Novel detection of *Leishmania* RNA virus-1 (LRV-1) in clinical isolates of *Leishmania Viannia panamensis*

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Abstract

American tegumentary leishmaniasis (ATL) comprises a discrete set of clinical presentations endemic to Latin America. *Leishmania* RNA virus-1 (LRV-1) is a double stranded RNA virus identified in 20-25% of the *Leishmania Viannia braziliensis* and *L. V. guyanensis*, however not in *L. V. panamensis*. This is the first report of LRV-1 in *L. V. panamensis* and its associations with clinical phenotypes of ATL. Unique surplus discard clinical isolates of *L. V. panamensis* were identified from Public Health Ontario Laboratory (PHOL) and the *Leishmania* Clinic of the Instituto de Medicina Tropical “Alexander von Humboldt” between 2012 and 2019 and screened for LRV-1 by real-time PCR. Patient isolates were stratified according to clinical phenotype. Of 30 patients with *L. V. panamensis*, 14 (47%) and 16 (53%) patients had severe and non-severe ATL, respectively. Five (36%) of 14 severe cases and 2 (12%) of 16 non-severe cases were positive for LRV-1, respectively. No differences in sex were observed for clinical phenotype and LRV-1 status. Although an association between LRV-1 status and clinical phenotype was not demonstrated, this is the first description of the novel detection of LRV-1 in *L. V. panamensis*, a species that has been documented predominantly in Central America.

**Keywords:** mucosal leishmaniasis; cutaneous leishmaniasis; *Leishmania Viannia panamensis*; Latin America; *Leishmania* RNA Virus-1 (LRV-1).

**Word Count:** Abstract-197/250 words, Text-2802/4000 words
Introduction

American tegumentary leishmaniasis (ATL) includes cutaneous leishmaniasis (CL), mucocutaneous (MCL), and mucosal leishmaniasis (ML), and affects 1-2 million people in the Americas (Reithinger, 2007). Localized CL (LCL) is generally a self-healing disease characterized by ulcerative, nodular, or verrucous lesions on the skin caused by members of the Leishmania complex and endemic to many parts of the world including Brazil and Peru (Reithinger, 2007; Aronson et al., 2016). Other clinical manifestations of CL include inflammatory CL where ulcers are associated with erythema, purulent exudate, pain and/or lymphatic involvement and more recently, atypical cutaneous leishmaniasis (ACL), which has been documented in an endemic region of Brazil (Guimares et al., 2016). To add, other forms include diffuse cutaneous leishmaniasis (DCL) with multiple non-ulcerative nodules (Reithinger, 2007), and disseminated leishmaniasis (DL), defined as maculopapular lesions identified in two or more anatomical sites ranging from 10-300 in number (Guimares et al., 2016). ML is a form of the disease affecting mucous membranes such as the nose, mouth, pharynx and larynx, more often attributed to sequela of the initial CL infection in Latin America, while MCL involves both cutaneous and mucosal lesions (Reithinger, 2007). This diverse phenotypology reflects a complex relationship between host, parasite, and vector factors (extensively reviewed in (Reithinger, 2007)), with strong geographic- and species-specific preponderances to cutaneous manifestations of disease.

To add to this complexity of ATL pathogenesis, the presence of a double stranded RNA virus, Leishmania RNA virus-1 (LRV-1), has been identified in up to a quarter of certain strains of Leishmania Viannia spp., including L. V. braziliensis and L. V. guyanensis. LRV-1 found in New World Viannia strains are identified as LRV-1, with 14 subtypes (LRV-1-1-LRV-1-14) predominantly found in the Amazon basin (Hartlet et al., 2012; Ginouves et al., 2016). Genetic
diversity between LRV-1 and parasite species exists, however the viruses from the same parasite species have shown less heterogeneity (Catanhede et al., 2018). In South America, it is believed that 10-15% of LCL will progress to either MCL or ML months to years after healing of the initial LCL lesion (Ives et al., 2011; Ronet et al., 2011; Valencia et al., 2014). It is hypothesized that the presence of LRV-1 will advance CL to MCL/ML stemming from an over-active immune response leading to severe immunopathological tissue infiltration and destruction (Ogg et al., 2003; Ives et al., 2011; Ronet et al., 2011; Kariyawasam et al., 2017).

