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Article

Windows into Canine Leishmaniasis: From Veterinary Diagnosis to Promising In Vitro Models to Target Parasite-Secreted Lipid Bound Vesicles

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Simple Summary: Extracellular vesicles, like extracellular vesicles released by *Leishmania* parasites (we usually called *L*EVs), are lipid bound vesicles secreted by cells into the extracellular space. Our results based on our observations in the last decade of studies are double aimed, considering macro clinical and micro histopathological aspects of canine leishmaniasis and the molecular composition of *L*EVs associated with immune host-modulation. Why? Lipids are essential molecular components of extracellular vesicles, but now the knowledge about lipid composition of *L*EVs, associated mechanism and functions is very limited. We can just confirm that this production by *Leishmania* exists, as its permanent modulation to be infective, "safe" and multiplying, under stimuli conditions (temperature, medium, contact with cells, etc.) e.g., growing the production of protein while down the lipidic trafficking is one very possible hypothesis would be useful for clinical and histopathological studies. In this way, comparative studies are necessary to highlight no concluded knowledge, thus we hope our findings will call the attention of researchers to consider new hypothesis by *in vitro* and *in vivo* observations like the clinical case reports to broaden the knowledge of canine leishmaniasis and the necessary comparative studies of zoonotic co-infections in a pos-pandemic future.

Abstract: Leishmaniases are zoonotic vector-borne diseases caused by a wide variety of *Leishmania* species with complex transmission cycles involving different reservoirs, potential new hosts and vectors. Similarly, to other eukaryotes, *Leishmania* release extracellular vesicles (*L*EVs) to play important initial interactions that are crucial to modulate the subsequent systemic immune response on the establishment of infection in humans and others hosts like dogs. Recent studies in endemic areas of Brazil concluded that canine infections were predominantly due to *L. amazonensis* and not restricted to *L. infantum* (syn. *Leishmania chagasi*). Under these premises, the diagnosis of leishmaniasis needs to be improved with the identification of current etiological agent and the histopathologic features, highlighting the differential diagnosis and the molecular components of *L*EVs. In this way, the dual aim of that study is to register collected observations of natural canine infections, enhancing the relevance of differential diagnosis in companion animals and inserting *in vitro* results in the field of *L*EVs that still research gaps to be filled to understand the mechanisms and biological aspects involving the parasite-host interactions. Therefore, improve these studies of

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Parasitology research is important for diagnostic, prognostic, treatment advances and continuing need for global prevention, control, elimination/eradication of these parasitic infections.

Keywords: *Leishmania* infectivity in mammalian hosts; inserts of leishmaniases and coinfections in companion animals; clinical and histopathologic features of natural zoonotic canine leishmaniasis; cell communication by parasite-derived extracellular vesicles; *in vitro* models advance to target parasite-secreted lipid bound vesicles

1. Introduction

Leishmania is a successful genus of parasitic Protozoa of medical and veterinary importance divided into Old and New World species adapted to large diversity of hosts/reservoirs such as dogs (the main reservoir of *Leishmania infantum*), and other vertebrates like domestic cats, wild and synanthropic animals, being transiently infectious in humans (Figure 1) [1]. According to World Health Organization (WHO), their high ability to infect multiple hosts has facilitated the spread of *Leishmania* by female phlebotomine of several species of *Phlebotomus* and *Lutzomyia* sandflies vectors, distributed and segregated geographically in 4 eco-epidemiological regions of the world: Americas, East Africa, North Africa and West and South-east Asia (Figure 1) [1–5].

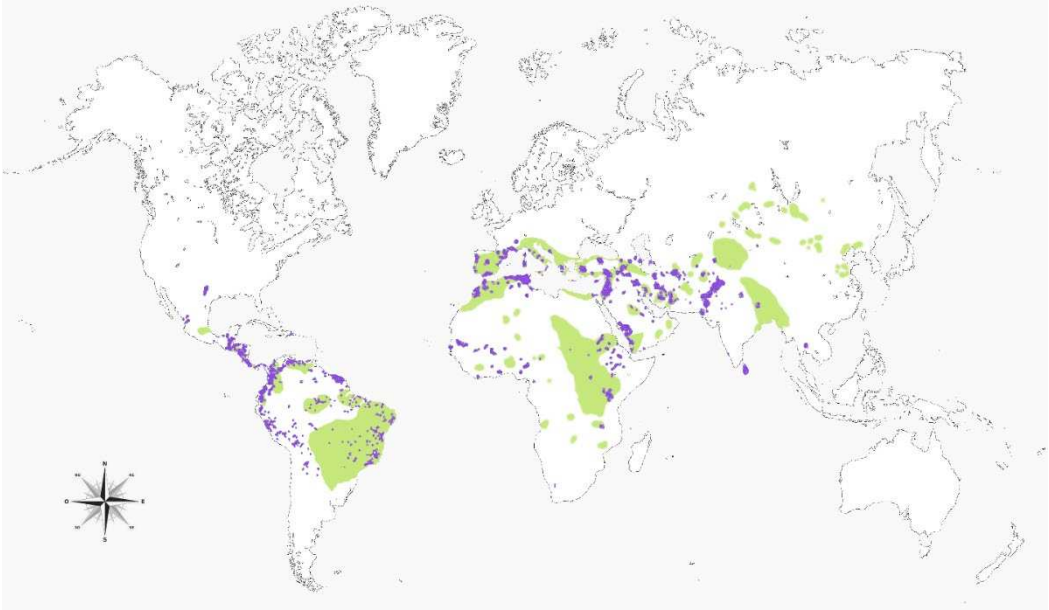

Figure 1. Endemic areas of zoonotic leishmaniases worldwide: visceral leishmaniasis (green areas) and cutaneous leishmaniasis (purple dots). Both types present rapid adaptation expanding geographic ranges influenced by risk factors, with new epidemiological scenarios emerging in previously disease-free areas. Increased transmission of leishmaniasis and higher incidence of disease, as well as its emergence/re-emergence in recent years may be related to many factors, among which we highlight the socio-economic conditions, climate and environmental change, closer contact between pets and wild ecosystems and the parasite resistance to drugs and insecticides vectors in use [1–4].

Leishmania present a digenetic life cycle with zoonotic transmission between mammals and sandflies (Figure 2) [1,3,5,6]. However, is also possible to occur infection through transfused blood products, from blood donors which are carriers of infection, transplacental and venereal transmission, and hardly any case studies about direct dog-to-dog *Leishmania* transmission by wounds/dog bites [6–10]. It is therefore important that we draw attention to this matter, considering the transmission/risk of transmission like a clinical key for the veterinarian to establish the signs and pathological abnormalities of *Leishmania* infection, despite the difficult to establish incubation period,

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even though intracellular parasites appear after several weeks or months (typically that seems shorter for cutaneous forms) (Figure 2) [11,12]. Concurrently with the spread of leishmaniasis recently studies reveal the most popular pet animals worldwide are at risk of acquiring coinfection with others emerging zoonotic parasites, virus among other infectious agents which also require further studies on their extracellular vesicle production, and the simultaneous cascade effects of their molecular interaction on the host immune system (Appendix A) [1–15]. In that sense, coinfected companion animals, such as dogs and cats, are important elements of *Leishmania* lifecycle and at same time for the transmission of others disease agents of medical and veterinary importance, that can cause sensible deterioration in health, leading to secondary infections or increased morbidity, and even death of these animals (e.g., weight loss, anemia, and low immune resistance) [16–18].

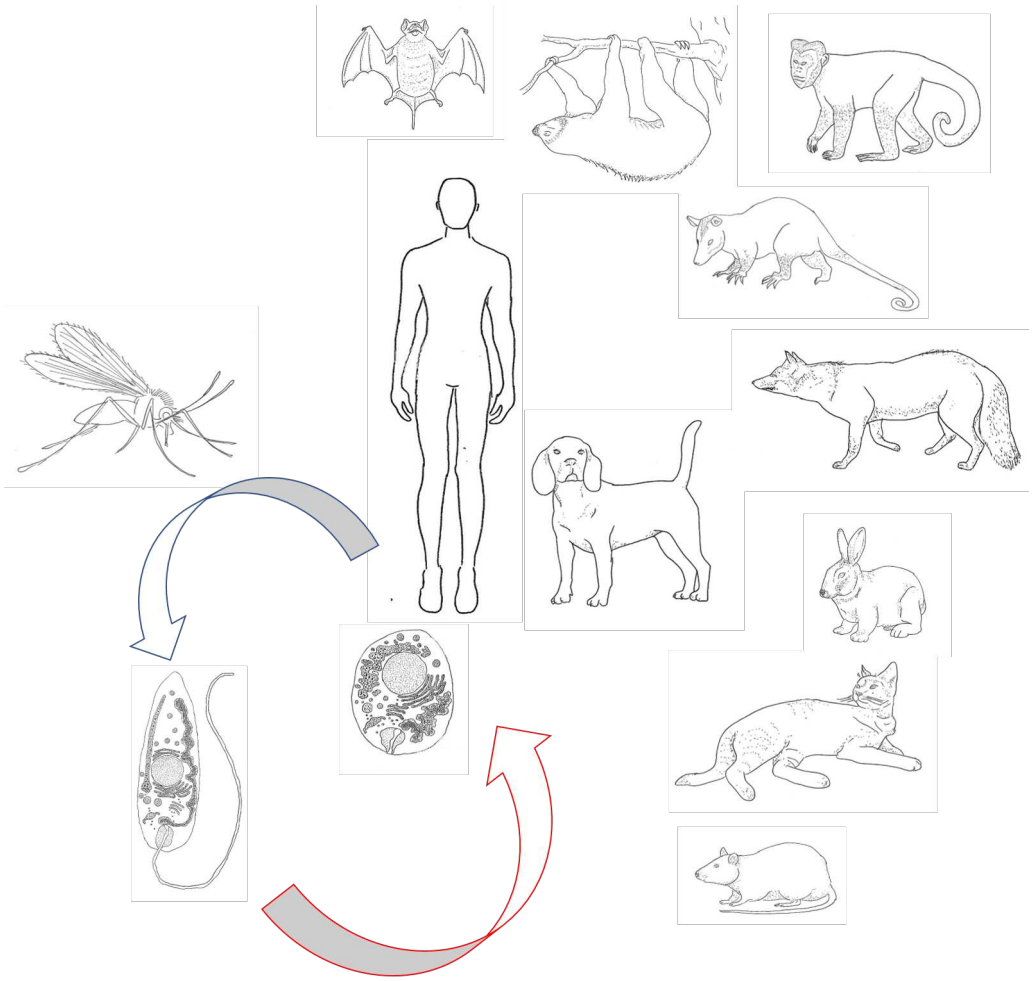

Figure 2. What hypotheses should be still being considered from the *Leishmania* zoonotic cycle? a. Hosts have different biochemical, immunological, behavioral, and other defense mechanism to ward off parasites, and many features of parasites are adaptations for overcoming these defenses; b. The different levels of *Leishmania* virulence depend on the evolution of both host and parasite; c. The susceptibility of potential new hosts and vectors can vary under interconnected factors; d. Even more impressive than the life cycles of *Leishmania* are their many defenses against the antibodies of vertebrate hosts, the proteins that recognize and bind to foreign proteins and other molecules of polydisperse complex biological samples (e.g., extracellular vesicles surrounded by a lipid layer of the parasite membrane) [1–19].

