RESEARCH ARTICLE

EVALUATION OF COMMERCIAL ELISA, INDIRECT IMMUNOFLUORESCENCE TEST AND qPCR FOR THE DIAGNOSIS *OF LEISHMANIA INFANTUM* IN ASYMPTOMATIC DOGS FROM BOUIRA, NORTHEAST ALGERIA

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ABSTRACT

Serological and molecular tests are important tools to determine and control Canine visceral leishmaniasis (CanL). The absence of pathognomonic symptoms and asymptomatic dogs make clinical diagnosis difficult. This study aims to determine the best combination for the diagnosis of *Leishmania infantum* in asymptomatic dogs. Ninety-four blood samples were collected from dogs in the province of Bouira, in Algeria and assessed with IFAT, ELISA, and qPCR. The sensitivity and specificity for each technique were evaluated in comparison with the indirect fluorescent antibody test (IFAT), which is considered the best-suited test to determine the presence of *L. infantum*. In addition, the agreement between these tests was assessed. The use of Cohen's Kappa coefficient demonstrated a moderate agreement between IFAT and qPCR (k= 0.475), and fair between the IFAT and ELISA (k=0.297). The sensitivity of the molecular test was higher (55.56%) than that of the ELISA (33.33%). According to those results, it is better to combine two tests for the diagnosis of CanL, especially in asymptomatic dogs, which is generally not included in the CanL control program.

Keywords: Canine visceral leishmaniasis, *Leishmania infantum*, zoonosis, diagnosis

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INTRODUCTION

Leishmaniasis is one of the most neglected diseases in the word. It occupies the third place of the most important vector-borne diseases after malaria and lymphatic filariasis (Inceboz, 2019), and is caused by an intracellular protozoan, *Leishmania* spp in mammals including humans. More than twenty species are belonging to the genus *Leishmania* (Bennai et al., 2018; WHO, 2020). For their transmission, *Leishmania* parasites involve a female Diptera from the family of *Psychodidae,* belonging to the *Phlebotomus* genus in the Old Word, and *Lutzomiya* genus in the New Word (Mouloua et al., 2017).

In humans, there is a wide variety of clinical forms of leishmaniasis. The most common in the world is cutaneous leishmaniasis (CL), local or diffuse, caused mainly by *Leishmania major*, *L. tropica* and *L. infantum* in the Old World, and generally by *L. braziliensis*, *L. chagasi* and *L. Mexicana* in the New World (De Vries et al., 2015). Visceral leishmaniasis (VL), known as kala azar, which can be fatal, is caused by *L. donovani* in Asia and Africa, and by *L. infantum* in the Mediterranean basin, South and Central America and in the Middle East and Central Asia (Dantas-Torres et al., 2019). The annual incidence of VL and CL has been estimated at 0.2 to 0.4 million cases and 0.7 to 1.2 million cases, respectively (Alvar et al., 2012). Another form of leishmaniasis deriving from kala azar, corresponds to the post kala azar dermal leishmaniasis (PKDL). It is caused by *L. donovani*. The mucocutaneous leishmaniasis (MCL) is the fourth form. It is caused by *L. major* and *L. tropica* (Inceboz, 2019).

Animals (dogs, cats, and rodents) play an important role in the transmission cycle of certain forms of leishmaniasis between them and secondary, to people, hence a zoonotic form (ZL). Indeed, *L. infantum* is the common and important cause of canine leishmaniasis (CanL), which can be fatal to humans. There are at least 2.5 million infected dogs in the Mediterranean basin (Moreno and Alvar, 2002). The visceral, cutaneous and mucocutaneous leishmaniasis types can be found in canine species (Inceboz, 2019; Palumbo, 2010).