LRV-1 has been documented in 20-25% of clinical isolates of L. V. guyanensis and L. V. braziliensis found in Brazil and Peru and has been associated with first-line treatment failure (Ives et al., 2011; Bourreau et al., 2016). Studies have also indicated higher levels of LRV-1 in metastasizing versus non-metastasizing strains of L. V. guyanensis, which were correlated to increased levels of proinflammatory cytokines and chemokines including TNF-α, IL-6, CXCL10, CCL4, and CCL5 after recognition by toll-like receptor 3 (TLR3) in human and murine studies (Ives et al., 2011). On the other hand, in a human macrophage model, LRV-1 in L. V. braziliensis was correlated to lower expression levels of TNF-α, IL-6, IL-1β, CXCL10, and increases in superoxide dismutase (SOD), although these differences were not noted in analysis of 5 L. V. panamensis strains (Kariyawasam et al., 2017). Given that LRV-1 may be predict and correlate to more severe clinical manifestations of ATL, we aimed to understand its prevalence in clinical isolates of L. V. panamensis, a species in which LRV-1 is not well described, and the possible epidemiologic association between severe and non-severe phenotypes of ATL.
Materials and methods

Specimen Enrolment

Unique surplus discard clinical specimens of *L. V. panamensis* were identified from Public Health Ontario Laboratory (PHOL) and the *Leishmania* Clinic of the Instituto de Medicina Tropical "Alexander von Humboldt", Lima, Peru between 2012-2019 (Figure 1). Biobanked isolates were confirmed as *L. V. panamensis* by multiplex real-time PCR targeting *Leishmania* 18S rRNA, following clinical testing, which included microscopic examination of Giemsa-stained smears and/or culture by certified medical lab technologists.

Clinical Data

De-identified clinical data of source patients collected from test requisitions and case record forms were stratified into types of 'severe' and 'non-severe' phenotypes, where a severe phenotype was defined as either mucosal involvement (MCL/ML); or inflammatory ulcers (ulcers with associated erythema, purulent exudate, pain with or without lymphatic involvement) or multifocal/disseminated ulcers (ulcers in ≥ 2 anatomic sites and ≥ 4 in number) as per the Infectious Diseases Society of America guidelines (Aronson *et al.*, 2016), understanding that the pathogenesis underpinning mucosal versus severe cutaneous manifestations of *Leishmania* infection are quite different. A non-severe phenotype was defined as LCL of < 4 ulcers in number (Aronson *et al.*, 2016).

DNA Extraction

DNA extraction was performed using the Qiagen DNA Mini Kit (Qiagen, MD) using 200 µL of cultured specimen with a final elution volume of 60 µL. In the case of primary clinical specimens...
including filter paper lesion impressions (FPLIs), biopsies, and cytology brushes, specimens were soaked in 200 µL of TE prior to extraction to achieve sufficient volume and DNA concentration and eluted in 60µL nuclease-free water.

**RNA Extraction**

RNA was extracted from cultured promastigotes using the Cells Protocol of the QIAamp RNA Mini Kit and eluted with 50µL of RNase-free Water. RNA was extracted from tissue biopsy and cytology brushes using the Fibrous Tissue Protocol from the Qiagen RNeasy Micro Kit with the addition of carrier RNA and eluted with 14 µL RNase-free water. RNA was extracted from FPLIs with the QIAmp RNA Blood Mini Kit and eluted with 30 µL RNase-free water. An in-column DNase treatment was included using the Qiagen rDNase Set as per manufacturer’s protocol.

**cDNA Synthesis and Purification**

cDNA was performed using 10 µL of RNA in combination with the Superscript II Reverse Transcriptase and random hexamers (Kariyawasam et al., 2017). PCR purification was performed using the Qiagen QIAquick PCR Purification Kit and eluted with 60µL nuclease-free water.

**Species Identification**

Species identification was performed using the following gene targets by end-point PCR: internal transcriber space 1 (ITS1), ITS2, cysteine proteinase B (CPB), heat shock protein 70 (HSP70), mannose phosphate isomerase (MPI), zinc-dependent metalloproteinase (GP63), and confirmatory Sanger sequencing (Schonian et al., 2003; de Almeida et al., 2011; Wortmann et al., 2011; Kariyawasam et al., 2017). Restriction fragment length polymorphism (RFLP) analysis was
performed on each product of end-point PCR (de Almeida et al., 2011; Kariyawasam et al., 2017; Wortmann et al., 2011).

