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Non-Invasive Molecular Diagnosis of Canine Visceral Leishmaniasis Using Conjunctival Swab Samples

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1. Introduction

Leishmaniasis is a neglected tropical disease caused by protozoan parasites of the genus *Leishmania,* which is a public health problem in tropical and subtropical regions of the World. The parasite is transmitted to mammals by the bite of naturally infected species of female sand fly vectors (Diptera, Psychodidae: Phlebotominae). The geographic distribution of leishmaniasis is limited by distribution of the sand fly vectors. Vectors of the genus *Lutzomyia* are responsible for transmitting the disease in the New World and the genus *Phlebotomus* in the Old World [1].

Leishmaniases comprise a complex of diseases caused by at least 22 species of *Leishmania* which are obligatory intracellular parasites surviving within phagolysosomes of the mononuclear phagocytes of mammal host. These parasites produce a wide spectrum of diseases, depending both on the species that initiating infection and on the immunological status of the host, among other factors. The disease ranges from: simple cutaneous lesion developing at the site of the sand fly bite, with skin ulcers that usually appears on exposed part of the body, such as the face, arms and legs, that could be heal within a few months, leaving scars; mucocutaneous form with lesions that can partially or totally destroy the mucous membranes of the nose, mouth and throat cavities and surrounding tissues and producing extensive disfiguring, being difficult to treat; diffuse cutaneous that is rare, but more serious complication which occurs when the immune system fails to react effectively to infection with multiple non ulcerative nodules and visceral leishmaniasis, also known as kala azar, that is characterized by high fever, substantial weight loss, swelling of the spleen and liver, and anemia. If left untreated, the visceral leishmaniasis can have a fatality rate as high as 100% within two years. The disease

presents high morbidity in tegumentary leishmaniasis and high mortality levels in visceral leishmaniasis [2].

Reported from 98 countries, in six continents, the leishmaniases are responsible for the second-highest number of deaths due to parasitic infection globally and are still one of the world's most neglected diseases, affecting largely the poorest of the poor, mainly in developing countries. It is associated with malnutrition, displacement, poor housing, illiteracy, gender discrimination, weakness of the immune system and lack of resources. Approximately 0.2 to 0.4 and 0.7 to 1.2 million of visceral leishmaniasis and tegumentary leishmaniasis cases respectively, occur each year, with 350 million people in worldwide living at risk to be infected, based on World Health Organization data. Leishmaniasis is the third most important vector-borne disease, and the estimated disease burden places it second in mortality and fourth in morbidity among tropical infections [3].

More than 90% cases of visceral leishmaniasis occur in six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil [3]. Certainly, these data are underestimated, since not all affected nations have a system of compulsory notification of cases, and even those countries where the leishmaniasis is a reportable disease there are logistical problems that increase the imprecision of the estimations [4].

The distribution of tegumentary leishmaniasis is more extensive, occurring in three epidemiological regions, the Americas, the Mediterranean basin and Western Asia from the Middle East to Central Asia. Ten countries have 70-75% of cases worldwide: Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru. Mortality data were extremely sparse and generally represent hospital-based deaths only [3].

Several studies have reported the expansion of leishmaniasis worldwide and occurrence of cases in endemic regions have been recurrent and the global number of cases has increased in recent decades. The main reasons given for such increases are related to environmental changes, agricultural development, migration of non-immune people to endemic areas, and, in part, by improved diagnosis, but are also due to other factors such as inadequate reservoir or vector control. More recently, an increased detection of disease associated with opportunistic HIV infections and visceral leishmaniasis, especially in intravenous drug users in Southwestern Europe and other endemic areas was verified. A recent report by the World Health Organization [4] indicated that people with AIDS have become the largest risk group for human visceral leishmaniasis in Southern Europe, and that their co-infection is expected to present an increasing problem in areas were HIV and human visceral leishmaniasis overlap, especially in Brazil, Africa, and India. In areas endemic for visceral leishmaniasis, many people have asymptomatic infection. A concomitant HIV infection increases the risk of developing active visceral leishmaniasis by between 100 and 2320 times [5]. In Southern Europe, up to 70% of cases of visceral leishmaniasis in adults are associated with HIV infection. Sum up this the urbanization, deforestation, the emergence of antileishmanial drug resistance, economic development itself is also increasing exposure, tourism, wars and in some areas military training in forest or desert [6].

A large number of different *Leishmania* species cause tegumentary disease belonging to both subgenera *Leishmania* and *Viannia* (which are present only in the America). Epidemiological studies have revealed that except *Leishmania tropica,* all of other species that cause tegumentary leishmaniasis are zoonoses that initially affect animals (rodents, sylvatic edentates, marsupials and non-human primates) and eventually humans [7].

Visceral leishmaniasis or kala azar, is almost always fatal if not treated. In the Americas as well in Mediterranean Basin, Middle East, West Africa and Central Asia, visceral leishmaniasis is caused by *Leishmania infantum* (=*L. chagasi*) and is a zoonosis with an animal reservoir and occasional human infections. However, visceral leishmaniasis in India and Africa caused by *Leishmania donovani* has an anthroponotic cycle with inter-human transmission. Despite the ecological differences, the disease is very similar in their clinical manifestations. It is characterized by targeting visceral organs and results in the development of syndromes comprised of irregular fever, substantial weight loss, splenomegaly, hepatomegaly, hypergammaglobulinemia, pancytopenia, anemia and hypoalbuminaemia witch is associated with edema and other features such as malnutrition [8].

Patients treated and cured for visceral leishmaniasis caused by *L. donovani* may subsequent develop Post-kala azar dermal leishmaniasis (PKDL). Post-kala azar dermal leishmaniasis is an unusual dermatosis that develops as a sequel in 5–15% of cured cases of kala azar after months or years of treatment in India [9].

