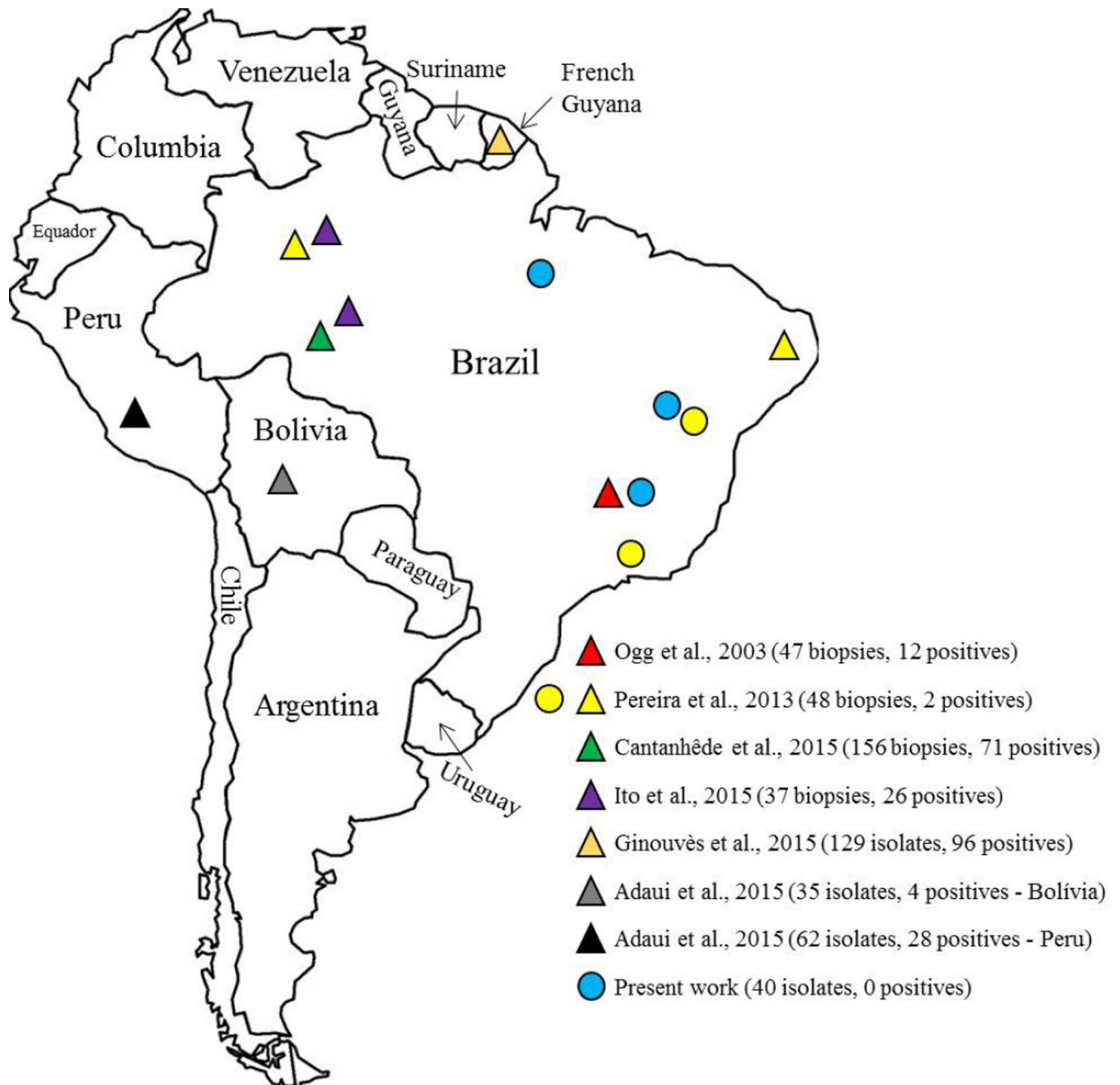
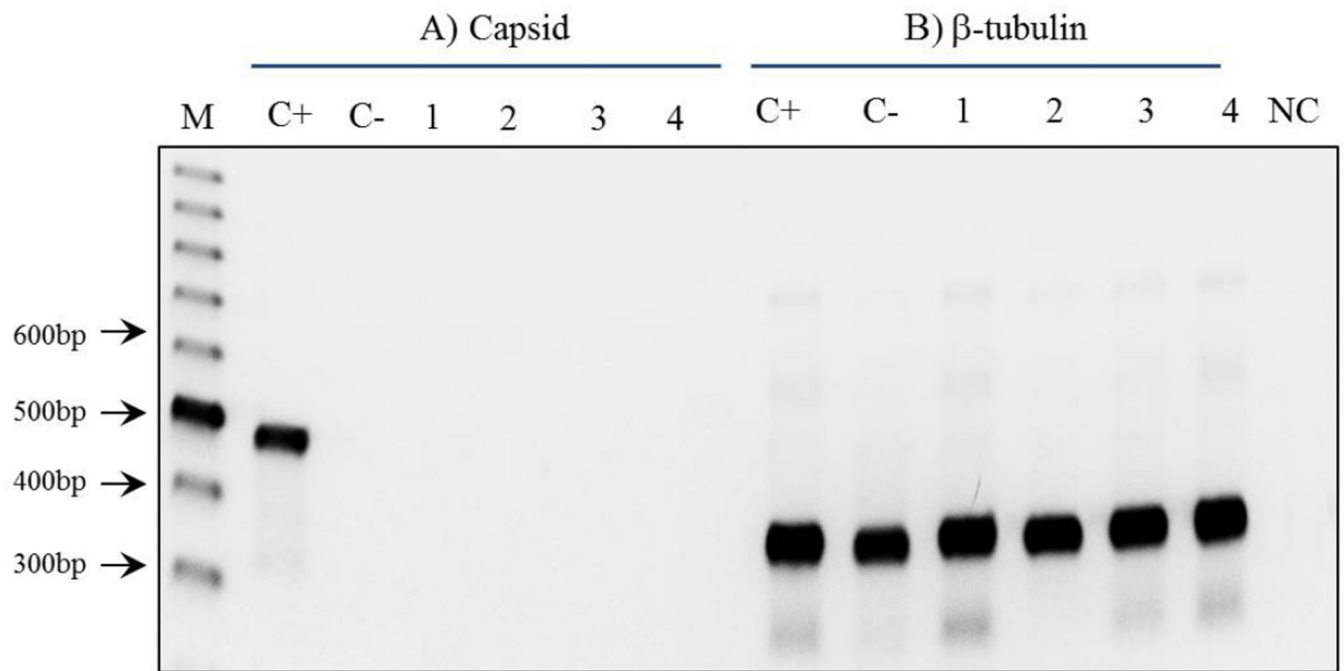