Sanger Sequencing

Sanger Sequencing was performed using 1 µL of PCR product, 2 µL of Big Dye, 3 µL of Buffer, and 2 µL of 10µM of primer and cleaned accordingly (Kariyawasam et al., 2017). Products were then centrifuged for 2 minutes at 2000g prior to being loaded onto the Applied Biosystems 3730xl DNA Analyzer. Data were standardized using the Sequencing Analyzer program and BLAST search engine was used to analyze the sequence (Kariyawasam et al., 2017).

LRV-1 Detection and Quantification

LRV-1 was detected in isolates of L. V. panamensis by real time PCR using two primer sets, set A and set B, respectively, as depicted in Figure 1 (Schmittgen et al., 2008; Zangger et al., 2013; Kariyawasam et al., 2017). Leishmania kinetoplastid membrane protein 11 (kmp11) was used as a reference for quantification where sufficient RNA volume for quantification permitted this analysis (Tarr et al., 1988; Kariyawasam et al., 2017). Each isolate was run in triplicate and contained the L. V. guyanensis ATCC® (American Type Culture Collection®) 50126™ (MHOM/BR/75/M4147) positive control to perform relative quantification using the 2−ΔΔCt method (Kariyawasam et al., 2017; Ogg et al., 2003; Schmittgen et al., 2008; Zangger et al., 2013). If kmp11 was not detected, a pre-amplification step was performed as per Perfecta Pre-Amp Supermix guidelines. In the case that kmp11 remained undetected after pre-amplification, the 18S rRNA gene was used as a reference and a relative quantification was performed using the 2−ΔΔCt method (Wortmann et al., 2001; Schmittgen et al., 2008; de Almeida et al., 2011; Zangger et al., 2013).

**Analysis**

Descriptive statistics (proportions, mean with SD, median, range) were calculated for all variables. Differences between categorical variables were compared using Fisher’s exact test or Chi-square analysis. Continuous variables were compared by Kruskal-Wallis Test or student’s t-test. Significance was set at p<0.05. Data were analyzed using GraphPad Prism (GraphPad, CA).

**Results**

**Clinical and Demographic Data**

Of 208 specimens from patients with confirmed ATL, 30 (14.4%) isolates were identified as *L. V. panamensis* (Figure 1). Demographic and parasitological factors for the 30 *L. V. panamensis* isolates from patients with ATL enrolled and analyzed are summarized in Table 1. Eighteen (60%) patients were male, while 12 (40%) were female and the median age was 35 years (range 9 - 80 years). Sixteen (53.3%) isolates were derived from patients with LCL, while 14 (46.7%) were from patients with inflammatory/multifocal CL, and zero (0%) patients with MCL/ML. Fourteen (47%), 7 (23%) and 4 (13%) had travel history to or resided in: Costa Rica, Peru and Ecuador, respectively.

**LRV-1 Prevalence and Copy Number by Phenotype: Primary Outcome**

A total of 7/30 (23%) isolates contained LRV-1 while 23/30 (77%) did not. Five of 14 (36%) isolates of patients with inflammatory/multifocal phenotypes were LRV-1 positive while 2/16 (12%) isolates from patients with the non-severe phenotype were LRV-1 positive (Table 1).
Clinical Phenotype and LRV-1 Prevalence by Demographics: Secondary Outcomes

Median ages of patients were distributed across phenotypes as follows: 35 years (range 9 – 80 years) for those with inflammatory/multifocal CL and 34.5 years (range 17 - 64 years) for those with LCL, respectively (p=0.17) (Table 1). One (50%) child had an inflammatory/multifocal phenotype (n=2); 9 (35%) individuals in the 18-65 years age bracket manifested inflammatory/multifocal CL (n=26), while those >65 (100%) exclusively manifested the inflammatory/multifocal CL phenotype (n=4). Male sex (n=65/78) was distributed across phenotypes as follows: 33% (n=6/18) with inflammatory/multifocal CL and 67% (12/18) with LCL (p=0.17). Twelve females were included in the analysis, of which 8 (67%) had the inflammatory/multifocal and 4 (33%) had the LCL phenotypes, respectively.

Median age of patients whose isolates were LRV-1 positive and caused inflammatory/multifocal CL and LCL were: 35 years (range 9-80 years) and 35 years (range 17-80 years), respectively (p=0.91). LRV-1 positivity was not associated with median age, whereby patients whose isolates were LRV-1 positive had a median age of 35 years (range 9 - 71 years) compared to LRV-1 negative patients whose median age was 35 years (range 17 - 80 years) (p=0.91). However, LRV-1-positivity was detected in only 1 (25%) isolate from patients >65 years (n=4); 5 (21%) isolates from patients aged 18-65 years (n=24); and 1 (50%) isolate from patients <18 years (n=2) (p=0.21).

