The maze pathway of coevolution: a critical review over the *Leishmania* and its endosymbiotic history

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Abstract: The description of the genus *Leishmania* as causative agents of leishmaniasis occurred during this modern age. But evolutionary studies suggest that the origin of *Leishmania* can be traced back to the Mesozoic era. Subsequently, during its evolutionary process, it sustained a worldwide dispersion predating the breakup of the Gondwana supercontinent. It is assumed that this parasite evolved from monoxenic Trypanosomatidae. Phylogenetic studies locate the dixenous *Leishmania* in a well-supported clade, recently named subfamily Leishmaniinae, which includes also monoxenous trypanosomatids. Virus-Like Particles were reported in many species of this family. So far, several *Leishmania* species have been reported as infected by *Leishmania* RNA Virus (LRV) and Leishbunyavirus (LBV). Since the first descriptions of LRV decades ago, differences in its genomic structure have been highlighted, leading to the designation of a LRV1 in *L.* (*Viannia*) species and a LRV2 in other *L.* (*Leishmania*) species. There are strong indications of virus infecting *Leishmania* spp. ability to enhance parasitic survival both in human and experimental infections, through highly complex and specialized mechanisms. Phylogenetic analyzes of these viruses have shown that their genomic differences correlate with the infected parasite species, suggesting a coevolutionary process. Herein, we will present unpublished results regarding the relationship *Leishmania* – endosymbiotic *Leishmania* viruses and will explore what has been described in the literature, and what is known about this association that could contribute to discussions about the worldwide dispersion of *Leishmania.*

Keywords: *Leishmania*, *Leishmania* viruses, Phylogeny, Coevolution, endosymbiont protozoan viruses

1. Introduction

The origin of the *Leishmania* parasite dates back to the Mesozoic era, and its dispersion to the continents, still hypothetical, considers different scenarios [1]. The diversification of this group of dixenous parasites occurred in different continents and nowadays the *Leishmania* genus comprises by dozens of different species widespread all over the world, pathogenic or not to the human, and which, by themselves, present complexities that are still not fully understood. There is some discussion on the taxonomy of *Leishmania*, and in this study, we will adopt the proposal of Kostygov *et. al.*, [2] and Espinosa *et. al.*, [3], naming four *Leishmania* subgenera: *L*. (*Leishmania*), *L*. (*Viannia*), *L*. (*Sauroleishmania*), and *L*. (*Mundinia*).

Despite the effort to unravel the mechanisms of pathogenicity, to determine risk of infection and to develop new treatments and vaccines against the parasite, there are still gaps in the state of the art to be explored. For example, one of the most well-defined aspects of the parasite, the *Leishmania* life cycle, has been updated by recent and important discoveries that influence the parasite's dispersion ability [4]. An amazing field to be explored regards to the effects of the endosymbiotic *Leishmania* virus' presence, its relationship with the *Leishmania* cell and further clinical and epidemiological consequences. *Paraleishmania hertigi* and *P. deanei*, former *Leishmania hertigi* and *Leishmania deanei* [2,5] were the first members of the Subfamily Leishmaniinae [6] pointed as hosting virus-like particles [7]. Still, from that moment on, no more studies were performed aiming to characterize VLPs from these species. Only nine years later, *Leishmania (Viannia) guyanensis* and *Leishmania (V.) braziliensis* were both described as hosting viruses that were molecular characterized [8], naming the *L. guyanensis* virus as LR1 and the one found in *L. braziliensis* as LR2. Both LR1 and LR2 were thought to be constituted by single-strand DNA [8], but soon after it was demonstrated to be a circular dsRNA and was renamed as LRV[9]. In the following years, LRV was described in 12 isolates of *L. braziliensis* and *L. guyanensis* from the Amazon region [10]. The next year, LRV was identified in one *Leishmania* (*Leishmania*) *major* isolated from a human patient in the former Soviet Union [11]. As far as we know, there are no studies looking for viruses in *L*. (*Sauroleishmania*) species,

and only one species from *L.* (*Mundinia*): *L. martiniquensis*, was found harboring the Leishbunyavirus (LBV).

Recent reports focused on *Leishmania* and the viral endosymbiont LRV first arose from questions not directly related to the virus, but rather to the Toll-Like Receptors and its association with the variable immunological responses to the *Leishmania* infection [12]. Important data was gathered since then. Both viral families, Leishbunaviridae and Totiviridae, influenced phenotypic expression of *Leishmania* infection in the vertebrate host, although biological aspects from *Leishmania* harboring virus *vs* virus free *Leishmania* remain to be elucidated.

Thus, current data, in association with reports from decades ago, led us a step further in the understanding of this peculiar, dynamic, and million-years-old parasite. Some studies were recently published searching and characterizing viruses in *Leishmania* parasites and in different members of the Trypanosomatidae family, suggesting endosymbiotic viruses as an ancient acquisition by these protozoans. Here, our main objective is to summarize previous and recent reports that characterize *Leishmania* viruses and the impact of this endosymbiosis, and from this, analyze their relationship with the parasite species that host them. We will also report unpublished results that reinforce the hypothesis of a symbiotic relationship between LRV1 and parasites of the subgenus *Leishmania* (*Viannia*).

2. The *Leishmania* Viruses

Virus-like particles (VLPs) in parasitic protozoan were first described in *Entamoeba histolytica* in the 1960s [13]. After that, several studies reported similar structures in many unicellular eukaryotes, such as *Giardia lamblia, Trichomonas vaginalis*, and members of the Trypanosomatidae family, including *Leishmania* spp. and *Trypanosoma* spp. For some additional protozoan, however, there are only studies reporting VLPs based on electron microscopy approaches, but not by molecular methods. The International Committee on Taxonomy of Viruses (ICTV) recognized only the family Totiviridae gathering *Leishmania* viruses [14]. But this, as well as families collecting viruses from other trypanosomatids, must be updated considering recent viruses discovery and characterization [15].

Totiviridae consists of five genera: *Giardiavirus*, *Leishmaniavirus* (LRV), *Trichomonasvirus*, *Totivirus,* and *Victorivirus*. According to ICTV, *Leishmania* RNA virus 1 (LRV1) and *Leishmania* RNA virus 2 (LRV2) belong to the *Leishmaniavirus*

genus. LRV was assumed as infecting *Leishmania* spp. only, with two species identified as LRV1 and LRV2, but recently it was found in *Blechomonas* spp., a monoxenous trypanosomatid parasitizing fleas [15].

