Elevated baseline expression of seven virulence factor RNA transcripts in visceralizing species of *Leishmania*: a preliminary quantitative PCR study

Avinash Naraiah Mukkala*, Ruwandi Kariyawasam*, Rachel Lau, Braulio M. Valencia, Alejandro Llanos-Cuentas and Andrea K. Boggild iD

Abstract

Introduction: Leishmaniasis is a neglected tropical disease that manifests as three major disease phenotypes: cutaneous, mucocutaneous, and visceral. In this preliminary study, we quantified virulence factor (VF) RNA transcript expression in *Leishmania* species, stratified by geographic origin and propensity for specific disease phenotypes.

Methods: Cultured promastigotes of 19 *Leishmania* clinical and ATCC isolates were extracted for total cellular RNA, cDNA was reverse transcribed, and qPCR assays were performed to quantify VF RNA transcript expression for *hsp23*, *hsp70*, *hsp83*, *hsp100*, *mpi*, *cpb*, and *gp63*.

Results: Comparison of visceralizing species (Leishmania donovani, Leishmania chagasi, and Leishmania infantum) versus non-visceralizing species [Leishmania (Viannia) spp., Leishmania tropica, Leishmania major, Leishmania mexicana, and Leishmania amazonensis] revealed a significantly greater pooled transcript expression for visceralizing species (p = 0.0032). Similarly, Old World species demonstrated significantly higher VF RNA transcript expression than New World species (p = 0.0015). On a per-gene basis, species with a propensity to visceralize ubiquitously expressed higher levels of gp63 (p = 0.005), cpb (p = 0.0032), mpi (p = 0.0032), hsp23 (p = 0.0039), hsp70 (p = 0.0032), hsp83 (p = 0.0032), and hsp100 (p = 0.0032).

Conclusion: Here, we provide quantitative, preliminary evidence of elevated VF RNA transcript expression driven largely by the visceralizing causative species of *Leishmania*. This work highlights the extensive heterogeneity in pathogenicity mechanisms between *Leishmania* species, which may partly underpin the fatal progression of visceral leishmaniasis.

Keywords: clinical phenotype, *Leishmania*, qPCR, virulence factor, visceral leishmaniasis

Correspondence to:
Andrea K. Boggild
Institute of Medical Science, University of Toronto, Toronto, ON, Canada

Department of Medicine, University of Toronto, Toronto, ON, Canada

Tropical Disease Unit, Toronto General Hospital, Room 13EN-218, 200 Elizabeth Street, Toronto, ON M5G 2C4, Canada.
andrea.boggild@utoronto.ca

Avinash Naraiah Mukkala
Institute of Medical Science, University of Toronto, Toronto, ON, Canada

Ruwandi Kariyawasam
Division of Diagnostic and Applied Microbiology, University of Alberta, Edmonton, AB, Canada

Alberta Precision Laboratories–Public Health Laboratory (ProvLab), Edmonton, AB, Canada

Rachel Lau
Public Health Ontario Laboratory, Toronto, ON, Canada

Braulio M. Valencia
Viral Immunology Systems Program, Kirby Institute, University of New South Wales, Kensington, NSW, Australia

Alejandro Llanos–Cuentas
Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru

Facultad de Salud Pública y Administración, Universidad Peruana Cayetano Heredia, Lima, Peru

*Both authors contributed equally to this work.

Introduction

Leishmaniasis is a vector-borne neglected tropical disease (NTD) that manifests as three major clinical syndromes: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), and mucocutaneous/mucosal leishmaniasis (ML).[1] Caused by over 20 species of protozoan parasites from the *Leishmania* genus, the disease presents with a wide array of symptoms that are influenced by both species-specific and host immunological characteristics.[1,2]

Leishmaniasis is transmitted by the bite of over 90 species of female *Phlebotomus* and *Lutzomyia* sand flies, with over 70 animal species confirmed to be natural reservoir hosts.[2] The World Health Organization (WHO) estimates an annual new case burden of 700,000–1 million, resulting in 20,000–30,000 deaths each year from all forms of leishmaniasis.[2] Of that substantial global disease burden, VL is estimated to account for about 50,000–90,000 new cases per year.[2]

Widely recognized as a disease of poverty, leishmaniasis is endemic to many subtropical and tropical parts of the world.[2,3] VL, in particular, is considered to be the most deadly form as parasites are able to evade host immune clearance and metastasize to internal organs.[1,4] Nearly 147 million people are at risk of contracting VL in Southeast Asia alone.[4] VL is endemic to parts of Southern Europe, North Africa, and West and Central Asia, with the Indian subcontinent suffering the brunt of the disease burden in pediatric populations aged 1–4 years.[2] VL has a wide but recognizable range of presenting symptoms including fever, anorexia, weight loss, abdominal distension, extreme weakness, and hepatosplenomegaly.[2] Of note, *Leishmania*-HIV co-infection is known to increase the overall susceptibility to VL in endemic regions, as well as the rate of treatment failure and relapse.[1,2] Symptomatic VL, if left untreated, can lead to death within 2–3 years due to severe wasting, multiorgan dissemination, hemorrhagic diathesis, and secondary infections.[2] As such, recent efforts by The Special Programme for Research and Training in Tropical Diseases, hosted by the WHO, have begun to shift focus onto regional VL elimination initiatives in the Indian subcontinent.[4] Conversely, ML is exclusively found in Latin America with a significant burden in Brazil, Peru, and Bolivia.[1]