L. infantum maintains a zoonotic cycle mostly involved canine host, and canine visceral leishmaniasis is also a veterinary problem. The recommended control methods for the disease have only been partially effective. The continued endemicity of zoonotic visceral leishmaniasis, its recent appearance in urban areas of Latin America [10] and its increasing importance as an opportunistic infection among persons infected with human immunodeficiency virus, indicate that present control methods for the disease are ineffective and that new control strategies are needed. Prevention of the disease in dogs appears to be the best approach for interrupting the domestic cycle of zoonotic visceral leishmaniasis. Not all *L. infantum* infections lead to overt clinical disease. In Brazil were described ratios of 8-18 incident asymptomatic infections to 1 incident clinical case [11].

Zoonotic visceral leishmaniasis is an important emerging parasitic disease of humans and dogs. The most feasible approach would seem to be a canine vaccine that protects dogs from developing disease and from becoming peridomestic reservoirs of the parasite. There are two vaccines developed in Brazil, but not used for routine immunization against zoonotic visceral leishmaniasis. There is clear evidence that in the zoonotic visceral leishmaniasis the parasites are maintained through the bite of infected female phlebotomine sand flies, and the prevalence of disease has been expanding throughout the world [12].

Many studies suggest that *L. infantum* in urban and peri-urban settings is a phenomenon reported in several countries when zoonotic visceral leishmaniasis is endemic. Dogs are the only confirmed primary reservoir of infection. Meta-analysis studies confirm that infectiousness is higher in symptomatic infection; infectiousness is also higher in European than South

American studies [13]. A high prevalence of infection has been reported from an increasing number of domestic and wild mammals; updated host ranges are provided.

The domestic dog is the most important domestic reservoir in urban and rural areas [14]. The dogs have intense cutaneous parasitism, favoring infection of vectors and play an important role in the epidemiological chain of human visceral leishmaniasis. Therefore, although visceral leishmaniasis remains more prevalent among dogs than humans, the presence of infected dogs may increase the risk for human infection in some situation [15]. There is a close canine relationship with human in both rural and urban areas, and canine cases usually precede human cases.

The strategies of the control of leishmaniasis have varied very little for decades, but in recent years there have been exciting advances in diagnosis, treatment and prevention. These include an immunochromatographic dipstick for diagnosing visceral leishmaniasis; the licensing of miltefosine, the first oral drug for visceral leishmaniasis; and evidence that the incidence of zoonotic visceral leishmaniasis in children can be reduced by providing dogs with deltamethrin collars. In the context of zoonotic visceral leishmaniasis measures of control transmission vary according to local epidemiology [10].

The canine visceral leishmaniasis is clinically alike to human infection, but with dermal lesions normally found in infected-symptomatic dogs. The infection in dogs carries a wide-ranging clinical signs related to high antileishmanial antibody levels and lack of a cell-mediated response. *Leishmania* are intracellular parasites and, under immunodeficiency conditions, they multiply and migrate from lymphoid tissue to other organs, displaying severe clinical and pathological changes which could leads to animal death. Although, infections in endemic areas are high, not all dogs infected develop the disease, in some dogs several clinical signs of disease appear in short time after infection. Part of dog remains infected for a long time, but without clinical signs of disease. There are evidences that the host's genetics could play a major role in susceptibility or resistance [16]. Canine visceral leishmaniasis has a high prevalence of infection, involving as much as 63-80% of the population [17, 18] and is accompanied by a lower rate of apparent clinical disease.

Dogs have also been found to be infected with other *Leishmania* spp., and their role in these infections is probably more than incidental. In the wide geographical range of *L. infantum*, there are many contrasting situations, depending on whether the dogs are domestic, stray or feral and on the animals' place in society. Naturally infected asymptomatic dogs have been demonstrated to be easily infective to sand flies under experimental conditions (xenodiagnosis). Therefore, the role they may play in the cycle should not be underestimated, as more than 50% of all infected dogs are asymptomatic carriers. This is due to the high degree of parasitism on the skin of the infected animal and greater susceptibility to disease in many of them [19]. Thus, occurrence of infection in endemic regions of sand flies is facilitated, and the dog plays a decisive role in keeping the disease cycle transmission under favorable conditions, with high population density vector and dogs, the infection spreads rapidly and extensively in the population of vectors and also in the canine population [20, 21].

In the Mediterranean basin, human cases and canine cases are treated with antileishmanial drugs. In this area the use of individual measures to protect dogs from sand fly bites using insecticides are common practices, but no public health surveillance and control interventions such as applied, for example, in Brazil are in place [22]. Despite years of effort using control measures the number of infected dogs in South-western Europe alone are approximately at 2.5 million [23] and the number of infected dogs in South America also is estimated in millions. In Latin America the strategies of control of visceral leishmaniasis are based in the diagnosis of human and canine visceral leishmaniasis, treatment of human cases, control of infected dogs using immunological test to diagnose *L. infantum* infections and to cull putative infected animals and also vector control by spraying insecticides with residual action. Animal reservoir control through environmental management is expensive and difficult to implement; the efficacy of dog culling is questionable [24].

In the context of visceral leishmaniasis prophylaxis, the rapid and accurate diagnosis of infected dogs is critical for the control. The correct diagnosis is essential for detection of *L. infantum* infection in both symptomatic and asymptomatic dogs. Reliable clinical signs of canine visceral leishmaniasis are not obvious until late in the disease. The precise diagnosis of canine visceral leishmaniasis is complex and must be performed combined to parasitological, immunological and molecular tests [25]. Although, the disease burden persists due to technical, managerial, financial and political constraints [24].

Current diagnosis methods of zoonotic visceral leishmaniasis are based on parasite demonstration in tissue stained smears. *Leishmania* amastigotes can be demonstrated in impression smears made from fine needle aspirates of lymph nodes, spleen or bone marrow, and stained with Giemsa or a quick stain such as Rapid Panotic (Laborclin®). In dogs impression smears can also be made from dermal lesions, such as those found on the tip of the ear, after scraping of the skin. The material is used for culture, cytology or examination of the parasites presence. Although demonstration of even a single amastigote upon microscopic examination of tissue smears is considered sufficient for positive diagnosis of disease, the sensitivity of the tissue examination varies, being splenic aspirate more sensitivity, than bone marrow or the lymph node aspiration. The specificity is high, but the sensitivity, except in the case of spleen aspirate, is low. However, spleen aspiration can be complicated by life threatening hemorrhages in ~0.1% of the cases. The identification of amastigotes requires considered expertise and training and is subject to the ability of the observer [26]. Diagnosis may also be established by the inoculation of hamsters with infected tissues and monitoring their clinical signs. This technique is used mostly in research parasite identification, requires considerable expertise and training and in our own experience is can be laborious and time consuming.