Dog constitutes the main reservoir/ host of *L. infantum*. It is frequently exposed to relapse after clinical recovery, which makes it a veritable target for reinforced and offensive preventive and vaccine prophylaxis, especially in endemic areas (Bourdoiseau, 2015). However, early diagnosis remains one of the means to control the infection. It is often based on the origin of the epidemic and clinical signs in dogs (Solano-Gallego et al., 2011). Moreover, the absence of pathognomonic symptoms and asymptomatic dogs makes clinical diagnosis of CanL difficult, which requires the use of laboratory techniques such as parasitological, immunological and molecular tests (Ribeiro et al., 2018). In veterinary medicine, molecular biology is increasingly used as a diagnostic tool because of its high sensitivity and specificity. Serological tests, such as the enzyme linked immunosorbent assay, are frequently used in epidemiological surveys (Ait-Oudhia et al., 2009). However, cross-reactions with *Leishmania* species responsible for the cutaneous form and other haemoparasites constitute one of the limitations of serological tests (Ferreira et al., 2007; Porrozzi et al., 2007). In contrast, serological test can be very useful in some situations, such as in Brazil and to control CanL. Positive results of serological tests obtained for suspected dogs represent a criterion to indicate their euthanasia (Ribeiro et al., 2018). However, the absence of a gold standard test makes difficult the evaluation of the diagnostic methods for CanL (Solano-Gallego et al., 2014). Our epidemiological survey conducted in the district of Bouira aims

to determine the best combination for detecting *Leishmania infantum* in asymptomatic dogs.

MATERIALS AND METHODS

Sampling and study area

The study was carried out in the district of Bouira (36° 17'0.708" N 3° 59' 16.234" E). It is located in Kabylia, where a rate of 36% of seropositive dogs by CanL has been recently reported (Medkour et al., 2020). The district of Bouira is characterized by a hot and dry climate in summer and cold and rainy in winter. The temperature varies from 0° C to 33° C and is rarely lower than -4 °C or higher than 37 °C. The region has an annual precipitation of at least 432 mm, for 216 days a year on average. The district of Bouira experiences a dry climate with estimated humidity of 69%.

In the period from March to April 2019, 94 dogs (52 males and 42 females with 21 dogs younger than 12 months, 59 dogs between 12 months and 48 months and 14 dogs of 60 months and older) were randomly selected. Three groups of dogs were defined depending on their particular activities and functions: guard dogs (n= 19), farm dogs (n= 7) and hunting dogs (n= 68). All samples were collected from apparently healthy dogs.

A volume of 6 ml of blood was collected from the forelimb vein of each animal, and equally divided into EDTA and dry tubes. The separated sera were obtained by centrifugation of the clotting dry tube at 3000 g for 10 min, then they were transferred to cryotubes. All samples were stored at – 20°C until analysis.

Laboratory analysis

The presence of *L. infantum* in asymptomatic dog samples was determined by serological and molecular tests. Indirect enzyme linked immunosorbent assay (ELISA) (ID screen Leishmaniasis Indirect, IDvet France) was realized in the UMR BIPAR (Maisons- Alfort, France), and the indirect immunofluorescence intibody test (IFAT) and molecular assays (qPCR) were realized at the Institut Hospitalier Universitaire IHU-Mediterranée Infection laboratory, Marseille, France. The doubtful results are considered negative.

Serological assays

For ELISA, following the manufacturer's instructions, 96 well plate pre-coated with *L. infantum*-purified antigen were incubated 45 minutes ± 5 min at 37° C with dog sera and standards, all diluted at 1/10 in sample buffer. After incubation, the plates were washed three times with wash buffer, and coated with an anti-dog IgG- peroxidase (HRP) conjugate diluted in a sample buffer. A second incubation was realized for 30 minutes at 37°C. The microwells were then washed three times and a reaction was revealed using a substrate solution (TMB) and the colorimetric reaction was terminated by stop solution. The plates were read at 450 nm, on the Multiskan fc reader and analyzed with Skanit Research Edition 4.1 software. For each sample, the S/P percentage was calculated according to the formula:

$$SP\% = \frac{\text{OD } sample - ODNC}{\text{OD } PC - ODNC} \times 100.$$

The interpretation of canine sera is as follows:
a positive result: $SP\% \geq 50\%$;
a doubtful result: $40\% < SP\% < 50\%$;
a negative result: $SP\% \leq 40\%$.