VL, ML, and CL are each caused by different species in the *Leishmania* genus, with some species displaying multiple clinical presentations.[1,2] VL is often caused by *Leishmania donovani*, *Leishmania infantum*, and *Leishmania chagasi*; ML is caused by *Leishmania Viannia braziliensis*, *L. V. panamensis*, and *L. V. guyanensis*, largely found in Latin America; CL is caused by a large array of both New World (NW) and Old World (OW) species, some of which include *Leishmania mexicana*, *Leishmania amazonensis*, *Leishmania tropica*, and *Leishmania major*.[1,3]

Virulence factors (VFs) are components of a pathogen that facilitate its ability to inflict disease in a host but are often not requisite for general viability.[5,6] VFs can increase disease capability of the *Leishmania* parasite by increased expression, host cell invasion and stress tolerance, and modulation of the host immune system.[5,6] VFs have been identified across a wide range of *Leishmania* species, many of which are often ubiquitously expressed across species.[5,6] VF RNA transcripts have been shown to increase post-macrophage

infection in a study focusing on NW species of the *Viannia* subgenus.[7] To add, some *Leishmania* VFs include molecular chaperones such as heat-shock proteins (HSP), which aid in pathogen temperature tolerance;[5–11] cysteine peptidases (e.g. CPB), which are known to have immunomodulatory properties;[5,6,12–14] metallopeptidases (e.g. GP63), which protects the parasite against serum-mediated opsonization and cleaves antimicrobial peptides (AMP);[5,6,15] isomerases (e.g. MPI), which are required for high-demand glycoconjugate synthesis involved in the biosynthesis of other VFs such as GP63, lipophosphoglycan (LPG), and glycoinositolphospholipids (GIPL).[5,6,16] Endogenous VFs in *Leishmania* contribute to the pathogenicity of the parasite, but there are more immunopathogenic mechanisms as well.

Localized and organ-specific immunogenic 'tropisms' are a common theme in VL models.[17–19] Compared to vector and host determinants of VL development, parasite-specific determinants are often what differentiate VL from ML and CL.[17] Parasite-specific determinants of VL development include differences in species-specific genes, polymorphisms, pseudogenes, and VF expression levels (both at the transcriptional and translational levels).[17] Here, we quantify relative baseline VF RNA transcript abundance in both NW and OW *Leishmania* species, across the following VFs: *gp63*, *cpb*, *mpi*, *hsp 23*, *hsp 70*, *hsp 83*, and *hsp 100*, and compared across species by clinical phenotype propensities (VL *versus* non-VL), in order to elucidate emergent patterns in parasite-specific VL determinants.

Materials and methods

Cultured Leishmania spp.

Cultured isolates of *Leishmania* were obtained from the American Type Culture Collection® (ATCC®) and the Public Health Ontario Laboratory (Toronto, Canada) *Leishmania* biobank. The following species of *Leishmania* were used: ATCC® strains of *Leishmania Viannia braziliensis* ATCC®50135™ (MHOM/BR/75/M2903), *L. (V.) guyanensis* ATCC®50126™ (MHOM/BR/75/M4147), *L. (V.) panamensis* ATCC®50158™ (MHOM/PA/71/LS94), *L. amazonensis* ATCC®50159™ (IFLA/BR/67/PH8), *L. chagasi* Cunha and Chagas ATCC®50133™ (MHOM/BR/74/PP75), *L. donovani* (Laveran

and Mesnil) Ross ATCC®50212™ (MHOM/IN/80/DD8), *L. infantum* Nicolle ATCC®50134™ (MHOM/TN/80/IPT-1), *L. major* ATCC®50122™ (MHOM/IL/67/JERICHO II), *L. mexicana* (Biagi) Garnham ATCC®50157™ (MHOM/BZ/82/BEL21), and *L. tropica* (Wright) Luhe ATCC®50129™ (MHOM/SU/74/K27) and nine clinical strains including *L. (V.) braziliensis* ($n = 1$), *L. (V.) panamensis* ($n = 4$), *L. tropica* ($n = 3$), and *L. infantum* ($n = 1$) (Table 1). Age and sex of the host were collected, where possible. A full list of isolates and de-identified clinical characteristics is found in Table 1 ($n = 19$). Promastigotes were regularly subcultured in Tobie's medium with Locke's overlay at ambient room temperature on a weekly basis. For the purposes of RNA extraction standardization, cultures were counted using a hemocytometer.