The clinical signs of leishmaniasis are directly related to the immune response of the infected dog, however it is estimated that more than half of infected dogs do not manifest clinical signs of the disease [20]. Despite the development of some veterinary vaccines, the lack of effective vaccines and

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drug treatments for humans has allowed the increase of this major health problem worldwide [1–12]. These parasites have a high level of genetic variability *in vivo* and a propensity for rapid evolution *in vitro*, establishing infection by heterogeneous extracellular vesicles released containing many molecules to modulate the host immune responses, what we generically refer to as *Leishmania* extracellular vesicles or *L*EVs in our last review published (*Leishmania* 360°: Guidelines for Exosomal Research, by Gabriel at al. 2021) [1–21]. However, there is increasing interest in the isolation and biological and functional characterization of the lipoproteic vesicles released by *Leishmania* (*L*EVs), given their apparent potential for the development of effective diagnostic and therapeutic approaches, including the prediction of the outcome of the interaction between cells [1–22]. While advances in tech have produced new tools to progress in extracellular vesicle biology and their application, the mechanisms of the selective packaging of *L*EVs are still poorly understood, and there is no consensus on the differential isolation and characterization of these extracellular vesicles or the ultrasensitive detection of specific subtypes, biomarkers and biogenesis [1–21]. Recent studies indicated that the lipid composition of extracellular vesicles influences their biogenesis, cargo sorting, interactions with target cells, and functional effects on recipient cells [22–25]. In this way, emerging focus on lipids in *L*EVs that still research gaps to be filled to understand the mechanisms and biological aspects involving the *Leishmania*-host interactions [1–22]. We note that lipid bodies are organelles distributed in the cytoplasm of eukaryotic cells largely associated with lipid storage in the past, however actually recognized like dynamic and functionally active organelles, involved in a variety of functions such as lipid metabolism, cell signaling, and inflammation (identified inside dermal macrophages and actively formed within heart macrophages following infection with *Trypanosoma cruzi*) [22,23]. Given these considerations, we present this work, with emphasis on the diagnosis of natural canine infections and to insert *in vitro* results related to endocytosis and exocytosis process of *Leishmania*, complementing the set of studies about *L*EVs we are developing during the last years in the scope of the PhD thesis of author and to collaborate with the approved Minimal information for studies of extracellular vesicles (MISEV2023): from basic to advanced approaches by The International Society for Extracellular Vesicles, providing a reference base for future applied nanotechnological research towards in One Health.

2. Materials and Methods

2.1. Quantification Method of Lipid Bodies in Promastigote of Leishmania (L.) Amazonensis

BODIPY™ 493/503 (4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene Molecular Probes Invitrogen™) is a lipophilic fluorophore used for the identification of neutral lipids (reserve lipids) present in abundance in the form of intracellular lipid bodies, being used for quantification, analysis of these structures by flow cytometry [24]. and morphological observation by fluorescence microscopy. For the quantification of lipids, promastigotes of *L.* (*L.*) *amazonensis* (strain MHOM/BR/26361) were maintained in culture in RPMI 1640 medium, supplemented with 10% SBF at 27ºC. After 24, 48 and 72 h of culture the cells were washed with PBS pH 7.2 and incubated with BODIPY™ 493/503 at a concentration of 10 µg/mL for 30 minutes, in the absence of light [25]. After incubation, the cells were analyzed in the flow cytometer BD FACSCanto IITM. A total of 10,000 events were collected for each sample and the average flowering intensity data were analyzed using the GraphPad Prism program (version 6.01).

2.2. Transmission Electron Microscopy of Macrophages Infected with Leishmania (Leishmania) Amazonensis

Peritoneal macrophages from mice (2x106/mL) were cultured and infected with *L.* (*L.*) *amazonensis* 1:10 promastigotes in the stationary phase [26]. After 72 h of infection, the cells were washed with PBS pH 7.2, followed by fixation for 1 hour with 2.5% glutaraldehyde type II (70%), 4% paraformaldehyde, 2.5% sucrose, in 0.1 M sodium cacodilate buffer, pH 7.2, after fixation, the cells were incubated in solution with 1% osmium tetroxide and potassium ferrocyanide (0.8%) for 1 hour. After this period, the cells were dehydrated in a growing series of acetone for 10 minutes (50%, 70%,

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90% and 3 times in 100%). After dehydration, the cells were slowly impregnated in Epon® resin (2:1, 1:1 and 1:2 - 100% acetone: Epon®). Subsequently, the material was placed in pure Epon® for 6 hours and finally polymerized at 60ºC for 48 hours. The blocks were cut in ultramicrotome (Leica EM UC6) and the sections obtained were contrasted in 5% uranyl acetate and lead citrate and observed in JEOL Transmission Electronic Microscope.

2.3. Transmission Electron Microscopy of Tissues from Male Balb/c Mice Infected with Leishmania (Leishmania) Amazonensis

Male Balb/c mice (between 8 to 10 weeks of age) [27], were infected subcutaneously with approximately 1x106 of *L. (L.) amazonensis* promastigote stationary forms, euthanized after 6 weeks and the lesion tissues were processed for TEM Analysis, followed by fixation for 1 hour with 2.5% glutaraldehyde type II (70%), 4% paraformaldehyde, 2.5% sucrose, in 0.1 M sodium cacodilate buffer, pH 7.2, after fixation, the cells were incubated in solution with 1% osmium tetroxide and potassium ferrocyanide (0.8%) for 1 hour. After this period, the cells were dehydrated in a growing series of acetone for 10 minutes (50%, 70%, 90% and 3 times in 100%). After dehydration, the cells were slowly impregnated in Epon® resin (2:1, 1:1 and 1:2 - 100% acetone: Epon®). Subsequently, the material was placed in pure Epon® for 6 hours and finally polymerized at 60ºC for 48 hours. The blocks were cut in ultramicrotome (Leica EM UC6) and the sections obtained were contrasted in 5% uranyl acetate and lead citrate and observed in JEOL Transmission Electronic Microscope.

2.4. Leishmania Diagnosis: Some Findings of Natural Canine Infection

We present in this section the information's case report of "Jimmy", a healthy dog (Shih Tzu), male, 1 year old that had presented eye infection during clinical examination.

2.4.1. Careful Anamnesis, Clinical and Routine Laboratory Tests

To set the standards, the following recommended monitoring of clinicopathological parameters were considered (clinical history; complete physical examination; identification of clinical signs according the 4 clinical stages: mild, moderate, severe and very severe disease; laboratory tests: complete blood count CBC, biochemical profile, serology, molecular techniques) [11].

2.4.2. Impression Cytology of the Infected Ocular Surface

Impression cytology to remove the superficial layers of the ocular surface epithelium following the minimally invasive method established, easy to perform, and yields reliable information about the area sampled with minimal discomfort to the patient. The method was applied to obtain a conjunctival cytological sample analyzed by light microscopy examination [28].

2.5. Molecular Analysis of Canine Clinical Samples by Polymerase Chain Reaction PCR

Following Galvão et al. tested protocols, 5mL of canine blood samples were collected by venipuncture using dry and EDTA-containing tubes for PCR analysis and the secretions from auditive duct, mucous membrane of the anus, oral mucosa, whole blood, preputial and ocular of "Jimmy" were obtained using sterile cotton swab (Neolab®) and frozen until DNA was extracted (Preliminary studies = blood samples and ocular swabs from 130 dogs: 67 males (51.5%), 60 females (46.1%) and three animals (2.4%) had no gender information, adding the samples from "Jimmy" the final total = 131 animals from the endemic areas of State of Pará-Amazon Region-Brazil – area: 1.284.042 km, situated between the coordinates 04°20'45" N and 09°50'27" S, and 46°03'18") [29,30]. The extraction of DNA for the molecular diagnosis from the canine blood samples was made according to the manufacturer's recommendations of AxyPrep ™ Blood Genomic DNA Miniprep Kit (Axygen) commercial kit and the technique with NaCl to extract the DNA from secretions by swabs (final volume of 20 µL). After extraction, molecular tests were performed out through PCR targeting the small subunit ribosomal rRNA (SSU rRNA) gene and extra-chromosomal DNA kinetoplastid

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DNA (kDNA) *Leishmania* sp specific and *Leishmania infantum* specific (Table 1). For detection to target the small subunit ribosomal rRNA (SSU rRNA) gene *Leishmania* sp.specific the first PCR was performed with primers S4 (5′-GATCCAGCT GCAGGTTCACC-3′) and S12 (5′-GGTTGATTCCGTCAACGGAC-3′). Reactions were performed with a final volume of 25 µl containing 1X PCR buffer, 2 mM MgCl2, 0.2 mM dNTPs, 0.2 µM of each primer and 1 U Taq DNA polymerase. DNA was first denatured at 94º C (3 minutes) and then cycled 35 times at 94º C (1 minute), at 50º C (1 minute), and at 72º C (1 minute). A final extension of 7 min was performed at 72º C. The fragment (520 pb) produced by S4/S12 PCR was used in a nested PCR with primers S17 (5′-CCAAGCTGCC CAGTA GAAT-3′) and S18 (5′-TCGGG CGGAT AAAACCC-3′), genus *Leishmania* specific. The reaction was undertaken on the same conditions as those described above. The S4/S12 PCR product was denatured at 94º C (4 minutes) and cycled 30 times; each cycle took place at 94º C (1 minute), at 55º C (1 minute), and at 72º C (30 seconds). Oligonucleotides S17 and S18 produced a 490 bp fragment. *L. infantum*-specific primers *Leish* 1 (5′-AACTTTTCTGGTC CTCCGGGTAG-3′) and *Leish* 2(5′-ACCCCCAGTTTCCCGCC-3′) were used to amplify a 120-base-pair fragment of the *Leishmania* kinetoplast DNA minicircle. PCR was conducted in a 25 ml final reaction mixture containing PCR buffer 1, 0.150 mM dNTPs, 2 mM MgCl2, 0.2 mM of each primer and 1U Taq Polymerase. The thermal cycling profile was as follows: 94 º C (3 minutes), followed by 35 cycles at 94º C (30 seconds), 60º C (30 seconds) and 72º C (30 seconds); with a final extension at 72º C (5 minutes). Products of small subunit ribosomal rRNA gene (490pb) and kDNA (120 bp) were applied to 1.5% and 2.5% agarose gel, respectively, in Tris-Acetate-EDTA buffer, stained with Diamond Nucleic Acid Dye (Promega Corporation, USA), and later submitted to electrophoresis and visualized under ultraviolet light. And for the PCR reactions an Applied Biosystems thermocycler (Model 2720) was employed [29,30].

Table 1. Olinucleotides used for identification of amplified DNA fragment size and identification of *Leishmania* by molecular biology assays of PCR-SSUr and PCR kDNA.