Recently, a new genus belonging to the Leishbuviridae family was proposed, the Leishbunyavirus (LBV). The family Leishbuviridae includes the genus *Shilevirus* which infects *Leptomonas moramango*, a monoxenic trypanosomatid [15] and also the LBV, which was found in *Leishmania martiniquensis*, a human pathogen producing a symptomatic range from severe visceral disease to asymptomatic infections, belonging to the subgenus *Leishmania* (*Mundinia*). The virus was denominated LmarLBV1 and represents the only non-LRV virus infecting a *Leishmania* species so far [16]. The subgenus *L.* (*Mundinia*) has been established recently and remains understudied. It is composed by newly emerging, human-infecting *Leishmania* species as well as non-human pathogens, distributed worldwide. It has been assumed that this subgenus represents the earliest diverging branch within *Leishmania*, possibly transmitted by a different vector [17].

3. Exploiting characteristics of *Leishmania*-infecting virus

Leishmaniavirus species, LRV1 and LRV2, were associated, respectively, with the *Leishmania* (*Viannia*), found exclusively in the American Continent, and to the Old World *Leishmania* (*Leishmania*) species [18–22]. LBV, found initially in monoxenous trypanosomatids belonging to the subfamily Leishmaniinae as well as in the dixenous plant-parasitizing *Phytomonas* spp. [15], has also been detected in *Leishmania martiniquensis* [16] and possibly *Trypanosoma* spp. [15].

The Totiviridae family encompasses a broad range of viruses characterized by isometric virions, ranging from 30 to 40 nm in diameter, each containing a non-segmented double-strand RNA (dsRNA) genome with overlapping open reading frames encoding (ORFs) a capsid protein (CP) and a RNA-dependent RNA polymerase (RdRp) [23]. LRV is a member of this family presenting around 5.3 kb RNA [24,25]. The classification of LRV in the Totiviridae was due to its replication characteristics [26]. The low level of similarity (less than 40%) detected by comparing the nucleotide sequences from *L.* (*Viannia*) and *L. major* viruses enable to classify them into two different species, LRV1 and LRV2. A variation in the arrangement of the gene sequences is also observed between LRV1 and LRV2 [21,27]. LRV1 has an overlap

between the regions encoding the viral capsid protein and the RNA polymerase, a particularity not observed for LRV2.

Leishbunyavirus belongs to the order Bunyavirales and is characterized as a virus exhibiting a negative-sense single-stranded RNA [28], organized in three genomic segments. The large segment encodes a viral RdRp, a surface glycoprotein precursor is encoded in the medium segment and a nucleoprotein is encoded by the small segment [29]. Virions are usually 90 to 100 nm in diameter. The medium or the small segments might present other ORFs involved in counteracting the host antiviral response, which may be present in both segments [30,31]. Infectivity and formation of viral particles in bunyaviruses depend on glycoproteins, type I transmembrane proteins that are proteolytically processed and glycosylated in the endoplasmic reticulum [31]. LmarLBV1 is a Bunyavirus and is the first non-LRV described infecting *Leishmania* [15,32].

Like other viruses, LRV and LBV need the resources of eukaryotic cells to sustain their metabolism. Furthermore, dsRNA molecules are not produced by eukaryotic hosts, which have several mechanisms for detecting and inactivating these molecules. The dsRNA viruses replicate within the capsid. Thus, the dsRNA genome is never exposed in the cytoplasm, which is an essential mechanism for evading the host cell's activation and antiviral action. Transcription of the dsRNA genome by RdRp takes place within the virus. The positive-strand acts as messenger RNA (mRNA), giving rise to new viral particles, while the negative strand serves as a template for mRNA transcription [33].

4. A brief history on detection and dispersion of LRV1, LRV2 and LBV

LRV1 from the reference strain for *L. guyanensis* (MHOM/M4147) represents the first virus from kinetoplastids characterized by molecular approaches. A few years later, the first study was conducted screening the presence of LRV in *Leishmania* spp. strains from different geographical areas. Based on hybridization analysis, twelve LRV1 types – LRV1-1 to LRV1-12 – were defined, and it was shown for the first time that LRV1 could infect *L. braziliensis*, *L. guyanensis* and various *Leishmania* strains from the Amazon Basin [34]. Comparative cDNA sequence analysis of LRV1-1 and LRV1-4 showed 77% of identity, corroborating differences previously observed between these two types [27]. Furthermore, the comparison of two genomic regions

from seven LRV types lead to the description of two new types, LRV1-13 and LRV1-14, detected in *L. braziliensis* strains isolated from human patients from Bolivia [35].

In the early 1990s, parallel to the detection of LRV1 in two *L.* (*Viannia*) species, addressed the discussion on whether the geographic distribution of *L.* (*Viannia*) spp. bearing LRV1 could be restricted to the Amazon Basin [34], despite a widespread circulation of *L. braziliensis* in the American continent. Later on, two other studies evaluated LRV1 in *L. braziliensis* from clinical samples and in *L. braziliensis* strains from Southeastern Brazil. All were negative [36,37] supporting the hypothesis of a restricted circulation to the Amazon Basin of *Leishmania* spp. bearing LRV1. Such findings exclude the possibility of a rigorous association between the presence of LRV1 and the severity of tegumentary leishmaniasis, since there are also severe leishmaniasis cases outside the Amazon Basin [20].

Recently, additional *L.* (*Viannia*) species were reported as infected by LRV1. Positive LRV1 samples were detected in tegumentary lesions from patients living in the western Brazilian Amazon region and infected by *L. (V.) lainsoni* and *L.* (*V.*) *shawi* living in the western Brazilian Amazon region [20]. Later on, it was demonstrated and characterized LRV1 in the reference strain of *L. shawi* (MCEB/BR/1984/M8408), a strain isolated from monkey [38] and in the present study we report a human strain from the Amazonas state presenting LRV1 (Table 1). A *L. naiffi* strain from the Amazonas state in Brazil was also reported as positive for LRV1 [39]. Here, we screened LRV1 in a set of 17 *L. naiffi* strains deposited at the *Leishmania* Collection of the Fundação Oswaldo Cruz (CLIOC) using the protocol described by Cantanhêde et al. [20,38] detecting 11 (64.7%) LRV1-positive strains (Table 1). We also screened LRV1 in *L.* (*V.*) *lainsoni* strains, considering that our previous study detected LRV1 in clinical samples from patients infected by this species. The screening confirmed this species as host for LRV1 (Table 1). All results aforementioned corroborated the assumption that LRV1 is restricted to *Leishmania* strains circulating in the Amazon Basin. However, we cannot rule out that the apparent narrow geographical distribution of LRV1 might be a result of biased survey. Studying *Leishmania* spp. from Costa Rica we detected *L.* (*V.*) *guyanensis* strain positive for LRV1, reinforcing a recent finding indicating the circulation of LRV1 in this area [40]. Table 1 resumes the LRV positive samples.