Leishmania *species identification and confirmation*

DNA was extracted using QIAamp DNA Mini Kit Blood (Qiagen, Germantown, MA, USA). *Leishmania* genus 18S real-time polymerase chain reaction (PCR) was performed as previously described.[20] Species identification included analysis of the internal transcribed spacer 1 (ITS1), ITS2, CPB, HSP70, and MPI targets by PCR, restriction fragment length polymorphism (RFLP) analysis, and Sanger sequencing.[21,22] PCR-RFLP analysis of the ITS1 region can only differentiate *L. V. braziliensis* from the other species within the *Viannia* subgenus (*L. V. guyanensis*, *L. V. peruviana*, *L. V. panamensis*, *L. V. lainsoni*). Thus, PCR-RFLP and sequencing analysis of the CPB, HSP70, MPI, and ITS2 regions were required to differentiate species within the *Leishmania Viannia* subgenus and to provide a confirmation of the species identified in the initial *ITS1* assay. Purified PCR product was used for Sanger sequencing as per Big Dye protocol (Life Technologies, Carlsbad, CA, USA). Sequence products were purified and analyzed using the Applied Biosystems 3130xl Genetic Analyzer. Data were standardized using the Sequencing Analyzer program and the BLAST search engine was used to analyze sequences.

RNA extraction, cDNA synthesis, and purification

RNA was extracted from 0.5 ml of baseline pure culture (up to 5×10^5 promastigotes) using QIAmp RNA Mini Kit (Qiagen, Germantown, MA, USA). In-column DNase treatment was included in all extractions as per manufacturer's protocol. cDNA was synthesized using Superscript II Reverse Transcriptase and random hexamers (Life Technologies, Carlsbad, CA, USA), followed by purification with QIAquick PCR Purification Kit (Qiagen, Germantown, MA, USA) and eluted with 60 μl of nuclease-free water. cDNA aliquots were stored at −20°C until quantitative real-time polymerase chain reaction (qPCR) assays were conducted.

VF RNA transcript expression qPCR assays

In this study, VFs are defined as proteins associated with disease severity in the *Leishmania* literature.[5–16] Transcript expression of the following VFs: *hsp23*, *hsp70*, *hsp90*, *hsp100*, *mpi*, *cpb*, *gp63*, and 18S, was performed on the ABI 7900HT real-time instrument using previously published primers and probes.[7] A real-time qPCR was set up in triplicate using 12.5 μl 2× Taqman Universal Master Mix (ThermoFisher Scientific, Carlsbad, CA, USA), 250 nM final concentration of forward and reverse primers, 10 nM probe, and 5 μl of cDNA from pure culture, in a total volume of 20 μl for each respective target.[7] Amplification was performed with the following conditions: Uracil DNA-glycosylase (UDG) activation at 50°C for 2 min, polymerase activation at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, and 60°C for 1 min.[7]

Expression quantification and statistical analysis

Transcript expression was quantified using the $2^{-\Delta Ct}$ method (a modified version of the $2^{-\Delta\Delta Ct}$ method, i.e., relative abundance of target *versus* reference housekeeping gene 18S).[7,23] Expression values of all seven VFs were added together to give a 'pooled VF' quantification.[7] Transcript expression values were grouped into four categories, for which categorical hypothesis testing was performed: VL *versus* non-VL, and OW *versus* NW (Table 1). Before statistical testing, all groupings were screened for normality *via* the D'Agostino and Pearson Normality Test. Normally distributed groupings underwent parametric unpaired *t*-tests and, non-normal groupings underwent non-parametric Mann–Whitney *U*-test, on a per-gene and pooled basis. A log transformation was performed to better graphically visualize the data

Table 1. Characteristics of cultured clinical and ATCC isolates of *Leishmania..*

ID No.	Species	Source of isolate	Passage number	Sex of patient	Age of patient	VL or non-VL	Geographic location
1	*L. chagasi*	ATCC® 50133™	P2	Unknown	Unknown	VL	New World
2	*L. infantum*	Clinical 1	P2	Male	74	VL	Old World
3	*L. infantum*	ATCC® 50134™	P3	Unknown	Unknown	VL	Old World
4	*L. donovani*	ATCC® 50212™	P2	Unknown	Unknown	VL	Old World
5	*L. major*	ATCC® 50122™	P3	Unknown	Unknown	Non-VL	Old World
6	*L. tropica*	ATCC® 50129™	P6	Unknown	Unknown	Non-VL	Old World
7	*L. tropica*	Clinical 2	P0	Male	33	Non-VL	Old World
8	*L. tropica*	Clinical 3	P7	Male	10	Non-VL	Old World
9	*L. tropica*	Clinical 4	P0	Unknown	Unknown	Non-VL	Old World
10	*L. V. braziliensis*	ATCC® 50135™	P13	Unknown	Unknown	Non-VL	New World
11	*L. V. braziliensis*	Clinical 5	P12	Male	22	Non-VL	New World
12	*L. V. panamensis*	ATCC® 50158™	P12	Unknown	Unknown	Non-VL	New World
13	*L. V. panamensis*	Clinical 6	P12	Male	80	Non-VL	New World
14	*L. V. panamensis*	Clinical 7	P8	Male	17	Non-VL	New World
15	*L. V. panamensis*	Clinical 8	P9	Male	9	Non-VL	New World
16	*L. V. panamensis*	Clinical 9	P3	Male	71	Non-VL	New World
17	*L. V. guyanensis*	ATCC® 50126™	P20	Unknown	Unknown	Non-VL	New World
18	*L. mexicana*	ATCC® 50157™	P2	Unknown	Unknown	Non-VL	New World
19	*L. amazonensis*	ATCC® 50159™	P6	Unknown	Unknown	Non-VL	New World

VL, visceral leishmaniasis.