Gene	Methods	Oligonucleotides	Base Pair (BP)	Identification
SSUr	PCR	R221- 5′-GGTTCCTTTCCTGATTTACG-3′ R332 -5′-GGCCCGTAAAGGCCGAATAG-3′	603	Kinetoplastid
	Nested	R223 -5′-TCCCATCGCAACCTCGGTT-3′ R333 - 5′-AAAGCGGGCGCGGTGCTG-3′	490	*Leishmania* sp.
SSUs	PCR	S4 - 5' GAT CCA GCT GCA GGT TCA CC 3' S12 - 5' GGT TGA TTC CGT CAACGG AC3'	520	Trypanossomatids
	Nested	S17 - 5' CCA AGC TGC CCA GTA GAA T 3' S18 - 5' TCG GGC GGA TAA AAC ACC 3'	358	*Leishmania* sp.
*k*DNA	PCR	Leish 1 - 5′ AACTTTTCTGGTCCTCCGGGTAG -3′′ Leish 2 - 5′ ACCCCCAGTTTCCCGCC -′- 3	120	*L. infantum*

After obtaining the amplicons, the PCR products were purified for the *k*DNA gene and nested-PCR for the SSU-rDNA gene using EXO-SAP PCR DNA (GE Healthcare), in the Sequencing was performed with the aid of the ABI 3500XL automatic DNA analyzer Applied Biosystems ™ in combination with BigDye® Terminator v3.1. The Sequences nucleotides obtained were edited and aligned using the BioEdit software. After comparison with other GenBank sequences (BLASTn = Basic Local Alignment Survey of nucleotides), the SSU rDNA sequence was aligned with the set of *Leishmania* sequences available at GenBank NCBI NIH using CLUSTAL program W 1.6, which aims to identify the single nucleotide polymorphisms (SNPs) and insertion-deletions (INDELS). Relationships phylogenetic studies were constructed with Neighbor-Joining, the p distance method

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was used to calculate the genetic distances, the monophyletic groups have been supported by the Bootstrap 2000 method, using MEGA 6 program. And to test whether a relationship exists between the diagnostic methods exists by a contingency table the BioEstat 5.0 software was used for results considered as relevants ($p<0.05$) [29,30].

3. Results

3.1. Endocytosis and Exocytosis Process of Leishmania: Infected Macrophage by Promastigotes, Infected Mice Tissues by Amastigotes and the Quantification of Lipid Bodies in Promastigote of Leishmania (L.) Amazonensis

The knowledge of specific cargo molecules identified within *L*EVs suggest their function as adjuvant-like in the immune responses, with a possible key advantage for the infection establishment and disease progression, inducing at the same time quantitative and qualitative changes in the protein content of infected host cells extracellular vesicles (Figure 3 and Figure 4). However, many challenges remain in the extraction, purification and analysis of polydisperse isolates containing *L*EVs.

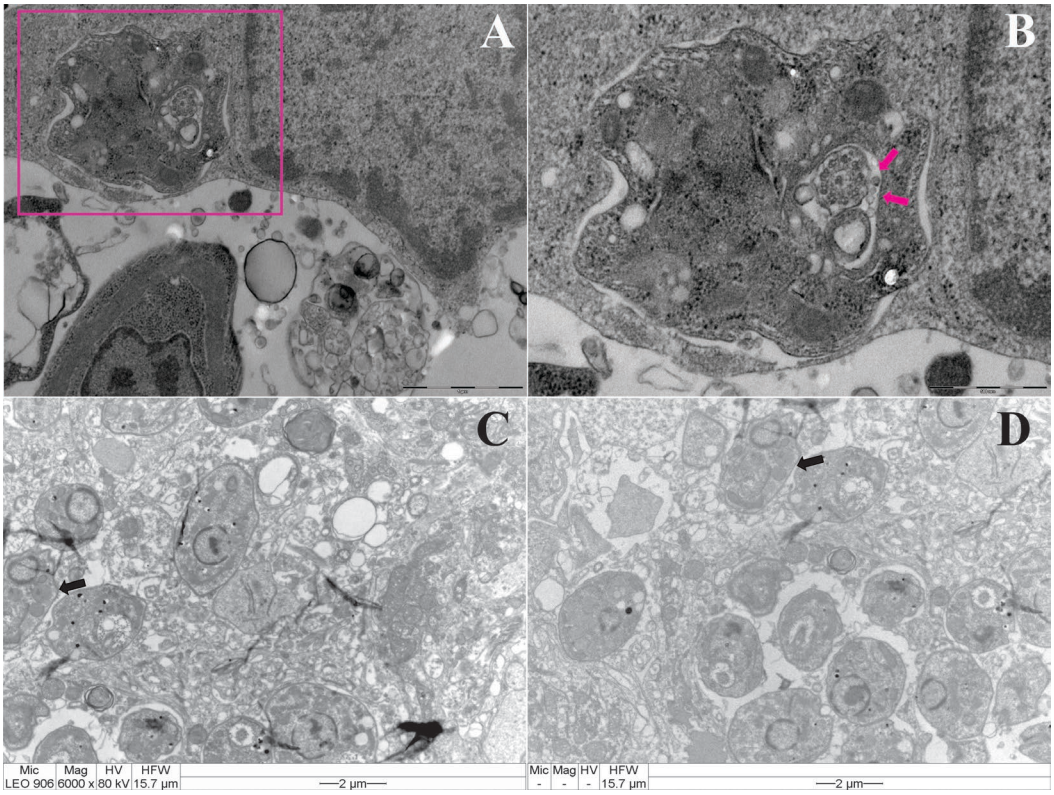

Figure 3. (**A**) Macrophage infected with *Leishmania* (*Leishmania*) *amazonensis* (MHOM/BR/2009/M26361 strain) examined by TEM Scale-bar 1 µ. (**B**) Tubular structures and vesicles (arrows) participating in the endocytosis and exocytosis process can be seen in the flagellar pocket (FP) of promastigote. Scale-bar 500 nm. (**C**,**D**) Transmission Electron Microscopy of tissues from Male Balb/c mice infected with *Leishmania (Leishmania) amazonensis*: lipid-like structures (arrows). Scale-bar 2 µm.

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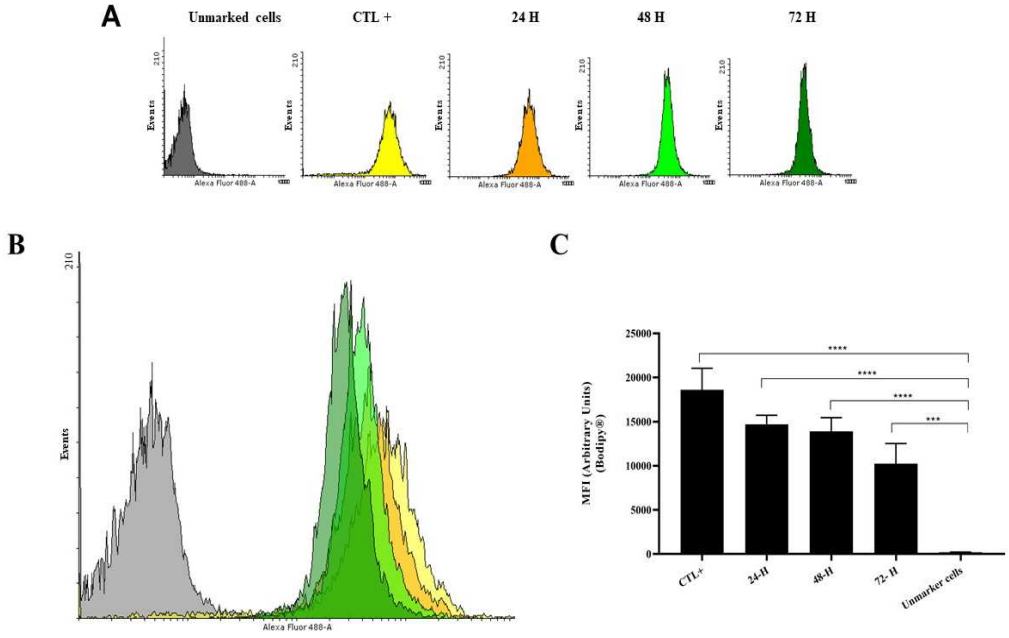

Figure 4. Quantification of lipid bodies in *Leishmania* (*L.*) *amazonensis* at different culture times; (**A**) Histogram of each experimental group; (**B**) Overlay histogram demonstrating increased fluorescence intensity of BODIPY™ 493/503-labeled cells at 24, 48 and 72 hours of culture and (**C**) Graphic representation of BODIPY™ 493/503-labeling in *Leishmania* promastigotes. *** $p < 0.001$ (ANOVA and Tukey post-hoc test).

3.2. Results of PCR Tested for the Biological Samples Collected from 130 dogs of Amazonian Endemic Area and the Results Obtained from Our Case Report "Jimmy"

The variables sex, age and race regarding the presence of infection were analyzed. In none of them was a statistical association found, however 47dogs between 1-8 years old, (36.2%) had positive results for *Leishmania* infection (Table 2).

Table 2. Distribution of *Leishmania* sp. in the different age groups.

AGE	INFECTION (%)		TOTAL
	PRESENT	ABSENT	
0 to 12 months	14 (10.7)	10 (7.7)	24
1 to 8 years	47 (36.2)	40 (30.8)	87
Over 8 years	7 (5.3)	6 (4.6)	13
Not informed	4 (3.1)	2 (1.5)	6
Total of animals	72	58	130

When the infected animals were evaluated for the presence of at least two symptoms suggestive of leishmaniasis, it was observed that 65/72 (90.3%) of the dogs were symptomatic and 7/72 (9.7%) asymptomatic, in addition to the presence of 28 symptomatic animals without infection (Table 3).

Table 3. Prevalence of *Leishmania* sp. in symptomatic and asymptomatic leishmaniotic dogs.

INFECTION	SYMPTOMATIC (%)	ASYMPTOMATIC (%)	TOTAL
Present	65 (50)	7 (5.4)	72
Absent	28 (21.5)	30 (23.1)	58
Total	93	37	130

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χ^2 = 25,80, p-value<0,001; Odds Ratio 9,95; p-value<0,001, IC 3,9-25,3.

Regarding the detection of the parasite's DNA in the different biological samples (blood and swabs), 58 (44.61%) dogs were positive in blood and 54 (41.53%) positive dogs in the swab samples, thus 43/130 (33.1%) samples were positive in both types of samples, 15/58 (25.9%) of the samples were positive blood samples and 11/72 (15.3%) were detected positive in the swab's samples (Table 4).

Table 4. Detection of the DNA of *Leishmania* sp. in the different types of biological samples.

Biological Sample	Blood (+)	Blood (-)	Total
Swab (+)	43 (33.1%)	11 (8.5%)	54
Swab (-)	15 (11.5%)	61 (46.9%)	76
Total	58	72	130

(+) Positive; (-) Negative. χ^2= 43,44; p-value <0,001.

For the detection of *Leishmania* sp. In the animals studied, different types of oligonucleotides were used, one of which is specific for the detection of *k*DNA and the others for SSUr-rDNA. In the present study, when sensitivity of detection of the parasite's DNA, carried out by the different oligonucleotides, A higher number of positive samples was detected through the marker for the *k*DNA in contrast to SSUr-rDNA. The success in detecting the *Leishmania* infection was between the markers and there was no statistical difference regarding the efficiency of markers on each other, indicating that both have similar sensitivity (Table 5).

Table 5. Detection of *Leishmania* sp. DNA in animals through markers for *k*DNA and SSUr-rDNA chromosomal.

