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Leishmania RNA Virus 1 (LRV-1) in *Leishmania* (*Viannia*) *braziliensis* Isolates from Peru: A Description of Demographic and Clinical Correlates

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Abstract. RNA virus 1-1 (LRV-1-1) is a dsRNA virus identified in isolates of *Leishmania* (*Viannia*) *braziliensis* and thought to advance localized cutaneous leishmaniasis (LCL) to mucocutaneous or mucosal leishmaniasis (MCL/ML). We examined the prevalence of LRV-1 and its correlation to phenotypes of American tegumentary leishmaniasis caused by *L*. (*V.*) *braziliensis* from Peru to better understand its epidemiology. Clinical isolates of *L*. (*V.*) *braziliensis* were screened for LRV-1 by real-time polymerase chain reaction (PCR) and stratified according to the phenotype: LCL (< 4 ulcers in number) MCL/ML; inflammatory ulcers (erythematous, purulent, painful ulcers with or without lymphatic involvement) or multifocal ulcers (≥ 4 in ≥ 2 anatomic sites). Proportionate LRV-1 positivity was compared across phenotypes. Of 78 *L*. (*V.*) *braziliensis* isolates, 26 (54.2%) had an inflammatory phenotype, 22 (28%) had the MCL/ML phenotype, whereas 30 (38.5%) had LCL. Mucocutaneous or mucosal leishmaniasis was found exclusively in adult male enrollees. *Leishmania* RNA virus 1 positivity by phenotype was as follows: 9/22 (41%) with MCL/ML; 5/26 (19%) with an inflammatory/multifocal cutaneous leishmaniasis phenotype; and 7/30 (23%) with LCL ($P = 0.19$). *Leishmania* RNA virus 1 positivity was not associated with age ($P = 0.55$) or gender ($P = 0.49$). Relative LRV-1 copy number was greater in those with MCL/ML than those with inflammatory/multifocal CL ($P = 0.02$). A direct association between LRV-1 status and clinical phenotype was not demonstrated; however, relative LRV-1 copy number was highest in those with MCL/ML. Future analyses to understand the relationship between viral burden and pathogenesis are required to determine if LRV-1 is truly a contributor to the MCL/ML phenotype.

INTRODUCTION

American tegumentary leishmaniasis (ATL) includes cutaneous leishmaniasis (CL), mucocutaneous (MCL), and mucosal leishmaniasis (ML), affecting one to two million people in the Americas.[1] Localized CL (LCL) is generally a self-healing disease characterized by ulcerative, nodular, or verrucous lesions on the skin caused by several *Leishmania* spp. and endemic to many parts of the world, including Peru.[1–2] 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 CL, which has been documented in an endemic region of Brazil.[3] To add, other forms include diffuse CL with multiple non-ulcerative nodules,[1] and disseminated leishmaniasis, defined as maculopapular lesions identified in two or more anatomical sites ranging from 10 to 300 in number.[3] Mucosal leishmaniasis 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, whereas MCL involves both cutaneous and mucosal lesions.[1] This diverse phenotypology reflects a complex relationship between host, parasite, and vector factors[1] (extensively reviewed in ref. 1), 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 *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Viannia*) *guyanensis*. *Leishmania* RNA virus 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.[4,5] Genetic diversity between LRV-1 and parasite species exists; however, the viruses from the same parasite species have shown less heterogeneity.[6] It is hypothesized that the presence of LRV-1 will advance 10–15% of CL to MCL/ML stemming from an overactive immune response leading to severe immunopathological tissue infiltration and destruction.[7–11]

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.[7,12] Studies have also indicated higher levels of LRV-1 in metastasizing versus non-metastasizing strains of *L*. (*V.*) *guyanensis*, which were correlated with increased levels of proinflammatory cytokines and chemokines, including tumor necrosis factor (TNF-α), interleukin 6 (IL-6), interferon-gamma inducible protein 10 (CXCL10), chemokine ligand 4 (CCL4), and chemokine ligand 5 (CCL5) after recognition by toll-like receptor 3 in human and murine studies.[7] On the other hand, in a human macrophage model, we have documented that LRV-1 in *L*. (*V.*) *braziliensis* was correlated with lower expression levels of TNF-α, IL-6, IL-1β, and CXCL10 and increases in superoxide dismutase.[11] Given that LRV-1 may predict and correlate with more severe clinical manifestations of ATL and given that Peru is one of the top worldwide contributors of CL, ML, and MCL, we aimed to understand its prevalence in clinical isolates of *L*. (*V.*) *braziliensis* and the possible epidemiologic association between different clinical phenotypes of ATL from Peru.

