Deciphering *Leishmania*: Bridging Molecular Biology and Serology for Enhanced Diagnostic Precision

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2023/2024

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Internship report carried out as part of the master's degree in Cellular and Molecular Biology

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2023/2024

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Resumo

Leishmania é um protozoário parasitário que infecta humanos e animais em todo o mundo. A leishmaniose é classificada como uma Doença Tropical Negligenciada (DTN) e seu diagnóstico constitui um grande desafio. Portanto, a deteção e diagnóstico eficientes são importantes para o controlo e tratamento da doença, sendo a PCR quantitativa (qPCR) muito sensível e específica. No entanto, a sua eficiência depende de fatores como a qualidade do DNA e o kit de PCR em tempo real utilizado.

Este estudo analisou 49 amostras caninas divididas por três matrizes, sangue, soro e medula óssea para detetar *Leishmania* spp. usando diferentes técnicas serológicas e moleculares. Técnicas serológicas como ensaio imunoenzimático (ELISA) e teste de imunofluorescência (IFAT) apresentaram variabilidade na deteção de anticorpos, enquanto os "kits" de extração de DNA mostraram algumas diferençasde desempenho. Os melhores valores de pureza foram observados com o "kit" daPromega, enquanto o kit MagMax mostrou um valor maior de amostras positivas. Além disso, o fato de o sangue conter maior qualidade e concentraçãode DNA do que o soro refletiu num valor de deteção mais elevado.

Este estudo enfatiza a importância da escolha adequada de "kits" de extração e qPCR, e sugere melhorias nos protocolos de extração para obter resultados mais fiáveis.

Palavras-chave: Doença transmitida por vetores; Ensaio imunoenzimático; Extração de DNA; Leishmaniosis; *Leishmania* spp.*;* One Health; PCR em tempo real; serologia; soro; Zoonose.

Abstract

Leishmania is a parasitic protozoan that infects humans and animals worldwide. Leishmaniosis is classified as a Neglected Tropical Disease (NTD), and its diagnosis constitutes a great challenge. So, efficient detection and diagnosis are important for disease control and treatment, with qPCR being very sensitive and specific. However, his efficiency depends on factors, such as DNA quality and the qPCR kit used.

This study analysed 49 canine samples divided by three matrices, serum, blood, and bone marrowto detect *Leishmania* spp. using different serological and molecular techniques. Serological techniques like enzyme-linked immunosorbent assay and immunofluorescence antibody test (ELISA and IFAT) presented variability in antibody detection, while DNA extraction kits showed some differences in performance. The Promega kit had the best purity values, whereas the MagMax kit showed a higher value of positive samples. Also, the fact that blood contains more DNA quality and concentration than serum was reflected in a higherdetection value.

This study emphasises the importance of appropriate extraction and qPCR kits and suggests improvements in extraction protocols to have more reliable results.

Keywords: DNA extraction; Enzyme-linked immunosorbent assay; *Leishmania* spp.; Leishmaniosis*;* One Health; Real-time PCR; serology; serum; Vector-Borne Disease; Zoonosis.

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1. Introduction

Leishmania is a parasitic protozoan belonging to the family *Trypanosomatidae* and genus *Leishmania*, which can infect humans and animals [1]. The genus is dimorphic, possessing two principal forms: the amastigote form is intracellular and found in the vertebrate host, which is the flagellated form present in the vector [1]. It is transmitted mainly through the bite of female phlebotomine sand fly vectors [2]. Macrophages and neutrophils take up promastigotes. They then differentiate into amastigote forms, rupturing the macrophages to infect the surrounding macrophages. The life cycle of the *Leishmania* parasites is shown in Figure 1. They possess a kinetoplast, a distinctive form of mitochondrial DNA [3]. Leishmaniosis is an infectious disease affecting humans and animals worldwide in temperate, subtropical, and tropical regions. It can occur in three forms: Visceral Leishmaniosis (VL), Cutaneous Leishmaniosis (CL), and Mucocutaneous Leishmaniosis (MCL) [1,3]. VL is considered the most severe and essential form out of these three, in humans and dogs because dogs are themain reservoir of *Leishmania* spp*.,* developing Canine Leishmaniosis (CanL) andcan be a risk to human infection, being considered fatal to both [1,4]. Human andanimal diseases associated with these pathogens have great significance for theWorld Health Organization (WHO), as they are included in the 2030 agenda for the elimination of neglected tropical diseases (NTDs) [5].

1.1. Biological cycle of *Leishmania* spp*.*

As mentioned earlier, *Leishmania* spp. has two forms: an amastigote form found in the mammalian host and a promastigote form found in the vector [6]. When the female sand fly bites a mammalian host, the infection begins. When the parasites reach the skin, macrophages are induced to phagocytose the metacyclic promastigotes [7]. Once inside these cells, promastigotes turn into amastigotes

inside the parasitophorous vacuoles, which multiply by binary division and infect other cells [8]. When other sand flies feed on infected blood from the reservoir, they are infected by macrophages with amastigotes. Inside the gut of sand flies, amastigotes turn into procyclic promastigotes. In dogs, unlike other mammals, *Leishmania* infects the blood, lymph, and other organs [9]. Figure 1 illustrates the cycle of the *Leishmania* parasites.

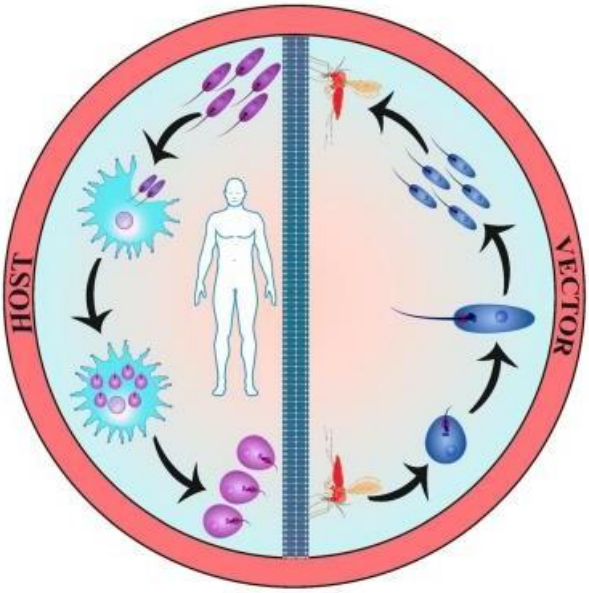

Figure 1. Life Cycle of *Leishmania* spp.

1.2. Geographic Distribution and Risk Factors

According to the World Health Organization (WHO), it is estimated that 50000 to 90000 new cases of VL occur worldwide, mainly in Brazil, East Africa and India. On the other hand, CL cases occur in the Americas, Mediterranean basin, Middle East and Central Asia with 600000 to 1000000 cases estimated to occur worldwide. Finally, 90% of MCL cases occurred in Bolivia, Brazil, Ethiopia and Peru. In Figure 2, we can see the countries where VL and CL are most affected. These countries are endemic; in other words, *Leishmania* cases occur regularly. However, certain risk factors may increase the chance of sporadic cases of *Leishmania* in certain countries. For example, socioeconomic conditions such as

poor sanitary conditions may increase sandfly breeding. In addition, diets lacking specific important body components (protein, iron, etc.) may increase the risk. Lastly, population mobility and climate change are connected because climate change affects the spread of leishmaniosis but also tends to make people move to other places where the transmission is higher.

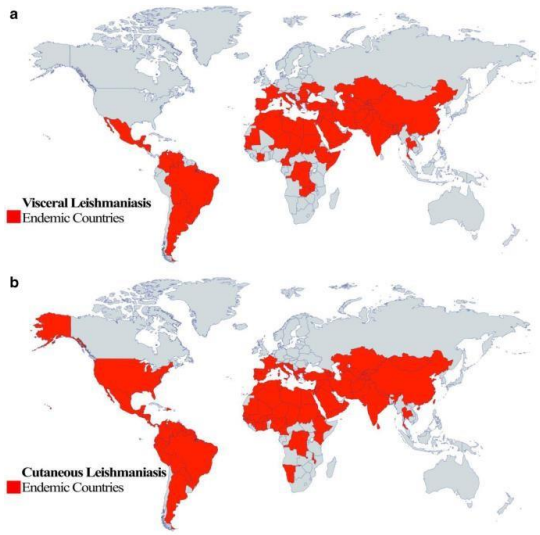

Figure 2. Red indicates where these two forms of leishmaniosis appear.

1.3. Principal Ways of Transmission

The most common type of transmission of Canine *Leishmania* is the bite of female phlebotomine sandflies. However, there is evidence of *Leishmania* infection in regions where phlebotomine sandflies do not exist; therefore, some articles report that sexual and transplacental transmission can occur in mammalsand humans [10]. Sexual transmission happens more often when the male dogis infected. Vertical or transplacental transmission occurs when dogs have genitallesions from Leishmaniosis; posteriorly, the newborn puppies are infected [10]. Also, direct transmission from dog to dog through bites or wounds is suspected, as well as transmission through blood transfusion, organ transplantation, and

contaminated objects [10,11]. Suspicions about Leishmaniosis transmission by other arthropods, like fleas, were also reported [12]. These transmission ways are crucial in areas where the phlebotomines are absent [13].