	***k*DNA (Minicircle)**		**Chromosomal (SSUr-rDNA)**	
	Blood	**Swab**	**Blood**	**Swab**
POSITIVE	47 (36.2%)	38 (29.2%)	38 (29.2%)	32 (24.6%)
NEGATIVE	83 (63.8%)	92 (70.8%)	92 (70.8%)	98 (75.4%)

Phi coefficient r_φ (*k*DNA Blood x SSUr-rDNA Swab) p-value 0.05.

The amplified products of the SSUr-rDNA segment of the 18S RNA obtained were sequenced to determine the phylogenetic relationships of the samples. With the initiators R223/R333 the best sequences were 26 out of 72 samples, all of which grouped in the *donovani* complex, resembling *L. infantum* (Figure 5).

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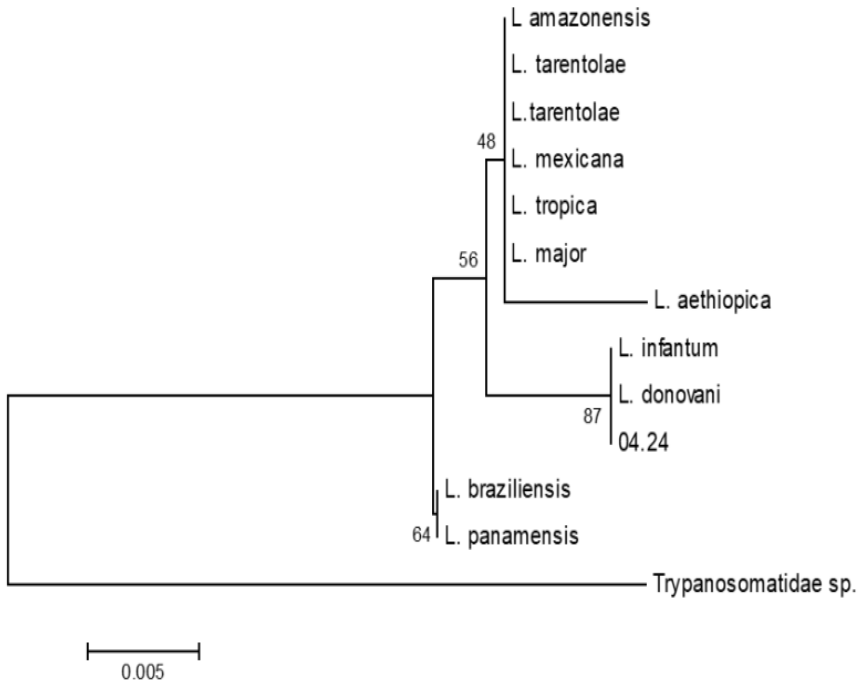

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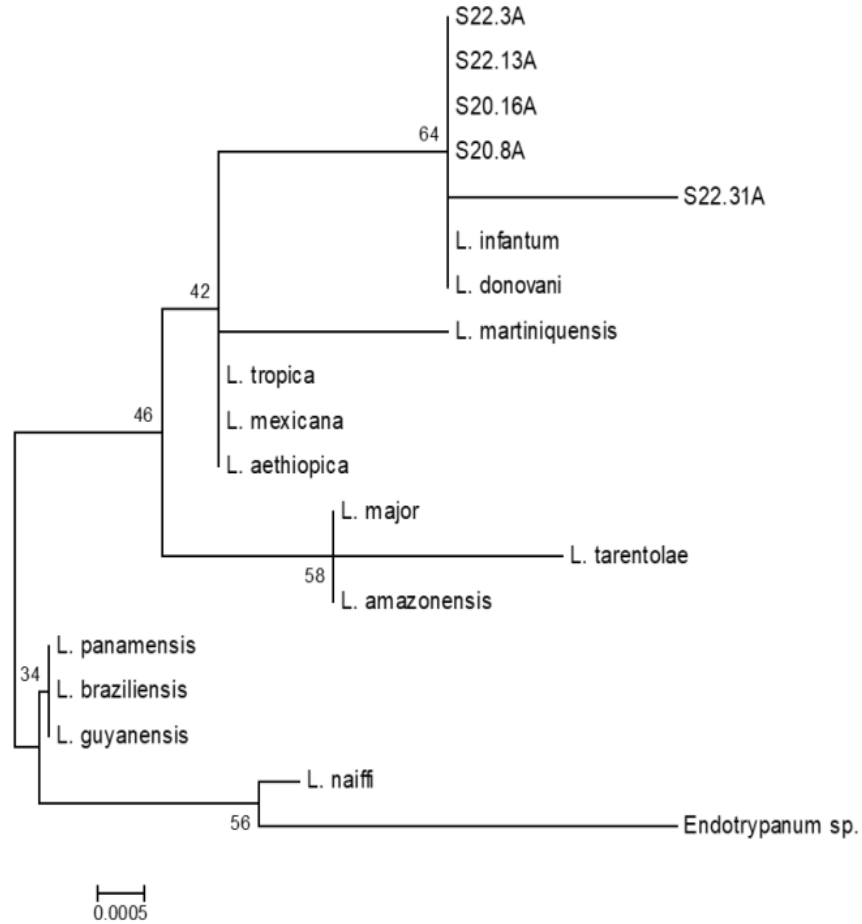

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Figure 5. Neighbor-joining method is proposed for reconstructing phylogenetic trees from evolutionary distance data. (**A**) In alignment for the construction of the phylogenetic tree, the sequence obtained was compared with the sequences from Genbank; (**B**) The amplified products of the S17/S18 initiators were 47 of the 72 sequences analyzed, most of which grouped and resemble *L. infantum*. In addition, it was observed that four samples (S20.16, S22.3, S22.13 and S22.31) show genetic variability. It should be noted that the S22.31 sample has a higher genetic distance in contrast to the others.

The samples of the study had showed nucleotide variation in the multiple alignments, and the presence of degenerated nucleotides (Y = C/T and R = A/G). These variations occur with a frequency of 1 to 3 in the analyzed segment, which are located between positions 121 to 316 of the sequencing (Table 6). Just one sample shows a pyrimidine transition mutation (C→T) at position 202. At this point is important to highlight that these samples come from different areas of State of Pará. The evolutionary analyses were conducted in MEGA 6, and the augmentation products of tree primers with A-markers. R223/R333, and B. markers S17/S18 (Figure 6).

Table 6. The canine samples with nuclotidic variation.

Code	Position	Degenerate Nucleotide	Nucleotides
S20.16	262	Y	C/T
S22.3	251, 316	Y	C/T
S22.13	122, 228, 313	R	A/G
S22.31	202		T

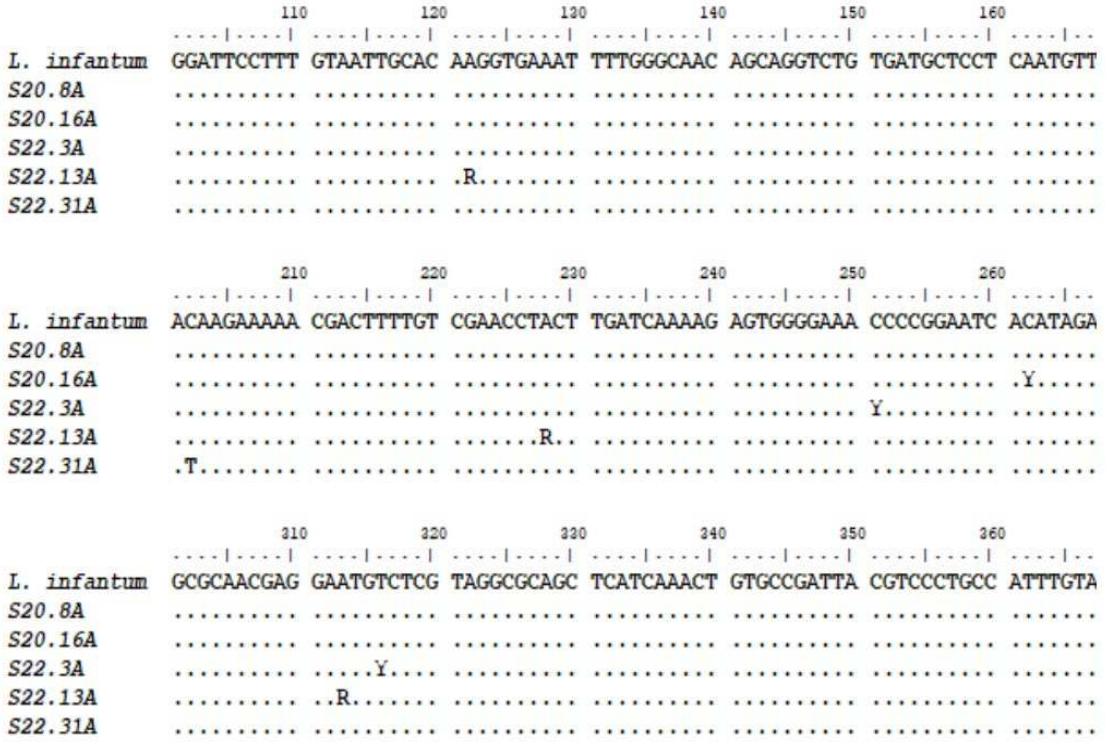

Figure 6. Multiple alignment of the sequences with nucleotide variation of the amplified products of the S17/S18 primers.

3.3. Leishmania Diagnosis: Some Findings of a Natural Canine Infection after Anamnesis, Clinical and Routine Laboratory Tests

Apparently, "Jimmy" was a young healthy dog with just one eye infection identified during clinical examination (Figure 8). Despite this, conjunctival cytological and additional biological

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samples analyzed by routine laboratory tests had confirmed the diagnosis of leishmaniasis. Important to refer that there is no register of previous vaccination against leishmaniasis in this clinical case, however the region is endemic (Figure 7).

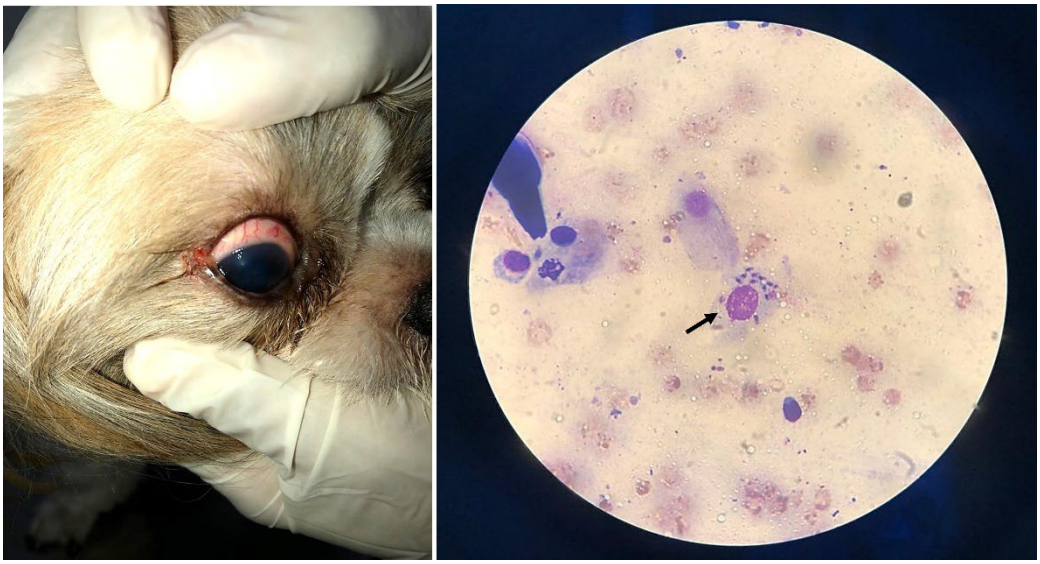

Figure 7. Ocular infection in canine leishmaniasis and light microscopy examination of a conjunctival cytological sample showing a macrophage containing multiple Leishmania amastigotes (arrow) (Author's photos G.R.G).