MATERIALS AND METHODS

Ethics approval. 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.

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Specimen enrollment. Unique surplus discard clinical specimens of *Leishmania* spp. were identified from Public Health Ontario Laboratory and the *Leishmania* Clinic of the Instituto de Medicina Tropical Alexander von Humboldt, Lima, Peru, between 2012 and 2018 (Supplemental Table 1, Figure 1). Biobanked isolates were confirmed as *Leishmania* spp. 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 laboratory technologists.

Clinical data. De-identified clinical data of source patients collected from test requisitions and case record forms were stratified into the following phenotypes: MCL/ML (simultaneous cutaneous infection and/or destruction of the mucosa), inflammatory ulcers (ulcers with associated erythema, purulent exudate, and 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,[2] understanding that the pathogenesis underpinning mucosal versus severe cutaneous manifestations of *Leishmania* infection are quite different. Localized cutaneous leishmaniasis was defined as of < 4 ulcers in number.[2]

DNA extraction. DNA extraction was performed using the Qiagen DNA Mini Kit (Qiagen, Germantown, 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) (ThermoFisher, Waltham, MA), biopsies, and cytology brushes (VWR, Radnor, PA), specimens were soaked in 200 µL of TE (ThermoFisher Scientific) before extraction to achieve sufficient volume and DNA concentration and eluted in 60 µL nuclease-free water (ThermoFisher Scientific).

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

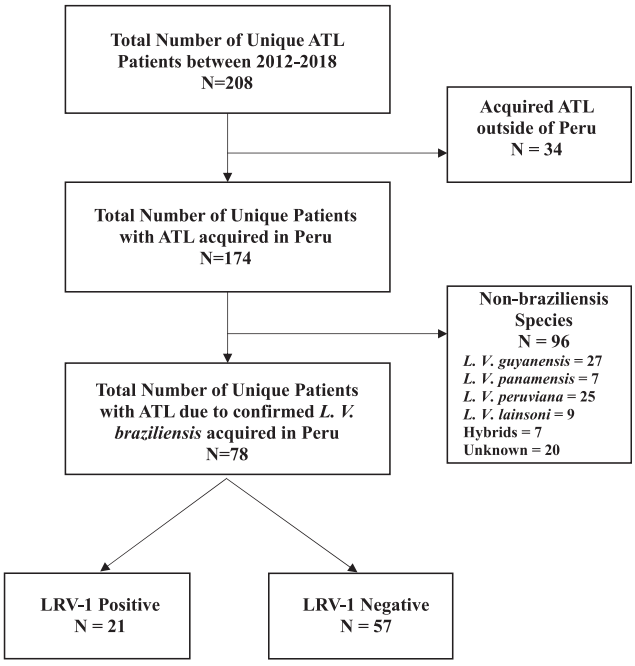

FIGURE 1. Workflow of sample identification and stratification of patients with confirmed *Leishmania* (*Viannia*) *braziliensis*.

cDNA synthesis and purification. cDNA was performed using 10 µL of RNA in combination with the Superscript II Reverse Transcriptase and random hexamers (ThermoFisher Scientific).[11] PCR purification was performed using the Qiagen QIAquick PCR Purification Kit (Qiagen) and eluted with 60 µL nuclease-free water (ThermoFisher Scientific).

Species identification. Species identification was performed using the following gene targets by end-point PCR: internal transcriber space 1 (ITS1), ITS2, cysteine proteinase B, heat shock protein 70, mannose phosphate isomerase, zinc-dependent metalloproteinase (GP63), and confirmatory Sanger sequencing.[11,13–15] Restriction fragment length polymorphism analysis was performed on each product of end-point PCR.[11,14–15]

Sanger sequencing. Sanger sequencing was performed using 1 µL of PCR product, 2 µL of BigDye, 3 µL of buffer, and 2 µL of 10 µM of primer (ThermoFisher Scientific).[11] The following cycling conditions were used on the Veriti ABI Thermal Cycler (Applied Biosystems, Waltham, MA): 1 minute at 96°C, 25 cycles of 10 seconds at 96°C, 5 seconds at 50°C, and 4 minutes at 60°C. The product was cleaned using 45 µL of SAM Solution and 10 µL of beads set on a shaking incubator for 30 minutes.[11] The products were then centrifuged for 2 minutes at 2,000 *g* before being loaded onto the Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems). Data were standardized using the Sequencing Analyzer program and Basic Local Alignment Search Tool (BLAST) search engine was used to analyze the sequence.[11]

