Parasitology

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Research Article

Cite this article: Kariyawasam R, Lau R, Valencia BM, Llanos-Cuentas A, Boggild AK (2024). Novel detection of *Leishmania* RNA virus-1 (LRV-1) in clinical isolates of *Leishmania Viannia panamensis*. *Parasitology* **151**, 151–156. https://doi.org/10.1017/S0031182023001221

Received: 5 June 2023
Revised: 16 November 2023
Accepted: 26 November 2023
First published online: 30 November 2023

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

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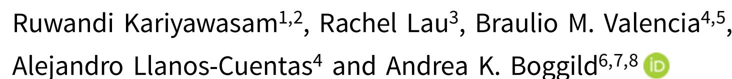
Novel detection of *Leishmania* RNA virus-1 (LRV-1) in clinical isolates of *Leishmania Viannia panamensis*



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Abstract

American tegumentary leishmaniasis 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 the 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 polymerase chain reaction. 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.

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 *et al.*, 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 *et al.*, 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, which has been documented in an endemic region of Brazil (Guimares *et al.*, 2016). To add, other forms include diffuse cutaneous leishmaniasis with multiple non-ulcerative nodules (Reithinger *et al.*, 2007), and disseminated leishmaniasis, defined as maculopapular lesions identified in 2 or more anatomical sites ranging from 10 to 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 *et al.*, 2007). This diverse phenotypology reflects a complex relationship between host, parasite and vector factors (extensively reviewed in Reithinger *et al.*, 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 to 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).

https://doi.org/10.1017/S0031182023001221 Published online by Cambridge University Press

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 *vs* non-metastasizing strains of *L. V. guyanensis*, which were correlated to increased levels of proinflammatory cytokines and chemokines including tumour necrosis factor (TNF)-α, interleukin (IL)-6, chemokine ligand 10 (CXCL10), chemokine ligand 4 (CCL4) and chemokine ligand 5 (CCL5) after recognition by Toll-like receptor 3 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, although these differences were not noted in the analysis of 5 *L. V. panamensis* strains (Kariyawasam *et al.*, 2017). Given that LRV-1 may 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 the Public Health Ontario Laboratory (PHOL) and the *Leishmania* Clinic of the Instituto de Medicina Tropical 'Alexander von Humboldt', Lima, Peru between 2012 and 2019 (Fig. 1). Biobanked isolates were confirmed as *L. V. panamensis* by multiplex real-time polymerase chain reaction (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 $\geqslant$2 anatomic sites and $\geqslant$4 in number) as per the Infectious Diseases Society of America guidelines (Aronson *et al.*, 2016), understanding that the pathogenesis underpinning mucosal *vs* 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, USA) 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 Tris-EDTA buffer (TE) prior to extraction to achieve sufficient volume and DNA concentration and eluted in 60 μL nuclease-free water.

RNA extraction

RNA was extracted from cultured promastigotes using the Cells Protocol of the QIAamp RNA Mini Kit and eluted with 50 μL 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 60 μL 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, heat shock protein 70, mannose phosphate isomerase, zinc-dependent metalloproteinase 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 analysis was performed on each product of end-point PCR (de Almeida *et al.*, 2011; Wortmann *et al.*, 2011; Kariyawasam *et al.*, 2017).

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 10 μM of primer and cleaned accordingly (Kariyawasam *et al.*, 2017). Products were then centrifuged for 2 min at 2000 ***g*** prior to being loaded onto the Applied Biosystems 3730xl DNA Analyser. Data were standardized using the Sequencing Analyser program and BLAST search engine was used to analyse 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 2 primer sets, set A and set B, respectively, as depicted in Fig. 1 (Schmittgen and Livak, 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 $2-\Delta\Delta$Ct method (Ogg *et al.*, 2003; Schmittgen and Livak, 2008; Zangger *et al.*, 2013; Kariyawasam *et al.*, 2017). 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 $2-\Delta\Delta$Ct method (Wortmann *et al.*, 2011; Schmittgen and Livak, 2008; de Almeida *et al.*, 2011; Zangger *et al.*, 2013; Bourreau *et al.*, 2016; Kariyawasam *et al.*, 2017; Schonian *et al.*, 2003).