(Figures 1 and 2). All groupings for categorical statistical analysis are outlined in Table 1. All statistical analyses were conducted using GraphPad Prism v5.04 software (GraphPad Software Inc, La Jolla, CA). Significance was set at $p < 0.01$.

## Results

Clinical and demographic characteristics

Where available, median age was 22 years old (range, 7–80 years), with isolates being derived from predominantly male hosts (Table 1). Three of 19 (16%) isolates were derived from pediatric patients (age <18), 5 of 19 (26%) isolates were derived from adult patients (age >18), and 10 of 19 (53%) ATCC® isolates were from an unknown host origin (Table 1). Eight of 19 (42%) patients were male, and the rest of the isolates were derived from an unknown host sex (Table 1).

VF RNA transcript expression: VL versus non-VL species

A significant difference was found between VL and non-VL species on a per-gene and pooled basis among all VFs. On a pooled VF basis, all VL species consistently and significantly expressed higher VF RNA transcript levels than non-VL species ($p = 0.0032$) (Figure 1(a)); moreover, the
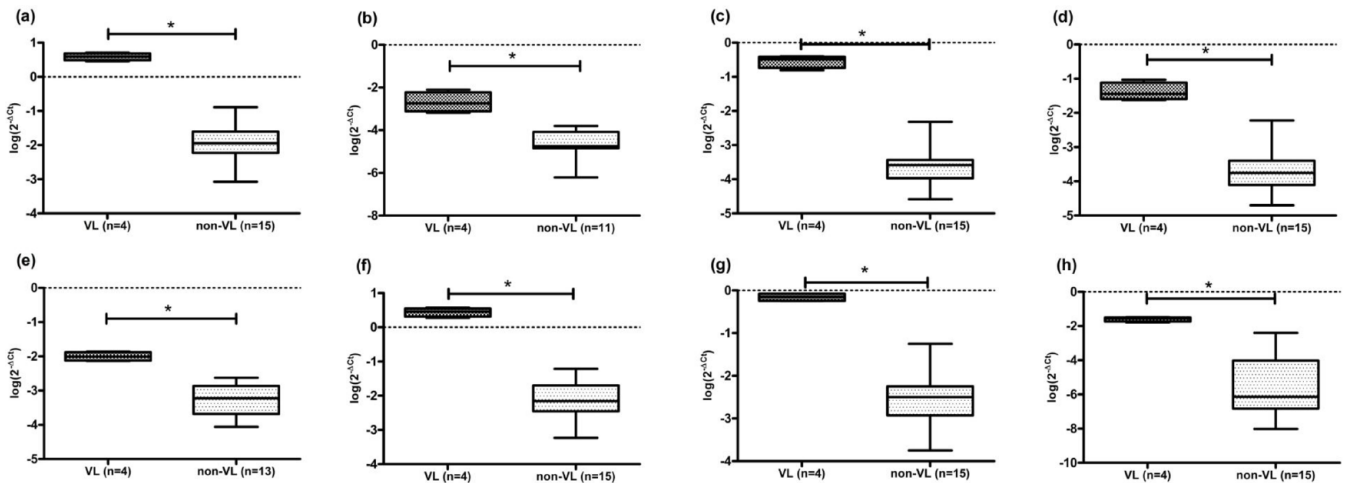
Figure 1. VF RNA transcript expression in visceralizing *versus* non-visceralizing species.
Log transformed, and $2^{-\Delta Ct}$ (i.e. relative abundance of VF target against reference 18S) quantified VF RNA transcripts represented as box plots (median with maximum and minimum whiskers) between VL and non-VL groupings of species compared by *t*-test. For the VL *versus* non-VL comparison, with the following VFs: (a) pooled ($p = 0.0032$), (b) *gp63* ($p = 0.005$), (c) *cpb* ($p = 0.0032$), (d) *mpi* ($p = 0.0032$), (e) *hsp23* ($p = 0.0039$), (f) *hsp70* ($p = 0.0032$), (g) *hsp83* ($p = 0.0032$), and (h) *hsp100* ($p = 0.0032$). *$p < 0.01$. VF, virulence factor; VL, visceral leishmaniasis.