Other several methods are described to diagnosis human and canine visceral leishmaniasis and the most employed are immunodiagnostic tests for antibody detection. Serodiagnosis is particularly useful in zoonotic visceral leishmaniasis, since humans and dogs present hyper-gammaglobulinemia. Dogs with canine visceral leishmaniasis infection, either symptomatic or asymptomatic, will almost always demonstrate a specific humoral response. The serological test used crude antigen preparations and they are limited in both specificity and assay reproducibility, and there are cross-reactions with other *Leishmania* species and with *Trypano-*

soma cruzi. Weak responses in some dogs and presence of antibodies in some healthy individuals are inherent limitations with antibody based diagnostics according our own observations. The use of crude *Leishmania* antigens is thought to underestimate the prevalence of canine visceral leishmaniasis [27, 28].

In Latin America, mainly in Brazil, the serological test is extensive used as part of control campaigns to remove seropositive animals for euthanasia often not with the agreement of the owners. The Brazilian Ministry of Health, through the Control Program of Visceral Leishmaniasis, has instituted specific measures to control of the disease using immunofluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA). To date, however, the actions of this program have had little impact. Control strategy based on the euthanasia of seropositive dogs depended on mass serological surveys usually with blood samples collected generally on filter paper. According reliable diagnostic test is essential for detection of *L. infantum* infection. This complex panorama certainly generates significant changes in the measures of accuracy of the serological tests and this negative outcome has been ascribed to delays in detecting and eliminating infected dogs, the tendency to replace infected dogs by susceptible puppies, the low sensitivity of the available serological methods, the high incidence of infected dogs and the presence of cross reactions in the used IFAT and ELISA methods [29, 30]. Thus, to remove seropositive dogs is insufficient as a measure for eradicating visceral leishmaniasis in dogs. However, the force of transmission of infection among dogs can be reduced by such programs. The results of this intervention study suggest that the elimination of the majority of seropositive dogs may affect the cumulative incidence of seroconversion in dogs temporarily and may also diminish the incidence of human cases of visceral leishmaniasis [31].

IFAT has low specificity, demand highly trained personnel, it is time consuming and expensive, thus is not adaptable to large-scale epidemiological studies (although it is used in Latin America) and the requirement of sophisticated laboratory conditions prohibit its application in the field. ELISA is the most commonly used test for immunodiagnosis of canine visceral leishmaniasis. The antigen used are traditionally derived from promastigotes cultivated *in vitro* and consist of a repertoire of a least 30 somatic antigens and several surface components. It results that most immunodiagnostic methods have been hampered by problems of cross-reactivity of species within the trypanosomatids as well microorganisms phylogenetically distant [32]. According WHO (2010) [5] serology existing tools (IFAT and ELISA) are difficult to decentralize, direct agglutination test (DAT) can be used at the periphery but needs cold chain and shaking during transportation frequently hampers the antigen. Parasitological existing tools (spleen, bone marrow and lymph node aspirates) are either invasive methods and difficult to decentralize (spleen and bone marrow) or of low sensitivity (lymph nodes).

The recombinant antigens rK9, rK26, rK39 used in the ELISA test seemed to be most suited for point-of-care diagnosis of symptomatic cases of dogs but lack sensitivity for asymptomatic ones [32, 29]. The K39 test was not able to detect active infection in dogs with low IFAT titers, in the range of 1:40 to 1:320. Other tests such as DAT, agglutination screen test (FAST), that is a DAT modified, rapid tests like the immunochromatographic-dipstick TRALD (Test Rapid *Leishmania donovani*) and other using the recombinant rK9, rK26 and rK39 proteins of *L. infantum* are used in the routine diagnosis in several countries but not in Latin America [33]. The direct agglutination test, in which stained parasites are agglutinated by serum antibodies, is popular in Iran and Africa, but variation between batches and the high cost of commercially

available antigen are limiting factors. The most recent platform for serodiagnosis by immu-nochromatography technology is the Dual Path Platform (DPP®), which may in the near future replace other immunochromatographic tests [31]. In Brazil, the Dual Path Platform test began to be incorporated to epidemiological routines [34].

Due to the problems presented by serological tests, different molecular methods have been evaluated for leishmaniasis diagnosis and Polymerase Chain Reaction (PCR) is presently the principal method for molecular diagnosis of *Leishmania.* PCR-based methods for detecting *Leishmania* species in clinical samples have been developed which amplify rRNA and miniexon genes, kinetoplast DNA (kDNA) and repeated nuclear DNA sequences. These methods are of variable specificity; some are generic and can detect all *Leishmania* species while other methods identify the infecting *Leishmania* parasite to the species level. These techniques mostly have a high sensitivity although some, such as PCR with a subsequent hybridization increase the sensitivity of the assay [35]. The real-time PCR was recently introduced for detection and typing of *Leishmania* [36, 37, 38] with the advantages of speed and reduced risk of sample contamination, since monitoring of amplification is conducted as the reaction proceeds. Many studies have reported that real-time PCR has greater sensitivity than conventional PCR for canine visceral leishmaniasis diagnosis and is reproducible in diagnostic routines [39]. PCR based assays are being suggested as useful methods to detect subclinical infections and as a possible addition to serological methods to definitively diagnose inconclusive cases that show low antibody titers or cross reactivity [26].The molecular diagnostics using PCR has demon-strated high sensitivity shown by different studies, than other conventional diagnostic techniques for diagnosis of the canine visceral leishmaniasis, on other hand, PCR methods can vary according to the biological sample examined [40]. This technique has several advantages over other detection methods, especially in the field situation. PCR advantages included, the ability to use very small amounts of target material, the fast detection of *Leishmania* in all material that are used for diagnosis, including skin biopsies, touch preparations, aspirates from lymph nodes, bone marrow, spleen, buffy coat and blood spots collected on filter paper and can be used to simultaneous detection and typing of the parasite [26].