***Leishmania* RNA virus 1 detection and quantification.** *Leishmania* RNA virus 1 was detected in isolates of *L*. (*V.*) *braziliensis* by real time PCR using two primer sets, set A and set B, respectively (Figure 1).[11,16–17] *Leishmania* kinetoplastid membrane protein 11 (kmp11) was used as a reference for quantification where sufficient RNA volume for quantification permitted this analysis.[11,18] A SYBR Green assay was set up using 1× SYBR Select Master Mix, 250 nM final concentration of forward and reverse primers, and 5 µL of cDNA in a total volume of 20 µL.[11] The ABI 7900HT real time instrument was set to the following conditions: uracil-DNA glycosylase (UDG) activation at 50°C for 2 minutes, polymerase activation at 95°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute.[11] A dissociation step of 95°C for 15 seconds, 60°C for 15 seconds, and another 95°C for 15 seconds was added at the end to generate a melting curve to check for specificity of amplification. Each isolate was run in triplicate and contained the *L*. (*V.*) *guyanensis* American Type Culture Collection® (ATCC®) 50126™ (MHOM/BR/75/M4147) positive control to perform relative quantification using the $2^{-\Delta\Delta Ct}$ method.[10,11,16,17] If kmp11 was not detected, a pre-amplification step was performed as per the 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 $2^{-\Delta\Delta Ct}$ method.[11–17] Relative LRV-1 copy number was calculated using the methods outlined by Zangger and colleagues, and further

described in the following paragraphs.[16] The "gold" standard source of LRV-1 in this analysis as well as the Zangger article is *L. V. guyanensis*.[16] We acknowledge that there are inter-species differences in LRV-1 viral load; however, we do not have an LRV-1 clone to calculate an absolute copy number. Moreover, by normalizing the relative abundance to the *L. V. guyanensis* MHOM/BR/75/M4147 strain, which is readily available from the ATCC, we are able to maintain consistent analysis across experiments and studies. Where copy number is recorded as N/A, this indicates an inability to calculate LRV-1 copy number because of a non-amplifiable kmp11 reference gene.

Data analysis. Descriptive statistics (proportions, mean with SD, median, and range) were calculated for all variables. Differences between categorical variables were compared using Fisher's exact test or chi-squared analysis. Continuous variables were compared by Kruskal–Wallis test or Mann–Whitney U test. Significance was set at $P < 0.05$. Data were analyzed using GraphPad Prism (GraphPad, La Jolla, CA). Relative LRV-1 copy number was calculated using the $2^{-\Delta\Delta Ct}$ method, whereby the gold standard LRV-1–containing strain, *L.* (*V.*) *guyanensis* ATCC 50126 (MHOM/BR/75/M4147), was used as a positive reference control for each PCR run containing LRV-1–positive *L.* (*V.*) *braziliensis* isolates.[10,11,16,17]

RESULTS

Clinical and demographic data. Of 208 specimens from patients with confirmed ATL, 78 (38%) isolates were identified as *L.* (*V.*) *braziliensis* acquired in Peru, by local Peruvians ($n = 76$, 97%) or travelers to Peru ($n = 2$, 3%) (Figure 1, Supplemental Table 1). One hundred thirty (62%) patients were excluded because of acquisition of ATL outside of Peru and/or infection with a non-*braziliensis* species (Figure 1). Sixty-five (83%) patients were male, whereas 13 (17%) were female (Tables 1 and 2). Median age was 34 years (range 2–76 years) (Tables 1 and 2). Thirty (38.5%) isolates were derived from patients with LCL, whereas 26 (33%) were from patients with inflammatory/multifocal CL and 22 (28%) were from patients with MCL/ML (Tables 1 and 2).