LRV-1 Copy Number

Relative LRV-1 copy number was calculated for 3/7 (43%) isolates positive for LRV-1. The mean relative copy number was identified in 3 isolates from patients with the inflammatory/multifocal phenotype was $1.09 \times 10^{-4} \pm 1.06 \times 10^{-3}$ (median $1.09 \times 10^{-3}$, range $6.029 \times 10^{-6} - 2.2$ copies).
Discussion

Severity of ATL has been hypothesized to be associated with the viral endosymbiont LRV-1 for decades, with the first report of LRV-1 isolated from a human with cutaneous satellite lesions and lymphatic involvement after visiting Suriname (Tarr et al., 1988). Since this initial report, there have been significant advancements and availability of molecular diagnostic tools to further investigate and understand the role of LRV-1 in ATL, and further accrual of data in humans (Ogg et al., 2003; Pereira et al., 2013; Valencia et al., 2014; Catanhede et al., 2015; Ito et al., 2015; Adaui et al., 2016; Bourreau et al., 2016; Ginouves et al., 2016; Macedo et al., 2016). It has been shown that LRV-1 and *Leishmania* parasites have co-evolved with clustering of both the virus and the parasite in specific geographic locations. Given the species-specific and geographic correlates of observed phenotype in ATL, LRV-1 has the potential to contribute to the diagnosis, treatment, and prognostic decision-making in the care of ATL patients (Catanhede et al., 2018). Using clinical strains of *L. V. panamensis* in this study, we examined the overall prevalence of LRV-1 and its possible correlation to clinical phenotypes in a species previously not recorded to contain the virus.

While no direct relationship between LRV-1 positivity or negativity with 2 discrete phenotypes was observed, only patients manifesting inflammatory/multifocal CL had a quantifiable viral load.

By analyzing LRV-1 status in 30 isolates of *L. V. panamensis* causing various clinical phenotypes of ATL, an overall 23% prevalence was identified, which is within the range reported previously from studies of strains in Latin America, specifically in *L. V. guyanensis* and *L. V. braziliensis* (Salinas et al., 1996; Wortmann et al., 2001; Pereira et al., 2013; Catanhede et al., 2015; Ito et al., 2015; Adaui et al., 2016; Macedo et al., 2016). LRV-1 has been loosely described in other species, particularly *L. amazonensis* and *L. naiffi*. It has been shown that LRV-1 is not...
preferentially associated with a specific phenotype (Adaui et al., 2016), although this study identified 36% inflammatory/multifocal CL patients were LRV-1 positive compared to 12% of LCL patients with no patients being identified with ML/MCL in this population. While these proportions were not statistically different, it is possible that with a larger-scale prospective study, a meaningful difference in the LRV-1 prevalence could emerge. Furthermore, it is possible to understand if LRV-1 in *L. V. panamensis* contributes to the ML/MCL phenotype, however this has not been documented in literature. The relationship of both LRV-1 prevalence and viral burden to clinical manifestations and observed phenotype warrant additional work in larger cohort of patients with ATL, specifically in patients with inflammatory/multifocal CL.

Although there was no age difference observed in LRV-1 isolates, the detection of the virus was documented in areas of Central America including Costa Rica, Belize and Panama, where LRV-1 has historically not been detected. MCL/ML was not identified in this cohort of *L. V. panamensis*, perhaps in this patient population, inflammatory/multifocal CL is considered the most severe phenotype achievable in this species, given that LCL was restricted to patients <65 years of age. Perhaps there is progression to inflammatory/multifocal CL after LCL (Reithinger et al., 2007; Jara et al., 2016). One possible explanation for why LRV-1 may be less likely to occur in older patients who are from endemic settings is the recurrent, lifelong exposure, which could enable the parasite to harness the endogenous RNAi activity of the Viannia subgenus to eliminate the virus over time (Brettman et al., 2016). In this study, all but one isolate from patients over age 65 (n=4) were found to be LRV-1-positive, and one isolates from patients under age 18 were LRV-1-positive. Advanced age is associated with poorer T-cell response and a Th2-biased response, in particular (Salam et al., 2013), which in the case of ATL, is correlated to poorer immunologic control of infection and persistence of the amastigote in the phagolysosome (Hartlet et al., 2012).
Similarly, the Th1-to-Th2 ratio has been demonstrated to be lowest in childhood and adolescence, with a peak during mid-adulthood, and slight decline thereafter (Chang et al., 2016). Th2 predominance over Th1 is also an important factor in the progression of severe disease (Tripathi et al., 2007; Hartley et al., 2013; Maspi et al., 2016; Moafi et al., 2017). Understanding the potential behavioral, socioeconomic, and biological underpinnings of the age distributions of LRV-1 noted in this analysis will be, ultimately, important to accurate interpretation of the viral role in ATL pathogenesis.