The results obtained from the samples of infected animal confirmed the *Leishmania* infection (Appendix B).

3.4. Results of PCR for the Biological Samples Collected from "Jimmy"

PCR is based on the amplification of the number of copies of fragment(s) of Deoxyribonucleic Acid - DNA and/or Ribonucleic Acid of the researched microorganism (*Leishmania* and others). The POSITIVE result indicates the presence of the microorganism in the biological material analyzed, while the NEGATIVE result indicates its absence. This test may present, although rarely, false-negative and false-positive results, which is a characteristic of the method. Animals with low parasitemia are more subject to false-negative results, especially when the test is performed from a peripheral blood sample.

The biological samples were collected and analyzed according to established protocols used in Molecular Biology of the Biomolecular Technology Laboratory of Institute of Biological Sciences of Federal University of Pará UFPA Brazil. The followed results were obtained:

- Auditive duct swabs: (PCR) *Leishmania* sp. NEGATIVE
- Mucous membrane of the anus swabs: (PCR) *Leishmania* sp. POSITIVE
- Oral Mucosa swabs: (PCR) *Leishmania* sp. POSITIVE
- Whole Blood*: (PCR) *Anaplasma platys* NEGATIVE
- Whole Blood*:(PCR) *Babesia vogeli* NEGATIVE
- Whole Blood*: (PCR) *Ehrlichia canis* NEGATIVE
- Whole Blood*: (PCR) *Leishmania* sp. POSITIVE
- Whole Blood*: (PCR) *Mycoplasma* sp. NEGATIVE
- Whole Blood*: (PCR) *Rangelia vitalli* NEGATIVE
- Preputial secretion swabs: (PCR) *Leishmania* sp. POSITIVE
- Ocular Swab: (PCR) *Leishmania* sp. POSITIVE

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*The whole blood sample was collected into Ethylenediaminetetraacetic acid (EDTA) tubes. PCR test results that are at odds with the clinical status of the animal should be repeated with a new biological sample. Indirect immunofluorescence assay (IFAT) and quantitative real-time PCR (qPCR) are alternative methods that aid diagnosis.

4. Discussion

The clinical signs of leishmaniasis are directly related to the immune response of the infected dog and we can account the disease into four stages based on serological status, clinical signs, laboratory findings and type of therapy and prognosis for each stage [12–31]. Of course, these aspects are result of *L*EVs interactions with the hosts. In susceptible animals, organisms can spread from skin to the local lymph node, spleen and bone marrow in a few hours [31]. Considering the present molecular results, *L. infantum* infection is not associated to gender or age of the dogs, also was not considered coinfections associated with the positive cases. No difference was found between the types of biological samples studied (blood and conjunctival swab) in the detection of *Leishmania* sp. However, for the greater reliability of the results, PCR is indicated for various types of samples, given the importance of the *Leishmania* infection, mainly due to its high zoonotic potential. Detection of infection by amplifying *k*DNA segments and SSUrrDNA showed similar sensitivity, indicating that both can be used with for the diagnosis of canine leishmaniasis [29,30]. The sequencing of 18S rRNA segments allowed the identification of *L. infantum* in all cases, and four animals were infected by strains with genetic variability, with three heterozygous samples and one with one with one pyrimidine (C→T) transition-type mutation. Following clinical knowledge: in resistant dogs, the parasite remains restricted to the skin and draining lymph node [31]. According to these considerations the first-choice samples should be used for PCR are: bone marrow, lymph node, spleen, skin and conjunctival swabs and others, however samples of blood, buffy coat and urine are considered less sensitive [12]. In parallel, the study of *L*EVs of prokaryotes and eukaryotes has aroused considerable interest in the scientific community, due to the possible potential for the development of diagnostic and therapeutic methodologies [1]. Despite advances in *Leishmania* studies, the selective mechanisms of *L*EVs are still poorly understood, there is no consensus on the differential characterization or ultrasensitive detection of their specific subtypes, biomarkers or their biogenesis and how this knowledge can be effective for faster diagnosis and prevention [1]. Elucidating the molecular mechanisms and strategies by which parasites employ e.g., lipids to secure parasite survival may provide advances for the identification and development of novel antiparasitic drug targets and therapies and early diagnosis [32]. Actually, the diagnostic methods for canine leishmaniasis include: parasitological (cytology/histology; immunohistochemistry and culture); molecular (conventional, nested and real-time PCR, considered the most sensitive technique) and serological quantitative (IFAT and ELISA) and quantitative (rapid tests) [12]. However individual case reports can add new parameters for the accuracy of diagnosis, to confirm the coinfection and the range of differential diagnoses – or if there the animal remains healthy or develops a mild, self-limiting illness [31]. Considering the canine leishmaniasis, these hardy dogs mount a weak antibody response, but a strong and effective Th1 response may have low antibody titers but produce IFN-γ in response to antigens parasitic, generate type I granuloma, mount a strong response of hypersensitivity of the late type, and eventually destroy the parasites [31]. The resistance to *Leishmania* has a strong genetic component; for example, dogs of the Podengo breed Ibicenco (Ibizan Hounds – antique hunter of rabbits) appear to be resistant to this parasite. There is also an association between resistance and certain MHC class II haplotypes, as well as certain Slc11a1 (Nramp) alleles in dogs [33]. Thus, the Ibizan Hounds may be an interesting canine model for the investigation of protective anti-*Leishmania* immune response [33]. Results of recent research show relevant differences between the cytokine serum profile and the data published for other canine breeds, and several genetic fixed variants in genes related to immune response, regulation of immune system, and genes encode cytokines and its receptors [34]. The most relevant genes that present such fixed polymorphisms were IFNG and IL6R [34]. Other variants with frequencies equal or above 0.7 were

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found in the genes ARHGAP18, DAPK1, GNAI2, MITF, IL12RB1, LTBP1, SCL28A3, SCL35D2, PTPN22, CIITA, THEMIS, CD180 [34]. Epigenetic regulatory genes as HEY2, L3MBTL3 show also intronic polymorphisms [34]. Future studies will reinforce why the regulation of immune response is different in the Ibizan hound dogs compared to other breeds [34]. By other side, some dogs develop severe and generalized nodular dermatitis, lymphadenitis granulomatous, splenomegaly and hepatomegaly, exhibiting activation of polyclonal (occasionally monoclonal) B lymphocytes involving all four classes of IgG, as well as hypergammaglobulinemia, and develop lesions associated with hypersensitivity types II and III [31]. Additionally, excessive production of immunoglobulin can lead to the development of an immune-mediated hemolytic anemia, thrombocytopenia and the production of antinuclear antibodies [31]. The chronic deposition of Immune complexes can result in glomerulonephritis, uveitis, and synovitis, leading to failure renal and death [31]. The significant elevation of ant histone antibodies is a feature of some dogs with glomerulonephritis associated with leishmaniasis. There is a positive correlation between the levels of these ant histone autoantibodies and the protein/creatinine ratio once that antibodies increase the likelihood of the development of glomerulonephritis [31]. In susceptible animals, the organisms can spread from the skin to the local lymph node, spleen, and bone marrow within a few hours [31]. In resistant dogs, the parasite remains restricted to the skin and draining lymph node – either the animal remains healthy or develops a mild, self-limiting disease. In contrast, susceptible dogs mount a Th2 response characterized by high antibody levels but poor cell-mediated immunity [31]. These differences were attributed to the activities of IL-10-producing Treg lymphocytes. Furthermore, the parasite can actively suppress transcription of the IL-12 gene, ensuring that the Th2 response predominates [31]. Of course, all these immune challenges affect the balance between progression to clinical disease and maintaining sub-clinical disease. During a chronic infection, a progressive disease develops in susceptible dogs [31]. Vaccines and immunotherapies targeted at recovering or maintaining T and B cell function can be important factors in mending the immune balance required to survive canine leishmaniosis [35]. In the veterinary practice, animals with clinical leishmaniosis can present suggestive signs, but dogs with subclinical infection or infected but clinically healthy present neither clinical signs, nor clinicopathological abnormalities, however, have a confirmed *Leishmania* infection [12]. In other words, it is important to consider that the use of anti-*Leishmania* therapeutic protocols is known to reduce the parasite load and hence infectiousness by treated animals, however presenting only temporary efficacy [3]. Dogs are an extraordinary heterogeneity in phenotype through the establishment of pure breeds; a change which has largely occurred over the past 200 years. With such selective inbreeding comes recognition that there is likely to be great diversity in the functioning of the immune system between breeds [36]. This has been clear for many years, based on the unique susceptibility of dog breeds to immune-mediated, infectious disease [35]. The immune response is crucial in the unfolding of the infectious process and in the establishment of the disease front, the mechanisms of adaptive and innate immunity of dogs [37,38]. Understanding the mechanisms of the immune system of the hosts is an important factor to comparatively elucidate what happens to the individual's organism during the progression of the disease [39]. Following the knowledge of Immunology Veterinary, the Pattern Recognition Receptors (PRRs) are a class of receptors that can directly recognize the specific molecular structures on the surface of pathogens [31]. The most important of the soluble C-type lectins is mannose-binding lectin (MBL) present at high levels in serum and which has multiple carbohydrate-binding sites that bind to oligosaccharides such as N-acetylglucosamine, mannose, glucose, galactose and N-acetylgalactosamine [31]. Although the binding is relatively weak, the multiple binding sites confer high functional activity [31]. Thus, MBL binds very strongly to different pathogens (e.g., parasites such as *Leishmania),* playing an important role in the activation of the complement system [31]. The surface of phagocytic cells is also covered by many PRRs that can interact with their ligands on the surface of infectious agents [31]. Another important mechanism that promotes contact between pathogens and neutrophils suspended in plasma is the "binding" between the pathogen and the leucocyte [31]. If, however, the pathogen is trapped by a neutrophil and another immune cell, it can be quickly ingested by phagocytosis [31].