Clinical phenotype by demographics: Secondary outcomes. Median ages of patients were distributed across phenotypes as follows: 40.5 years (range 20–82 years) for those with MCL/ML, 31 years (range 13–68 years) for those with inflammatory/multifocal CL, and 31 years (range 2–76 years) for those with LCL ($P = 0.72$) (Table 1). No children or adolescents had an MCL/ML phenotype; those in the < 18 years age bracket manifested LCL ($n = 4$, 50%) or inflammatory/multifocal CL ($n = 4$, 50%) exclusively (Table 3). Male gender ($n = 65/78$) was distributed across phenotypes as follows: 100% ($n = 22$) with MCL/ML, 69% ($n = 18/26$) with inflammatory/multifocal CL, and 83% (25/30) with LCL ($P = 0.02$) (Table 2). No females in the analysis had MCL/ML, whereas 31% and 17% of those with the inflammatory/multifocal and LCL phenotypes, respectively, were female (Table 2). To summarize the clinical phenotype by demographics data, MCL/ML was found exclusively in adult male enrollees.

LRV-1 prevalence by clinical phenotype: Primary outcome. A total of 21/78 (27%) isolates contained LRV-1, whereas 57/78 (73%) did not (Figure 1, Tables 1 and 2). *Leishmania* RNA virus 1 was detected in nine (41%) isolates causing MCL/ML, five (19%) isolates causing inflammatory/multifocal CL ulcers, and seven (23%) isolates causing LCL, respectively ($P = 0.21$) (Table 2). *Leishmania* RNA virus 1 positivity was distributed across phenotypes as follows: 43% (9/21) of LRV-1–positive isolates were found in MCL/ML, 24% (5/21) of LRV-1–positive isolates were found in inflammatory/multifocal ulcers, and 33% (7/21) of LRV-1–positive isolates were found in LCL ($P = 0.19$) (Table 3). However, LRV-1 positivity was detected in only one (10%) isolate from patients > 60 years ($n = 10$), 20 (33%) isolates from patients aged 19–59 years ($n = 60$), and zero (0%) isolates from patients < 18 years ($n = 8$) ($P = 0.0591$) (Table 3).

Relative LRV-1 copy number (abundance). Relative LRV-1 copy number (abundance) was calculated for 17/21 (81%) isolates positive for LRV-1. Mean relative copy number of LRV-1 for isolates causing ML/MCL ($n = 7$) was 21.6 ± 14.6 copies (median 4.7, range 9.0×10^{-2}–103.5 copies), whereas for inflammatory/multifocal CL ($n = 4$), it was $5.5 \times 10^{-2} \pm 2.5 \times 10^{-2}$ copies (median 5.8×10^{-2}, range 2.9×10^{-3}–1.0×10^{-1} copies), and for LCL ($n = 6$), it was 8.3 ± 4.1 (median 7.4, range 8.5×10^{-3}–27.2 copies) ($P = 0.11$) (Figure 2A, Supplemental Table 1). Relative copy expression of LRV-1 in isolates causing MCL/ML was higher than that in LRV-1–positive isolates causing inflammatory CL ($P = 0.02$) (Figure 2B, Supplemental Table 1). There was no difference in relative copy expression of LRV-1 in isolates causing MCL/ML versus all CL ($P = 0.30$) and LCL ($P = 0.94$) (Figure 2A and B, Supplemental Table 1). To summarize, LRV-1 relative copy number in MCL/ML isolates was 392.5-fold higher than in isolates causing inflammatory/multifocal CL.

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.[18] Since this initial report, there have been significant advancements and availability of molecular diagnostic tools to further

TABLE 1
Demographic data for 78 patients with *Leishmania* (*Viannia*) *braziliensis* isolates acquired in Peru by clinical phenotype

Characteristic	Total, *N* (%)	Mucocutaneous or mucosal leishmaniasis ($n = 22$), N (%)	Inflammatory/multifocal CL ($n = 26$), N (%)	Localized CL ($n = 30$), *N* (%)	*P*-value
Gender					0.02
Male	65 (83)	22 (100)	18 (69)	25 (83)	
Female	13 (17)	0 (0)	8 (31)	5 (17)	
Median age, years (range)	34 (2–82)	40.5 (20–82)	31 (10–70)	31 (2–76)	0.10
Leishmania RNA virus 1 status					0.21
Positive	21 (27)	9 (41)	5 (19)	7 (23)	
Negative	57 (73)	13 (59)	21 (81)	23 (77)	

CL = cutaneous leishmaniasis.