1.4. Biological Matrix

The decision to use blood, serum, or bone marrow samples depends on the intended purpose. However, laboratories typically prefer blood samples. The reason for this preference is that blood contains more specific components compared to serum. Additionally, blood contains nucleated cells, such as white blood cells, which contain DNA. [14]. Serum is a liquid derived from blood that clots, so it does not have cellular components or clotting factors, and it has much less DNA when compared to blood samples; the extraction needs an extra step, which is DNA enrichment, to concentrate the DNA on the bottom. Therefore, laboratories often utilise blood samples to detect potential diseases. However, bone marrow is the optimal matrix for detecting *Leishmania* due to its high sensitivity and elevated parasitic load. Despite this, bone marrow is not commonly used in laboratories because the procedure is invasive for the dog. [15].

1.5. Clinical Signs and Diagnosis

The clinical signs associated with this disease can vary widely, from subclinical (no noticeable symptoms) to a severe, life-threatening infection that can affect multiple organs, including the skin, kidneys, spleen, liver, and eyes. Some common clinical signs include skin and ocular lesions, weight loss, muscle atrophy, exercise intolerance, signs of renal failure, lymphadenomegaly, and many others [1,16]. Cats also develop a clinical disease, Feline Leishmaniosis (FeL), and may potentially have dermal and visceral manifestations [1]. In addition, the percentage of carriers in endemic zones is far higherthan that of symptomatic carriers [2,17].

As a fatal disease, prompt and accurate diagnosis in humans, dogs, and cats is crucial for determining appropriate treatment. Diagnosis takes into account the epidemiological origin and the range of clinical signs. However, many humans, dogs, and cats may be asymptomatic, necessitating laboratory confirmation. Various diagnostic techniques are employed, including parasitological, immunological, and molecular methods [4,16,18].

Parasitological methods were the *gold standard* for detecting leishmaniosis and were influential in eco-epidemiological studies [3]. Parasite identification by isolation on a specific culture medium, followed by microscopic examination of tissue biopsy specimens, bone marrow, blood, or splenic aspirates, is important to help the diagnostic approach. To improve the problems of parasitological methods, immunological methods were developed and based on the presence of specific humoral responses [19]. These antibody detection methods have been developed to diagnose leishmaniosis using the interaction between hosts and parasites [3]. Some of these methods were ELISA and IFAT. They have been used for Leishmaniosis diagnosis. However, serological tests have critical limitations, for example, cross-reactions with *Trypanosoma* parasites, cutaneous leishmaniosis species, and other hemoparasites, as well as low sensitivity and specificity that may lead to inconsistent results [4,16,20]. So, it was necessary to develop different techniques to reach higher levels of sensitivity and specificity. Therefore, there has been an increase in the use of molecular techniques, such as qPCR, due to their high sensitivity and specificity, and they can estimate the parasite load in addition to monitoring treatment efficacy [1,4].

1.6. Diagnosis

1.6.1. Parasitological

This method of diagnosis was the *gold standard* for diagnosing leishmaniosis, but nowadays there is no widely accepted *gold standard* [21]. When using this method, bone marrow or splenic aspirate samples are the most used ones, but

other biological matrices can also be used. For this type of diagnosis, a microscope with direct light is needed for direct visualisation of the amastigotes in Giemsa-stained smears [22]. *Leishmania* amastigotes are commonly observed within macrophages and have a blue cytoplasm, red nucleus, and purple-pink-stained kinetoplasts [23]. This method is used mainly because of the low cost, simplicity of procedures, and ability to identify parasites at the genus level [22]. Sensitivity and specificity can vary significantly, but generally, sensitivity ranges from 54% to 100%, while specificity is approximately 100%, particularly in spleen and lymph node aspirate samples [21,22,24]. However, it all depends on the operator's expertise and the quality of the smears [21,22,24].

Another method is in vitro isolation of *Leishmania* parasites, where conditions must be created to turn amastigotes into promastigotes. Here, we found a technique with high specificity and sensitivity that requires sterile conditions and is too complicated [25].

1.6.2. Serological

CanL is typically diagnosed by serologic methods since parasitological methods have some limitations [3,26]. There are several serologic tests on the market, but we only mention two of them: the IFAT and ELISA [24]. They work by detecting the presence of anti-leishmanial antibodies, so these techniques are more often used to detect VL. However, it is challenging to detect CL because of low sensitivity and poor humoral response [25]. IFAT test is considered the reference technique by the World Organization for Animal Health (WOAH) to detect anti-leishmanial antibodies or antigens using the promastigotes [27]. This test utilises a fluorescent marker attached to an antibody, forming a reporter molecule that is easy to measure and bind to a target molecule with high specificity [3]. The primary benefit of IFAT lies in its capability to distinguish between true positives and false positives [24]. According to a report, this method presents sensitivity values between 28.4% and 92% and specificity between 83.3% and 94.4% [24]. The ELISA test uses the same principle as the IFAT test, and it is one of the most

sensitive and specific methods for detecting *Leishmania* [27]. However, the sensitivity can vary depending on the antigen used [24,28]. One study showed that rK39 was a better antigen than rK26 and rK9 [28]. This permits the detection of small quantities of antigens in a sample. The sensitivity and specificity of ELISA-rk39 are approximately 88-99% and 81-92%, respectively. The major problem is cross-contamination with *Trypanosoma* parasites and *Leishmania* species.

1.6.3. Molecular

Molecular methods have been used to improve the sensitivity and specificity and overcome parasitological and serological problems, such as qPCR and Multilocus sequence typing (MLST). These techniques are fast and can be usedon different matrices, such as bone marrow aspirates, blood, urine, spleen, and conjunctival swabs, to detect the presence of *Leishmania* spp. [29-32]. In addition, they have a low risk of contamination [3]. Over the last decade, various real-time PCR assays have been developed to identify appropriate tools for detecting different *Leishmania* species. Real-time PCR enables themeasurement of nucleic acid template quantities by assessing PCR kinetics throughout the amplification cycles, allowing for rapid DNA quantification througha calibration curve [33,34]. TaqMan technology involves a probe with a fluorescent dye at one end and a quencher dye at the other. The probe will bind to a specific sequence in the target DNA. DNA strands are separated with heat, then the temperature is lowered to permit primers and the probe to bind to the DNA. Along the process, the Taq polymerase enzyme extends the DNA strands,and when it encounters the probe, it begins to hydrolyse. This process emits a fluorescence signal [22,35]. This method is more effective in confirming a diagnosis of CanL, especially when serology results are inconclusive, such as incases where the dog has not yet been seroconverted [36, 37]. However, qPCR has disadvantages, such as the slightly elevated cost and the fact that a positiveresult doesn't necessarily indicate the existence of an infection [35]. According to

a report, this technique showed a sensitivity of 91.2% compared to 50.6% on microscopy for VL [38]. Another study showed a similar sensitivity and 100% specificity [39]. Sometimes, a combination of techniques, such as PCR and ELISA, could help diagnose inconclusive cases that exhibit low antibody titters [40].

1.7. Treatment and Prevention of *Leishmania* Infection

As there are many species of *Leishmania*, treatment is a difficult task. The treatment of leishmaniosis in infected dogs and cats is prolonged and is frequently successful if the dog or cat is not in a progressive stage. Long-term monitoring is needed to evaluate if there is no relapse if the disease does not progress, or if the treatment has side effects. The most commonly used drug is allopurinol, which acts by interfering with the purine pathway and parasite RNA synthesis [16]. It has low toxicity and can be used in combination with other drugs. Antimonial compounds are also used as monotherapies; however, they are morecommonly combined with allopurinol. Since these compounds were introduced in1945, some reports have started to mention drug-resistant strains [41]. Over the years, an increasing number of drugs have been introduced, including miltefosine and meglumine antimoniate. The first was the first oral drug to interfere with lipid metabolism. It can also be used alone or with other medications [42]. The second is an antimonial compound that inhibits the enzyme activity necessary for the parasite's survival and replication. In humans, chemotherapy is the primary treatment, and no vaccine is available against *Leishmania* spp [43].

In the past, to control the spread of leishmaniosis, government policies in some South American countries resulted in the euthanasia of millions of dogs [44]. However, a more effective approach to preventing this disease from progressing or even to prevent the host from catching *Leishmania* must be taken, aiming to control sandflies or using insecticides that repel sandfly bites, such as permethrin, which leads to an anti-feeding and knockdown effect in sandflies and an 84%

protective effect in dogs [43,45]. Unlike humans, there are vaccines against leishmaniosis in dogs that reduce the risk of disease development [44]. The use of vaccines may be the best way to control infections. There are two ways of prevention: direct methods aimed at infectious dogs and indirect methods aimed at the vectors. In direct control methods, infectious dogs can be removed by euthanasia, which is always an ethical debate because the dog can be a false positive for example, and is not accepted in developed countries. As explained before, treating the dog can be a way to help the animals and should be the first option to prevail.