PCR based assays can disclose the presence of parasite DNA very early on, even before seroconversion [21]. The detection of *Leishmania* DNA based on PCR represents an alternative for visceral leishmaniasis diagnosis with highly sensitive, specific and versatile methods [10]. PCR are consistently been shown to be better than microscopy or parasite culture, particularly in samples with low parasite loads [6]. As stated above, several types of canine clinical samples have been used for diagnosis with these techniques [41, 42, 43, 44]. The skin is the principal access point for the phlebotomines and hence represents the principal pathway for infection. Different studies have demonstrated high positive indices for PCR using skin samples of dogs with different clinical signs, and it has been suggested that these techniques based on use of ear skin could be the best procedure for diagnosing canine visceral leishmaniasis [44]. The collection of this sample, however, is painful, bloody and invasive, requiring local anesthesia and aseptic manipulation. The parasites show natural tropism towards lymphoid tissues and many studies have shown that these tissues are a good source for *Leishmania* DNA detection. PCR performed using bone marrow has shown high sensitivity [45], but the procedure of bone marrow sampling is very invasive and traumatic. Complete anesthesia of the animal is necessary, which usually results in opposition by dog owners. Lymph node are one of the

preferred internal tissues for *L. infantum* multiplication and lymph node aspirates also allow high sensitivities by PCR [45, 46], but again the collection procedure is invasive, offering risk of infection for the animal and demands very well trained personnel. These limitations make these samples unsuitable for large-scale surveys. Blood is considered a less invasive sample type. The evaluation of blood for the diagnosis of canine visceral leishmaniasis by PCR is still contradictory. Some studies provided evidence that this clinical sample showed a good performance by PCR [42, 47, 48, 49]. In contrast, other authors encountered problems with the use of blood related to DNA preparation, high frequency of PCR inhibitors in dog blood, variations of the parasite load in the course of infection and low sensitivity [40, 41, 50].

Besides to provide high sensitivity, the ideal source of biological material for molecular diagnosis of canine visceral leishmaniasis would be a non-invasive, painless and easily obtained sample, which could be more accepted by the dog-owners and obtained outside veterinary centers. The conjunctival swab sample is acquired by a non-invasive procedure that uses a sterile swab for sampling the dog conjunctiva (Figure 1) and fulfills these criteria.
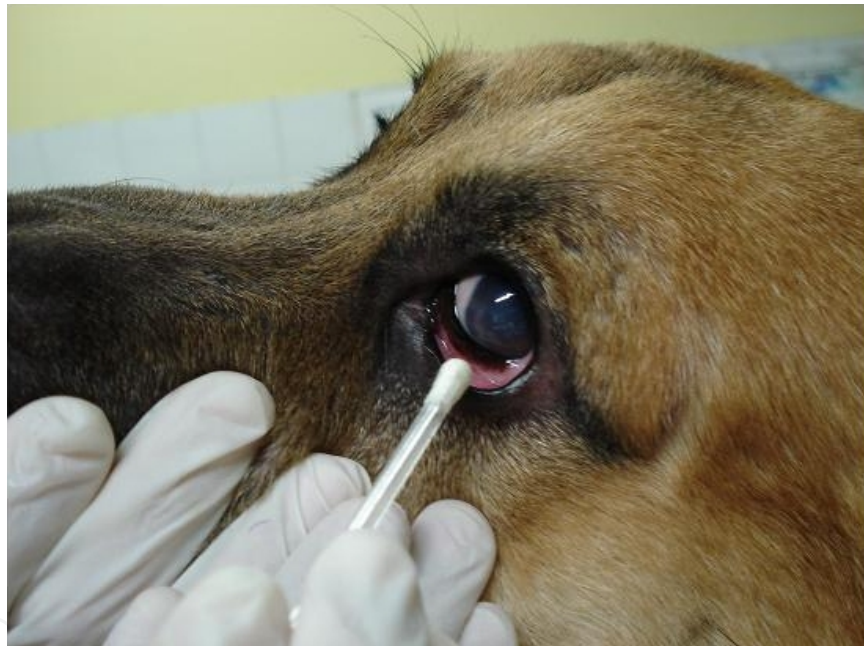

Figure 1. Conjunctival swab sampling method

2. Conjunctival swab

Previous studies had initially pointed that the conjunctiva of infected dogs was a good source of *Leishmania* DNA. Berrahal et al. (1996)[51] investigating asymptomatic carriers was able to identify 15 of 16 dogs (93.8%) by PCR using 5mg of conjunctiva biopsies. In the same study 9 of 14 asymptomatic dogs (64.3%) were positive for skin samples PCR. All these dogs were negative for ELISA and IFAT but immunoblotting detected specific antibodies in 66% of the

animals. Solano-Gallego et al. (2001)[17] identified by PCR 32 out 100 dogs (32%) using conjunctiva biopsies while in the same group 17 out of 95 animals (17.8%) had positive bone marrow and 51 out of 100 (51%) presented positive skin. Reithinger et al. (2002)[52] in a study dealing with tissue tropism and parasite dissemination in two domestic dogs was also able to detect by PCR and histology *Leishmania (Viannia)* spp. in conjunctiva biopsies of both animals.