Analysis

Descriptive statistics (proportions, mean with S.D., median, range) were calculated for all variables. Differences between categorical

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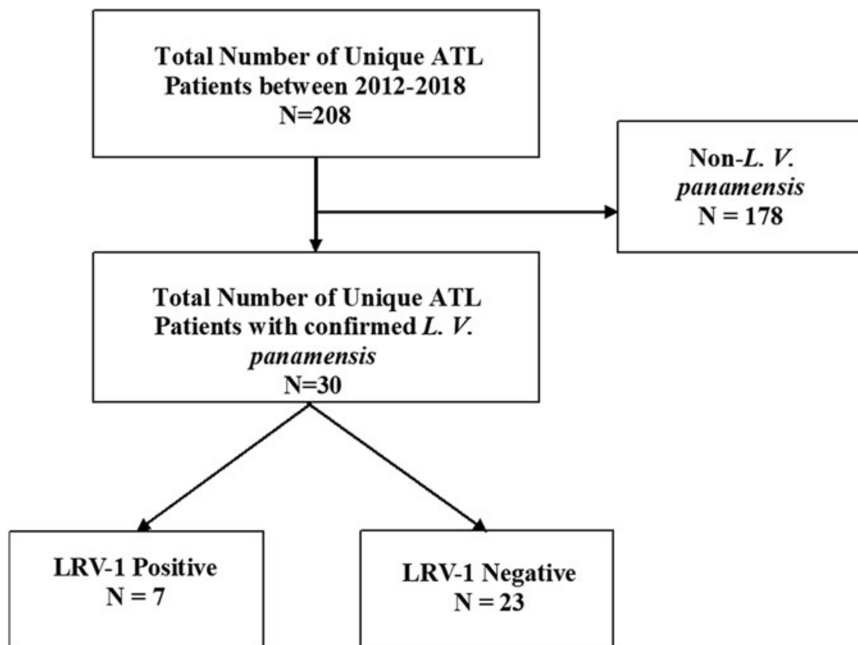

Figure 1. Workflow of sample identification and stratification of patients with confirmed *L. (V.) panamensis.*

variables were compared using Fisher's exact test or χ^2 analysis. Continuous variables were compared by Kruskal–Wallis test or Student's *t*-test. Significance was set at $P < 0.05$. Data were analysed using GraphPad Prism (GraphPad, CA, USA).

Results

Clinical and demographic data

Of 208 specimens from patients with confirmed ATL, 30 (14.4%) isolates were identified as *L. V. panamensis* (Fig. 1). Demographic and parasitological factors for the 30 *L. V. panamensis* isolates from patients with ATL enrolled and analysed 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 that was $1.09 \times 10^{-4} \pm 1.06 \times 10^{-3}$ (median 1.09×10^{-3}, range 6.029×10^{-6} to 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; Cantanhede 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

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Table 1. Demographic data for 30 patients with *L. (V.) panamensis* isolates by clinical phenotype

Characteristic	Total *N* (%)	Inflammatory/multifocal CL ($n = 14$) *N* (%)	LCL ($n = 16$) *N* (%)	*P* value
Sex				0.14
Male	18 (60)	6 (43)	12 (75)	
Female	12 (40)	8 (57)	4 (125)	
Median age, years (range)	35 (9–80)	35 (9–80)	34.5 (17–64)	0.17
Travel history				0.42
Costa Rica	14 (47)	5 (36)	9 (65)	
Peru	7 (23)	1 (7)	6 (38)	
Ecuador	4 (13)	1 (7)	3 (19)	
Belize	1 (3)	1 (7)	0 (0)	
Brazil	1 (3)	0 (0)	1 (6)	
Panama	1 (3)	0 (0)	1 (6)	
Unknown	2 (7)	0 (0)	2 (13)	
LRV-1 status				0.20
Positive	7 (23)	5 (36)	2 (13)	
Negative	23 (77)	9 (64)	14 (87)	

of ATL patients (Cantanhede *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 and negativity with 2 discrete phenotypes was observed, only patients manifesting inflammatory/multifocal CL had a quantifiable viral load.

By analysing 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.*, 2011; Pereira *et al.*, 2013; Cantanhede *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 warrants 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 1 isolate from patients over age 65 ($n = 4$) were found to be LRV-1-positive, and 1 isolate from patients under age 18 was 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 behavioural, 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 travellers from Latin America to Canada or live in Peru, with a majority of travellers having gone to Costa Rica. Prospective enrolment of larger cohorts that might enable more even distribution of returning travellers 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

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immunological age correlates of LRV-1-positivity while controlling for behavioural, 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 availability statement. All data and other materials necessary are included in the article.

Author contributions. R. K. contributed to study design; data collection, analysis and interpretation; and was primarily responsible for drafting the manuscript. R. L. contributed to study design; data collection, analysis and interpretation; and to manuscript revision and critical appraisal. B. M. V. and A. L.-C. contributed to study design; data collection and interpretation; and to manuscript revision and critical appraisal. A. K. B. 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.

Financial support. This work was supported by Public Health Ontario *via* the Project Initiation Fund and the University of Toronto *via* an Early Career Department of Medicine award. Dr Boggild is supported as a Clinician Scientist by the Departments of Medicine at the University of Toronto and University Health Network.

Competing interests. None.

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.

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