Table 1 – Strains of L. (Viannia) positive for LRV1

ID	*Leishmania* Strain ID	Parasite Specie	*Leishmania* International Code	Geographic Origin
1	IOCL 3316	*Leishmania naiffi*	MHOM/BR/2011/58-AMS	Pará/BR
2	IOCL 3515	*Leishmania naiffi*	MHOM/BR/2013/49UAS	Amazonas/BR
3	IOCL 3516	*Leishmania naiffi*	MHOM/BR/2013/63DDL	Amazonas/BR
4	IOCL 3517	*Leishmania naiffi*	MHOM/BR/2013/65HCC	Amazonas/BR
5	IOCL 3518	*Leishmania naiffi*	MHOM/BR/2013/66CPS	Amazonas/BR
6	IOCL 3519	*Leishmania naiffi*	MHOM/BR/2013/51FRS	Amazonas/BR
7	IOCL 3520	*Leishmania naiffi*	MHOM/BR/2013/62FJFM	Amazonas/BR
8	IOCL 3523	*Leishmania naiffi*	MHOM/BR/2013/25EPF	Amazonas/BR
9	IOCL 3524	*Leishmania naiffi*	MHOM/BR/2013/45JOM	Amazonas/BR
10	IOCL 3525	*Leishmania naiffi*	MHOM/BR/2013/56EGP	Amazonas/BR
11	IOCL 3574	*Leishmania naiffi*	MHOM/BR/2015/352 FMS	Amazonas/BR
12	IOCL 1545	*Leishmania shawi*	MCEB/BR/1984/M8408	Pará/BR
13	IOCL 3481	*Leishmania shawi*	MHOM/BR/2013/18 LTA MLF	Amazonas/BR
14	IOCL 3398	*Leishmania lainsoni*	MHOM/BR/2012/AP60A	Rondônia/BR
15	IOCL 3804	*Leishmania guyanensis*	MHOM/CR/2019/108-GML	Costa Rica

In 1993 a virus was identified in an Old World *Leishmania* species, *L. major*, and was designed as LRV2-1. It was described as immunologically distinct when compared to LRV1-1 and LRV1-4 [11]. The complete sequence of the virus, found in *L. major* promastigotes MHOM/SU/1973/5-ASKH, was published two years later, and it showed that the most relevant characteristic distinguishing the genomic structure of LRV2 from LRV1 and other Totiviruses is the non-overlapping of capsid and RdRp genes [21].

LRV2 was detected in *L. major* [21], *L.* (*L.*) *infantum* [22], *L.* (*L.*) *aethiopica* [18,41], and *L.* (*L.*) *tropica* [42]. Two studies conducted in Iran, in a zoonotic focus of cutaneous (CL) and including visceral leishmaniasis (VL) patients, reported that the virus was detected in two different parasite specimens: one *L. infantum* strain derived from a VL patient unresponsive to treatment using meglumine antimoniate, and one *L. major* strain from a great gerbil, *Rhombomys opimus* [22]. More recently, a survey was conducted in isolated promastigotes from 85 CL human patients from Iran. Eighty-three were identified as *L. major* and 2 as *L. tropica*. Fifty-nine (69.4%) presented LRV2 and one out of the two *L. tropica* isolates was also positive for LRV2 [42]. *L. tropica* was

first demonstrated infected by LRV2 in a survey conducted in Turkey, in which 7 LRV2 positives out of 24 *L. tropica* strains were identified [43].

Lately it was described in three (out of 3 examined) *L. major* strains in Turkey [43] and in two *L. major* stains isolated from CL patients from Uzbekistan. Sequence analysis indicated a high similarity among the two LRV2 from Uzbekistan, which were closely related to the LRV2 found in the *L. major* strains ASKH documented in Turkmenistan [21,41]. Thus, the presence of LRV2 in *L. major* is possibly frequent and widespread.

Recently, a study demonstrated for the first time *L.* (*M.*) *martiniquensis* infected by endosymbiotic virus, a Leishbunyavirus. The molecular characterization showed a genomic arrangement with three segments and sequences similar to Leishbunyavirus, which was first described infecting the monoxenous *Crithidia* spp., a trypanosomatid member of the subfamily Leishmaniinae. But from the best of our knowledge, the work published by Grybchuk and colleagues in 2018 [15] presented the most comprehensive study on Leishbunyavirus. Summarizing what was shown, Leishbunyavirus so far represent the most widespread and species-rich group of RNA viruses from trypanosomatids. This virus was found in *Crithidia* spp. from Ecuador, Ghana and Russia, and *Leptomonas moramango* from Madagascar, monoxenous trypanosomatid strains isolated from different hosts. Furthermore, using metatranscriptomic data from dipterans and horse leech for viral and trypanosomatids surveys they proposed this group of viruses associated to the subfamily Strigomonadinae and also to *Trypanosoma* spp.

Back to *Leishmania*, it is interesting that a so geographically dispersed and multiple-host virus was detected in a *L.* (*Mundinia*) species, the earliest branch within the genus *Leishmania*, probably originated before Gondwana' breakup [44,45]. Another interesting feature of this group of parasites is concerned to its geographical dispersion and the diversity of vertebrates incriminated as hosts, including humans. Not less interesting, this group of parasites are probably not transmitted by sandflies. Comparative genomic analysis show interesting differences in *L. (Mundinia)* from other *Leishmania* species [17].

5. LRV and Leishbunyaviruses modulating *Leishmania* spp. phenotype

Virus-infected *Leishmania* spp., either with Leishbunaviridae or Totiviridae viruses, show altered phenotypic expressions. Several studies have been proposed to

understand this impact, mainly involving the LRV1 endosymbiont on the biology of different *L*. (*Viannia*) strains. The reason for that might be the enigmatic pathophysiology of CL, and the intriguing hypothesis that LRV confers either a state of hypovirulence or hypervirulence on the host-parasite interaction [46].

Several groups have speculated on the influence of LRV1 on the parasite´s virulence, and years have passed without major studies on the biological impact of LVR1 on *Leishmania* parasites [47]. The concern with LRV1 as a determinant of parasitic virulence reappeared in a 2011 study by Ives and colleagues using clones of *L*. *guyanensis* clinical isolates. Samples were classified due to their tendency to metastasize, ranging from highly metastatic (M+) to non-metastatic (M-), using hamsters as the animal model. Authors found that the mucosal-lesions-associated clone *L*. *guyanensis*, carring the virus (LgM+), increased the endogenous immune response in an unregulated manner, promoting an increase in inflammatory cytokines. These clones resulted in a phenotype of severe destruction of the nasopharyngeal mucosa when inoculated in mice, despite the significant reduction in the number of parasites. Macrophages infected with a virus showed a phenotype similar to macrophages infected with parasites (LgM+), with increased expression of chemokines and cytokines such as CXCL10, CCL5, Tumor Necrosis Factor-alpha (TNF-α), and interleukin 6 (IL-6) also demonstrating that LRV1 alone, induced the intensification of the inflammatory response to *Leishmania* antigens [12].