mean pooled VF transcript expression for all VL species was 159-fold higher than the mean pooled VF transcript expression for all non-VL species. Across all analyzed VF transcripts, VL species expressed significantly greater RNA transcripts than non-VL species: *gp63* (79.2-fold; $p = 0.005$) (Figure 1(b)), *cpb* (556-fold; $p = 0.0032$) (Figure 1(c)), *mpi* (78.5-fold; $p = 0.0032$) (Figure 1(d)), *hsp23* (12.9-fold; $p = 0.0039$) (Figure 1(e)), *hsp70* (178-fold; $p = 0.0032$) (Figure 1(f)), *hsp83* (107-fold; $p = 0.0032$) (Figure 1(g)), and *hsp100* (84.5-fold; $p = 0.0032$) (Figure 1(h)). The following VFs could not be quantified in the following species: *gp63* could not be amplified from *L. tropica* specimens, and *hsp23* could not be amplified from either *L. mexicana* or *L. amazonensis*.

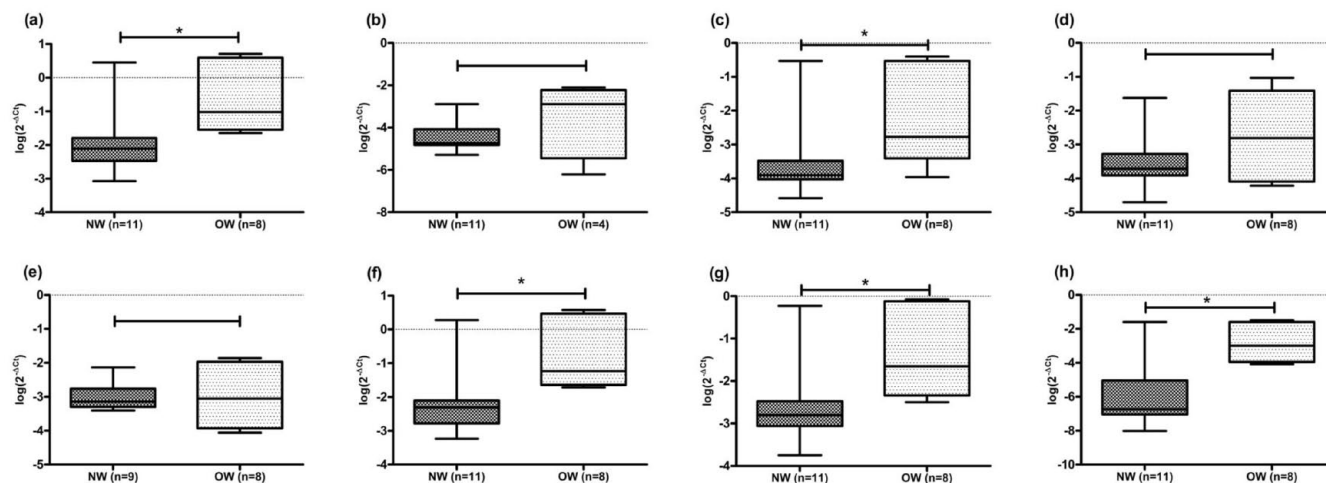### VF RNA transcript expression, Old World *versus* New World species

A significant difference was found between OW and NW species on a per-gene and pooled basis among most VF transcripts. On a pooled VF basis, OW species significantly expressed higher VF RNA transcript levels than NW species (6.24-fold; $p = 0.0015$) (Figure 2(a)). Across most analyzed VFs, OW species expressed significantly greater RNA transcripts than NW species: *gp63* (17.8-fold; $p = 0.2149$) (Figure 2(b)), *cpb* (4.29-fold; $p = 0.0093$) (Figure 2(c)), *mpi* (9.01-fold; $p = 0.3020$) (Figure 2(d)), *hsp23* (2.83-fold; $p = 0.9626$) (Figure 2(e)), *hsp70* (6.84-fold; $p =$

0.0015) (Figure 2(f)), *hsp83* (5.26-fold; $p = 0.0057$) (Figure 2(g)), and *hsp100* (4.45-fold; $p = 0.0020$) (Figure 2(h)). Much of these differences are largely driven by the VL-causative species, when taking both Figures 1 and 2 into account.

Discussion

The immense mechanistic diversity with which the *Leishmania* parasite infects its hosts, along with the large range of clinical presentations, makes the pathogenesis quite complex.[1,5,15] For the development of VL, parasites must (1) traverse from the skin to visceral organs and (2) survive the host environment.[17] Some parasitological factors for the development of VL are as follows: species-specific genes, pseudogenes, gene mutations and polymorphisms, and most importantly, the expression of VFs.[17] Our preliminary data suggest that higher baseline levels of VF RNA transcripts are significantly correlated with *L. donovani*, *L. infantum*, and *L chagasi*, which may partly explain the aggressive and fatal development of VL. Recent evidence suggests that even within a given species, geographically distinct populations have extremely divergent genomic and metabolomic pathogenesis mechanisms, particularly in the Indian subcontinent (ISC1) *L. donovani* population.[24]

The ability to tolerate host temperature differentials is crucial for survival and infection of