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Thus, neutrophils can undergo a form of cell death called NETose as an alternative to apoptosis or necrosis [31]. After activation by CXCL8 or lipopolysaccharides, neutrophils can release the contents of their nuclei, with extrusion of large strands of decondensed nuclear ADN and associated proteins in the extracellular fluid [31]. This forms networks of extracellular fibers called "neutrophil extracellular traps" (NETs) [29]. The NETs are abundant at sites of acute inflammation. These networks trap and kill several pathogens such as *L. amazonensis*) [31]. NETs can be very important in containing microbial invaders by acting as physical barriers, capturing large numbers of parasites and thus prevent its spread) [31]. When promastigote forms of this parasite are injected by sandflies in the skin of a dog, they are quickly phagocytosed by the neutrophils [31]. When neutrophils go into NETose, parasites are released and then engulfed by macrophages and dendritic cells, in which organisms become differentiate in amastigotes. *Leishmania* amastigotes are intracellular parasites obligators that divide in macrophages until the cells rupture, and when released into the body, they are phagocytosed by adjacent cells [31]. Depending on the degree of host immunity, parasites can be restricted to the skin (skin disease); alternatively, dendritic cells may migrate to the lymph nodes or enter the circulation and lodge in the internal organs, leading to visceral spread of the disease. Although the disease is widely spread in endemic areas, most dogs is resistant to *Leishmania*, and only 10% to 15% develop the visceral form of the disease [31]. Macrophages are the major host cell for *Leishmania* and the effector cells to limit/ to allow the adaptative growth of these parasites into infected macrophage phagolysosomes (intracellular form) [31]. Its resistance to intracellular destruction is the result of multiple mechanisms, including genetic factors - comparative studies of 245 macrophage genes demonstrated that 37% were suppressed by *Leishmania* infection [31]. *Leishmania* lipophosphoglicans delay the maturation of the phagosome, preventing the production of NO and inhibiting the response of macrophages to cytokines [31]. These parasites also reduce the presenting of macrophage antigen by suppressing the expression of the class II major histocompatibility (MHC), when the parasites stimulate chronic inflammation. They are thus characterized by granulocytic invasion, this is followed by macrophages, lymphocytes and NK cells that collectively form granulomas [31]. Additionally, one important factor that determine the success or failure of an infection is the availability of iron [31]. Innate resistance to many intracellular organisms such as *Leishmania* is controlled, in part, by a gene called Slc11a1 (short for solute carrier family 11), member 1a; formerly called Nramp1) [31]. *Leishmania* can evade the host's immune response and ensure their survival and completion of their life cycles. In general, antibody-mediated immune responses protect against extracellular protozoa, while cell-mediated one's control intracellular protozoa [31]. Parasitic protozoa employ some sophisticated techniques to ensure its survival in the face of an animal's immune response [31]. The Th1-mediated responses that result in macrophage activation are important in many diseases caused by protozoa, in which organisms are resistant to intracellular destruction [31]. One of the most significant routes of destruction in the M1 cells is the production of nitric oxide (NO) [31]. The nitrogen radicals formed by interaction of NO with oxidants are lethal to many intracellular protozoa [31]. However, protozoa are also experts at surviving inside macrophages; for example, *Leishmania* and *T. cruzi* can migrate to safe intracellular vacuoles by blocking the maturation of phagosome. *Leishmania* and *T. cruzi* can suppress the production of oxidants or cytokines [31]. The ontogeny of the canine immune organs was reviewed, it is known hematopoietic and immune cells arise from a common bone marrow stem cell. Thereafter, B cells undergo maturation in the fetal liver and bone marrow, which represent successive primary lymphoid organs. B cells maturation involves the acquisition of BCR and selection to ensure that only B cells that express functional BCR (positive selection) and do not ligate self-antigens (negative selection) survive. On the other hand, immature T cells are exported to the thymus for final maturation. Although the puppy was considered immunocompetent between 6–12 weeks of age, it is not possible to accurately predict the onset of immunocompetence, since it depends on the presence of MDA [40]. Increased life span allowed the recognition of age-related higher susceptibility to infectious, inflammatory, autoimmune, and neoplastic diseases. Age-related changes include impairment of the cell-mediated immune response, as demonstrated by the reduction of proliferative

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response of blood lymphocytes to mitogens and the reduction of cutaneous delayed type hypersensitivity [40]. Moreover, there is a decline in the humoral immune response probably related to the decreased functionality of Th cells. The ability to mount humoral immune responses seems to prevail, as demonstrated by the persistence of protective vaccine antibody titers and respond to booster vaccination with elevation in titer [40]. Although the currently adopted triennial re-vaccination program (instead of the prior annual re-vaccination), offers adequate protection to young and adult dogs, however, may not confer protection to geriatric dogs [40]. Older dogs commonly present an impairment of immune responses to novel antigenic challenges, such as infections and vaccines, which probably is related to the reduction of the peripheral pool of naïve T cells and low diversity of the repertoire of T cell receptors [40]. The key genetic elements of immune responsiveness lie within the genes of the MCH; present as the dog leukocyte antigen (DLA) and feline leukocyte antigen (FLA) systems in the species under discussion. This would suggest that specific dog breeds have genetically determined immune function, and recent studies concern breed-specific serological response patterns to vaccination. Such genetic background is also likely to impinge on maturation of the immune system in these species [36]. In dogs, C-reactive protein (CRP) is the main acute phase protein, and its levels increase about a hundred times in infectious diseases such as leishmaniasis, babesiosis, parvovirus and colibacillosis. Acute phase protein levels increase moderately in canine inflammatory bowel disease. Concentrations of CRP, haptoglobin, and Serum amyloid A (SAA) protein (apolipoproteins associated with high-density lipoprotein/ HDL in plasma) are significantly elevated in the cerebrospinal fluid and serum of dogs with corticosteroid-responsive arthritis and meningitis. In pregnant dogs, the levels of haptoglobin, ceruloplasmin and fibrinogen are moderately higher [36]. Some studies showed, dogs in the asymptomatic and symptomatic groups with an outcome of heterogeneity in Cu, Zn, and Fe concentrations compared with the control group, emphasize the important roles of trace elements (TEs) in leishmaniasis [40]. Suggesting, TEs could be assessed as a prognosis factor in leishmaniasis, and/or an adjuvant for the treatment of leishmaniasis [41]. Susceptible dogs mount a Th2 response characterized by high levels of antibodies but showing a poor cell-mediated immunity [31]. These differences were attributed to the activities of IL-10-producing Treg lymphocytes [31]. In addition, also is consensus that the parasite can actively suppress IL-12 gene transcription, ensuring that the response Th2 predominates [31]. In that connection, a chronic and progressive disease develops in susceptible dogs as well as the macrophages loaded with parasites accumulate, with continuous multiplication in the organism, spreading throughout the body, and resulting in disseminated infection [31]. But, despite their antigenicity, parasitic protozoa manage to survive in their host using multiple evasion mechanisms acquired over many millions of years of co-evolution [1–31]. Looking to block these evasion mechanisms many studies had focused on immune expected effects of vaccines for canine leishmaniosis and some steps for the next-generation therapies following *L*EVs research advances. At this moment effective vaccines are available against canine leishmaniasis. Those that are considered the most effective use purified fractions of *Leishmania*, including enriched fraction of glycoproteins, also called fucose-mannose ligands [31]. This vaccine not only prevents the development of the disease, but also serves as an immunotherapeutic agent, producing clinical improvement in dogs with disseminated disease [31]. An alternative vaccine containing excretory and secretory products of *L. infantum* promastigotes with an adjuvant Muramyl dipeptide also seems to work well. Experimental vaccines, including attenuated and ADN have shown promising results for Veterinary Medicine [31]. For additional *Leishmania* control mechanisms and tools are needed, including new drugs, vaccines, diagnostics, and vector control agents and strategies [1–42]. Considering these important points, for the future of prevention and treatment of leishmaniases we can include all advances resulting from the *L*EVs research in the world that represent variety of advantages over live biotherapeutics (next-generation therapies) [42]. Over the past years, isolation and analysis of extracellular vesicles from *Leishmania* is challenging. The protocols are not standardized yet, like we refer in our Guidelines for exosomal research published by Gabriel et al. 2021 [1–42], as many protocols with potential effect on the outcome are used and published. It is

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important to refer that several protocols are labor-intensive, requiring costly equipment, increase risks for loss of heterogeneous extracellular vesicles and do not discriminate well between exosomes and contaminating structures such as lager vesicles and protein/lipid aggregates [43]. We referred also that direct isolation performed with magnetic beads for multiomic analysis (size, concentration, and phenotype by Spectradyne particle analysis, western blot analysis, LC-MS analysis, and flow cytometry). can be used for *L*EVs research, requiring minimal hands-on activities and provides highly pure exosomes with minimal loss of material. It also enables future automation opportunities in different formats and others simple, rapid, and reliable bead-based exosome isolation methods based on the strong anion exchange (SAX) principle, using both automated KingFisher (for rapid and efficient isolation of exosomes is compatible with the KingFisher Duo Prime, Flex, and Apex systems) and manual protocols improved in the last years in the Ketil Winther Pedersen's Lab (Figure 8) [44].

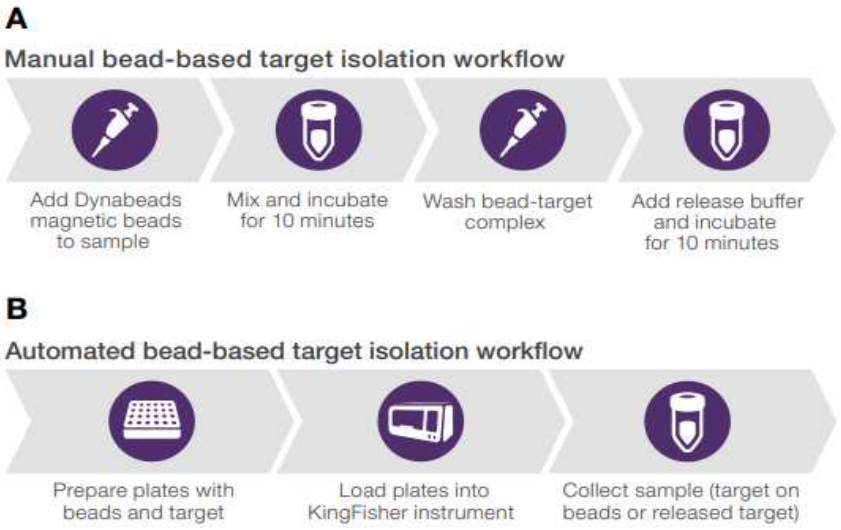

Figure 8. Exosome isolation workflow using a manual (**A**) or automated (**B**) method can be used for all *in vitro* and *in vivo* extracellular vesicles researches to highlight its biogenesis, composition and molecular interactions with host immune system [44].