The first work using the conjunctival swab was performed by Strauss-Ayali et al. (2004)[50]. Ninety-eight dogs were examined in this study and divided in four groups. The group A included 24 seropositive symptomatic animals; in the group B were incorporated 65 seronegative dogs; the group C was formed by six male five-month-old beagle dogs that were experimentally infected with *L. infantum*; and the group D included nine seronegative beagle dogs with no clinical signs of leishmaniasis. In the group A, 83% of the conjunctival samples from either the right or the left conjunctivas were positive by ITS1-PCR and 92% of the dogs were found positives when results from both eyes were combined. Skin scrapings obtained from two areas of the back and from skin lesions were positive for 29%, 41% and 46% of the samples, respectively. Sixty five percent of the dogs were found positive if all skin tests were combined. ITS1-PCR from buffy coat and blood were positive for 57% and 17% of the dogs in that order. Spleen and lymph node aspirates were PCR positives for 77% and 67% of the dogs, respectively. Positives cultures were obtained from 61% and 37% of the spleen and lymph node samples and 74% of the dogs were positive considering the sum of results of both cultures. All group B seronegative dogs were found to be negative by ITS1-PCR in both conjunctival samples and buffy coat. In this study a sensitivity of 92% and a specificity of 100% were found for detection of *L. infantum* DNA in naturally symptomatic infected dogs by using conjuntival swab samples. The experimentally infected dogs (group C) were evaluated every two weeks for 90 days. Forty five days after infection 5 (83%) of the 6 dogs had at least one conjunctival sample found to be positive by PCR while the correspondent optical density values for ELISA were still below the cutoff value. After 60 days of infection the number of positive dogs by conjunctival PCR remained the same but all dogs have seroconverted. At 75 and 90 days after infection, at least one conjunctival sample from 100% of the dogs was found to be positive. Conjunctival, spleen and buffy coat samples were PCR negative for group D control animals (seronegative beagle dogs) and spleen cultures were also negative for *L. infantum.* The study demonstrated that sensitivity obtained by the conjunctival swab PCR was superior to that obtained by culture or by PCR using invasively obtained samples. The work also showed that conjunctival PCR was positive in the experimentally infected dogs before the seroconvertion and was superior to serologic testing for early diagnosis.

A study evaluating the conjunctival swab for canine visceral leishmaniasis diagnosis by the kDNA PCR-hybridization method in a Brazilian endemic region of leishmaniasis in Belo Horizonte, Minas Gerais State, was accomplished by Ferreira et al. (2008)[41]. In the kDNA PCR-hibridization method the PCR amplified products (a 120 bp conserved region of kDNA minicircles) are hybridized with minicircle cloned probes labeled with ^{32}P radionuclide. The study also evaluated two procedures of DNA extraction from conjunctival swabs: phenol chloroform and boiling. The efficiency of the two DNA extraction methods was first evaluated, *in vitro,* using cotton swabs seeded with different numbers of *L. infantum* promastigotes. By

using the phenol chloroform method of DNA extraction, the kDNA PCR-hybridization procedure was able to detect down to a single parasite per swab, while the limit of detection for the boiling method was 25 parasites. Afterward, two groups of 23 seropositive symptomatic dogs were evaluated. Conjunctival swab samples were obtained from both eyes of each animal. The DNA extraction was performed by the phenol chloroform method in group 1 and by boiling in group 2. Blood was also collected from each animal so that $30\mu L$ were spotted onto filter paper and 1.0ml was treated to obtain the buffy coat. The DNA extraction from the buffy coat and filter paper was accomplished by identical procedures in both groups using commercial kits. After hybridization step, the positivities calculated for conjunctival swab combining the results of the right (RC) and left conjunctivas (LC), were 91.3% and 65.2% for groups 1 and 2, respectively. The kDNA PCR-hybridization positivities calculated for the RC and LC separately were 73.9% (17/23) for RC and 91.3% (21/23) for LC in group 1, 52.2% (12/23) for RC and 56.5% (13/23) for LC in group 2. The results obtained for buffy coat and filter paper were 21.7% (5/23) and 30.4% (7/23) in the group 1, 34.8% (8/23) and 43.5% (10/23) in group 2, respectively. All the seronegative control dogs were negative for the kDNA PCR-hybridization assay in conjunctival swab, filter paper and buffy coat. The highest frequency of positivity was obtained by the association between conjunctival swab samples and DNA extraction by phenol chloroform.

Di Muccio et al. (2008)[53] evaluated the conjunctival swab for the early detection of *Leishmania*-Dog contacts in a group of Italian dogs. The following samples were also examined: peripheral blood for IFAT serology, bone marrow and lymph node aspirates for culture, and bone marrow and peripheral blood buffy coat for molecular analysis. Fifty three sets of samples were obtained from 38 dogs. The conjuntival swab (from left and right conjunctivas) PCR was positive for 50 samples (94.3%) while bone marrow plus peripheral blood PCR was positive for 41 samples (77.4%). The IFAT and cultures (bone marrow and lymph node) were positive for 66% and 38.9% of the samples in that order. The PCR sensitivity obtained from conjunctival swab samples proved to be superior to that of systemic samples and much higher than antibody detection. The study highlights that conjunctival swab positives included some asymptomatic animals, as well as drug-treated infected dogs converted to negative at the bone marrow PCR.

The sensitivity of four molecular methods for conjunctival swab samples was compared by Pilatti et al. (2009)[54] in a group of seropositive symptomatic animals. The following methods were used: kDNA PCR-hybridization, kDNA seminested PCR (kDNA snPCR), internal transcribed spacer 1 nested PCR (ITS-1 nPCR) and *Leishmania* nested PCR (LnPCR). All methods had two steps: a first amplification followed by hybridization or by a new amplification (nested or semi-nested).Two methods (kDNA PCR-Hybridization and kDNA snPCR) used primers targeted to the minicircles of kinetoplast DNA (kDNA) and the other two methods to the coding (LnPCR) and intergenic noncoding regions (ITS-1 nPCR) of ribosomal rRNA genes. For all methods DNA samples of $1.0\mu l$ were used. The kDNA PCR–Hybridization was positive for 22/23 dogs (95.6%) and for 40/46 samples (86.9%), considering the right and the left conjunctivas. kDNA snPCR was positive for 21/23 dogs (91.3%) and for 40/46 samples (86.9%). The ITS 1 nPCR and LnPCR were both able to detect the parasites in 17/23 dogs (73.9%)

and respectively 29/46 (63%) and 30/46 (65.2%) samples. The positivities of the kDNA based methods were significantly higher. The authors credited this result to the fact that there are ~10, 000 kDNA minicircles per parasite, while the SSU rRNA and ITS-1 targets have less than 200 copies per cell. In this study the conjunctival swab associated with the most sensitive kDNA PCR based assays showed sensitivities above of 90% for symptomatic dogs.