Thereafter, several studies explored the participation of LRV in the clinical evolution of the disease. Our group demonstrated that the relative risk of developing mucosal lesions in patients with TL and LRV1 was three times higher compared to patients infected with parasites without LRV1 [20]. Moreover, the presence of LRV1 was associated with therapeutic failure cases in patients infected by *L*. *guyanensis* [48] and in patients infected with *L*. *braziliensis* [49]. However, other reports did not correlated LRV with distinct clinical phenotypes of TL [36,46] neither to treatment failure [50,51].

Assuming a mutualistic relationship between LRV and *Leishmania* spp. it is expected that *Leishmania* harboring LRV1 could display a better performance and fitness than virus-free strains facing certain environmental challenge. Routine evaluation of cultures maintained at CLIOC indicates two patterns of growth among *L*. *guyanensis* strains. These observations led to an experimental analysis of the growth rate between two *L*. *guyanensis* strains, one positive (IOCL 3357; LgLRV1+) and one

negative (IOCL 3521; LgLRV1-) for LRV1, using the protocol previously described [20,38]. It was observed that LgLRV1+ survived longer and despite the environmental stress faced by parasites during *in vitro* cultivation, maintaining viable parasites even in a nutrient-depleted environment, without medium replacement [52]. The *L. guyanensis* LRV1 negative strain showed a similar profile to that of the LgLRV1+ until the 7th day of culture, although with a greater number of parasites on the 5th and 6th days of culture. However, on the 7th day, the number of parasites declined, and after the 9th day, no viable parasites were detected (Figure 1A). A study that evaluated the reference strain for *L. guyanensis* (MHOM/BR/1975/M4147), LRV1+ also detects viable parasites until the end of the monitoring of the culture [53].

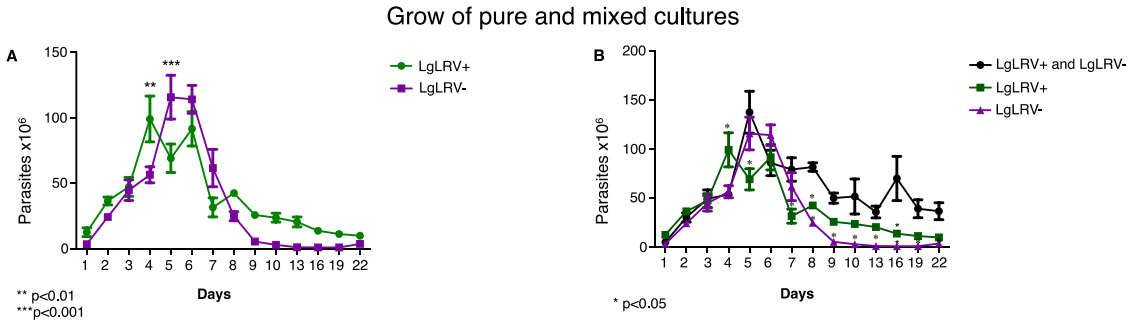

Figure 1. Growth curve of *Leishmania guyanensis* strains infected or not with the *Leishmania* RNA Virus in single or mixed culture. *L. guyanensis* strains were cultivated in Schneider´s medium (Vitrocell Embriolife, Campinas, SP, BR) supplemented with 20% fetal bovine serum (FBS) (Vitrocell Embriolife, Campinas, SP, BR), starting with $1x10^6$ parasites/ml for single cultures or $0.5x10^6$ parasites/ml of each for mixed cultures. Strains employed: *L. guyanensis* LRV1 positive (LgLRV+) - MHOM/BR/2011/S83 and *L. guyanensis* LRV negative (LgLRV-) - MHOM/BR/2013/57FRMS.

Studies have reported data of LRV+ and LRV- parasites under the same environment, e.g., growing in the same culture medium [12,54]. We determined the growth curve of a mixed culture containing LgLRV1+ and LgLRV1-, starting the culture with 0.5×10^6 parasites/ml of each strain (Figure 1B). The individual culture of LgLRV1- strain showed no viable parasite at day 9 of culture. In the mixed LgLRV1-/LgLRV1+ culture, however, the number of viable parasites in culture at day 9 was similar to that observed in the single culture for the LgLRV1+ strain (Figure 1A) suggesting either i) the counted parasites corresponded strictly to LgLRV+ cells or ii) cocultivation enhances LVR- parasites' ability to survive.

Mixed cultures with positive and negative LRV1 parasites were already reported, although apparently always with less negative than positive parasites [55], suggesting that few LgLRV1- parasites may remain viable for a long time when co-

cultivated with LgLRV1+ parasites. It is plausible though, that *Leishmania* spp., as well as described in *T. brucei* [56], synthesize and secrete compounds in the shared environment, affecting population density and the parasites' behavior, measured, for example, by growth rate in culture. It is possible that in addition to mechanisms such as cell-cell contact and secretion factors, the exosome secretion, recently demonstrated for LRV1+ parasites, also contributes to this interaction [57–59].

Studies using mice infected by *L. guyanensis* LRV1+ demonstrated a higher parasite burden in lesions produced by these parasites than those produced by *L. guyanensis* LRV1- [12,60,61]. The immunization of mice with a vaccine produced from the LRV1 viral capsid protein decreases the burden of parasites in lesions after a new infection with *L. guyanensis* LRV1+ [62]. We analyzed samples from 83 TL patients, evaluating the parasite burden and the presence of LRV1, using methodologies that we have described in previous studies [20,63]. We found a high frequency of samples with a high parasite load and positive for LRV1 (Figure 2), confirming an association between LRV1 presence and parasite load ($p = 0.001603$). No association, however, was observed between viral and parasite load. This corroborates data showing occurrence in the same patient, of parasites with and without LRV and with a variable number of turning particles *per* cell of the parasite [54]. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Centro de Pesquisa em Medicina Tropical/CEPEM (CAAE: 54386716.1.0000.0011).

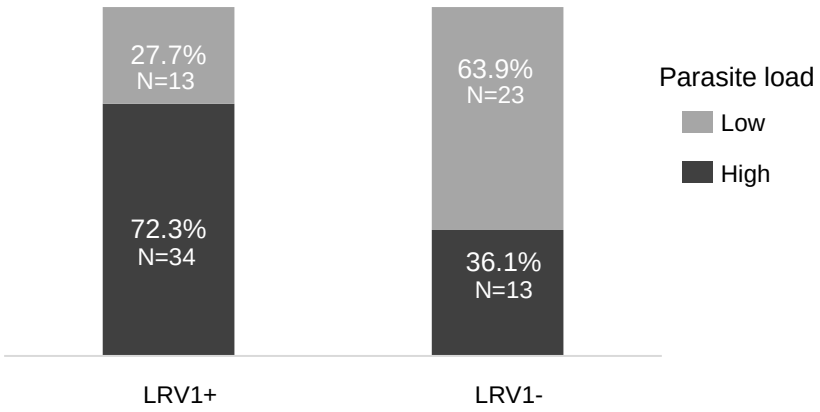

Figure 2. Relative frequencies of the parasite load group and LRV1 occurrence in samples from TL patients. The quantification of the parasite load and the detection of LRV1 were performed as previously described [20,63]. LRV1+ = positive samples for LRV1. LRV1- = negative samples for LRV1.