Figure 2. VF RNA transcript expression in old world *versus* new world species of *Leishmania*.
Log transformed, and 2$^{-\Delta Ct}$ (i.e. relative abundance of VF target against reference 18S) quantified VF RNA transcripts represented as box plots (median with maximum and minimum whiskers) between OW and NW groupings of species compared by *t*-test. For the NW *versus* OW comparison, with the following VF transcripts: (a) pooled ($p = 0.0015$), (b) *gp63* ($p = 0.2149$), (c) *cpb* ($p = 0.0093$), (d) *mpi* ($p = 0.302$), (e) *hsp23* ($p = 0.9626$), (f) *hsp70* ($p = 0.0015$), (g) *hsp83* ($p = 0.0057$), and (h) *hsp100* ($p = 0.002$). *$p < 0.01$. VF, virulence factor; VL, visceral leishmaniasis.

Leishmania.[5–11] Molecular chaperones, such as HSPs, are crucial for maintaining cellular homeostasis in infective thermotolerance.[5–11] Under heat stress, which invariably occurs during the process of mammalian infection, HSP23 and HSP70 co-localize, upregulating their expression to meet host temperature requirements.[7–9] HSP70 is the most conserved protein in all organisms, and plays essential roles in prevention of peptide denaturation in *Leishmania*, which is especially important in the high-temperature mammalian host environment.[5,9] HSP83 (an HSP90 homolog) facilitates both virulence *via* drug resistance and thermotolerance.[9,11] HSP100 is involved in the refolding of misfolded proteins, particularly under environmentally stressed conditions.[9,10] Our preliminary study quantified the RNA transcripts for *hsp 23, 70, 83 (90)*, and *100* to better understand species-specific expression correlates, which may well elucidate clinical presentation propensities in the diverse pathogenesis of leishmaniasis. Our preliminary data suggest that for all four HSPs, VL-causative species of *Leishmania* consistently and significantly express larger quantities of baseline VF RNA transcripts. The substantial and significant 13-, 178-, 107-, and 85-fold higher levels of *hsp23, 70, 83, 100*, respectively, noted in the VL species investigated herein corroborates this differential. Furthermore, in this study, VL-causative species drove much of the difference between the OW and NW groupings. Therefore, there is potentially some speculative mechanistic

underpinning to this correlation: *L. donovani, L. infantum*, and *L. chagasi* have such high baseline expression of all four HSPs to perhaps better infect thermally challenging environments in the internal organs of mammalian host organisms. For example, HSP23 plays a crucial role in adaptation to mammalian host temperatures in *L. donovani*, as it is significantly upregulated in amastigotes, as evidenced in both RNA and protein studies.[8] For HSP100, the expression levels correlate to stage-specific roles, whereas HSP70 is often constitutively kept at high levels.[8] Therefore, HSPs show a dual role in *Leishmania* pathogenesis: selective or universal.[10] Hence, initially high levels of HSPs may expedite and enhance parasite propensity to produce the VL phenotype.

Cysteine peptidases, such as CPB, are VFs in *Leishmania* that are known to have significant cytokine immunomodulatory effects as well as parasite–host interaction regulation.[5,6,12–14] Three distinct genes exist: CPA, CPB, and CPC, all belonging to the same group designated Clan CA, Family C1.[13] CPB has also been implicated in the regulation of other VFs' expression, such as GP63.[12] Zinc-dependent metalloprotease GP63 is a surface antigen expressed on all *Leishmania* species at the promastigote stage.[6] It is involved in parasite adherence to macrophages and circumvention of complement-mediated lysis.[12,15] GP63 activates protein tyrosine phosphatases to reduce NO production, thus facilitating parasite

persistence in the macrophage vacuole.[14] Our preliminary data suggest that VL-causative species express 79- and 556-fold higher quantities of *gp63* and *cpb* RNA transcripts at baseline, respectively. Since CPB is required for GP63-mediated parasite persistence in macrophages,[12] our preliminary data's similar correlation pattern for these two VFs supports their mechanistic contribution to pathogenesis in VL-causative species. Finally, MPI is an enzyme involved in the reversible conversion of fructose-6-phosphate and mannose-6-phosphate required for biosynthesis of various glycoconjugates.[16] MPI is involved in constructing other VFs such as GP63, LPG, and GIPL.[5,6,16] Again, our preliminary data demonstrate *mpi* expression in 79-fold greater quantities in VL species than in other isolates, implying a possibly high demand for pre-infection glycoconjugate production. This increased baseline necessity for glycoconjugates may tie into the increased expression of *gp63* transcripts.