Samples were also analyzed by IFAT, which is used to detect *L. infantum* specific antibodies. Coated plates with *L. infantum* commercial antigens (Zoetis, France) were incubated with diluted sera at 1:50 for 30 min at 37°C. After incubation, they were washed twice with PBS and once with distilled water. Then IgG anti-dog conjugated with fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch Europe Ltd. Cambridge House, UK) was added into each microwell after dilution at 1:200. A second incubation for 30 min at 37°C was done in the dark. The washing procedure was repeated as described above. Some drops of mounting medium Fluoprep (bioMérieux, France) were added on the coverslips to read plates using a fluorescence microscope. Positive and negative controls had been added to validate the results. Positive control was a known serum of French dog infected by *L. infantum* (at the dilution 1:1600), and negative control was a known serum of dog non-infected by *Leishmania* species from IHU-Méditerranée Infection lab collection. All samples were examined by two different investigators to confirm observations. Samples were considered positive at 1:50 dilution, and all positive samples positive results were further investigated using serial dilution at 1:100, and 1:200, 1:400, 1:800 and 1:1600. Positive sera at dilution of 1:1600 were considered highly positive.

Molecular assays

DNA was extracted from 200 µl of dog blood samples using a commercial DNA extraction kit (QIAmp DNA Mini Kit®, (Qiagen,Courtaboeuf, France) and performed on BIOROBOT EZI (Qiagen, Courtaboeuf, France) per the manufacturer's recommendations. The extracted DNA was eluted in 200 µl of distilled water and then stored at –20 °C. All samples were screened using a qPCR targeting the 18S rRNA gene of *Leishmania* spp (Medkour et al. 2020) as well as specific *L. infantum* qPCR targeting the kinetoplast DNA (kDNA) (Mary et al., 2004). qPCR reactions were performed in a final volume of 20 µl including 10 µl of Eurogentec Master Mix Roche, 3 µl of distilled water DNAse and RNAse free, 0,5 µl FAM- labeled probe (concentrated at 5µM), 0.5 µl for each forward and reverse primers (concentrated at 20 µM), 0.5 µl of UDG and 5 µl of the DNA template. The amplification was performed in a CFX96 Real-Time System (Bio-Rad Laboratories, Foster City, CA, USA) according to Roche Protocol: incubation at 50°C for two minutes, 40 cycles of denaturation at 95 °C for 5 s and annealing-extension at 60 °C for 30 s. These two steps are preceded by the first denaturation for 5 minutes, at 95 °C. Known *Leishmania* DNAs were added as positive control and master mixture was added as negative control. Samples were considered positive when the cycle threshold (Ct) waslower than 38.

Statistical analysis

Statistical analyses were performed using XLSTAT (V 2020.3.1). The accuracy parameters of the assays (sensitivity, specificity, positive predictive value and negative predictive value) were calculated using two-by-two contingency tables, and IFAT was considered the best-suited test. The χ^2 *test* was calculated to determine if a difference in efficiency exists and $p< 0.05$ was considered significant. The agreement between ELISA, IFAT and qPCR to detect *L. infantum* was evaluated by the use of Cohen's Kappa coefficient (k). The strength of the agreement between these techniques is assessed as follows: no agreement (< 0), slight agreement (0–0.2), fair agreement (0.2-0.4), moderate agreement (0.4- 0.6), substantial agreement (0.6- 0.8) and almost perfect agreement (0.8 – 1) (Landis and Koch, 1977).

RESULTS

All samples were randomly selected from 94 dogs living in an endemic area of CanL and tested using ELISA, IFAT and qPCR. The comparative test parameters (sensitivity, specificity, positive and negative predictive values) were calculated and represented in Table 2.