TABLE 2
Demographic data for 78 patients with *Leishmania* (*Viannia*) *braziliensis* isolates acquired in Peru by LRV-1 status

Characteristic	Total, *N* (%)	LRV-1 positive (*n* = 21), *N* (%)	LRV-1 negative (*n* = 57), *N* (%)	*P*-value
Gender				0.50
Male	65 (83)	19 (91)	46 (81)	
Female	13 (17)	2 (9)	11 (19)	
Median age, years (range)	34 (2–82)	29 (20–68)	35.5 (2–82)	0.55
Clinical phenotype				0.19
Mucocutaneous or mucosal leishmaniasis	22 (28)	9 (43)	13 (22)	
Inflammatory/multifocal CL	26 (33)	5 (24)	21 (37)	
Localized CL	30 (38.5)	7 (33)	23 (40)	

CL = cutaneous leishmaniasis; LRV-1 = *Leishmania* RNA virus 1.

investigate and understand the role of LRV-1 in ATL, and further accrual of data in humans.[5,9,10,12,19–23] 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 tegumentary leishmaniasis, LRV-1 has the potential to contribute to the diagnosis, treatment, and prognostic decision-making in the care of ATL patients.[6] In this study, we examined the overall prevalence and possible correlation to clinical phenotypes of LRV-1 in clinical strains of *L*. (*V.*) *braziliensis* acquired locally and exported from Peru, a highly endemic country for CL and MCL/ML. Our analysis reflects predominantly the specimens from patients both residing in and traveling to endemic areas of Peru and, thus, does not constitute a full survey of representative cases restricted to endemic highland and jungle areas of Peru, where the distribution of isolates and phenotypes may differ at a population level. Although we observed no direct relationship between LRV-1 positivity or negativity with three discrete phenotypes, we documented that patients manifesting MCL/ML had strains of *L*. (*V.*) *braziliensis* containing the highest relative copy numbers of LRV-1, a novel observation in this patient population.

We analyzed LRV-1 status in 78 isolates of *L*. (*V.*) *braziliensis* causing various clinical phenotypes of ATL from Peru and found an overall 27% prevalence, which is within the range reported previously from studies of strains in Latin America, specifically Peru.[5,19–24] It has been shown that LRV-1 is not preferentially associated with a specific phenotype,[20] although we herein determined that 41% of MCL/ML patients were LRV-1 positive, followed by LCL and inflammatory/multifocal CL at 23% and 19%, respectively. Unlike Cantanhêde et al.,[19] we noted no direct association of LRV-1 positivity prevalence with clinical phenotype; however, we documented an almost 400-fold higher relative LRV-1 copy number in isolates causing MCL/ML than isolates causing inflammatory/multifocal CL, potentially supporting a possible LRV-1 association with mucosal disease, in particular. Our findings extend what was originally documented by Ives and colleagues in a murine model of ATL where it was observed that LRV-1 quantity was several-fold higher in metastasizing strains of *L*. (*V.*) *guyanensis*.[7] The relationship of both LRV-1 prevalence and relative viral burden to clinical manifestations and observed phenotype warrant additional work in larger cohort of patients with ATL, specifically in patients with MCL/ML.

On average, LRV-1–positive isolates in this analysis originated from patients who were 6 years younger than those whose isolates were LRV-1 negative; however, those at the extremes of age in this analysis had very low rates of LRV-1 positivity. In addition, those with MCL/ML were an average of 8.5 years older than those with disease confined to the skin. No children or adolescents had either clinically manifest MCL/ML or LRV-1–positive *L*. (*V.*) *braziliensis* isolates causing their disease. Given that progression to ML typically occurs many years after LCL,[1,25] that patients with MCL/ML in this analysis were older is, in itself, unsurprising. 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.[26] In this study, all but one isolate from patients older than 60 years (*n* = 10) were found to be LRV-1 negative, and no isolates from patients younger than 18 years were LRV-1 positive. Advanced age is associated with poorer T-cell response and a Th2-biased response, in particular,[27] which in the case of ATL, is correlated to poorer immunologic control of infection and persistence of the amastigote in the phagolysosome.[4] Similarly, the T-helper-1 (Th1)-to-T-helper-2 (Th2) ratio has been demonstrated to be the lowest in childhood and adolescence, with a peak during mid-adulthood and slight decline thereafter.[28] Th2 predominance over Th1 is also an important factor in the progression to ML.[29–32] Understanding the potential behavioral, and socioeconomic and biological underpinnings of the age distributions of LRV-1 noted in this analysis will be, ultimately, important for accurate interpretation of the viral role in ATL pathogenesis.