At the same set of samples mentioned above, we screened the expression levels of 22 *Leishmania* genes (Table S1), grouping the samples accordingly to the presence of LRV1. The Heat Shock Protein 60 - HSP60 gene was preferentially expressed in the LRV1+ group, whereas the Trypanothione reductase - TRYR gene was preferentially expressed in the LRV1- group (Figure 3). The methodology for analyzing Real-time PCR data is available in the supplementary material.

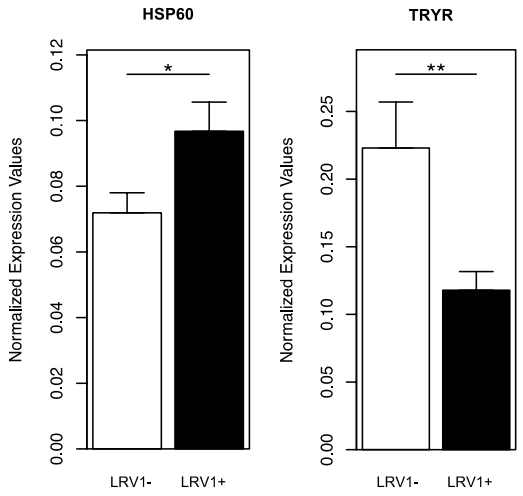

Figure 3. Differential *Leishmania* genes expressions between LRV1 positive (LRV1+) and LRV1 negative (LRV1-) samples groups. Gene expression assays were performed on the Fluidigm® platform (Biomark™ HD, San Francisco, USA) - Gene Expression 96.96 IFC, using Fast TaqMan Assays. The collection of clinical samples and the processing to obtain cDNA were performed as previously described [20]. For the gene expression assay, the protocols indicated by the manufacturer were followed. HSP60 - Heat-Shock Protein 60 gene. TRYR - Trypanothione reductase gene. Fluorescence accumulation curves of Rn intensity were used for relative quantification of the gene expression by the second derivative method. ACTIN and S8 genes were used for normalization. Hypothesis tests were performed by Non-parametric T-tests with 10,000 permutations. * = P-value < 0.05 and ** = P-value < 0.001.

The HSP60 gene encodes the heat shock protein 60 and is expressed in greater quantity in the mitochondria [64]. This chaperonin expression is influenced by stressful conditions, where the oxidative challenge generated by host's immune system and also that imposed by anti-leishmanial drugs are good examples [65]. In the first case, its production could be conditioned to an effective immune response and its increase could avoid the parasite's proteins to be denatured by oxidative stress generated by the parasite proliferation, with a consequent increase in HSP60 [66]. The LRV1+ group exhibited both, an increased expression of the HSP60 and a higher parasite load in LRV+. It is possible that higher HSP60 expression favors parasite's survival, although the direct causal effect of such association needs further studies. Alternatively, it is plausible that the increase in HSP60 expression could represent a consequence to the stress caused by the presence of LRV1, which ultimately enhances parasite tolerance to different stressful conditions, leading to a high parasite load at the lesion site.

The analysis of cutaneous lesions samples assuming LRV1 negative and positive groups, regardless the parasite load, a significant higher expression in the LRV- group for 4 *Leishmania* genes was observed: Gamma-glutamylcysteine synthase - GSH, Mitosis activating protein kinase - MAPK, Peroxiredoxin - PRX and Trypanothione reductase - TRYR putative genes. On the other hand, while the Thiolase-like gene - THIOL was preferentially expressed in the LRV+ group (Figure 4). MAPK negative regulation was linked to therapeutic failure in *L. donovani* infection [67], and cases of therapeutic failure have been associated with the presence of LRV1 in patients infected by *L. braziliensis* [49] and by *L. guyanensis* [48]. Since the clinical follow-up of the patients was not possible, the association with treatment outcome could not be conducted. Furthermore, the increase in THIOL expression was observed in the LRV1 + group, which is composed mainly by samples with a high parasitic load. Thiolase are relevant in lipids metabolism an important carbon source of intracellular forms [68]. Such observations suggest that LRV1 infection could promote upregulation of THIOL gene and downregulation of MAPK which would contribute to the parasite proliferation and persistence.

Both GSH and TRYR were overexpressed in the LRV1- group, with a value of $p \leq 0.01$, indicating an agonism of these molecules. GSH encodes Gamma-glutamylcysteine synthase and has the function of glutathione and trypanothione biosynthesis and TRYR guarantees reduced-trypanothione turn over [69,70], being important to parasite's survival under the oxidative stresses from the host cell [71,72]. However, the association between increased levels of expression among antioxidative defense related targets and LRV- samples was intriguing. Possibly reflecting the complexity within mechanisms related to stress defense that can be also shaped by LRV infection.

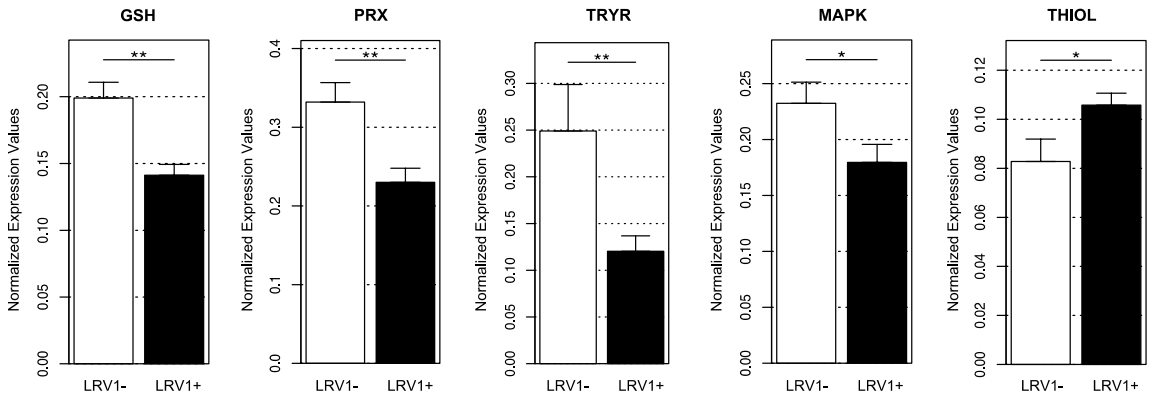

Figure 4. Differential *Leishmania* genes expressions in cutaneous lesions samples. Samples were grouped considering LRV1 detection: LRV1 positive (LRV1+) and LRV1 negative (LRV1-). The protocols used were the same as those described in Figure 3. GSH= Gamma-glutamylcysteine synthase gene, PRX= Peroxiredoxin gene, TRYR= Trypanothione reductase gene, MAPK= Mitogen activated protein kinase, THIOL= Thiolase protein-like protein gene. Fluorescence accumulation curves of Rn intensity were used for relative quantification of the gene expression by the second derivative method. ACTIN and S8 genes were used for normalization. Hypothesis tests were performed by Non-parametric T-tests with 10,000 permutations. * = P-value < 0.05 and ** = P-value < 0.001.