Of the 94 samples, 17 (18%) were positive by ELISA, 9 (10%) were positive by IFAT and 3 (3%) by qPCR **(**Table 1**)**.

The IFAT has resulted in different IgG titers: 3.19% (3/94) were positive at 1:50 dilution; 4.26% (4/94) were positive at 1:100 dilution; 1.06 % (1/94) was positive at 1:200 and only 1.06% (1/94) was positive at 1:400 dilution.

When considering IFAT as a reference test, 5/94 (5.32%) were positive with both IFAT and ELISA, and 4/94 (4.26%) were positive with IFAT but negative with ELISA. Only 3/94 (3.19%) were positive with both IFAT and qPCR. Eighty-five samples were negative when comparing IFAT and qPCR, and 73/ 94 were negative when comparing IFAT and ELISA (Table 1).

Table 1 Contingency table for the detection of *L. infantum* antibodies in dogs' sera by commercial ELISA and qPCR in comparison with IFAT *(n =94)*

		IFAT			
		Positive	Negative	Total	%
Elisa	Positive	5	12	17	**17 (18%)**
	Negative	4	73	77	**77 (82%)**
		9 (10%)	**85 (90%)**	94	
qPCR	Positive	3	0	3	**3(3%)**
	Negative	6	85	91	**91 (97%)**
		9 (10%)	**85 (90%)**	94	

A moderate agreement in detecting *L. infantum* in dogs was reached between IFAT and qPCR (k= 0.475). However, the results indicate a fair level agreement between IFAT and ELISA (k= 0.297). In both cases, we found a statistically significant difference between these methods (χ^2>3.841, P < 0.05 (Table 2).

The results of the diagnostic performance of tests are described in Table 2. The sensitivity and specificity of ELISA were 55.56 % and 85.88 %, respectively. qPCR obtained a high specificity (100%).

The positive predictive values (PPV) were 29.41% and 100% with ELISA and qPCR, respectively, and the negative predictive values (NPV) were 94.81% and 93.41% with ELISA and qPCR, respectively.

Table 2 Results of diagnostic performance of ELISA and qPCR for detection of *L. infantum* in asymptomatic dogs in comparison with IFAT

	ELISA	qPCR
Sensitivity (%)	55.56	33.33
Specificity (%)	85.88	100
Positive predictive value (%)	29.41	100
Negative predictive value (%)	94.81	93.41
Kappa coefficient	0.297	0.475
P value	< 0.002	< 0.0001
χ^2	9.43	29.27

DISCUSSION AND CONCLUSION

The Mediterranean Basin, including Algeria, is an endemic area for visceral leishmaniasis, and dogs constitute a potential reservoir for humans (Bourdoiseau, 2015). The geographical position and the climate of the district of Bouira maintain the cycle of *L. infantum*. Samples were collected from rural areas, where water points are frequent. In Algeria, the sand fly vectors are represented mainly by *Phlebotomus perniciosus* and *P. longicuspis* (Izri et al., 1990; Killick-Kendrick, 1990)

In most cases CanL is either asymptomatic or accompanied by no specific symptoms, which make a diagnosis difficult (Ribeiro et al., 2018). In those cases, laboratory techniques are requested to determine the infection by *L. infantum*. In this study, we aimed to evaluate the sensitivity and specificity of ELISA and qPCR in comparison with IFAT as a reference test to determine the best combination for detecting *L. infantum* infection in asymptomatic dogs. The randomly selected dogs were living in an endemic area of CanL.

All parasitological, serological and molecular tests need to be interpreted according to their limits and advantages (Ribeiro et al., 2018). Some studies consider that IFAT is a technical reference in diagnostic laboratory practices (Paltrinieri et al., 2010). It is commonly used as the best-suited test in clinical and research studies (Maia and Campino, 2008; Solano-Gallego et al., 2009). In some Latin American countries such as Colombia and Brazil, IFAT is considered the reference test to determine the presence of *Leishmania*-specific antibodies (Herrera et al., 2019). However, even if IFAT is one of the most common serological tests, the study of Persichetti et al. (2017) suggested that the diagnosis of clinical leishmaniasis with ELISA was better than with IFAT. Besides, with IFAT a cross-reaction between *Leishmania Spp* and *Trypanosoma cruzi* can occur (Luciano et al., 2009).