TABLE 3
Leishmania RNA virus 1 (LRV-1) status and clinical phenotype according to age bracket in 78 patients with *Leishmania* (*Viannia*) *braziliensis* acquired in Peru

		Clinical phenotype		
Age bracket	LRV-1 positivity (*n* = 21), *N* (%)	Mucocutaneous or mucosal leishmaniasis (*n* = 22), *N* (%)	Inflammatory/multifocal (*n* = 26), *N* (%)	Localized cutaneous leishmaniasis (*n* = 30), *N* (%)
< 18 years (*n* = 8)	0 (0)	0 (0)	4 (50)	4 (50)
19–59 years (*n* = 60)	20 (33)	18 (30)	18 (30)	24 (40)
> 60 years (*n* = 10)	1 (10)	4 (40)	4 (40)	2 (20)

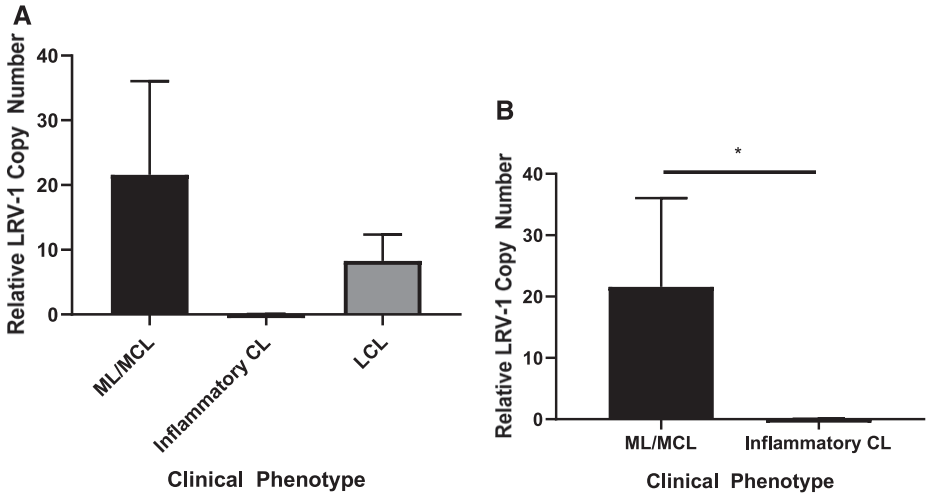

FIGURE 2. Mean relative *Leishmania* RNA virus 1 (LRV-1) copy number in isolates of *Leishmania* (*Viannia*) *braziliensis* by clinical phenotype of American tegumentary leishmaniasis compared by Kruskal–Wallis test (**A**). Mean relative LRV-1 copy number in isolates of *L*. (*V.*) *braziliensis* causing mucocutaneous or mucosal leishmaniasis (MCL/ML) and inflammatory/multifocal CL compared by Mann–Whitney test (**B**).

Limitations of this descriptive analysis of LRV-1 prevalence among *L*. (*V.*) *braziliensis* isolates originating from Peru include the comparatively small number of isolates from each age group (children, young adults, and older adults), which may have biased our interpretation of the data. Prospective enrolment of larger cohorts that might enable more even distribution of age brackets would be worthwhile. It is also possible that significantly different proportions of LRV-1 positivity by phenotype might have emerged with a larger cohort. Although our limited budget did not permit such a large-scale analysis, our findings are nevertheless important as, even in this smaller cohort, they document the higher relative viral load in *L*. (*V.*) *braziliensis* isolates causing MCL/ML and also suggest some interesting age preponderances that will be best interrogated using a combination of epidemiologic and basic scientific approaches going forward. Relative LRV-1 quantification in this study represents a relative copy number in relation to the ATCC *L*. (*V.*) *guyanensis* strain, where primary clinical samples are compared with a clonal line, which arguably has higher viral burden, given the oligoparasitic nature of clinical samples (which contain low amastigote burden, generally). All LRV-1–positive isolates in our analysis derive from a variety of primary clinical specimens, including cytology brushes, FPLIs, and a few cultured specimens. These isolates all reflect a mixed population and are not clonal lines; thus, our findings around relative LRV-1 copy number by strain should be interpreted cautiously. All estimates of relative LRV-1 copy number are based on methods that are highly dependent on the quality of procedures used to prepare samples and are based on a number of estimates. A truly accurate measure of LRV-1 copy number across strains will require development of improved methods. Another limitation of this analysis was our inability to resolve down to the final species-level *Leishmania* isolates from 20 individuals, some of whom may have been infected with *L*. (*V.*) *braziliensis*, which, again, may have influenced our findings. A prospective study following patients who are LRV-1 positive with CL over a significant time period and evaluating the likelihood of patients developing ML could shed light on the ability of LRV-1 to contribute to mucosal diseases while demonstrating the possibility of using antiviral therapy as a novel means of primarily or adjunctively treating patients.