To this end, multidisciplinary expertise cooperation for multiomic exosome research is very important to further our understanding of the *Leishmania*-host-cell interactions, a broad-scale analysis of the cargo of their production of extracellular molecules and create very promising perspectives for the development of innovative applications following the *Leishmania* virulence factors that include lipophosphoglycan (LPG), surface acid proteinase (GP63), glycoinositolphospholipids (GIPLs), proteophosphoglycan (PPG), A2 protein, the kinetoplastid membrane protein (KMP-11), nucleotidases, heat-shock proteins (HSPs), and transmembrane transporters, which support the survival and propagation of the parasite in the host cell [1]. Following the findings of expertise development, we propose for future laboratorial research to rapid bead-based isolation of *L*EVs for multiomic research to consider the techniques of manual bead-based target isolation and automated bead-based target isolation workflows to obtain *L*EVs isolation within 10 minutes using Invitrogen™ Dynabeads™, reinforcing our considerations of proposed guidelines for extracellular vesicles research *in vitro* and also *in vivo* from animal or human samples: from prevention, to diagnosis, prognostic and therapeutic [1–44]. In line, we can confirm the importance of emerging focus on lipids in *Leishmania* extracellular vesicles for the future studies, according to the evidence of our results. Thus, extracellular vesicles contain a lipid bilayer membrane that protects the encapsulated material, such as proteins, nucleic acids, lipids and metabolites, from the extracellular environment. These vesicles are released from cells via different mechanisms following to sense the lysosome-specific environment (pH and host temperature). During recent years extracellular vesicles have been studied as possible biomarkers for different diseases, as biological nanoparticles for drug delivery, and in

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basic studies as a tool to understand the structure of biological membranes and the mechanisms involved in vesicular trafficking. Lipids are essential molecular components of extracellular vesicles and essential in Apicomplexa parasites, but now our knowledge about the lipid composition and the function of lipids in these vesicles is limited (Appendix C) [45,46]. However, the interest of the research community in these molecules is increasing as their role in extracellular vesicles formation. Following the reproducible results for lipid bodies of *Leishmania* labeled with BODIPY™ 493/503 we believe that the crucial 72 hours period has showed clear decrease of lipids released. This regression is in accord with Zhang (2021) in the way that amastigotes acquire most of their lipids from the host although they retain some capacity for de novo synthesis, differently of promastigotes that rely on de novo synthesis to produce most of their lipids including glycerophospholipids, sterols and sphingolipids [45]. We can consider that lipid metabolism is of crucial importance for *Leishmania*, and changes to plasma membrane fluidity can be a new focus of cell-cell communication research between parasites and hosts [46]. Thus, we can consider some factors will be linked with these changes: the length of the fatty acid tail; the length of the fatty acid tail impacts the fluidity of the membrane; temperature; cholesterol content of the bilayer. And the degree of saturation of fatty acids tails [47]. Therefore, new protocols are needed to achieve a better efficacy in the prevention and clinical treatment, and they can include the relation of lipids released from parasites [1]. Different types of laboratory tests are available to diagnose parasitic infections, like conventional methods considered as gold standards and serological [48–50]. Several molecular diagnostic tools to detect parasites and new strains have been developed in the last decades [48–50]. Accurate diagnosis of zoonotic infections collaborates with the work of medical scientists, policy makers and public health officials planning to prevent the dissemination of these diseases and establishing a world-wide network of surveillance for the coinfection of parasitic infections [49,50]. Advanced techniques for the study and diagnosis of simultaneous infections are indicating that multi-parasitism is more common than single infections [51,52]. Evolutionarily, multi-parasite systems are ecologically dynamic, they involve key host species within multi-host parasite systems and their contribution to transmission [52]. The understanding of these complex relations is one of the highest priorities for biomedical sciences for the 21st century [53]. Clinically, coinfection of zoonotic parasites in companion animals may appear in its classical presentation, with acute aggressive evaluation or coming at long-standing infection, asymptomatic or sometimes non-specific, what difficulty the clinical diagnosis [52–54]. Veterinarians and clinical researchers should consider the health status and background of the patients (animals or humans) to apply a better parasite management program [53]. Considering certain factors like resource-mediated processes most often influencing how, where and which co-infecting parasites interact, and may dictate more intensive monitoring for effective treatment, while others may suggest a less aggressive approach [53–56]. Furthermore, innovative strategies applying the knowledge about extracellular vesicles in their specific profiles (e.g., proteic and lipidic data basis), on the studies of host-parasites mechanisms can be incorporated into immunotherapy to interfere with the dynamics of disease transmission and progression and the development of effective, safe and available vaccines against leishmaniasis in helping to protect puppies and dogs of different ages. 4 vaccines against canine leishmaniasis are available on the market, Leishmine® and Leish-Tec® in Brazil, CaniLeish® and LetiFend® in Europe (the first vaccine based on purified excreted//secreted antigens of *Leishmania* has been licensed in Europe since 2011) [1–57]. Challenges with these vaccines include current manufacturer recommendations which require the vaccination of seronegative dogs. In countries where disease is endemic in both dogs and people, identification of healthy uninfected animals is less than 100% accurate due to challenges with current diagnostics. Adverse events were mild and site specific, so use of vaccines in healthy sub-clinical dogs may warrant a change in current recommendations regarding vaccination/immunotherapy in infected healthy animals [35]. Despite the available studies on licensed vaccines for canine leishmaniasis, they are still considered insufficient, given the lack of standardization of the study design, methodological deficiencies and substantial differences in the characteristics of the study populations are some of the issues that impede comparative analysis between the available vaccines. In addition, research is needed on other

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aspects of vaccination: xenodiagnostic studies to assess the infectivity of vaccinated and infected dogs and an adequate assessment of the potential interference of vaccination in the diagnosis of *Leishmania* infection are some examples. In addition, long-term pharmacological surveillance should be maintained after licensing any vaccine to provide reliable information to relevant organizations and the public [57]. In this way are expectable, like we had indicated in previous publication of Guidelines for Exosomal Research, that more advances in the techniques and protocols for accuracy of isolation and characterization of *L*EVs and their activity on the host immune responses, including their lipid bilayer membrane that protects the encapsulated material, such lipids (essential molecular components of extracellular vesicles), from the extracellular environment. The knowledge about the lipid composition and the function of lipids in *L*EVs is very limited. Changes in the lipid profile and metabolism in both parasite and host during development of the disease depend on the presence of lipid bodies. Further *in vitro* and *in vivo* research are required to fully understand the relationship between the interactions between lipid metabolism of host and parasite, immune response, the prognosis of the disease and for the advances on the prevention and therapeutic of Leishmaniases [58].

5. Conclusions

According to WHO One Health is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals and ecosystems. There is a concern thoroughly documented relating to the close contact between companion animals and people with well-known positive aspects of human-animal interactions. However, in due time, put forward new hypothesis, and new models as well as reformulate aspects of analytic techniques, and claim that it is an open field to future research in favor of solutions for public health front the emerging/reemerging diseases. In this context, despite many advances, e.g., we continue fighting to find human vaccines against *Leishmania* and others neglected diseases. Considering that companion animals carry the risk of transmitting zoonoses to humans, multidisciplinary contributions between medical scientists and veterinarians able to accurately diagnose (e.g., molecular nano diagnostics) of zoonotic protozoal and helminth infections are necessary, prevention efforts may include a world-wide network of surveillance for the coinfection in continuous adaptation in front of global environment changes. Unfortunately, *Leishmania*-infected dogs continue to be parasite reservoirs for sandfly vectors, and co-factors can improve the risk of coinfection of *Leishmania* with other pathogens. Thus, further studies focusing on the clinical diagnosis and epidemiological aspects of coinfection of zoonotic diseases in endemic/non-endemic regions are necessary for better control them in companion animals, as a source of infection is important and maybe underrated. Highlight crucial molecular mechanisms into this nano universe related with immunomodulation to understand how the extracellular vesicles of different agents of diseases interact with the host cells is urgent. Why should one scrutinize evidence of mechanisms extracellular vesicles in One Health? We propose one hypothesis we suggest for future opportunities to extend that search on comparative studies considering multi-infections' studies. Against the background, and considering lipids are essential in Apicomplexa parasites, we propose with our findings to enlarge the interest of the research community in lipids released by *Leishmania* as their role in host-parasites extracellular interactions, following advances expertise development of rapid bead-based isolation of *L*EVs for multiomic research using Invitrogen™ Dynabeads™, reinforcing our considerations of our previous proposed guidelines for *Leishmania* extracellular vesicles research *in vitro* and also *in vivo* from animal or human samples (e.g., infected tissues) and including the extracellular vesicles from the etiologic agent, host specific interactions, consequences for the health of hosts (e.g., asymptomatic host), their clinical and laboratory features on differential diagnosis, and find new approaches for nano targeted therapies and vaccines production.

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Figure 9. Homeless animals that involuntarily represent a hazard for public health: an open field for studies to accurate differential diagnosis in companion animals and highlight pathogen-host interactions and research gaps of extracellular vesicles in immunomodulation (Author's photos Á.M.G.).

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Appendix A

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Leishmaniasis affects humans, domestic companion animals (dogs and cats) and wild animals worldwide, involving reservoir and hosts such as rodents, marsupials, edentates, monkeys and wild canids [48–61]. However, many pathogens' species potentially threaten canine/feline health, while some dog and cat parasites are strictly associated to these animals, some zoonotic emerging infectious diseases can be transmitted naturally to humans [18–62]. More recent surveys reported from several countries in the world indicate that zoonotic parasites (*Leishmania* sp, *Babesia* sp, *Toxoplasma* sp, *Neospora* sp. and *Dirofilaria* sp.) are associated with infections and coinfections in companion animals [16–63]. Protozoa and helminths are two major groups of organisms acting as etiologic agents of animal and human diseases with variable severity, especially for immunocompromised hosts resistance [62–66]. Parasitic infections caused by protozoa and helminths infect predominantly puppies, kittens, geriatric, chronically sick or immune-compromised animals and perhaps pregnant animals [55]. Older dogs and cats are mainly immune after previous infections and seldom show symptoms, however, may still be a source of transmission of infection [16–55]. Companion animals living in crowded conditions and poor sanitation or with access to the outdoors, may have a high risk of direct transmission of protozoan infections (e.g., *Giardia* sp, *Trichomonas* sp, *Cryptosporidium* sp and *Cystoisospora* sp) [16–68]. The contact with infected rodents or ingest raw meat represent significative risk to these animals acquire infections caused by cyst-forming coccidia, i.e., *Neospora* sp, *Hammondia* sp, *Toxoplasma* sp and *Sarcocystis* sp [16–66]. Dogs and cats can also be infected with others gender and parasites species that release extracellular vesicles, like *Ancylostoma* sp *Angiostrongylus* sp [63–70]. Emerging infectious diseases may increase frequently in some regions, either due to increased importation of infected vertebrate hosts or by the establishment of pathogens and their vectors in previously non-endemic areas [56]. Cases of zoonotic infections such as leishmaniosis, babesiosis and dirofilariasis was detected in some non-endemic regions, mainly due to the expansion of the parasitic transmission area and their increasing occurrence in wildlife, which act as reservoir or hosts of their complex cycles [56–63]. Multi-species coinfections pose one of the greatest challenges to global health needing efforts to develop effective methods of infectious diseases control. Concurrently with the spread of leishmaniasis recently studies reveal that dogs and cats of endemic areas are at risk of acquiring coinfection with emerging zoonotic parasites. In this context, companion animals stand out as important hosts of *L. infantum, Babesia* sp., *Toxoplasma gondii*, *Neospora caninum* and *Dirofilaria immitis*. These parasites are responsible for infections, which are little known in the feline population in co-endemic areas, requiring more preventive attention of health authorities, research teams, veterinarians and companion animals' owners. Of course, considering how the world is going through important environmental changes, many other pathogens, including bacteria and virus, must be considered in future similar and complementary studies. In that Appendix, we propose to stimulate the scientific community to grow our research started by a rapid review of and continuing need for global control of simultaneous infection of *Leishmania* sp. and hemoparasites in companion animals. These results we had considered to support the laboratorial findings explored on case report of present study and to register the importance of comparative differential diagnosis (DDx) for future multidisciplinary research. That Appendix is a preliminary study applied for the doctoral thesis of the author Á.M.G. (medical veterinary and researcher of Medical Parasitology) considering the clinical experience of author, looking for: the coinfection prevalence in companion animals, outcomes of coinfection and superinfection with *Leishmania* and other hemoparasites of dogs and cats (Tables 7 and 8), suggesting that the evolutive history of hosts species and the causative agents of diseases must be explored in the future comparative studies of extracellular vesicles *in vitro* and *in vivo* to highlight its mechanisms and correlations and to demonstrate the importance of individual clinical studies for: the progression of disease (including the molecular immunomodulation), co-stimulatory and co-inhibitory pathways during coinfections, mechanisms of synergy in polymicrobial infections and the future of prevention and treatment [15–69]. For that complementary review of literature for the introduction of the present manuscript, we had considered the importance to suggest an upgrade study that currently show advances in the field of extracellular vesicles released by *Leishmania* and other zoonotic pathogens and their host immunomodulation under co-infection conditions to