LRV is found in both stages of the *Leishmania* life cycle, the promastigotes and the intracellular amastigotes [8,9,73]. But, despite several studies exploring the effect of LRV in the leishmaniasis pathogenesis, it is still unclear if the virus-effect is either the response of the vertebrate host to the viral infection or if the virus affects its own host - the *Leishmania* spp. - biology [47,74]. A recent study evaluated the effect of LRV1 on the pathogenesis of TL using an isogenic, high viral load clone of *L. guyanensis* LRV- (from the M4147 strain). Doing so, it was possible to evaluate the effect of the virus in inducing the innate immune response. This study deciphered the mechanism by which LRV1 promotes parasitic persistence and disease progression and showed that this occurred from the limited activation of inflammasome in macrophages. Such effect of LRV1 in modulating the immune response has also been demonstrated in human samples and was associated with mucosal leishmaniasis [75]. Also, as already mentioned, the presence of LRV1 as well as the viral load were pointed out as crucial factors in the severity and pathology of the disease [54]. A question remains, however, regarding the participation of LRV1 in modulating the immune response: It has been shown that the virus can be transported via exosomes [58], but at what point of infection is the LRV1 exposed to the host cell, signaling the cascade that leads to the most severe phenotype of the disease?

Like LRV1, LRV2 present in *L. aethiopica* strains isolated from humans (LRV2-Lae) showed potential in modulating the immune response in macrophages,

resulting in a hyper-inflammatory and TLR3-dependent response [18]. In *Leishmania tropica*, LRV2 was detected in about 30% of the strains analyzed [43]. *L. tropica* is an important etiological agent of cutaneous leishmaniasis in the Old World, and there are several reports of this species in cases of mucosal leishmaniasis [76–79].

In Ethiopia, as well as in Brazil, a portion of patients with cutaneous lesions commonly progress to severe forms of the disease, such as mucosal leishmaniasis [80]. The presence of LRV was associated, in those cases, with the development of the mucosal phenotype.

Despite the common influence of both LRVs types exert on the immune response, other characteristics were not shared between them. For example, the LRV2 present in *L. major* isolates did not affect the therapeutic response [50], as already reported in infections by *L. guyanensis* and *L. braziliensis* LRV1+ [48,49]. However, a report of *Leishmania infantum* harboring LRV2 describe a patient with visceral leishmaniasis who had not responded to three cycles of systemic treatment. Therefore, not enough evidence is available to associate the presence of the LRV2 with clinical phenotypes in VL caused by *L. infantum* [22].

The Leishbunyavirus detected in *L. martiniquensis* (LmarLBV1) seems also to influence the parasite pathogenicity. Using an isogenic clone of *L. martiniquensis* without LBV (LmarLBV1-depleted) the influence of the virus on the biology of the parasite was evaluated, specifically on its ability to infect murine macrophages. The results showed that the LmarLBV1-depleted strain was less infective compared to the LmarLBV1 strain, indicating the LmarLBV1 facilitates parasites' infectivity in vitro in the primary murine macrophages model [16].

6. The maze pathway of coevolution of *Leishmania* spp. and its infecting viruses

It is not fully known yet how *Leishmania* viruses were maintained and transmitted to *Leishmania* parasites. The most common mechanism for viral transmission in the Totiviridae family is possibly the vertical and/or horizontal (by cell fusion) propagation [81]. Infection of non-LRV1 infected *Leishmania* parasites failed or was transitory when electroporation was attempted [82]. Mature viral particles of LRV could be transmitted to new parasites by cell division [11,33] or via exosomes [58]. More than 30% of exosomes produced by a *L. guyanensis* strain carry viral particles, and inside exosomes LRV1 is able to resist unhospitable conditions until exosomes-enveloped LRV1 infect other parasites [58]. Extracellular transmission of

Totivirus in some protozoan parasites like *Giardia lamblia* [83] and in *L. guyanensis* via exosomes [58] has been documented. Although this transmission is probably rare, considering that virus-infected and non-infected parasites are still observed in the same culture [55]. It could not be rulled out that some parasites are resistant to the virus infection, a hyphothesis to be tested.

The lack of a detectable infectious phase of LRV suggest a long-lasting relationship between the virus and the parasites, representing a symbiotic association. Indeed, studies showed similar genetic intervals between the *Leishmania* species and LRV1 and LRV2 respectively sheltered [21,35]. Phylogenetic findings suggested that LRV acquistion by *Leishmania* parasites was prior to the divergence of Old and New World *Leishmania* parasites [35], but its interaction with the Trypanosomatid family is ancient as indicated by the finding of LRV in Blechomonas [15].

Viral particles, LBV and LRV were found in *Leishmania* species as well as in their closest phylogenetic clades *Endotrypanum* spp. and *Paraleishmania* spp. Loss and the acquisition of both LBV and LRV probably occurred early in the family Trypanosomatidae, but additional research with different specimens from this family is necessary to make a proper inference for this hypothesis. Considering the knowledge gathered so far, the relationship of LBV and members of the Trypanosomatidae is older than that observed for LRV. LBV appears in *Trypanossoma* spp., whether *Blechomonas* is the first genus of the family harboring LRV. Leishbunyavirus was detected in several members of the Trypanosomatidae family [15]. Although LRV, more specifically LRV3 and LRV4, was observed in *Blechomonas*, prior to the moment when Leishmaniinae split from other trypanosomatids, this virus emerged again in the *Leishmania* spp. branch. This could have coincided with the point in time when the dixenous life cycle emerged in Leishmaniinae, which could be supported by identification of VLPs in *Paraleishmania* and *Endotrypanum* [2] as LRV, although a characterization of these particles is still required. Another possibility which can be assumed is the re-emergence of LRV before the time of *L.* (*Viannia*) and *L.* (*Leishmania*) diversification, considering VLPs found in *Paraleishmania* and *Endotrypanum* as non-LRV. Comparative analyses of the *Leishmania* tree, based on RAPD, and the LRV trees, obtained by sequence analysis of either ORF3 or 5'-UTR, supported a long history of coevolution between LRV and the parasite-host strains, sustaining the hypothesis that LRV is an ancient virus *Leishmania* spp. [35] and probably has spread following its host diversification (Figure 5).