CONCLUSION

We have demonstrated that relative LRV-1 viral burden was highest in *L*. (*V.*) *braziliensis* isolates causing mucosal involvement in this cohort of ATL acquired in Peru. Age emerged as an interesting bias in this cohort, where LRV-1–positive isolates originated from younger patients on average, but proportionate representation of LRV-1 positivity was not observed across age groups, with those within the extremes of age having low rates of LRV-1 positivity in their *Leishmania* isolates. 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 MCL/ML.

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REFERENCES

1. Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S, 2007. Cutaneous leishmaniasis. *Lancet Infect Dis 7:* 581–596.
2. Aronson N, Herwaldt BL, Libman B, Pearson R, Lopez-Velez R, Weina P, Carvalho EM, Ephros M, Jeronimo S, Magill A, 2016. Diagnosis and treatment of leishmaniasis: clinical practice guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH). *Clin Infect Dis 63:* e202–e264.
3. Guimares LH et al., 2016. Atypical manifestations of cutaneous leishmaniasis in a region endemic for *Leishmania braziliensis*: clinical, immunological and parasitological aspects. *PLoS Negl Trop Dis 10:* e005100.
4. Hartlet MA, Ronet C, Zangger H, Beverley SM, Fasel N, 2012. *Leishmania* RNA virus: when the host pays the toll. *Front Cell Infect Microbiol 2:* 99.
5. Ginouves M, Simon S, Bourreau E, Lacoste V, Ronet C, Couppie P, Nacher M, Demar M, Prevot G, 2016. Prevalence and distribution of *Leishmania* RNA virus 1 in *Leishmania* parasites from French Guiana. *Am J Trop Med Hyg 94:* 102–106.
6. Catanhede LM, Fernandes FG, Ferreira GEM, Porrozzi R, Ferreira RGM, Cupolillo E, 2018. New insights into the genetic diversity of *Leishmania* RNA virus 1 and its species-specific relationship with *Leishmania* parasites. *PLoS One 13:* e0198727.
7. Ives A et al., 2011. *Leishmania* RNA virus controls the severity of mucocutaneous leishmaniasis. *Science 331:* 775–778.
8. Ronet C, Beverley SM, Fasel N, 2011. Muco-cutaneous leishmaniasis in the New World. *Landes Biosci 2:* 547–552.
9. Valencia BM, Adaui V, Chantry M, Alba M, Ramos A, Arevalo J, Llanos-Cuentas A, Boggild AK, 2014. Controversial role of *Leishmania* RNA virus as a determinant of pathogenicity in human leishmaniasis. *Int J Infect Dis 21S:* 165–166.
10. Ogg MM, Carrion R, de Carvalho Botelho AC, Mayrink W, Correa-Oliveira R, Patterson JL, 2003. Quantification of *Leishmania* virus RNA in clinical samples and its possible role in pathogenesis. *Am J Trop Med Hyg 69:* 309–313.
11. Kariyawasam R, Grewal J, Lau R, Purssell A, Valencia BM, Llanos-Cuentas A, Boggild AK, 2017. Influence of Leishmania RNA virus 1 on proinflammatory biomarker expression in a human macrophage model of American tegumentary leishmaniasis. *J Infect Dis 216:* 877–886.
12. Bourreau E et al., 2016. *Leishmania*-RNA virus presence in *L. guyanensis* increases risk of first-line treatment failure and symptomatic relapse. *J Infect Dis 213:* 105–111.
13. Schonian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HD, Presber W, Jaffe CL, 2003. PCR diagnosis and characterization of *Leishmania* in local and imported clinical samples. *Diagn Microbiol Infect Dis 47:* 349–358.
14. de Almeida ME, Steurer FJ, Koru O, Herwaldt BL, Pieniazek NJ, da Silva AJ, 2011. Identification of *Leishmania* spp. by molecular amplification and DNA sequencing analysis of a fragment of rRNA internal transcribed spacer 2. *J Clin Microbiol 49:* 3143–3149.