For indirect control methods, repellent tools such as collars with deltamethrin, spot-on with permethrin and imidacloprid, and spray with permethrin and pyriproxyfen demonstrate good performance against sandflies [44].

These tools prevent sand flies from feeding on dogs by repelling or killing the flies. [46]. According to previous studies, collars protect between 70% and above90% [46]. Spot-on has much higher protection, between 88% and 98%, and the spray technique showed values of approximately 71% [45,47].

1.8. *Leishmania*-Host interaction

1.8.1. Cellular Invasion and Infection

This interaction is not fully understood; however, it is known that the parasite can modulate some mechanisms to be more dangerous. However, to weaken the host, *Leishmania* promastigotes must enter the host. Once they enter, a process called metallocyclogenesis occurs, in which procyclic promastigotes turn to metacyclic promastigotes. Here, the mechanisms of the host, such asneutrophils, cytokines, and chemokines, begin the response to infection. As previously mentioned, *Leishmania* spp. can eliminate these mechanisms and parasite neutrophils phagocytised by macrophages. Subsequently, the infection was successfully performed [48,49].

1.8.2. Host immune response to *Leishmania* infection

As we already know, *Leishmania* parasites have two forms: an extracellular promastigote that lives in the sandflies and an intracellular amastigote form that colonises host macrophages. After colonisation, *Leishmania* parasites develop structures called parasitophorous vacuoles, where they grow and replicate, and delay cytotoxic responses [50]. Various host immune responses triggered by *Leishmania* parasites are responsible for different levels of disease severity. Macrophages and neutrophils are considered the primary cells that react to *Leishmania* infection, but several other immune cells, such as monocytes, dendritic cells (DCs), natural killer (NK) cells, CD4+ and CD8+ T cells, and effector molecules, such as cytokines [51]. The T helper cell (Th1) cytokine response leads to a classical macrophage activation profile (M1), characterised by the secretion of high levels of proinflammatory cytokines such as Interleukin-2, Immune interferon-y, and tumour Necrosis Factor-Alpha [51-53]. Macrophages are activated by IFN-γ and TNF-α, intensifying their defence mechanisms, including the production of nitrous oxide and reactive oxygen species (ROS) [54].

On the other hand, the T helper cell (Th2) cytokine response leads to an alternative macrophage activation profile (M2), characterised by inhibition of proinflammatory signals by interleukin-4 and interleukin-10 and a subsequent increase in parasite numbers and disease progression [51-53]. A previous study showed that a predominant Th1 cytokine response and M1 macrophage activation make mice resistant to infections. In contrast, other mice with Th2 cytokine and M2 macrophage activation profiles showed a susceptible phenotype that increased parasite growth [55].

Although the cellular immune response is the best defence against *Leishmania* spp., the humoral response also plays a crucial role. During infection, antibodies against antigens are triggered, especially the immunoglobulin G type; however, it is produced by other antibody types, such as IgM, IgE, and IgA [56]. These antibodies are produced by B cells that are activated by contact with antigens

[56]. IgM is detected in the initial stages of infection and activates the complement system. IgG is important for facilitating the phagocytosis of *Leishmania* by neutrophils and macrophages using a process called opsonisation [57]. IgA and IgE can contribute to defence against parasites. In addition, antibodies can inhibit the capacity of pathogens to infect the host cells. However, the efficiency of the humoral response is limited because the *Leishmania* parasites are intracellular. They can also influence the production of cytokines and affect the polarisation of T-cells [54]. For example, the production of IgG antibodies correlates with the immune response of Th1 and Th2 cells. High levels of IgG are related to the secretion of Th2 cytokines, whereas low levels of IgG are related to the secretion of Th1 cytokines [58].

1.9. Co-infections

Co-infections are also a significant concern because leishmaniosis and Human Immunodeficiency Virus (HIV) co-infection normally appear in the same areas of transmission [59]. Both diseases target the same immune cells, worsening their effects on the immune system [3]. HIV infects cells involved in host defence and weakens the immune system, making individuals more susceptible to infections such as leishmaniosis [60]. An elevated viral load accelerates the progression to AIDS and shortens the lifespan of individuals with HIV. Furthermore, HIV infection intensifies viral load, exacerbating the inadequacy of the CD4+ T-cell immune response, thereby worsening the severity of the disease [3]. In dogs, studies have shown that *Leishmania infantum* and *Ehrlichia canis* have a synergistic pathological effect that enhances clinical signs, has more haematological changes, and shows poor clinical improvement after treatment. Dogs with these two parasites alone showed fewer effects than other dogs [61, 62].

1.10. Problems in Portugal and the World

Using Portugal as an example, CanL is a concern, as already known, and is present throughout the country with a heterogeneous distribution. According to an article from 2012, where 3974 dogs from all districts were used, the seroprevalence of *Leishmania* in Portugal was 6,3% using only the Direct Agglutination Test (DAT) as a detection method [63]. Ten years later, another study was conducted to reassess the seroprevalence in Portugal, and the value doubled to 12,5%. DAT is again the chosen method [63-65]. This information leads us to conclude that *Leishmania* infection was neglected during this period, and it is vital to change and improve.

In Brazil and Iran, poor socioeconomic conditions, increased prevalence of leishmaniosis in the canine population, and dog ownership are risk factors for human leishmaniosis [66,67].

1.11. Objectives

The primary objective of this study was to establish a connection between molecular biology and serology and identify the most effective procedure for diagnosing *Leishmania* infections in humans and animals. Although there have been some studies with great results [1,3,4,16,20,68], the use of different extraction and PCR kits can yield greater results.

This study aimed to compare the testing techniques from these two study areas and assess their accuracy and reliability in detecting *Leishmania* infections. In addition, this study can be used to evaluate whether the results obtained in serum are as precise as those obtained from blood or bone marrow because, as previously mentioned, the low level of DNA in this matrix will be a challenge to

overcome. Another crucial aspect is the evaluation of the type of extraction that yields the best results in terms of the correlation with economic factors.

In this study, we preferred to use binding beads to extract DNA, but methanol extraction is the best method [31,32]. Methanol is typically used because of its high metabolite coverage and low cost. However, overly complex samples that hinder the detection of low-abundance metabolites can result from the broad specificity of this solvent [32].

Practical steps will involve conducting molecular biology and serological tests on diverse samples, including blood, bone marrow, and serum. Standardised protocols and meticulous documentation of the procedures are essential.

The results will be analysed quantitatively and qualitatively, enabling a comprehensive understanding of the differences and similarities between the evaluated diagnostic techniques. This study has the potential to significantly contribute to the advancement of *Leishmania* detection, inform more effective diagnostic practices, and identify optimised approaches for serum extraction.

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2. Materials and Methods

2.1. Clinical Samples

This study included 49 samples from dogs collected from veterinary medical centres (i.e. clinics and hospitals) from mainland Portugal's regions and submitted to Cedivet Veterinary Laboratory (Porto, Portugal). A laboratory requisition was received along with the samples, including the complete clinical information for each dog, particularly breed, sex, age, vaccination, prophylactic status, clinical suspicion/clinical signs, and requested analyses. These 49 samples were divided into two of bone marrow, twenty-three of blood, and twenty-four of serum as biological matrices. To preserve DNA, the samples were kept at a temperature of -20 °C until used for detecting antibodies by ELISA and IFAT or at 4 °C until used for qPCR.

2.2. DNA Extraction

DNA extraction is essential in molecular studies to analyse the parasite genome. Identifying and characterising specific genes in *Leishmania* provides crucial information about its pathogenicity, drug resistance, and potential therapeutic targets. Furthermore, DNA extraction plays a central role in developing specific and sensitive diagnostic methods, allowing for earlier detection of the infection. This study relies specifically on the detection of *Leishmania* spp. DNA by qPCR and immunological methods; therefore, DNA extraction is required. For this, we used three extraction kits, as described below. All the kits are based on cutting-edge magnetic bead technology. They ensure high-quality nucleic acidpurification from different biological matrices and are compatible with qPCR or other molecular techniques. All of them allow us to choose between manual and automated workflow; we use the automated method in the laboratory.