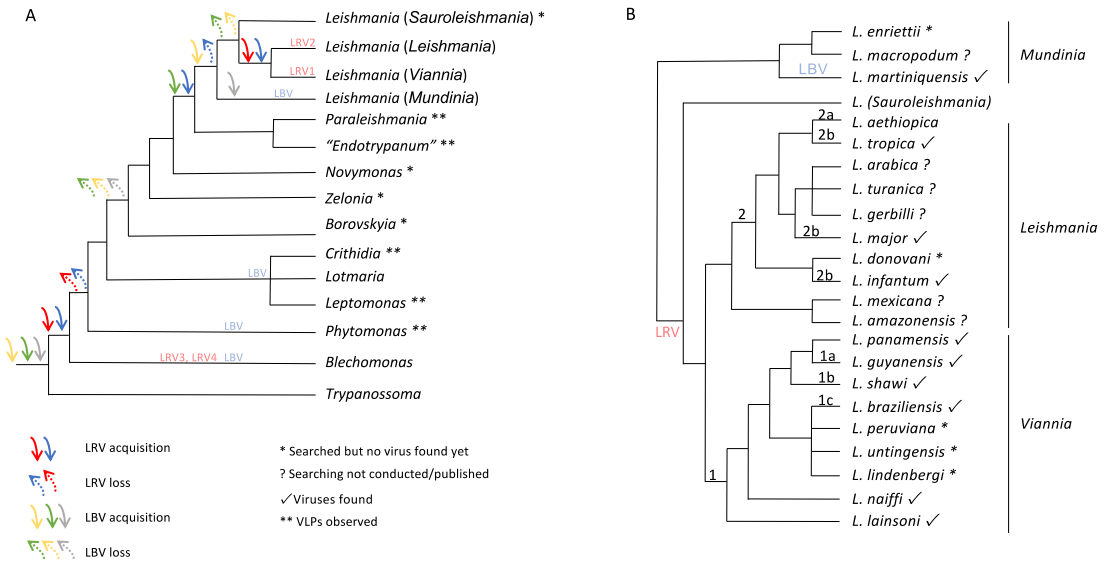

Figure 5. Schematic phylogenetic tree for the Family Trypanosomatidae (A) and the genus *Leishmania* (B) based on published data [2,3,15] showing possibilities of LBV and LRV acquisition by members of the family Trypanosomatidae and LRV dispersion across *Leishmania* species. Three scenarios are expected for LBV (green, yellow and grey arrows): Green - ancient acquisition, with possible loss (dashed green arrow) in the first Leishmaniinae split and new acquisition in the clade containing *Leishmania*, Paraleishmania and "Endotrypanum", followed by loss in the split of *Leishmania* (*Mundinia*) from the other three *Leishmania* subgenera. This scenario assumes LBV not infecting Novymonas, Zelonia and Borovskyia, and VLPs found in the clade Paraleishmania and "Endotrypanum" as LBV; Yellow - The same as green, but with the last acquisition happening in the split of *L.* (*Mundinia*) from the other *Leishmania* subgenera and subsequent loss in member of the other three *Leishmania* subgenera, assuming VLPs found in the clade Paraleishmania and "Endotrypanum" not as LBV; Gray - ancient with possible loss (dashed gray arrow) in the first Leishmaniinae split and new acquisition when *L.* (*Mundinia*) split from the other *Leishmania* subgenera. Scenarios expected for LRV: Blue - acquisition by a monoxenous trypanosomatid followed by sequential loss when another dixenous clade appear, and acquisition in the clade containing *Leishmania*, Paraleishmania and "Endotrypanum", followed by loss when *L.* (*Mundinia*) Split from the other *Leishmania* subgenera and a new acquisition by the clade *L.* (*Viannia*)/*L.* (*Leishmania*). This scenario assumes VLPs found in the clade Paraleishmania and "Endotrypanum" as LRV and the possibility of LRV infecting all *Leishmania* subgenera; Red - acquisition by a monoxenous trypanosomatid followed by sequential loss when another dixenous clade appear and a new acquisition by the clade *L.* (*Viannia*)/*L.* (*Leishmania*).

Phylogenetic studies have shown that the transition from a monoxenous to a dixenous state occurred at least three times in the family Trypanosomatidae, giving rise to parasites of vertebrates, like the *Trypanosoma* and *Leishmania* genus, and also to *Phytomonas*, a dixenous phytopathogenic genus. Therefore, monoxenous parasites of invertebrates were ancestors of dixenous pathogens [84]. Considering the phylogenetic

reconstruction of viruses found in many trypanosomatid using RdRp sequences, a well-supported clade for LBV was observed closely related to Phenuiviridae [15], a family including many viruses from insects, including the genus *Phlebovirus* which is transmitted by sandfly species - the *Leishmania* vectors [85].

Assuming monophyly in the *Leishmania* clade, and including also their sister clades *Endotrypanum* spp. and *Paraleishmania* spp., two different points in time appear when the inclusion of these viruses could have happened: first for the LBV in the subgenus *L*. (*Mundinia*) and then for the LRV, in *L*. (*Viannia*) and *L*. (*Leishmania*) with a latter diversification in LRV1 and LRV2 at the same time that these two *Leishmania* subgenera split [18,35]. The challenge now is to uncover the points when gain and loss of the viruses appear in the process of diversification of the trypanosomatid taxa. Different strains from the same taxon can be found infected and non-infected by a specific virus, but it is still unknown if the virus infection is an ancestral character or a derived one. The common ancestor for the *Leishmania* clade and their sister clades *Endotrypanum* spp. and *Paraleishmania* spp. could be virus-free and then independent viral acquisitions occurred. Different routes of both LBV and LRV acquisition and loss are possible in this protozoan group considering data gathered so far (Figure 5).

Alternatively, virus loss might have occurred independently and randomly. For strains from the same species is plausible that a given strain, or its ancestor, was infected and during binary division the virus was not equally transferred, resulting in both, infected and non-infected descendants. That hypothesis also explains the observation of virus-infected and non-infected parasites in the same culture. To explore such alternative, we will take LRV1 and *L*. (*Viannia*) as example. LRV1 was detected in most of the *L*. (*Viannia*) species: *L. guyanensis*, *L. braziliensis*, *L. shawi*, *L. naiffi*, *L. lainsoni* and *L. panamensis*. Sequence analysis ofLRV1 from *L. braziliensis*, *L. guyanensis* and *L. shawi* showed clusters gathering accordingly to the *Leishmania* species (Figure 5); the solely LRV1 sequence analyzed from *L. shawi* was placed among two LRV1 from *L. guyanensis* [38,55]. Curiously, the similarity between *L. shawi* and *L. guyanensis* was reported in many studies [86–88] and is also detected when LRV1-*L. guyanensis* and LRV1-*L. shawi* sequences are analyzed [38]. Microsatellite analysis of *L*. (*Viannia*) spp. indicated *L. guyanensis* is a distinct population within *L. (Viannia*) subgenus (by microsatellite analysis), with no distinguishable subpopulations. However, differences in the reactivity profile with monoclonal antibodies were detected, overlapping the geographical distribution of the

strains [89,90] and correlating with clusters formed after the LRV1 *L. guyanensis* sequences analysis [38].