Limitations of this descriptive analysis of LRV-1 prevalence amongst L. V. panamensis isolates include the comparatively small number of isolates, as well as enrolment from patients who are returning travelers from Latin America to Canada or live in Peru, with a majority of travelers having gone to Costa Rica. Prospective enrolment of larger cohorts that might enable more even distribution of returning travelers would be worthwhile. It is also possible that significantly different proportions of LRV-1 positivity by phenotype might have emerged with a larger cohort. While a limited budget did not permit such a large-scale analysis, the findings are important as, even in this smaller cohort, the presence of LRV-1 in a species with very limited literature and higher viral load in L. V. panamensis isolates causing inflammatory/multifocal CL, also suggests some interesting age preponderances that will be best interrogated using a combination of epidemiologic and basic scientific approaches going forward.

**Conclusions**

Continued exploration of LRV-1 prevalence across age groups, particularly in larger cohorts, with specific interrogation of immunological age correlates of LRV-1-positivity while controlling for behavioral, socioeconomic, and other possible biological contributors to the age biases observed
herein will be essential to understanding the relevance of this demographic variable to the host-parasite-viral interplay that governs phenotype. The role of LRV-1 as a predictive biomarker of disease severity remains unclear, however the mechanistic nature, particularly regarding the immune response, will prove useful to understanding overall ATL-LRV-1 pathogenesis particularly in patients with inflammatory/multifocal CL.

Data. All data and other materials necessary are included in the article.

Authors’ contributions. RK contributed to study design; data collection, analysis, and interpretation; and was primarily responsible for drafting the manuscript. RL contributed to study design; data collection, analysis, and interpretation; and to manuscript revision and critical appraisal. BMV and AL-C contributed to study design; data collection and interpretation; and to manuscript revision and critical appraisal. AKB conceived the study and contributed to study design; funding acquisition; data collection, analysis, and interpretation; and to writing and revising subsequent iterations of the manuscript. All authors serve as guarantors of the work.

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Competing interests. The authors have no interests to declare.
Ethical standards. Approval for this study was obtained from the Ethics Review Board of Public Health Ontario, the Research Ethics Board of University of Toronto, and the Institutional Review Board of Hospital Nacional Cayetano Heredia, Lima, Peru.
References


Cantanhede, LM, Silva, CF, Ito, MM, Felipin, KP, Nicolete, R, Salcedo, JMV, Porrozzi, R,


Wortmann, G, Sweeney, C, Houn, HS, Aronson, N, Stiteler, J, Jackson, J and Ockenhouse,


9-30.
Table 1. Demographic data for 30 patients with \textit{L. (V.) panamensis} isolates by clinical phenotype.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total N (%)</th>
<th>Inflammatory / Multifocal CL (n=14)</th>
<th>LCL (n=16)</th>
<th>P-value</th>
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<tbody>
<tr>
<td></td>
<td>Total N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
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<tr>
<td>Sex</td>
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<td></td>
<td></td>
<td>0.14</td>
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<tr>
<td>Male</td>
<td>18 (60)</td>
<td>6 (43)</td>
<td>12 (75)</td>
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<tr>
<td>Female</td>
<td>12 (40)</td>
<td>8 (57)</td>
<td>4 (125)</td>
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<tr>
<td>Median Age, years</td>
<td>35 (9-80)</td>
<td>35 (9-80)</td>
<td>34.5 (17-64)</td>
<td>0.17</td>
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<tr>
<td>(range)</td>
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<tr>
<td>Travel History</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Costa Rica</td>
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<td>5 (36)</td>
<td>9 (65)</td>
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<td>Peru</td>
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<tr>
<td>Positive</td>
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<td>5 (36)</td>
<td>2 (13)</td>
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<tr>
<td>Negative</td>
<td>23 (77)</td>
<td>9 (64)</td>
<td>14 (87)</td>
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Figure 1: Workflow of sample identification and stratification of patients with confirmed *L. (V.) panamensis*