Diverse expression of VF RNA transcripts is found within species with different propensity to cause a particular type of leishmaniasis.[7] VL-causative species tend to express significantly greater amounts of all quantified VFs, when compared with both all non-VL species and NW species. *L. donovani*, *L. infantum*, and *L. chagasi* are distinct in how much VFs they express, even in a preinfection, pure culture, baseline model, such as ours. Moreover, only 0.2–5% of the *Leishmania* functional genome is differentially expressed between the amastigote and promastigote life stages; consequently, the *Leishmania* genome is largely constitutively expressed with a limited set of genes showing stage-specific expression patterns.[25,26] Some genomic evidence suggests that *Leishmania* amastigotes are pre-adapted for intracellular survival and experience little alterations in gene expression at the RNA level.[26] Our preliminary data partially suggest this pre-adaption phenomena to be especially present in the VL species, *L. donovani*, *L. infantum*, and *L. chagasi*. However, this small number of differentially expressed genes may still be significant in predicting the clinical outcome of infection.[26] Recently, Shadab *et al.*[27] performed RNA-seq comparison in a murine macrophage model of virulent and avirulent *L. donovani* infection. This preliminary analysis demonstrated the vast differences in transcriptomic expression profiles between parasites, particularly macrophages infected with virulent

L. donovani strains showed marked anti-inflammatory responses and dysregulation of protein synthesis.[27] Furthermore, virulent *L. donovani* were shown to globally upregulate virulence and parasite survival factors.[27]

Therefore, a proposed novel model for VL development, as compared with CL or ML, is that VL species (*L. donovani*, *L. infantum*, and *L. chagasi*) tend to 'start off' their preinfection (baseline) status at a significantly more 'virulent' state with large quantities of available VFs, ready for usage when a host is present, whereas non-VL species tend not to be in a 'triggered' virulence state and perhaps not as parasitologically primed for visceral infection. It may well be that the *Viannia* subgenus species are more prone toward disease modulation *via* more host immunological responses. This elevated level of ubiquitous VFs in VL-causative species may help 'prime' the parasite for a more efficient and effective visceral infection of the host organism. Seemingly, VFs are expressed in a species and stage-specific manner, as previously demonstrated by increased RNA transcripts pre- and post-macrophage infection.[7] Hence, the uniquely severe pathogenesis of VL may partly be explained by elevated expression of VFs. This preliminary study requires extension and broader interrogation with larger sets of *Leishmania* species.

Limitations and next steps

Some confines for this work include the small number of cultured isolates and the fact that not all possible VF transcripts from all possible species were assessable (e.g. *Gp63* for *L. tropica* and *hsp23* for *L. mexicana* and *L. amazonensis*). Moreover, given limitations in biobank availability, some isolates were evaluated at different times of passage, which could have impacted our analysis and interpretation. When selecting VFs to target, those that tied directly to mechanistic virulence methods and were the most evidentially pathogenic were selected. This limitation may have biased some part of our interpretation of our data, but with likely minimal overall effect on our conclusions. We evaluated VF RNA transcript expression at baseline, but did not quantify protein expression; thus, it is unknown whether or not transcript abundance would correlate to protein abundance. This is especially crucial as much of the regulation in *Leishmania* occurs post-transcriptionally and even

post-translationally.[23,25,26] Further validation of our hypothesis is required with a comprehensive genome-wide VF screen, macrophage, or other model,[7] while taking the many immunomodulatory features of the host organism into account. Importantly, a more robust evaluation of multiple isolates from different species at the exact same time of passaging would be crucial for corroborating our findings from this preliminary work. It would also be interesting to characterize the energy requirements of high preinfection VF expression in VL *Leishmania* species in order to compare by propensity for particular clinical presentations.

In conclusion, we herein provide quantitative, preliminary evidence of elevated VF RNA transcript expression driven largely by the visceralizing causative species of *Leishmania*, which underscores the extensive heterogeneity in pathogenicity mechanisms between *Leishmania* species, and which may partly explain the fatal progression of visceral leishmaniasis.

Ethics approval and consent to participate
Approval for this study was obtained from the Research Review Board of Public Health Ontario Laboratory (RRB-12-052). As the biobank contains surplus discarded clinical specimens that are anonymized and delinked before analysis, no informed consent or human subjects considerations were required. Non-clinical specimens derived from the ATCC are commercial products sold for experimental purposes.

Author contribution(s)
Avinash Naraiah Mukkala: Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing – original draft.

Ruwandi Kariyawasam: Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing – original draft.

Rachel Lau: Data curation; Formal analysis; Investigation; Methodology; Project administration; Validation; Writing – review & editing.

Braulio M. Valencia: Investigation; Methodology; Project administration; Writing – review & editing.

Alejandro Llanos-Cuentas: Project administration; Supervision; Validation; Writing – review & editing.

Andrea K. Boggild: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Software; Supervision; Writing – original draft; Writing – review & editing.

ORCID iD
Andrea K. Boggild [iD] https://orcid.org/0000-0002-2720-6944

Conflict of interest statement
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

References
1. Reithinger R, Dujardin JC, Louzir H, *et al*. Cutaneous leishmaniasis. *Lancet Infect Dis* 2007; 7: 581–596.

2. World Health Organization (WHO). Leishmaniasis, fact sheet, https://www.who.int/en/news-room/fact-sheets/detail/leishmaniasis (2021, accessed 20 May 2021).