In our study, ELISA technique showed a moderate sensitivity (55.56 %) against 33.33 % of qPCR in comparison to IFAT. However, the specificity of ELISA was much lower (85.88%) than that of qPCR (100%). According to the results of Wang et al. (2011), molecular assays are more sensitive than ELISA, remaining the most suitable test for *L. infantum* diagnosis in blood samples, more suitable than serological tests in asymptomatic

dogs. PPV and NPV were analyzed to evaluate the effectiveness of the diagnostic tools used in this study. These parameters are affected by sensitivity and specificity of tests (Surawicz and Travel, 2008). According to our results, PPV of qPCR was optimal (100 %), with the specificity of 100% compared to ELISA (Table 2), which suggest that qPCR can be the best tool to detect *L. infantum* in asymptomatic dog samples. Further studies with a large number of samples are needed, especially in asymptomatic dogs because most studies were performed in clinically infected dogs, which make our results inconclusive.

In many endemic areas, the vaccination is induced against leishmaniasis. In Brazil, euthanasia is recommended for infected and seropositive dogs (Ribeiro et al., 2018). Serological tests cannot distinguish between vaccinated and naturally infected dogs, unlike the molecular approach, which is more relevant than serological tests (Marcondes et al., 2013).

In another study carried out on thirty-four dogs with skin lesions evoking American Tegumentary Leishmaniasis (ATL) in an endemic area of the state of Rio de Janeiro, it was suggested that ELISA was more sensitive to detect specific anti-*Leishmania* IgG with sensitivity and specificity of 97.1% (Ribeiro et al., 2007). Thus, the sensitivity and specificity may be different between several diagnostic tests. According to Camargo et al. (2010), even if the PCR technique is in huge demand for the diagnosis of CanL due to its high accuracy, this technique can reveal false-negative results in some cases. This study showed that the specificity of qPCR was 100 %, but it proved less sensitive. Despite this conclusion, molecular techniques are characterized by high sensitivity and specificity, and they are commonly used in veterinary diagnosis routine with the use of several biological samples, for instance, skin fragments, lymph node puncture and vein blood samples (Silva et al., 2017; Solano-Gallego et al., 2007; Solano-Gallego et al., 2011). However, blood samples are best analyzed with PCR for *L. infantum* diagnosis. Moreover, molecular assays have a non–invasive access to the sample (Wang et al., 2011).

A moderate agreement between IFAT and qPCR (k= 0.475) and a fair agreement between IFAT and ELISA (0.297) were measured. In the study of Ferreira et al. (2007), the agreement between ELISA and IFAT was satisfactory, which can mean that test agreement depends on various parameters, as it was mentioned by Dye et al. (1993). Ferreira et al. (2007) reported that in 20 % of detected cases of CanL during the incubation period or seroconversion, the levels of antibodies could be variable, which explained the low level of agreement between the serological tests used for the detection of asymptomatic dogs. The specificity of 100% for serological test was showed and ELISA proved more sensitive than IFAT (Ferreira et al., 2007).