2.2.1. MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit

DNA was extracted from all samples using the MagMAX™ Viral/Pathogen II Nucleic Acid Isolation Kit (thermofisher scientific). This kit requires 96 deep well plates. First, we prepared a 275 µL binding bead mix using 265 µL of Binding Solution and 10 µL of Binding Beads, which were homogenised. Then, for the processing plates, we prepare Wash Plate 1 by putting 500 uL of Wash Solution; for Wash Plate 2, we utilise 500 uL of 80% Ethanol Solution, and for the Elution Plate, we use 50 uL of Elution Buffer. Finally, we prepared the sample plate (KingFisher™ 96 Deep-Well Plate) by adding 275 µL to each specimen well and the negative control well. Then, 200 µL of the specimen was added to each specimen well, and 200 µL of nuclease-free water was added to the negative control well. Afterwards, we added 5uL of Proteinase K to each well. Finally, we processed the specimens on the KingFisher™ Flex Magnetic Particle Processor with a 96 Deep-Well Head (Figure 3). We loaded the prepared plates into position and started the run, which was completed after 22 minutes. Then, we removed

the elution plate and covered it with an adhesive film.

Figure 3. Image representing the KingFisher™ Flex Magnetic Particle Processor with 96 Deep-Well Head.

2.2.2. Promega One4All kit

We also used the *Promega One4All kit*, which requires 96 deep well plates. We prepared the processing plates and added 700 µL of wash buffer I to Wash Plate 1. Then, 300 µL of the same buffer was added to Wash Plate 2, and for Elution and Ethanol 80% plates, 110 µL of elution buffer and 250 uL of ethanol 80% were added. Finally, 300 µL of lysis buffer was added to the sample plate, and 200 µL of the sample was transferred. Next, 30 µL Proteinase K was added. It is important to mention that all these values were obtained for each well. To end this process, we ran the plates on the same extractor that will last 1h10; however,after 27 min, the software will pause and prompt the addition of Binding Buffer (350 µL Binding Buffer + 20 µL resin), and we can restart the run.

2.2.3. NzyTech Mag Universal Isolation kit

The process of this brand's kit is similar to that described above. Therefore, it requires 96 deep-well plates, and as with the others, we begin by preparing the processing plates. For Wash Plate 1, 500 µL of NZY Mag UniWash Buffer 1 and for Wash Plate 2, we added 500 µL of NZY Mag UniWash Buffer 2 were added. We added 90 µL of NZY Mag UniElution Buffer for the Elution Plate. Two mixes were added to 200 µL of the sample for the Sample Plate. A combination of Beads/Proteinase K Solution (20 µL beads + 10 µL Proteinase K) and a combination of Lysis/Binding Solution (350 µL Lysis Buffer + 350 µL Binding Buffer). These values are per well. Using the same extractor, we were able to start the run.

2.3. DNA Quantification

DNA Quantification and analysis were performed to determine the yield and quality of the DNA obtained [70]. Spex NanoSNAP (Figure 4) was used to measure the concentration and purity of the DNA using the A260/A280 and A260/A230 absorbance ratios. An A260/A280 ratio of approximately 1.8 is accepted as "pure" for DNA, and a ratio of approximately 2.0 is accepted as "pure" for RNA, and if it is lower, it may indicate the presence of protein as a contaminant. For the A260/A230 ratio, a value of 2.0 – 2.2 is accepted as "pure"DNA. To obtain the spectrophotometer read, 1 µL of the sample was sufficient, taking only 3 s.

Figure 4. Image representing the spectrophotometer used.

2.4. Serological Methods

2.4.1. ELISA

This study used ELISA to evaluate *Leishmania* antibody concentrations in serum. According to a report, the kit used in this study, CIVTEST CANIS LEISHMANIA (Hipra Laboratories), showed a sensitivity of 92.5% and a specificity of 100% [9]. The reagents from this kit must be stored at temperatures between 2ºC and -8ºC. For sample preparation, it must be diluted 1/20 in the Sample Diluent Solution, and all controls do not require dilution and should be tested in duplicate. After the reagents had cooled to room temperature, the process was started by adding 100 µL of each control (High Positive Control, Low Positive Control, and Negative Control) and 100 µL of 1/20 diluted samples to the appropriate wells. Following this, the samples were incubated at room temperature for 10 min. Then, we discarded the contents of the wells and washed the 96-well microplate with 300 µL of Washing Solution per well. Next, 100 µL of the Conjugate Solution was added to each well and incubated for 5 min at room temperature. We repeated the washing step mentioned before and then added 100 µL of Substrate Solution and incubated it at room temperature for 10 min to develop the chromogenic reaction. Finally, 100 µL of Stop Solution was added to each well, and the plate was read using a Microtiter Plate reader at 450 nm. To interpret the results shown

in Table 1, it is necessary to obtain the Rz value for each sample. The following equation was used to calculate this value.

$$Rz = \frac{SampleOD_{450}}{MeanOD_{450}LowPositiveControl}$$

Table 1. Interpretation of the results according to the manufacturer.

Rz value of the sample	Result
Rz ≤ 0.5	Negative
0.5 < Rz ≤ 0.7	Negative
0.7 < Rz ≤ 0.9	Negative
0.9 < Rz ≤ 1.1	Doubtful
1.1 < Rz ≤ 1.5	Low Positive
1.5 < Rz ≤ 2.0	Positive
2.0 < Rz ≤ 3.0	High Positive
Rz > 3.0	Very High Positive

2.4.2. IFAT

In this study, it was used to evaluate Leishmania antibody concentrations in serum. According to a previous report, the MegaFLUO LEISH kit showed a sensitivity of 99.4% and a specificity of 91.9% [77]. The components of the kit must be between +2 °C and +8 °C. We diluted the sera samples with PBS and placed the slides in the humid chamber. In separate antigen wells, one drop (20 µL) of the positive and negative controls was added to each slide. Then, 20 µL of the diluted serum samples were pipetted into different wells and incubated for 30 min at 37 °C. For the washing step, the slides were gently shaken for 5 minutes in PBS and then for 5 minutes with fresh PBS. The slides were then rinsed with

distilled water and gently tap water. The slides were then placed in a humid chamber, and one drop of FLUO FITC anti-dog IgG conjugate was dropped onto each well. After 30 min of incubation at 37 °C in the dark, the conjugate was photosensitive, and the washing step was repeated. Before the evaluation, we used a fluorescence microscope (Leica DM750 microscope with SFL100 light source for fluorescence illumination) at 400x magnification. We added some drops of Mounting Medium to the coverslips and placed them on the slides. To interpret the results shown in Table 2, a fluorescence microscope with a filter system for FITC and 400x magnification and the fluorescence patterns of the negative and positive controls were used as a reference.

Table 2. Interpretation of the results according to the manufacturer.

Positive fluorescence pattern ≥ 1:100	The *Leishmania* show an apparent, yellow-green fluorescence on their membrane and flagella area.
Cut-off fluorescence pattern / recommended cut-off: 1:100	The *Leishmania* (membrane, flagella) shows a weak yellow-green fluorescence.
Negative fluorescence pattern < 1:100	The *Leishmania* does not show any yellow-green fluorescence, they are coloured greyish-red!
Divergent fluorescent reaction	Strong fluorescence of single (< 5 %) small, strongly globular *Leishmania* with shortened or missing flagellum among most normally shaped non-fluorescent promastigotes must be considered non-specific reactions and, therefore, negative.

2.5. Real-time Quantitative Polymerase Chain Reaction (qPCR)

Different extraction kits extracted Genomic DNA from the blood and serum samples. The 96 deep-well plates were stored at -20 °C to preserve DNA, but before use, they were slowly defrosted at a temperature of 5 °C to avoid destroying the genetic material. *Leishmania* spp. qPCR Kit from NzyTech and LshSpp dtec-qPCR kit from Genetic PCR Solutions were used for this work, and the storage process was equal to that of the 96 deep-well plates.

qPCR was performed in a PCR chamber with caution to avoid contamination of the plates (Figure 5). For all samples, we used the NzyTech kit with a total volume of 20 µL, 10 µL of Lyo NZYSupreme qPCR master mix (2x), and 2 µL of Lyo *Leishmania* spp./IEC PPMix (10x), 3 µL of NTC (neutral), and 5 µL of the sample. For controls, 5 µL of NTC was added to the negative control and 5 µL of *Leishmania* spp. Positive Control for the Positive Staining. All experiments were performed according to the manufacturer's instructions for the *Leishmania* spp. qPCR kit (NzyTech).

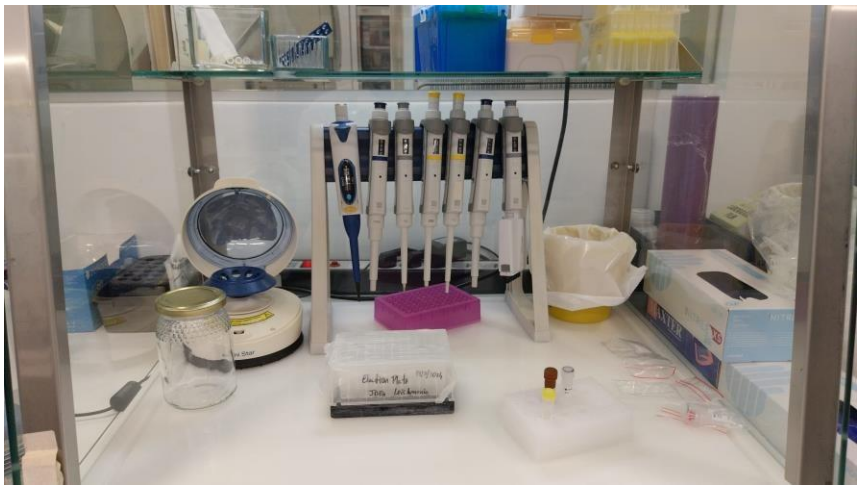

Figure 5. Image showing a PCR chamber.