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comparison purposes). Thus, the studies focused on the association between leishmaniasis and other parasitic diseases: *babesiosis, toxoplasmosis, neosporosis* and dirofilariasis in dogs and cats would highlight the interrelations and the evolutionary association of some pathogens and hosts like dogs and cats (companion animals), following the importance of the risk of zoonotic coinfections from companion animals. Further studies focusing on the clinical diagnosis, epidemiological aspects and the universe of released extracellular vesicles by pathogens during coinfection in endemic and non-endemic regions for better control the healthcare in companion animals, as a source of infection is important and maybe underrated. Moreover, multidisciplinary contributions between medical scientists and veterinarians able to accurately diagnose of zoonotic protozoal and helminth infections are necessary, prevention efforts may include a world-wide network of surveillance for the coinfection in continuous adaptation in front of global environment changes.

Table 7. Overview reports of coinfection between *Leishmania* sp. and hemoparasites of dogs.

Countries	Year of reports	Leishmaniasis cutaneous/ visceral	Other parasitic diseases	Diagnostic methods	N° of references
Brazil, Hungary, Italy, Spain, Portugal, Bulgaria	2013, 2014, 2015, 2016	*Leishmania* sp, a	babesiosis	IFAT, ELISA, PCR, DPP serological, qPCR	70, 71 *,72,73,74,75
Brazil, Iran, Albania	2011,2013,2014, 2015,2016	*Leishmania* sp, a, b	toxoplasmosis	IFAT, ELISA, PCR serological, parasitological	71,73,76,77,80,81
Brazil, Iran, Albania	2011, 2013, 2014, 2015	*Leishmania* sp a, b	neosporosis	IFAT, ELISA, PCR serological, parasitological	71,77,78,79,80,81,82
Hungary, Portugal, Albania	2015, 2016	*Leishmania* sp a	dirofilariosis	PCR serological	72,74,77,83

a) L. infantum, b) *L. Chagasi*. *Commonly differential diagnosis can also be difficult, and coinfection, particularly with *Babesia* sp or *Ehrlichia* species can occur.

Table 8. Overview reports of coinfection between *Leishmania* sp. and hemoparasites of cats.

Countries	Year of reports	Leishmaniasis cutaneous/ visceral	Other parasitic diseases	Diagnostic methods	N° of references
Spain	2012	*Leishmania* sp	babesiosis	seroreactivity, PCR	84 *
Brazil, Turkey**	2011, 2013, 2014, 2015	*Leishmania* sp a. b, c. d	toxoplasmosis	immunofluorescence serology, qPCR IFAT, MAT	81,85,86,87,88,89,90
Brazil	2011, 2014	*Leishmania* sp a	neosporosis	immunofluorescence	85,88
Portugal	2015	*Leishmania* sp	dirofilariosis	ELISA, DAT	91

a) L. infantum, b) *L. chagasi* c) *L. major* d) *L. Tropica*. *Commonly differential diagnosis can also be difficult, and coinfection, particularly with *Babesia* sp or *Ehrlichia* species can occur. **Coinfection of *L. major* and *L. tropica.*

Note. Multiple functional implications of extracellular vesicles in *Babesia*-host interactions support the potential that these vesicles have as agents in disease pathogenesis [92]. On the other hand, specific studies are necessary to full understanding of the molecular and genetic mechanisms by which *Ehrlichia* virulence factors induce differential regulation of host innate responses is thus of utmost importance [93]. In this context, the interaction with the secretion of vesicles by protozoa in coinfections and their specific actions on the immune system of

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different hosts are still keyless [1]. It is known that orphaned animals under varying stress conditions have a greater immunological susceptibility, which favors the development of infections and the ensuing complications, which include coinfections involving other pathogens [94].

Appendix B

Efficient and accurate diagnosis of canine diseases is of primary importance for veterinary clinical care (e.g., early detection of diseases, case confirmation and differential diagnosis with other infectious diseases), surveillance activities, outbreak control, pathogenesis, academic research, vaccine development, and clinical trials. Currently, the veterinarian can send animal samples for specialized laboratories to confirm the primary clinical suspicion. The present case report "Jimmy" didn't have clear symptoms of leishmaniasis and following that occurrence was necessary to apply its samples for laboratorial tests (Table 9).

Table 9. Laboratory findings from leishmaniotic dog with ocular infection.

Complete blood count - veterinary - Material: Whole blood in EDTA			
Value	**Results**	**Absolute Value**	**Reference**
Erythrocytes: millions/mm3	3,38 millions		Dogs 5,5- 8,5
Hemoglobin:	6,7 g/dl		Dogs 12,0- 18,0 g/dl
Haematocrit:	20%		Dogs 35-55%
Leukocytes: mm3	10.400/mm3		Dogs 6000 a 17000
Mean corpuscular volume (MCV):	58,1 fl		Dogs 60-70% fl
Mean Corpuscolar Hemoglobin (MCH):	19,8 pg		Dogs 19-23 pg
Mean corpuscular hemoglobin concentration (MCHM):	33,5 g/dl		Dogs 32-36 g/dl
Eosinophils:	4%	416/mm3	Dogs 2-10%
Basophils:	0%	0/mm³	Dogs 0%
Lymphocytes:	13%	1.352/mm³	Dogs 12-30%
Monocytes:	4%	416/mm³	Dogs 3-10%
Myelocytes:	0%	0/mm³	
Metamyelocytes:	0%	0/mm3	
Bands:	0%	0/mm³	Dogs 0-3%
Segmented:	79%	8.216/mm3	Dogs s 60-70%
Platelets: mm3	135.000%		Dogs 200 000 a 500 000
Method: Automated and Microscopic Analysis			
Note: ANISOCYTOSIS ++; HYPOCHROMIA ++			

ALKALINE PHOSPHATASE - VETERINARY	
Sample: SERUM Method: Dry Chemistry	
Result: 29,0 U/L	Reference value: Dogs 20 to 150 U/L

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URÉIA – VETERINÁRIO

Sample: SERUM Method: Dry Chemistry

Result: 29,0 MG/DL Reference value: Dogs 15 to 65 MG/DL

TOTAL PROTEINS AND FRACTIONS – VETERINARY

Sample: SERUM Method: Dry Chemistry

Total Proteins: 14,7 g/dl Reference value: Dogs 5,8 to 7,9 g/dl

Serum Albumin: 1,1 g/dl Reference value: Dogs 2,6 to 4 g/dl

Serum Globulin: 13,6 g/dl Reference value: Dogs 2,3 to 5,2 g/dl

CREATININE - VETERINARY

Sample: SERUM Method: Dry Chemistry

Result: 0,6 mg/dl Reference value: Dogs o 0,5 to 1,5 MG/DL

Method: Dry Chemistry

SERUM GLUTAMATE PYRUVATE TRANSAMINASE (SGPT)/ALANINE AMINOTRANSFERASE (ALT) - VETERINARY

Sample: SERUM Result:28,0 U/L Reference value: Dogs 10-88 U/L

CANINE LEISHMANIASIS (ELISA and IFAT)

METHOD: Enzyme-linked Immunosorbent Assays (ELISA)

Result: Reagent CUT OFF: 0214 Value of OD*: 0,664

Kit with License in the Ministry of Agriculture of Brazil – MAPA

Number: 7.434/2000, Batch control 004/18, Validity: 02/2019

METHOD: Indirect Fluorescent Antibody Test (IFAT)

RESULT: REAGENT 1/80

Kit with License in the Ministry of Agriculture of Brazil - MAPA

Number: 9347/2007, Batch control 228, Validity: 07/2019

Reference value: ELISA: NON-REAGENT: Optical Density with value below the Cut off. INDETERMINATE: Result with intermediate values, corresponding to the Gray Zone, where the Tests were not able to determine whether it is Reagent or Non-Regent. To determine the amplitude, the cutoff point 0.03 is subtracted. A new test is recommended 30 days after the last test, as it may correspond to the onset of serum conversion, nonspecific reactions, or immune system failure.

REAGENT: *Optical Density with value above the Cut off. RIFI: NON-REAGENT: Results without antibody titers. REAGENT: Result with a titre equal to or greater than dilution 1/40.

Appendix C

Lipid bodies (LBs) are intracellular accumulations of neutral lipids surrounded by a single membrane. These organelles are involved in the production of eicosanoids, which modulate immunity by either promoting or dampening inflammatory responses. *L. infantum*, the etiological agent of visceral leishmaniasis in Brazil, is an intracellular parasite that causes disease by suppressing macrophage microbicidal responses. C57BL/6 mouse bone marrow-derived macrophages infected with *L. infantum* strain LcJ had higher numbers of LB+ cells and total LBs than noninfected cultures. Large (>3 μm) LBs were present inside parasitophorous vacuoles (PVs). These results contrast with those of *L. infantum*-infected BALB/c macrophages, in which the only LBs are derived from parasite, not macrophage origin. Increased LBs in C57BL/6 macrophages in close association with parasites would position host LBs where they could modulate *L. infantum* infection [95]. These results imply a potential influence of the host genetics on the role of LBs in host-pathogen interactions. Overall, our data support a model in which the expression, and the role of LBs upon infection, ultimately depends on the specific combination of host-pathogen interactions [95]. In some recent findings, *Leishmania* uses the host's cholesterol to ensure macrophage phagocytosis and evade immune response [58]. Additionally, the host's lipid bodies have key roles in disease progression and development of the parasite inside the cell. This induces changes in the patient's serum lipid profile like hypertriglyceridemia and low HDL levels [58]. We had tried highlight the association between the title and the objectives and methodologies used. We had evaluated the findings making comparative conclusions both clinical and in vitro models with extracellular vesicles during the last 8 years during the doctorated research for the next publication and some inserts we had apply for this paper and contributing for the MISEV2023 will be published in few weeks. The auto control of lipid bodies production in promastigotes of *L. (L.) amazonensis* we consider its relevance and discuss in the discussion, its one point we continue in parallel research to publish others results – now is convenient to call the interest of researchers for the study of lipids and not just of proteic compounds. The tubular structures, vesicles in macrophages and infected tissues of mouse infected with *L. (L.) amazonensis* have been useful model for many studies to show relationship with extracellular vesicles and other mammals. In the review, we did include studies conducted on dogs and cats (companion animals) and other parasitic diseases to call the interest of research to comparative analysis of co action of extracellular vesicles of different agents of diseases for future studies.

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