Fig. 1.

LRV1 prospection studies in South America [13–15,20,22,23]. Legend: ▲, regions where LRV1 was detected; ●, regions where LRV1 was not detected.



Fig. 2.
Origin and number of isolates of *L. braziliensis* prospected in this study. * Origin not specified in Bahia State.

**Fig. 3.**

Prospection of LRV1 in *L. braziliensis* parasites from typical and atypical forms of cutaneous leishmaniasis. Legend: M: Molecular marker; C+: LVR1 positive control (*L. guyanensis* reference strain M4147); C-: LRV1 negative control (*L. braziliensis* reference strain M2903); Lanes 1–3 (Atypical isolates MG24, 330 and MG20), and lane 4 (typical isolate MG16); NC: Negative Control.

Table 1LRV1 detection in *L. braziliensis* isolates according to origin and type of lesion.

Isolate	Origin	Lesion Type	LRV1
RR30	Araçuaí/MG	Typical	Negative
M15991	Belém/PA	Mucocutaneous	Negative
M8401	Belém/PA	*	Negative
RR051	Belo Horizonte/MG	Typical	Negative
BH453	Caratinga/MG	Typical	Negative
BH450	Caratinga/MG	Typical	Negative
BH456	Caratinga/MG	Typical	Negative
BH454	Caratinga/MG	Typical	Negative
BH455	Caratinga/MG	Typical	Negative
BH451	Caratinga/MG	Typical	Negative
BH457	Caratinga/MG	Typical	Negative
BH452	Caratinga/MG	Typical	Negative
BH17	Caratinga/MG	Typical	Negative
BH459	Caratinga/MG	Typical	Negative
Luís	R.F. Caratinga/MG	Typical	Negative
RR41	Diamantina/MG	Typical	Negative
RR48	Esmeraldas/MG	Typical	Negative
RR037	Jaboticatubas/MG	Typical	Negative
RR010	João Pinheiro/MG	Typical	Negative
RR015	Lagoa Santa/MG	Typical	Negative
RR019	Martinho Campos/MG	Typical	Negative
RR49	Pará de Minas/MG	Typical	Negative
RR56	Pitangui/MG	Typical	Negative
RR45	Ribeirão das Neves/MG	Typical	Negative
RR43	Rio Vermelho/MG	Typical	Negative
RR052	Sabará/MG	Typical	Negative
RR42	Santa Luzia/MG	Typical	Negative
RR023	Santa Luzia/MG	Typical	Negative
MG23	São João das Missões/MG	Atypical	Negative
MG16	São João das Missões/MG	Typical	Negative
MG24	São João das Missões/MG	Atypical	Negative
MG20	São João das Missões/MG	Atypical	Negative
MG27	São João das Missões/MG	Typical	Negative
330	São João das Missões/MG	Atypical	Negative
340	São João das Missões/MG	Typical	Negative
347	São João das Missões/MG	Typical	Negative
390	São João das Missões/MG	Typical	Negative
416	São João das Missões/MG	Typical	Negative
RR71	Sarzedo/MG	Typical	Negative

Isolate	Origin	Lesion Type	LRV1
BA788	Not specified/BA	Typical	Negative

Legend: MG, Minas Gerais State; PA, Pará State; BA, Bahia State.

* Strain isolated from vector.