The first study investigating the efficacy of conjunctival swab PCR for visceral leishmaniasis diagnosis in naturally infected asymptomatic dogs was performed by Leite et al. (2010)[40]. Asymptomatic animals may represent a high percentage of infected dogs in areas of ende-micity and they serve as reservoir for vector transmission to susceptible animals and humans. Symptomatic dogs usually produce high levels of specific antibodies which can be easily detected, but the sensitivity of antibody detection is generally lower in early or in asympto-matic canine infections. In this report conjunctival swab sensitivity was compared to two less invasive samples potentially useful for massive screening of dogs: blood and skin biopsies. The study was performed with 30 asymptomatic dogs, all presenting serological and parasi-tological positive tests. The samples were analyzed by two PCR methods: kDNA PCR-hybridization and ITS-1 nPCR. Using conjunctival swab samples the kDNA PCR-hybridization was able to detected parasite DNA in 24/30 dogs (80%) using the right conjunctiva (RC) and 23/30 dogs (76.6%) with the left conjunctiva (LC). The positivity obtained combining RC and LC results was of 90% (27/30 dogs). A total of 17/30 dogs (56.7%) were positive by means of skin biopsies and 4/30 dogs (13.3%) with Blood. The assay of conjunctval swab samples by ITS-1 nPCR revealed that 25/30 dogs (83.3%) were positive when using RC and 20/30 dogs (66.6%) were positive when using LC. The conjunctival swab positivity obtained by ITS-1 nPCR combining RC and LC was of 83.3%. Via the same method 15/30 dogs (50.0%) were positive by skin biopsies and 17/30 dogs (56.7%) with blood. The kDNA PCR-hybridization and ITS-1 nPCR methods showed similar sensitivities for conjunctival swab and skin biopsy samples. On the other hand, for blood samples, the positivity of ITS-1 nPCR was significantly higher than the one obtained by the kDNA PCR-hybridization, indicating that sensitivity of PCR methods can vary according to the biological sample examined. This study demonstrated the conjunctival swab potential to detect *Leishmania* DNA in asymptomatic dogs and that the sensitivities obtained with asymptomatic animals were similar to the ones observed in previous studies for conjunctival swab PCR in symptomatic dogs [41, 50].

A research conducted by Gramiccia et al. (2010)[55], in a public kennel for stray dogs in Santa Maria Capua Vetere (Campania region, Southern Italy), evaluated the diagnostic performance of conjunctival swab associated to a nested PCR assay for both the early and the late detection of *Leishmania* contacts in dogs exposed to risk of transmission. The nested PCR assay was performed using primers addressed to the small subunit rRNA gene. Two groups of animals were used: (A) a cohort of 65 IFAT and conjunctival swab PCR negative dogs exposed to and followed up during a full sand fly season (July-November 2008), and (B) a cohort of 17 IFAT and conjunctival swab PCR negative dogs but positive at the peripheral blood buffy-coat PCR at July 2008. These animals were examined again in September and November 2008, by buffy coat PCR and in May 2009 along with conjunctival swab PCR. None of group A dogs converted to positive by conjunctival swab PCR or IFAT during the transmission season. In relation to

group B dogs, all remained IFAT seronegative till the end of the study, except for one animal. The results of buffy coat PCR showed an intermittent tendency with transient or full conversion to negative involving 4 and 11 dogs respectively, till November 2008. Eight two percent of the dogs (14/17) converted to negative on May 2009 by buffy coat PCR. However, the conjunctival swab PCR was negative for all dogs at November 2008, but 71% of the animals (12/17) converted to positive by this technique on May 2009. The positive control group, in which were included 10 asymptomatic dogs positives at high IFAT titres at the initial screening performed in 2008, when examined again in May 2009 presented 80% of the dogs positives by conjunctival swab PCR and 60% positive by buffy coat PCR. The conjunctival PCR was not found effective for the very early detection of infection, but this investigation demonstrated a slowly conversion of conjunctival PCR to positive in a high rate of dogs even in absence of seroconversion. The buffy coat PCR although could represent an early marker of leishmanial infection tends to be transient and prone to negative conversion. The authors considered the conjunctival swab PCR as a non-invasive alternative to current serological and molecular methods to asses *Leishmania* exposure in dogs.

Leite et al. 2011[56] carried out a comparison between the diagnosis by conjunctival swab PCR and serology in a group of 42 police dogs vaccinated against visceral leishmaniasis. The dogs belonged to the Military Police of the State of Minas Gerais (PMMG), Brazil. All dogs were vaccinated against visceral leishmaniasis with Leishmune® vaccine (Fort Dodge, Brazil) according to the manufacturer's protocol. The serologic assays were performed one year after vaccination independently by three laboratories: Laboratories 1 and 2 were private laboratories and Laboratory 3 was the National Reference Laboratory of Brazil. ELISA and IFAT were the serologic tests used. The laboratory 1 analyzed all 42 dogs and found 15 positive animals and 4 were identified as indeterminate. Laboratory 2 confirmed only 3 reactive dogs and 2 were classified as indeterminate. Laboratory 3 confirmed 7 reactive dogs and found 3 indeterminate animals. The consolidated serologic result was considered positive when ELISA and IFAT were simultaneously reagents or ELISA was non reagent and IFAT showed fluorescence at sera dilution of 1:80. The results were considered indeterminate when ELISA was non reagent and IFAT showed fluorescence at sera dilution of 1:40 or ELISA was reagent and IFAT was non reagent. Although the three laboratories used the same official diagnostic kits to perform the serologic assays, a significant difference in the results were verified among them. For this reason only the seven cases confirmed by Lab 3 (the National Reference Laboratory) were considered for euthanasia. The autopsy of the euthanized animals showed organ and tissue morphologic changes related to visceral leishmaniasis, except for one dog. The molecular diagnosis by PCR using the conjunctival swab procedure was performed in all 42 animals and was able to detect *Leishmania* DNA in 17 dogs. Comparing the PCR results with those obtained by serologic assay of Laboratory 1, PCR was positive for 10 reactive and one indeterminate case, but was negative for 5 reactive and 3 indeterminate cases. Conjunctival swab PCR was also positive for 5 non-reactive dogs, all of them asymptomatic. The reactive cases according to Laboratory 1 that were PCR-negative tested negative in the serologic assays of Laboratories 2 and 3, and may represent false positives. The same occurred with the three indeterminate cases from Laboratory 1 that were PCR negatives. For the Laboratories 2 and 3 the PCR was positive for all reactive and indeterminate cases. The PCR assay also confirmed all cases

simultaneously reactive in the serologic tests of two laboratories. The study suggested the use of molecular methods as complementary tools for an accurate diagnosis of canine visceral leishmaniasis and pointed that the conjunctival swab procedure could be especially useful as a confirmatory diagnosis for asymptomatic vaccinated dogs that test positive in the serologic assay, since a fraction of these animals might test positive due the vaccination.