The case of *L. braziliensis* is especially interesting as this species is widespread in the American Continent, but so far LRV1 was detected only in strains isolated from the Amazon region. By microsatellites analyses, LRV(-) *L. braziliensis* strains belong to a distinct population from those LRV1-infected *L. braziliensis* [91,92]. The intra-group diversity detected by the *L. braziliensis*-LRV1 sequences analysis is as high as the heterogeneity reported for this parasite species [93–95]. Two LRV1 clusters were demonstrated, corresponding to *L. braziliensis* from the western Amazon region, (one from Bolivia and one from Brazil); a *L. braziliensis*-LRV1 sequence from French Guyana was put in the middle, but with lower bootstrap support [38].

For *L. guyanensis*, *L. braziliensis*, other *L.* (*Viannia*) spp. and also species infected by LRV2, infected and non-infected parasite cells are detected within the same strain. The same occurs for strains from the same regions. This assortment might be a significant determinant of coevolution [96] assuming the degree of mixing, virus-free and virus-infected *Leishmania* spp. would increase *Leishmania* spp. exposure to viruses, therefore selecting for greater resistance and infectivity intervals. The characteristics of *Leishmania* and LRV could influence the probability of fluctuation in the direction of natural selection for a given phenotype over an evolutionary period of time (Fluctuating Selection Dynamics - FSD). Furthermore, it could also be possible that *Leishmania* and their viruses are in combat, causing both to select adaptive characteristics, leading them to coevolve (Arm Race Dynamics - ARD). The shift from FSD to ARD associated to population mixing is a possibility to be acknowledge [96]. Considering the infection by LRV in *Leishmania* species since the diversification of the subfamily Leishmaniinae, ARD could explain the lack of LRV- infected *L. braziliensis* outside of the Amazon Basin. If is the case, *L. braziliensis* and LRV1 differently developed resistance and infectivity (or strategies of infection), respectively. The raised hypothesis assumed the existence of *L. braziliensis* populations resistant to LRV infection. The methodology used to describe LRV transmission via exosomes [58] to uninfected *L. guyanensis* could be applied to test such assumption.

It seems that *L. braziliensis* parasites without LRV1 have been better adapted to the conditions encountered, especially in relation to the phlebotomine species, indicating that a bottleneck phenomenon occurred during the spread of *L. braziliensis*. Considering microsatellite analyses, there is one *L.* (*Viannia*) population in the Amazon

region comprising *L. braziliensis* strains and other *L.* (*Viannia*) species - *L. guyanensis* excluded. This diverse population is organized in subpopulations which match species identity [92]. In previous studies [20,34,38,55,97] and corroborated by the presented data LRV1 infection was described in many *L.* (*Viannia*) species. Remains unsolved, however, whether the LRV1 from these species are related to *L. braziliensis* LRV1. To address many of the points raised it is important to conduct phylogenetic studies of LRV1, LRV2, LBV and their hosts. It is noteworthy that the phylogenetic trees for LRV [35] and for LBV [15] must display congruent with those obtained for their hosts, suggesting coevolution and limitation to horizontal viral transmission.

7. Concluding Remarks

The hypothesis that parasites influence the quantity or geographical dispersion of their host is opposed by a more acceptable hypothesis arguing that successful or well-adapted parasites evolve to be harmless to their host. Although virus-like particles and viruses were first detected in *Leishmania* parasites some decades ago, the impact of this interaction and the diversity of these endosymbionts has recently drawn considerable attention, mainly by the virulence trade-off experimentally demonstrated in the context of *Leishmania* (*Viannia*) *guyanensis* and LRV1. However, theory regarding the evolution of interactions among different endosymbiotic viruses and *Leishmania* spp. is still in its emerging stages. Recent studies showed the discovery of several viruses in trypanosomatids, indicating the existence of an unknow viral diversity, which needs to be further investigated and which can bring us important evolutionary information. At least two viruses' families were already described as *Leishmania* spp. endosymbionts, but we still don't know if these viruses occur only in *Leishmania* spp. or if they can be detected in the invertebrate host of *Leishmania* spp., for example. It is plausible to assume a dynamic symbiotic relationship in this long-term interaction between LRV or LBV and *Leishmania* spp., but it is not clear yet the influence of either LRV or LBV on *Leishmania* biology. At least in some circumstances it seems that this interaction causes a stressful condition promoting a *Leishmania* spp. more tolerant to some environments and augmenting its replication rate. It is a fact that both viruses influence the leishmaniasis pathogenesis, but it is still unclear if this is a consequence of the vertebrate host response to the virus living in *Leishmania* spp. cytoplasm or a biological response of *Leishmania* spp. to the endosymbiotic viruses. The impact of a "parasitized parasite" in the initial moments of a natural infection is also an aspect that deserves

attention. The phenotype of higher pathogenicity linked to *Leishmania* spp. bearing viruses might be linked to an increased evolutionary fitness and considered to signal that the viral acquisition was beneficial to the parasite. But there is also the possibility of a better fitness for those less pathogenic, which could have the chance to produce asymptomatic infections, to be maintained longer in the vertebrate host and to be dispersed to new environments.

The screening of viruses in *Leishmania* spp. is still limited to few studies, but so far, the evidence indicated that LRV1 is restricted to the American Continent and associated to *Leishmania* (*Viannia*) species; that LRV2 is linked to the Old World and hosted by *Leishmania* (*Leishmania*). That LBV was detected only in *L. martiniquensis*, a species belonging to a subgenus not closely related to *L.* (*Viannia*) or *L.* (*Leishmania*). For both LRV1 and LRV2 there were different genotypes and a correlation with the parasitized *Leishmania* species. The consequence of *Leishmania*-LRV or *Leishmania*-LBV coevolution was probably dependent on coevolutionary dynamics, involving i) fluctuating selection affecting the frequency of some genotypes, especially those linked to resistance and infectivity [98] or fluctuations in the range of resistance and infectivity [99], and ii) antagonist coevolution turning towards increasing infectivity and/or resistance. There is an important imbroglio of evolution and ecology linked to the relationship between *Leishmania* spp. and LRV or LBV, providing to these interactions a direct impact on the evolutionary route.

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