Sensitivity and specificity can differ between the studies. Several factors can be at the origin of these variabilities such as the type of antigen or the protocol followed when using certain tools (Sundar and Rai, 2002). In all cases, further studies are needed to improve the sensitivity of the diagnostic tests for canine leishmaniasis, especially in asymptomatic dogs (Silva et al., 2014). Away from all appreciations about descriptive test parameters, asymptomatic dogs can be an infective source of sandflies and can therefore transmit the infection to humans (Alvar et al., 1994; Soares et al., 2011; Wang et al., 2011). Actually, the skin is the main target of sandflies. It is the site of inoculation of *Lesihmania* spp, which has been isolated from intact skin (Madeira et al., 2009; Silva et al., 2016). The incubation period for CanL is variable and can last from few months to several years (Reiner and

Locksley, 1995). After the incubation period, the dogs can remain asymptomatic and resistant to leishmaniasis, or they present with clinical signs such as the skin lesions, and can die if not cured (Dye et al., 1992). The sand fly takes its blood meal in the host from the skin, which is sufficiently irrigated, especially in the auricular region (Travi et al., 2001). It was demonstrated by Laurenti et al. (2013) that asymptomatic dogs were more infective to the Diptera female than symptomatic dogs, which could explain the possibility of transmission of *Leishmania* spp from dogs without clinical lesions, especially in an endemic area. The same authors reported the results of xenodiagnoses which showed a high proportion of infection by *Leishmania infantum chagas i*n vectors (38.5 %) that fed on asymptomatic dogs in comparison with the Phlebotomines that fed on symptomatic dogs (24.7%). In addition, the study demonstrated that five asymptomatic dogs among 14 examined by immunohistochemistry presented parasites in their skin.

With these conclusions and according to Madeira et al. (2009), asymptomatic dogs are considered a potential source of infection of sandflies, and can maintain the epidemiological cycle of CanL.

This study highlights for the first time *L. infantum* in asymptomatic dogs from Algeria, suggesting their involvement in the epidemiological cycle of the parasite, especially in an endemic area. According to our results, IFAT had a moderate agreement with qPCR for detecting *Leishmania infantum* antibodies in sera from dogs compared to ELISA, but the sensitivity was not as good. In epidemiological surveys and for the reliable results, especially in asymptomatic dogs, it is better to combine two tests for the screening and diagnostic of CanL. Serological and molecular tests are very helpful and widely used to determine and control the *Leishmania* infection, but serological tests remain more practical and easier to use. The choice of diagnostic tools depends on several parameters, including their sensitivity and specificity.

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CONFLICT OF INTEREST

None

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EVALUACIJA KOMERCIJALNOG ELISA TESTA, INDIREKTNOG IMUNOFLUORESCENTNOG TESTA I qPCR U DIJAGNOSTICI *LEISHMANIAE INFANTUM* KOD ASIMPTOMATSKIH PASA IZ BOUIRAE NA SJEVEROISTOKU ALŽIRA

SAŽETAK

Serološki i molekularni testovi predstavljaju važno sredstvo u određivanju i kontroli pseće visceralne lišmanijaze (CanL). Odsustvo patognomoničnih simptoma i asimptomatski psi otežavaju postavljanje dijagnoze kliničkim putem. Cilj ovog istraživanja jeste odrediti najbolju kombinaciju za dijagnosticiranje *Leishmaniae infantum* kod asimptomatskih pasa. Devedeset i četiri krvna uzorka su prikupljena od pasa u provinciji Bouira u Alžiru nakon čega su analizirani IFAT, ELISA i qPCR metodom. Senzitivnost i specifičnost svake od metoda je evaluirana u usporedbi s indirektnim testom fluorescentnim antitijelima (IFAT) koji se smatra najboljim za određivanje prisustva *L. infantum*. Pored toga se procjenjuje i slaganje između ovih testova. Korištenjem Cohen Kappa koeficijenta je pronađeno umjereno slaganje između IFAT testa i qPCR (k= 0.475) i slabo slaganje između IFAT I ELISA testova (k=0.297). Dokazana je veća senzitivnost molekularnog testa (55.56%) u odnosu na ELISA test (33.33%). Rezultati našeg istraživanja pokazuju da je za dijagnosticiranje CanL, posebno kod asimptomatskih pasa, bolje kombinirati dva testa, što obično nije uključeno u kontrolni program CanL.

Ključne riječi: Pseća visceralna lišmanijaza, *Leishmania infantum*, zoonoza, dijagnoza