Amplifications were performed in a QuantStudio5 with a first cycle of 2 min at 95

°C (polymerase activation), followed by 40 cycles of 5 s at 95 °C (denaturation) and 30 s at 60 °C (annealing/extension). Table 2 was used for the principal results of the data analysis.

Table 3. Interpretation of the results according to the manufacturer

Sample Target Cq < 40 (FAM)	Internal Extraction Control Cq < 28 (HEX/JOE/VIC)*	Negative Control Cq > 40	Positive Control Cq < 32	Result
+	+/-	-	+	POSITIVE result
-	+	-	+	NEGATIVE result

For the GPS kit, we used 9 µL of DNase/RNase-free water, 5 µL of GPS-mix, 1 µL of TargetSpecies dtec-qPCR-mix, and 5 µL of sample. For the controls, 5 µL of DNase/RNase-free water was added to the negative control, and 5 µL of the Positive Control tube was added. All experiments were performed according to the manufacturer's instructions using the LshSpp dtec-qPCR kit. All amplifications were performed in QuantStudio5 with an initial activation step at 95 °C for 2 min, followed by 40 cycles of 5 s at 95 °C (denaturation) and 20 s at 60 °C (hybridisation/extension). Table 3 was used for the principal results of the data analysis.

Table 4. Interpretation of the results according to the manufacturer.

Ctrl -	Samp	Ctrl	ExtCtr	IC/M-I	Interpretation
-	+ / -	+ / -	+ / -	+ / -	Experiment fails
+	+	+	+ / -	+ / -	PCR reagents contaminated
		-	+	+ / -	Possible contamination at extraction step
			-	+	**Positive sample**
+	-	-	-	+	**Negative sample**
				-	PCR inhibition

A Standard Curve for the NzyTech kit was also generated, adjusted to a concentration of 2×10^6 promastigotes per mL. This was important for calculating the efficiency of the TaqMan-qPCR. The Ct value represents the cycle number at which a sample reaction crosses the basal fluorescence threshold [69]. Lower Ct values indicate higher levels of the target sequence. The calibration curve obtained after several dilutions is shown in Figure 6, where we can see the equation line and determination coefficient (R^2).

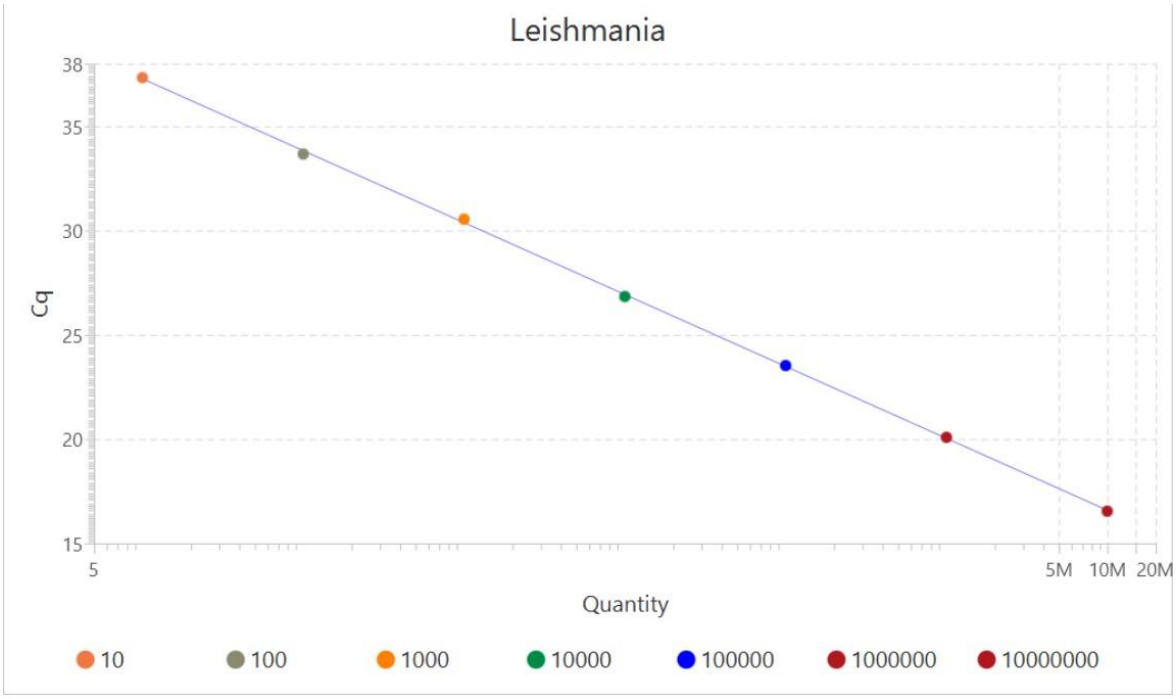

Figure 6. Calibration curve with equation line and R^2.

2.6. Serum samples

As mentioned, serum is derived from blood clots, so it does not have cellular components or clotting factors and has much less DNA. A manual DNA extraction technique was used to determine whether the concentrations and purity were improved.

The protocol started with preparing the lysis mix, using 140 µL of Lysis Buffer and

30 µL of Proteinase K. Then, 200 µL of serum sample and 170 µL of lysis mix were added in a sterile tube, and the mixture was pipetted up and down. After a 20-minute incubation to lyse the sample at room temperature, we bind the DNA. This step added 50 µL Binding Buffer and 6 µL Magnetic Beads and pipetted up and down. After a 2 - 2-minute incubation to allow the beads to bind to DNA, we placed the sample in the Rack (Figure 7) for 3 min until a pellet was formed. Then, without removing the tube from the rack, the supernatant was discarded, and we washed it without removing the tube from the rack. Here, 50 µL of the Wash Buffer was added and the tube was placed in the rack for 2 minutes. The supernatant was discarded without removing the tube from the rack, and the wash step was repeated. Finally, 50 µL of Elution Buffer was added and mixed very well to resuspend the beads. After a 2-minute incubation at room temperature, the sample was placed in the rack for 1 minute. Without removing the tube from the rack, the supernatant was transferred into a sterile tube and prepared for DNA quantification and qPCR.

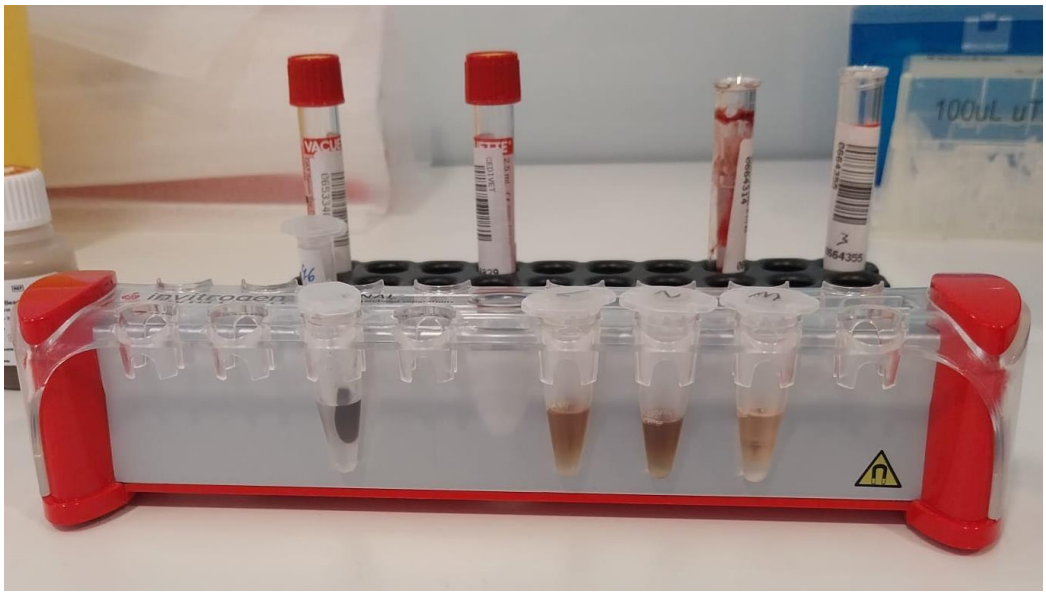

Figure 7. Image representing the rack.