A real-time PCR method based on TaqMan which amplifies a 122 bp fragment of the highly conserved kDNA minicircles of *L. infantum* was developed and standardized by Galleti et al. (2011)[57]. In order to evaluate the method, clinical samples of bone marrow, lymph node aspirates, blood and conjunctival swab were collected from 88 dogs for a total of 177 samples. Additional samples of spleen, kidney, lung and liver were also obtained from dead dogs. Twenty seven samples derived from 15 dogs tested positive in the assay. Three of these positive samples corresponded to conjunctival swabs, for which were found a mean number of parasites for PCR reaction of 29.4, 0.35 and 170. The author's opinion was that the conjunctival swab might be suitable for diagnosis of *Leishmania* infection only in case of high parasitic load, but improvements in the sampling and DNA extraction procedures could enhance the sensitivity obtained.

The diagnostic utility of conjunctival swab to detect *Leishmania* infection in a canine population of highly endemic area of leishmaniasis was investigated by Lombardo et al. (2012)[58]. One hundred sixty-three dogs, randomly recruited in various provinces of Sicily, were enrolled. The real-time PCR based on TaqMan (Applied Biosystems) using primers targeting the constant region of the minicircle Kinetoplast DNA, was the molecular assay used. Samples of blood, lymph node, conjunctival and oral swabs were obtained for the molecular assay. From 138 dogs the conjunctival swabs were collected from one eye (Group A) whereas from an additional 25 animals (Group B) both eyes were sampled. Indirect fluorescent antibody test (IFAT), delayed-type hypersensitivity reaction to leishmanin (DTH) and physical examination were also performed. The positivity found for serology and DTH were 27.0% and 73.8%, in that order. The positive PCR percentages for lymph node, conjunctival swab, oral swab and blood were: 24.5%, 22.1%, 8.7% and 5.5%, respectively. The positivity obtained for conjunctival swab in group B, in which both eyes were sampled, increased for 52.0%. The following median parasite load (parasites/ml) was found for each sample: conjunctival swab 10 (range 1-5000), oral swab 7 (range 2-100), lymph node aspirates 16, 500 (range 2-75, 000) and blood 7 (range 2-14). The similar positive PCR percentages obtained for lymph node aspirates and conjunctival swab, based on at least one conjunctival sample, reinforced the use of this non-invasive alternative for the detection of *Leishmania* infected dogs. The study did not show significant association between antibody titers and percentage of positive conjunctival swab PCR, but seropositive and lymph node PCR positives dogs showed a high likelihood to be positive by conjunctival PCR. No association was also found between clinical status and individual molecular results, in especial between the presence of ocular lesions and positive conjunctival PCR. Interestingly, the study demonstrated the presence of *Leishmania* DNA in oral swabs of dogs without any evidence of oral lesions. Therefore oral swab PCR was not a sensitive diagnostic method, the study emphasized that further studies should investigate the importance of this finding for risk of *Leishmania* transmission by licking or bites.

The work of Ferreira et al. (2012)[25] corroborated the conjunctival swab applicability for canine visceral leishmaniasis diagnosis. In this study the kDNA PCR-hybridization and the quantitative real-time PCR were used, respectively, for diagnosis and assessment of parasite load in clinical samples of 80 naturally infected dogs. The dogs were divided in two groups: without clinical manifestations (1) and presenting clinic signs associated with visceral leishmaniasis (2). All animals had positive ELISA and IFAT and/or parasitological positive test. The negative control group included 10 health dogs that tested negative in the serological and parasitological tests. The kDNA PCR-hybridization positive results rates for the clinical samples in the Group 1 were as follow: right conjunctiva, 77.5% (31/40); left conjunctiva, 75.0% (30/40); skin, 45.0% (18/40); bone marrow, 50.0% (20/40) and blood, 27.5% (11/40). By combining the results of both conjunctivas the positivity was 87.5% (35/40). For the group 2 the PCR-hybridization allowed the following results: right conjunctiva, 95% (38/40); left conjunctiva 87.5% (35/40); bone marrow, 77.5% (31/40) and blood 22.5% (9/40). A positivity of 95.0% (38/40) was obtained considering the positive results of both conjunctivas. For qualitative molecular diagnosis the conjunctival swab samples showed the best results for both dogs groups. The quantitative real-time PCR was performed using primers addressed to a fragment of a single-copy-number *L. infantum* DNA polymerase gene. Canine housekeeping β-actin gene was used as endogenous control. The results were defined as the number of parasites per 104 canine cells. For both groups the parasite burdens determinate by the quantitative real time PCR in conjunctival swab and bone marrow were statistically equivalent, by the other side the parasite load in the skin was higher than the other clinical samples. When compared between groups the parasite load from conjunctival swab in group 2 was higher than in group 1. The same relationship was found for bone marrow. However, no differences were observed in skin load between groups. The high parasite burdens detected in skin from both symptomatic and asymptomatic animals emphasized the role of infected dogs, especially the asymptomatic, as reservoir. The article considered the conjunctival swab sampling procedure suitable form molecular diagnosis of canine visceral leishmaniasis and suggested their widespread use.