3. Pace D. Leishmaniasis. *J Infect* 2014; 69(Suppl. 1): S10–S18.

4. Hirve S, Kroeger A, Matlashewski G, *et al*. Towards elimination of visceral leishmaniasis in the Indian subcontinent – translating research to practice to public health. *PLoS Negl Trop Dis* 2017; 11: e0005889.

5. Bifeld E and Clos J. The genetics of Leishmania virulence. *Med Microbiol Immunol* 2015; 204: 619–634.

6. Atayde VD, Hassani K, da Silva Lira Filho A, *et al*. Leishmania exosomes and other virulence factors: impact on innate immune response and macrophage functions. *Cell Immunol* 2016; 309: 7–18.

7. Kariyawasam R, Mukkala AN, Lau R, *et al*. Virulence factor RNA transcript expression in the Leishmania Viannia subgenus: influence of species, isolate source, and Leishmania RNA virus-1. *Trop Med Health* 2019; 47: 25.

8. Hombach A, Ommen G, MacDonald A, *et al*. A small heat shock protein is essential for thermotolerance and intracellular survival of *Leishmania donovani*. *J Cell Sci* 2014; 127: 4762–4773.

9. Requena JM, Montalvo AM and Fraga J. Molecular chaperones of leishmania: central players in many stress-related and -unrelated physiological processes. *Biomed Res Int* 2015; 2015: 301326.

10. Krobitsch S, Brandau S, Hoyer C, *et al*. *Leishmania donovani* heat shock protein 100: characterization and function in amastigote stage differentiation. *J Biol Chem* 1998; 273: 6488–6494.

11. Alexandratos A, Clos J, Samiotaki M, *et al*. The loss of virulence of histone H1 overexpressing *Leishmania donovani* parasites is directly associated with a reduction of HSP83 rate of translation. *Mol Microbiol* 2013; 88: 1015–1031.

12. Casgrain PA, Martel C, McMaster WR, *et al*. Cysteine peptidase B regulates *Leishmania mexicana* virulence through the modulation of GP63 expression. *Plos Pathog* 2016; 12: e1005658.

13. Mottram JC, Coombs GH and Alexander J. Cysteine peptidases as virulence factors of Leishmania. *Curr Opin Microbiol* 2004; 7: 375–381.

14. Silva-Almeida M, Pereira BA, Ribeiro-Guimarães ML, *et al*. Proteinases as virulence factors in *Leishmania* spp. infection in mammals. *Parasit Vectors* 2012; 5: 160.

15. Olivier M, Atayde VD, Isnard A, *et al*. Leishmania virulence factors: focus on the metalloprotease GP63. *Microbes Infect* 2012; 14: 1377–1389.

16. Garami A and Ilg T. The role of phosphomannose isomerase in *Leishmania mexicana* glycoconjugate synthesis and virulence. *J Biol Chem* 2001; 276: 6566–6575.

17. McCall LI, Zhang WW and Matlashewski G. Determinants for the development of visceral leishmaniasis disease. *PLoS Pathog* 2013; 9: e1003053.

18. Wilson ME, Jeronimo SM and Pearson RD. Immunopathogenesis of infection with the visceralizing Leishmania species. *Microb Pathog* 2005; 38: 147–160.

19. Faleiro RJ, Kumar R, Hafner LM, *et al*. Immune regulation during chronic visceral leishmaniasis. *PLoS Negl Trop Dis* 2014; 8: e2914.

20. Wortmann G, Sweeney C, Houng HS, *et al*. Rapid diagnosis of leishmaniasis by fluorogenic polymerase chain reaction. *Am J Trop Med Hyg* 2001; 65: 583–587.

21. Schönian G, Nasereddin A, Dinse N, *et al*. PCR diagnosis and characterization of Leishmania in local and imported clinical samples. *Diagn Microbiol Infect Dis* 2003; 47: 349–358.

22. de Almeida ME, Steurer FJ, Koru O, *et al*. Identification of *Leishmania* spp. by molecular amplification and DNA sequencing analysis of a fragment of rRNA internal transcribed spacer 2. *J Clin Microbiol* 2011; 49: 3143–3149.

23. Schmittgen TD and Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008; 3: 1101–1108.

24. Cuypers B, Berg M, Imamura H, *et al*. Integrated genomic and metabolomic profiling of ISC1, an emerging *Leishmania donovani* population in the Indian subcontinent. *Infect Genet Evol* 2018; 62: 170–178.

25. Depledge DP, Evans KJ, Ivens AC, *et al*. Comparative expression profiling of leishmania: modulation in gene expression between species and in different host genetic backgrounds. *PLoS Negl Trop Dis* 2009; 3: e476.

26. Cohen-Freue G, Holzer TR, Forney JD, *et al*. Global gene expression in Leishmania. *Int J Parasitol* 2007; 37: 1077–1086.

27. Shadab M, Das S, Banerjee A, *et al*. RNA-seq revealed expression of many novel genes associated with *Leishmania donovani* persistence and clearance in the host macrophage. *Front Cell Infect Microbiol* 2019; 9: 17.