2.7. Statistical Analysis

2.7.1. Sensitivity and Specificity

Statistically, sensitivity is calculated as true positives divided by the sum of true positives and false negatives. High sensitivity indicates that the test can effectively minimise false negatives. The specificity of a test is calculated as true negatives divided by the sum of true negatives and false positives. High specificity indicates that the test can effectively minimise false positives. We usedserology as the standard method and calculated using EXCEL and the following equations:

SENSITIVITY = $\frac{TP}{TP+FN}$ and SPECIFICITY = $\frac{TN}{TN+FP}$, TP means True Positive, FN means False Negative, TN means True Negative and FP means False Positive [90].

2.7.2. Descriptive Statistic

Descriptive statistics are important in organising and analysing the data collected during a study. In this work, we calculated the mean, median, and standard deviation of the qPCR Ct and the serology results using EXCEL to assist the calculations. Through these measures, we could see the data's variability and characteristics.

3. Results

This section will present the results from serological analysis and qPCR in a set of 49 samples. The results are divided into two sections: Serological analysis and

qPCR analysis.

3.1. Serological analysis

Table 5 shows the values obtained from ELISA, IFAT, and Microscopy. The technique varies between samples according to the requests from the owners. In the following Tables, [ID] S. means the sample is serum.

Table 5. Results from Serology Techniques.

ID	ELISA	IFAT titer	Microscopy
655772	2.5		
655727	4.8		
654998	2.7		
654829	4.0		
653828	5.8		
653346	2.7		
656319	2.7		
658108	0.9		
658890	1.2		
659018	4.2		
637333		1:40	
637264	0.3		
661740	5.1		

662412		1:1280	
662907	5.2		
662913	5.8		
664355	0.5		
664314	3.3		
664323			Positive
665770		1:1280	
666384			Positive
638169	0.3		
661510			Negative
669958	3.1		
669591		1:1280	
675704	6.4		

The results of ELISA from Table 5 vary between 0.3 and 6.4, with IFAT registered values of 1:40 and 1:1280. By Microscopy, we saw very high positives and one negative. Following Table 1, where we have the interpretation of the results, we acknowledge that we only had five negatives, and samples 662913 and 653828 presented higher values, meaning they are very positive.

3.2. qPCR analysis

The results from qPCR are shown in the following Tables. The samples were processed using different extraction and PCR kits to detect *Leishmania spp.* In

the first set of samples, we used Promega as an extraction kit and GPS as a qPCR kit (Table 6). Then, we extracted using the same kit but changing the qPCR one (Table 7).

Tables 6 and 7. Results from qPCR were obtained using Promega for extraction and GPS and NzyTech for PCR, respectively.

ID	Results	Ct	cDNA/mL
655772	-		
655772 S.	-		
655727	+	33.2	150.96
655727 S.	-		
654998	-		
654998 S.	+	39.6	2.12
654829	-		
654829 S.	-		
653828	+	28.6	3077.90
653828 S.	-		
653346	-		
653346 S.	-		

ID	Results	Ct	cDNA/mL
655772	-		
655772 S.	-		
655727	+	34.6	56.41
655727 S.	-		
654998	+	34.7	52.73
654998 S.	+	36.6	15.40
654829	+	35.7	27.87
654829 S.	-		
653828	+	28.2	4079.80
653828 S.	+	34.3	70.04
653346	-		
653346 S.	-		

As we can see, when the NzyTech kit was used, we had more positives than the GPS kit. We can also affirm that blood samples amplified more when compared to serum samples. 655772 and 653346 samples were highly positive on ELISA (Table 5); however, Tables 6 and 7 show that they were negative by qPCR. The lowest value of qPCR was shown on sample 653828, which is taken into account in Table 5, where this sample had the highest value by serology.

On the second set of samples, we experimented for the extraction, two kits, NzyTech and Promega kits, and the GPS and NzyTech kit for qPCR, respectively.

Table 8. Results from qPCR using NzyTech for extraction and GPS for PCR. Table 9. Results from qPCR using NzyTech for extraction and NzyTech for PCR. Table 10. Results from qPCR using Promega for extraction and NzyTech for PCR

ID	Results	*Results*	Ct	cDNA/mL	*Results*	Ct	cDNA/mL
656319	-	+	39.1	2.89	-		
656319 S.	-	-			-		
658108	-	+	37.3	9.65	+	37.7	7.14
658108 S.	-	-			-		
658890	-	-			-		
658890 S.	-	-			-		
659018	-	-			-		
659018 S.	-	-			-		

Table 5 shows that only 658108 was negative by ELISA and positive in Tables 9 and 10. However, we expected much better results here since qPCR is more sensitive and specific than serology.

After a not-so-good set of results, we decided to change the kit for extraction to see if the results were improved. So, we used the MagMax kit in the following tables.

Table 11. Results from qPCR using MagMax for extraction and NzyTech for PCR.

ID	Results	Ct	cDNA/mL
656319	+	39.9	1.6
658108	-		
653346	-		
637333	-		
659018	+	38.1	5.7
658890	+	37.8	6.6
655727	+	38.7	3.7

As we can see in Table 11, samples that were negative before (656319, 658890, 659018), using MagMax for extraction and NzyTech for qPCR, were positive. On the contrary, the sample 658108 was negative this time. However, all these Ct's are high and close to the limit (40).

Table 12. Results from qPCR using MagMax for extraction and NzyTech for PCR.

ID	Results	Ct	cDNA/mL
661740	+	33.6	112.0
662412	+	36.9	12.4
662907	+	35.2	38.7
661740 S.	+	37.7	7.0
662907 S.	-		
662913 S.	-		
653828	+	32.2	294.1
655772	-		
654829	-		
654998	+	36.6	15.0
656319 S.	-		
658108 S.	+	38.3	5.1
658890 S.	-		

The first six lines of Table 12 are a new set of samples, and as we have already seen a few times, blood samples have more significant results than serum samples. The lowest Ct was 32,214 from sample 653828, one of the highest values of ELISA, and the highest Ct was 38.373 from sample 658108 S., a surprise since it was negative by ELISA and it is serum, which makes it more difficult to amplify by qPCR. This positive sample using MagMax as an extraction

kit shows that this kit has potential. We continued to use the same kits for the following samples.

Table 13. Results from qPCR using MagMax for extraction and NzyTech for PCR.

ID	**Results**	**Ct**	**cDNA/mL**
664355	-		
664355 S.	-		
664314	+	37.7	7.2
664314 S.	-		
664323	+	28.7	2882.9

The 664355 sample was negative in Serology and qPCR, both in blood and serum. The 664314 sample was positive in ELISA and positive in blood but negative in serum. Sample 664323 from bone marrow showed the lowest value of Ct and was positive by Microscopy.

Table 14. Results from qPCR using MagMax for extraction and NzyTech for PCR.

ID	**Results**	**Ct**	**cDNA/mL**
665770	+	33.7	108.0
666384	+	31.9	344.5

Both samples were positive by both methods, as shown in Tables 5 and 14. 665770 was blood, and 666384 was bone marrow.

Table 15 shows only one positive sample by qPCR when we used the combination of MagMax and NzyTech. Sample 675704 was the highest positive by ELISA, and we can see in Table 4 that samples 637333, 637264, 638169, and 661510 were negative by serology.

Table 15. Results from qPCR using MagMax for extraction and NzyTech for PCR.

ID	Results	Ct	cDNA/mL
675704	+	38.0	6.1
675704 S.	-		
669958	-		
669591	-		
669591 S.	-		
669958 S.	-		
637264	-		
638169	-		
661510	-		

As we saw spread across the tables, the serum results are poor. So, intending to improve serum results, we used 664355 and 664314 samples to test the protocol shown in section 2.6. The results were inconclusive, so they did not amplify anything.

3.3. Variations in the quantity of DNA copies

The qPCR analysis results showed variations in the quantity of DNA copies per mL, depending on the extraction and qPCR kits used. Sample 653828 had a high parasitic charge in different analyses, with 3077.9 and 4079.8 copies of DNA/mL results. Sample 655727 showed a parasitic charge when extracted with Promega (150,96 and 56,41 copies of DNA/mL) and with MagMax (3.8 copies of DNA/mL), but it was much lower in this one. Sample 654998 varied from negative to positive, with 52.7 copies of DNA/mL depending on the kit used.

3.4. Quantification of DNA

DNA quantifications and purity checks were done using the NanoSNAP to anticipate if the qPCR would work. So, Table 16 shows the values of the first set of samples when the kit Promega was used.

Table 16. Results from concentration and purity of Promega kit. Conc means concentration (ng/µL)

ID	Conc	A260/280	A260/230
655772	36.1	1.76	1.46
655772 S.	9.2	1.20	0.73
655727	108.8	1.85	1.82
655727 S.	20.5	1.49	0.84
654998	25.2	1.71	1.19

654998 S.	5.6	1.18	0.73
654829	50.3	1.76	1.52
654829 S.	9.3	1.36	0.66
653828	62.0	1.70	1.69
653828 S.	3.9	0.64	-1.15
653346	65.8	1.85	1.89
653346 S.	9.6	1.13	0.61

As said before, 1,80 is the value for pure DNA for A260/280. For A260/230, 2,0 is the acceptable value. So, Table 16 shows that blood samples have a higher concentration and better purity.