An interesting study to evaluate the conjunctical swab diagnostic performance in different stages of infection and also for the follow up of dogs undergoing antileishmanial treatment was conducted by Di Muccio et al. (2012)[59]. To achieve the first objective 253 dogs from areas of endemicity from central Italy were submitted to a cross-sectional survey. For the second aim was performed a longitudinal study using 20 sick dogs under treatment. The molecular assay was a nested PCR using primes addressed to the small-subunit rRNA gene. Among the 253 animals the rates of *Leishmania* infection were 21.73% for conjunctival swab PCR, 21.34% for IFAT, 14.22% for popliteal lymph node cytological examination and 8.69% for buffy coat PCR. Seventy two dogs were positive by at least one test and considered positives for canine visceral leishmaniasis. Among these 72 dogs 76.38% were positive for conjunctival swab PCR, 75.0% for IFAT, 50.0% for lymph node cytological examination and 30.55% for buffy coat PCR. The conjunctival swab PCR showed the best performance and presented a high concordance in relation to IFAT (κ = 0.75). Test correlation with infection and clinical staging were analyzed in 54 IFAT seropositive dogs. Seven dogs were classified as exposed (low IFAT titer plus negative cytology and negative PCR), 38 as infected (low IFAT titer plus positive cytology and/or positive PCR but without clinical signs) and 9 as sick (high IFAT titer plus positive cytology

and with at least one clinical sign). The conjunctival swab PCR showed the best positivity in the infected group (84.2%, 32/38 dogs) followed by lymph node cytological examination (77.8%, 7/9 dogs) and buffy coat PCR (42.1%, 16/38). The positivity of conjunctival swab PCR was also high in the symptomatic group (77.8%, 7/9 dogs), the lymph node cytological examination detected 9/9 dogs (100%) and buffy coat PCR 33.3% (3/9 dogs). In the group exposed, none of the three methods was able to detect infection. Eighteen dogs were negative by IFAT and 16 of these dogs were positive by conjunctival swab PCR and 3 by buffy coat PCR. In the longitudinal study using 20 sick dogs, all of them were positive by IFAT and conjunctival swab PCR (100%) at the beginning of the study, whereas 17 tested positive in the lymph node cytological examination (85%) and 9 in the buffy coat PCR (45%). After three months, the therapy protocols promoted a total remission of clinical signs and decrease of antibody titers with reduction in the positivity rates for conjunctival PCR (30%), lymph node cytological examination (10%) and buffy coat PCR (5%). After six months of treatment was verified an increase in the positivity for conjunctival swab PCR (88.89%) and buffy coat PCR (44.44%) and a less marked increase in lymph node cytological examination positivity (22.22%), without reappearance of clinical signs or increase in serological titers. These results demonstrated that conjunctival swab PCR was sensitive for the early detection of relapses and suitable to monitor the evolution of infection after therapy.

Ferreira et al. (2013) [60] compared conjunctival and nasal swabs with other clinical samples in 62 naturally infected dogs (58 of them symptomatic). *L. donovani* complex specific primers addressed to kDNA minicircle conserved region were used. The following frequencies of positive results were obtained: nasal swab, 87% (54/62); conjunctival swab, 76% (47/62); skin biopsy, 81% (50/62); bone marrow biopsy, 90% (56/62). Positivity obtained using nasal swabs was statistically equivalent to those obtained with the other samples, but in this study the conjunctival swab showed a lower frequency of positivity than that calculated for bone marrow samples, probably due to the PCR protocol used. The parasite load was estimated by qPCR, using primers addressed to the parasite DNA polimerase gene and the canine β-actin gene as a housekeeping gene. The parasite load from conjunctival and nasal samples were equivalent, but lower than verified in bone marrow and skin samples. Oral and ear swabs were also evaluated in a smaller group of 28 animals. Positive results were: oral swab, 79% (22/28) and ear swab, 43% (12/28). The results of this study indicated that conjunctival, nasal and oral swabs were effective in detecting *Leishmania* in naturally infected dogs. The authors suggested that a combination of these samples would be useful in large-scale screening of dogs.

3. Conclusions

In the Mediterranean, Southern Europe and South and Central America, with approximately 500, 000 new human visceral leishmaniasis cases reported annually and millions of dogs infected, being dogs considered to be the major reservoirs for the disease, the accurate diagnosis in these animals is extremely important. Diagnosis of canine visceral leishmaniasis is performed mainly by direct parasitological methods that can yield false-negative results, either because of the very low number of *Leishmania* spp. organisms in clinical samples (bone

marrow and lymph nodes) or because morphological identification is difficult. In addition, these methods are invasive. Another problem mentioned is that serology is not sufficient as a criterion for eliminating infected dogs. Conventional serological techniques are limited by cross-reactivity with other parasitic diseases, because several technical procedures have not been standardised and due the low sensitivity of the available serological methods in the initial stages of infections.

In dogs PCR-based assay is currently the more sensitive and specific technique for detection of *Leishmania* and it allows using different clinical samples. The conjunctival swab is a non-invasive sample recently reported and up to moment few studies have been performed using this approach. Nevertheless, its high sensitivity and applicability for molecular diagnosis of canine visceral leishmaniasis have been confirmed, independently, by different research groups.

The studies demonstrated that the method allows the identification of infected dogs before the seroconversion and that conjunctival swab sensitivity for molecular diagnosis was superior or equivalent to obtained by invasive samples of either symptomatic or asymptomatic animals. The conjunctival swab was also proved useful to monitor the dogs during drug therapy. The molecular diagnosis using non-invasive samples such conjunctival swab is of great relevance in epidemiological studies when large numbers of dogs are sampled and also for clinical or experimental purposes, that implies repeated samplings. The standardization of this sampling procedure can help to become viable and widespread the molecular diagnosis of canine visceral leishmaniasis. The DNA extraction protocol and the sensitivity of PCR assay used are important variables to be considered in order to obtain the best results. Field studies in wide heterogeneous populations including seronegative and seropositive animals and works that follow up PCR positive seronegative dogs are still lacking and are very important for the method validation.

Molecular tests are yet comparatively expensive in relation to other diagnostic techniques available and require technological expertise, but considering the data presented above, a sensitive, specific and practical test could provide very cost-effective alternatives to currently available diagnostic tests, especially when used in mass-screening surveys.

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