15. Wortmann G, Sweeney C, Houng HS, Aronson N, Stiteler J, Jackson J, Ockenhouse C, 2001. Rapid diagnosis of leishmaniasis by fluorogenic polymerase chain reaction. *Am J Trop Med Hyg 65:* 583–587.
16. Zangger H et al., 2013. Detection of *Leishmania* RNA virus in *Leishmania* parasites. *PLoS Negl Trop Dis 7:* e2006.
17. Schmittgen TD, Livak KJ, 2008. Analyzing real-time PCR data by the comparative CT Method. *Nat Protoc 3:* 1101–1108.
18. Tarr PI, Aline RF, Smiley BL, Scholler J, Keithly J, Stuart K, 1988. LR1: a candidate RNA virus of *Leishmania*. *Proc. Natl. Acad Sci USA 85:* 9572–9575.
19. Cantanhede LM, Silva CF, Ito MM, Felipin KP, Nicolete R, Salcedo JMV, Porrozzi R, Cupolillo E, Ferreira RGM, 2015. Further evidence of an association between the presence of *Leishmania RNA Virus 1* and the mucosal manifestations in tegumentary leishmaniasis patients. *PLoS Negl Trop Dis 9:* e0004079.
20. Adaui V et al., 2016. Association of the endosymbiont double-stranded RNA virus LRV-1 with treatment failure for human leishmaniasis caused by *Leishmania braziliensis* in Peru and Bolivia. *J Infect Dis 213:* 112–121.
21. Macedo DH, Menezes-Neto A, Rugani JM, Rocha AC, Silva SO, Melo MN, Lye LF, Beverley SM, Gontijo C, Soares RP, 2016. Low frequency of LRV-1 in *Leishmania braziliensis* strains isolated from typical and atypical lesions in the State of Minas Gerais, Brazil. *Mol Biochem Parasitol 210:* 50–54.
22. Ito MM, Catanhede LM, Katsuragawa TH, Silva CP, Camargo LMA, Mattos RG, Vilallobos-Salcedo JM, 2015. Correlation between presence of *Leishmania* RNA virus 1 and clinical characteristics of nasal mucosal leishmaniosis. *Braz J Otorhinolaryngol 81:* 533–540.
23. Pereira L, Maretti-Mira AC, Rodrigues KM, Lima RB, Oliveira-Neto M, Cupolillo E, Pirmez C, Oliveira MP, 2013. Severity of tegumentary leishmaniasis is not exclusively associated with *Leishmania* RNA virus 1 infection in Brazil. *Mem Inst Oswaldo Cruz 108:* 665–667.
24. Salinas G, Zamonra M, Stuart K, Saravia N, 1996. *Leishmania* RNA viruses in *Leishmania* of the *Viannia* subgenus. *Am J Trop Med Hyg 54:* 425–429.
25. Jara M, Valencia BM, Adaui V, Alba M, Lau R, Arevalo J, Llanos-Cuentas A, Boggild AK, 2016. Quantitative Kinetoplast DNA Assessment during treatment of mucosal leishmaniasis as a potential biomarker of Outcome: a pilot study. *Am J Trop Med Hyg 94:* 107–113.
26. Brettman EA et al., 2016. Tilting the balance between RNA interference and replication eradicates *Leishmania* RNA virus 1 and mitigates the inflammatory response. *Proc Natl Acad Sci USA 113:* 11998–12005.
27. Salam N et al., 2013. T cell ageing: effects of age on development, survival & function. *Indian J Med Res 138:* 595–608.
28. Chang W-S, Kim E-J, Lim Y-M, Yoon D, Son J-Y, Park J-W, Hong S-J, Cho S-H, Lee J-S, 2016. Age-related changes in immunological factors and their relevance in allergic disease development during childhood. *Allergy Asthma Immunol Res 8:* 338–345.
29. Moafi M et al., 2017. Comparison of pro-inflammatory cytokines of non-healing and healing cutaneous leishmaniasis. *Scand J Immunol 85:* 291–299.
30. Tripathi P, Singh V, Naik S, 2007. Immune response to leishmanial: paradox rather than paradigm. *FEMS Immunol Med Microbiol 51:* 229–242.
31. Maspi N, Abdoli A, Ghaffarifar F, 2016. Pro- and anti-inflammatory cytokines in cutaneous leishmaniasis: a review. *Pathog Glob Health 110:* 247–260.
32. Hartley MA, Kohl K, Ronet C, Fasel N, 2013. The therapeutic potential of immune cross-talk in leishmaniasis. *Clin Microbiol Infect 19:* 119–130.