Table 17 represents the values of the second set of samples when we used two different extraction kits.

Table 17. Concentration and purity results of NzyTech and Promega kits. Conc means concentration (ng/µL)

	NzyTech			**Promega**		
ID	Conc	A260/280	A260/230	Conc	A260/280	A260/230
656319	18.9	1.55	0.62	49.9	1.87	1.56
656319 S.	17.6	1.48	0.61	4.6	1.74	0.68
658108	12.4	1.53	0.66	50.6	1.80	1.22
658108 S.	12.7	1.64	0.59	11.3	1.69	0.75

658890	16.5	1.43	0.52	41.2	2.02	1.74
658890 S.	16.4	1.59	0.60	16.7	1.34	0.64
659018	13.6	1.27	0.49	60.4	1.80	1.28
659018 S.	17.8	1.54	0.61	17.3	1.35	0.67

Looking at Table 17, we can observe that these values are a problem in the NzyTech kit. Even in blood samples, the values for purity and concentration are far from good. The Promega kit, the values are better, with two samples (658108 and 659018) having an excellent A260/A280 ratio of 1,80. So, to see if some of these samples could have better results, we measured purity and concentration on the MagMax kit.

Table 18. Concentration and purity results of MagMax kit. Conc means concentration (ng/µL)

ID	Conc	A260/280	A260/230
656319	35.3	1.94	0.41
658108	44.3	1.90	0.42
653346	160.6	1.65	0.41
637333	37.0	2.03	0.44
659018	77.5	1.84	0.53
658890	80.3	2.04	0.66
655727	102.0	1.39	0.79

Table 18 shows four blood samples that appear in Table 17, so we can compare them and observe that the values on MagMax are much better on those four samples.

Table 19. Concentration and purity results of MagMax kit. Conc means concentration (ng/µL).

ID	Conc	A260/280	A260/230
661740	39.7	1.70	0.44
662412	42.3	1.77	0.46
662907	54.2	1.81	0.53
661740 S.	5.7	2.51	0.15
662907 S.	10.0	1.28	0.24
662913 S.	15.7	1.17	0.68
658828	26.2	1.54	0.25
655772	29.1	1.41	0.23
654829	16.0	1.41	0.26
654998	24.5	1.59	0.32
656319 S.	23.2	1.39	0.23
658108 S.	30.3	1.41	0.29
658890 S.	17.0	1.37	0.19

Focusing on the first six samples, we can see that blood samples have the best A260/280 ratio, but both matrices need better A260/230 ratio values. 662907 sample had the best A260/280 ratio (1.81), and 661740 S. had the worst value

(2.51). In the concentration topic, the story repeats with serum samples having lower values. The values of the other seven samples in Table 19 were better than those in Tables 16 and 17.

Table 20. Concentration and purity results of MagMax kit. Conc means concentration (ng/µL)

ID	Conc	A260/280	A260/230
664355	29.5	1.91	0.47
664355 S.	8.7	1.57	0.12
664314	97.0	1.96	0.81
664314 S.	8.6	1.54	0.13
664323	4.4	1.81	0.97

Moving on to another group of samples, Table 20 shows what we already saw in almost all the other Tables. Surprisingly, the 664323 sample had the lowest concentration in the study. However, it also had one of the best Ct values.

Table 21. Concentration and purity results of MagMax kit. Conc means concentration (ng/µL).

ID	Conc	A260/280	A260/230
665770	52.95	1.79	0.71
666384	13.65	1.41	0.33

As mentioned before, sample number 666384 is from bone marrow, and the values were not as good as sample 664323, which is bone marrow too (Table 21).

Table 22. Concentration and purity results of MagMax kit. Conc means concentration(ng/µL).

ID	Conc	A260/280	A260/230
664355 S.	28.4	5.77	0.33
664314 S.	18.1	2.06	0.28

Table 22 presents the DNA quantification and purity of the samples that suffer manual extraction. Compared to Table 20, we had more DNA concentration, and 664314 S. had a good A260/A280 ratio. However, there still were lots of contaminants.

Table 23. Concentration and purity results of MagMax kit. Conc means concentration (ng/µL).

ID	Conc	A260/280	A260/230
675704	49.96	1.87	1.56
675704 S.	11.11	1.51	0.75
669958	50.67	1.80	1.22
669958 S.	14.26	1.48	0.67
669591	60.41	1.80	1.28
669591 S.	4.60	1.54	0.70

637264	40.69	1.69	1.27
638169	16.77	1.70	1.32
661510	17.31	1.75	1.49

In Table 23, we note that, similarly to the other Tables, the blood samples had relatively good ratios, while serum samples presented poor values of concentrations and ratios.

3.5. Sensivitity and Specificity

Table 23 summarises the sensitivity and specificity of the combination of kits used in this study. We can see that the combination of MagMax and NzyTech presented the highest blood sensitivity percentage (80%). NzyTech and NzyTech showed the lowest blood and serum sensitivity percentages (50%) but the highest specificity percentages on both matrices (100%). Some combinations did not present specificity because there weren't negative samples. MagMax and NzyTech had the best overall results, and when we used NzyTech for qPCR, we had better results.

Table 24. Results of sensitivity and specificity of the five kit combinations used in this study.

Extraction and qPCR	Matrix	Sensivitity %	Specificity %
Promega and GPS	Blood	60	NA
	Serum	55	NA

Promega and NzyTech	Blood	66.7	66.7
	Serum	57.1	100
NzyTech and GPS	Blood	50	100
	Serum	50	100
NzyTech and NzyTech	Blood	66.7	66.7
	Serum	50	100
MagMax and NzyTech	Blood	76	87.5
	Serum	53.3	75

NA, *Not Applicable (not enough samples)*

3.6. Descriptive Statistics

Table 23 shows us the positive results of Ct of kit combinations. MagMax + NzyTech was the kit that was most used, so its results are more reliable than the others, but we will mention all of them. Promega + NzyTech demonstrated the lowest Ct mean (33.8) but showed high variability, indicated by the elevated standard deviation (5.5).

As we can see in Table 24, ELISA was more requested than IFAT, so it has more reliable results, but we will consider all of them. ELISA presented a 3.2 mean and standard deviation of 2.0. The median (2.7) shows us that we have a good range of results, with a moderate dispersion around the mean.

Table 25. Descriptive Statistics for Ct on the five combinations used in this study

Kit	Count	Mean	std	min	Median	max
MagMax+NzyTech	13	36.4	2.9	28.8	37.7	39.9
NzyTech+GPS	1	37.7	-	37.7	37.7	37.7
NzyTech+NzyTech	2	38.2	1.2	37.3	38.2	39.1
Promega+GPS	6	34.7	2.9	28.2	34.9	36.6
Promega+NzyTech	3	33.8	5.5	28.7	33.2	39.6

Table 26. Descriptive Statistics of the serology results

	Count	Mean	std	min	Median	Max
ELISA	19	3.2	2.0	0.3	2.7	6.4
IFAT	4	1:970	1:619,5	1:40	1:1280	1:1280

4. Discussion

Leishmaniosis is a parasitic disease caused by protozoans from the *Leishmania* genus and affects millions of animals and people worldwide, especially in tropical regions. The detection and diagnosis of the infection are crucial for efficient control and treatment, and molecular techniques have gained some prominence because of their sensitivity and specificity. However, the efficiency of qPCR can be influenced by DNA quality and the PCR kit used.

So, in this study, we analyse 26 canine samples of serum, blood, and bone marrow to test the presence of *Leishmania* spp., using different serological and molecular methods. A comparison of the results from various combinations of extraction and qPCR kits gave us some good information about the efficiency and reliability of these methods.

The following discussion explores the implications of the results, approaching the efficiency of the methods and kits and differences observed between biological matrices, and suggests possible improvements in extraction protocols to gain more precision and reliability.

Serological techniques showed some variations in antibody detection against *Leishmania* spp*.,* ELISA presented values between 0.5 and 6.4, while IFAT presented titters of 1:1280 and 1:40. Microscopy was used to detect parasites in some samples. These methods are low-priced and relatively simple, so they are used often but have limitations in sensitivity and specificity [24,69].

On the other hand, we must talk about the performance of the extraction kits. Tables 16 to 23 show that the Promega kit showed the best DNA purity, with values of A260/A280 close to 1.80, indicating a low presence of protein contaminants [70]. A low presence of contaminants is essential for the accuracy of qPCR and to mitigate false positives or negatives. However, the MagMax kit was superior in DNA detection, with more positive samples. These results can be explained by the capacity to lead effectively with DNA extraction of complex samples, like serum. This kit can recover DNA more efficiently, with fewer losses during extraction, resulting in a much higher concentration of amplifiable DNA. Because the NzyTech kit has given bad results, we can see in Table 17 that both ratios are abysmal, meaning protein, phenols, and polysaccharides contamination [70]. So, the kit may not have an affinity for animal samples, or the methodology is less effective in serum samples since there are much more proteins and lipids. This was reflected in negative samples because the presence of contaminants can inhibit qPCR, reducing the detection of DNA. However, we needed to have the chance to use the kit in more samples for replicability.

When we started to look at the samples, we saw more positives in blood samples. Samples like 655727 and 654829 were positive by ELISA and positive by qPCR in blood but negative in serum. That can be explained by the greater concentration of DNA in blood, where the circulating free DNA (cfDNA) is frequently low in quality and quantity in serum. However, a study said that TaqMan-qPCR detected only 13% positivity in blood samples, so neither matrices were the best for getting good results [16].

Using qPCR for *Leishmania* diagnosis can be an excellent tool for an early diagnosis [71]. To access and comprehend the differences between extraction and qPCR kits, it is better to discuss them apart. So, in Tables 6 and 7, we have Promega as the extraction kit and GPS and NzyTech as qPCR kits, respectively. All the samples from those Tables were positive for ELISA. However, only three out of six were positive in Table 6 and four out of six in Table 7 (654998 and 653828 were positive in both matrices). As said, these results presented positive serology; therefore, these negative results on qPCR may be due to possible interference with DNA extraction or the sample not having protozoan DNA [21]. Also, a study showed that despite qPCR presenting a great sensitivity right after the transmission, it decreases to 50% with the progress of the infection [72].

Despite the NzyTech kit having the worst values of concentration and purity, when we move to Tables 8 to 10, and knowing that samples 658108 and 658890 were negative by ELISA, we observe in Table 9 that sample 658108 was positive with a Ct of 37.3 in blood. Then, in Table 10, we see that sample 658108 was again positive, and 656319 was positive in both techniques. These results for sample 658108 can mean that the dog had contact with the disease and had the infection, but he defeated it, or it is a false positive. The problem of false positives is that it could lead to treatment the dog doesn't need. The fact that sample 656319 was positive may be due to the change of qPCR kit since we used the NzyTech one. Sample 659018 was negative by qPCR because serologydetects antibodies, which remain in blood long after the initial infection, and qPCR detects the pathogen's DNA; however, if the disease has progressed, the qPCR might not detect the pathogen [36]. Also, cross-reactivity with similar pathogens in serology can produce wrong values, resulting in a false positive but a true negative by qPCR [73]. Also, false positives can be a problem for obvious reasons, and they can lead to the death of the dog because it will not have accessto treatment.

Table 11 shows four positives out of seven samples; samples 658108, 658890, and 637333 were negative in serology. Only 658890 was positive by qPCR, which could mean that the dog was exposed to *Leishmania* spp., is a false positive, or has not yet been seroconverted. As said before, the agent is expected

to have low DNA detection without infection in endemic zones. MagMax is the better kit according to our data; the fact that sample 658108 was positive using NzyTech as an extraction kit and negative by MagMax could mean that the freeze-thaw cycle between extractions may have degraded the sample or the result from MagMax is a true negative [74]. However, it was positive in the serumso that some human error could have happened. Sample 653346 was always negative by qPCR and positive by ELISA and, according to studies, molecular techniques are better than serological techniques [16]. So, an error occurred when ELISA was performed and generated a false positive.

Table 12 shows four new samples, all very high positive by serology. 661740 amplified in both matrices, as you can see; on the contrary, sample 662907 only amplified in blood, and sample 662913 didn't amplify in serum. We repeated samples 655772, 654829, and 654998 in blood with MagMax to see if we would have better results since they were positive for ELISA. However, this was not the case; sample 655772 was negative again. Maybe the infection was in the initial stage, and the DNA was low, or if it is in the final stage, the concentration of *Leishmania* in blood could be low and replicated in other tissues [75]. Sample 654998 was positive, showing that the techniques were well applied and that the dog was infected. 654829 was positive when extracted by Promega, but this time, it was negative. The freeze-thaw cycles between extractions may have degraded the material genetic of the samples, leading to poor extractions or because the values of A260/A280 and A260/A230 were not so good [74].

Tables 13 and 14 show us five samples (664355, 664314, 665770, 666384, and 664323), 664355 was negative, and the others were positive by serology. After qPCR, we had 664355 negative in blood and serum, 664314 positive in blood but negative in serum, 665770 positive in blood, 666384 positive in bone marrow, and 664323 being one of the lowest Ct of this study. This concordance suggests an infection in the last four dogs. Also, samples 666384 and 664323 were from bone marrow, and they presented some of the best values of Ct; this is according to studies that say that PCR performed at bone marrow showed good sensitivity, indicating that these samples are a suitable source of DNA for molecular diagnosis. However, the collection of the aspirates could be

traumatic or painful to the dog [15]. A study reported that although blood is less invasive when compared to bone marrow, it presented the lowest positive detection value [40,76,77]. Other studies said that blood worked well, but other authors found problems with the presence of PCR inhibitors in blood, variations in parasite load, and low sensitivity [78,79]. Additionally, blood functions as a transport system for the protozoan rather than a reservoir organ. This implies that the protozoan's presence in the blood may be temporary and not reflective of the overall parasite load in the body, potentially causing discrepancies between serology and qPCR results [15].

Table 15 showed only one positive sample with a Ct of 38, which is very high since that was the highest positive by ELISA. This indicates that the dog was infected, but we should have had the lowest Ct if the ELISA result had been correct. Next, samples 669958 and 669591 were also positive, as shown in Table 5, but were negative in qPCR. In this case, we can explain the results by saying that the infection was in an initial stage and the DNA was low, or if it is in the final stage, the concentration of *Leishmania* in blood could be low and replicated in other tissues [75]. Also, the probable existence of DNases can degrade the DNA even at low temperatures, and the cycles of freezing and defrosting may have degraded and fragmented the DNA [74,80]. Regarding the manual serum extraction, the higher value of A260/A280 in sample 664355 S. and the lower values of A260/A230 suggest protein and other chemical compound contamination [81].

As said earlier, sensitivity and specificity were calculated using serological methods referencing our calculations. Sensitivity is calculated by dividing the total positives by the sum of total positives and false negatives [82]. Starting with Promega and GPS, a sensitivity of 60% in blood suggests that this combination could detect the presence of *Leishmania* spp. but with limitations. Some poor lysis or a lower DNA extraction efficiency could explain these results. In serum, the sensitivity was slightly lower, showing the same problems, which generally means that DNA is lower in serum. Promega and NzyTech presented marginallybetter results, suggesting that the NzyTech qPCR kit is more effective than the GPS one. Then, we changed the extraction kit to NzyTech, and we can see in

Table 24 that this kit might not be as efficient as Promega. However, we can see again that the NzyTech qPCR kit had better results. Only four samples were used for this one, so we only have a little replicability and reliability; it must be tested in more samples. The combination of MagMax and NzyTech showed 76%sensitivity in blood, demonstrating that it was the most effective because it had superior lysis and purification methods. The serum value was about the same asthe others (53.3%).

Specificity is calculated by dividing total negatives by the sum of total negatives and false positives [82]. Promega and GPS had NA because there weren't any negative samples by serology in the set of samples used. Promega and NzyTech were used on the sample samples as the combination before, plus four more. We had a specificity of 66.7% in blood and 100% in serum, suggesting reduced false positives. Combinations with NzyTech as an extraction kit showed 100% specificity, except in NzyTech and NzyTech in blood (66,7%). MagMax and NzyTech could be the most reliable combination, with high specificity, indicating effective elimination of false positives. However, this study should have more negative samples for better and more reliable specificity results. High sensitivity and specificity values are essential to avoid false negative results, which can underestimate the infection proportion in canine populations in endemic areas, and falsepositive results [35]. It is important to note that we only had 26 samples, so moresamples are needed to relate to the canine population.

Finally, the lab work contributed to acquiring competencies for a future job in the area and having some knowledge of serology.

5. Conclusion

The results of this study highlight the complexity and the challenges in *Leishmania* DNA detection from blood, serum, and bone marrow samples. The comparison between different extraction and qPCR kits showed that the

combination of MagMax and NzyTech had the best results in terms of sensitivity and specificity. However, the presence of inhibitors and the variability in extracted DNA quality are still crucial challenges. The Promega kit had better DNA purity, and MagMax had higher DNA detection values.

Optimised protocol implementation and the use of blood or bone marrow samples can increase the precision and reliability of *Leishmania* diagnosis. Also, further research with larger sample sizes and diverse conditions is needed to validate these findings.

Overall, in this study, we saw that despite qPCR offering high sensitivity and specificity, the extraction and qPCR kits chosen impact its reliability. This work may give a solid base for future improvements in diagnostic protocols, contributing to more precise and efficient diagnosis and, consequently, for better management of leishmaniosis.

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