A Novel Exo-antigen-based ELISA for the Detection of Canine Leishmaniasis

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Abstract. Dogs which are infected with leishmania parasites serve as major reservoir hosts for zoonotic visceral leishmaniasis. The incidence of zoonotic visceral leishmaniasis is rising in many countries. This may be associated with the continuing drift of people and their pets from rural areas into peri-urban settings, particularly at the fringe of large cities. At the same time, there is evidence of adaptation of sand fly vectors to these urban settings. This has created an alarming situation because, even though domestic and stray dogs may be infected, many remain asymptomatic but are still highly infectious to the sand fly vectors and thus pose a serious threat to human health. Over half of the infected dogs have asymptomatic infections and current assays are not sensitive enough under field conditions to distinguish asymptomatic from symptomatic dogs. There is an urgent need for a specific and sensitive screening tool for use in the field. We have previously demonstrated that promastigote exo-antigen-based ELISAs can be used in the specific diagnosis of human visceral leishmaniasis (HVL). A cocktail of exo-antigens prepared from three species (L. infantum, L. donovani, and L. major) was used to develop and optimize a canine ELISA assay. Serum samples from dogs with a variety of pathological conditions but living in a non-leishmania endemic area were used as negative controls and their reactivity was used to determine a cut-off value for the ELISA. Samples from dogs residing in a leishmania endemic area were tested in parallel using direct agglutination (DAT), immunofluorescence (IFAT), and ELISA. The ELISA results correlated closely (100%) with the clinical symptoms, and were elevated in one asymptomatic dog. This sample was also found to be positive by IFAT. Based on its sensitivity and specificity, the cocktail exo-antigen-based ELISA may prove useful, even at 1:2,000 serum dilutions, for screening dogs in different geographical regions of the world.

INTRODUCTION

In both the New World and Old World settings dogs are an important reservoir host in zoonotic visceral leishmaniasis.1–5 Dogs as reservoirs are directly responsible for dissemination of HVL in 50 of 62 countries where HVL is endemic.6 In the Old World, around the Mediterranean basin and North Africa both human and canine visceral leishmaniasis is caused by Leishmania infantum. In the New World L. chagasi and other species are responsible. However, this interrelationship is absent in other countries, notably India. About 100,000 to 500,000 new cases of HVL occur every year and dogs have a major role in causing these new cases. More than half the infected dogs do not show any overt clinical symptoms.7 Despite the clear relationship between canine and human leishmaniasis, accurate statistics on the rate of infection in the reservoir host are lacking. There have been attempts to use serology8,9 and PCR10 for determining whether or not a particular dog is carrying infection. Earlier work8,9,11,12 indicated that dogs serologically respond following exposure to Leishmania parasites. This is the basis for epidemiological screening of dogs. However, lack of sensitivity of currently available serological tests has been reported in the literature. Critical appraisal of past work suggests that there is some scope to improve assay specificity as well as sensitivity provided appropriate antigens are used in the immunoassay. The lysate antigens that have been used by earlier workers are known to generate false positive results.4 The antigens that are used in ELISA should be specific to Leishmania parasite species. Recently we demonstrated that promastigote exo-antigens are sensitive for specific detection of antibodies during HVL.13,14 By using a different mix of secreted promastigote antigens, we developed an ELISA for serological screening of reservoir dogs for potential exposure and for carrying reservoir infection. This assay was evaluated against conventional direct agglutination (DAT) and immunofluorescence (IFAT) tests. The Exo-Ag–based ELISA correlated well with the clinical signs and was found to be more sensitive than DAT and IFAT.

MATERIALS AND METHODS

Chemicals and reagents. Buffer salts were purchased from BDH; Evans blue from Fluka, Buchs, Germany; gelatin from Difco, Detroit, MI; 0.2 M β-mercaptoethanol, and goat anti-dog IgG-HRP conjugate from Sigma, St. Louis, MO; multiwell spotted Leishmania antigen slides, goat anti-dog IgG-FITC conjugates were obtained from bio-Mérieux, Marcy-l’Étoile, France. Protein-free and serum-free maintenance medium were purchased from GIBCO-BRL USA.

Leishmania promastigote secreted antigens. Three Leishmania isolates were used: (i) L. infantum (IPT-1) originating from the Liverpool School of Tropical Medicine, England was obtained from Professor John Ellis of the University of Technology Sydney; (ii) L. donovani (WR130L); and (iii) L. major (WR1075) originated from the Walter Reed Army Institute of Research, Silver Spring Maryland. Respective promastigote stages were cultured separately at 1 × 10^7/mL concentration in protein-free medium in roller tissue culture flasks for 3 days at 26°C. The cultures were centrifuged at 3,500 g for 15 minutes and the supernatant was re-centrifuged and the resultant clear supernatant was further filtered through 0.45 μm pore size membranes to obtain the secreted exo-antigens. The protein content of the filtered supernatant was determined by the mass absorbance (OD 280 nm) in a spectrophotometer and aliquots stored in sterile polycarbonate vials at 4°C until use.13 The secreted antigens were used singly or in a cocktail format for coating the ELISA wells.

Preparation of ELISA test plates. Flat-bottom, 96-well microtiter plates (Greiner) were coated with a known amount of
antigen diluted in a coating buffer. Exo-antigens are known to contain a complex mixture of protein and glycoproteins. After 16 hrs of immobilization, the unbound antigens were removed and the wells were blocked with 0.5% casein for 1 h. The blocking solution was decanted, and the plates were stored in aluminum foil with silica. Because dogs are infected with different species of Leishmania in different geographic areas, the ELISA assay was designed to detect as broad a spectrum of antibodies against all possible Leishmania species. In view of this, exo-antigens from single promastigotes were initially tested and subsequently a cocktail (combination of L. infantum, L. donovani, and L. major) antigen was evaluated.

Enzyme-linked immunosorbent assay (ELISA). An indirect ELISA was performed by incubating the diluted serum samples in antigen-coated wells. A series of washings removed the unbound samples. The goat anti-dog IgG-HRP conjugate was placed into the wells and the resulting antigen and antibody complex visualized by the addition of TMB chromogen. The intensity of color is reciprocal to the amount of antibody bound to the immobilized antigen on the solid phase. After developing the color, the plates were read (450/620 nm) in a dual-beam plate reader and the absorbance of each sample was determined.

Checker board assays were performed by using reference positive (Ref+ve) and negative (Ref-ve) samples to determine the optimal coating concentration of the various antigens, along with the optimum serum dilution. Several parameters were examined to optimize the canine leishmania antibody detection ELISA.

Final Can-Leish IgG ELISA procedure. The ELISA wells were coated with a cocktail antigen prepared by pooling equal proportion of exo-antigens of L. infantum, L. donovani, and L. major promastigotes. After blocking with 0.5% casein, the wells were dried and sealed in aluminum foil and kept dry with silica. Test sera from endemic areas were diluted 1:2,000 in PBS/T and incubated at 37°C for 1 h. After a series of washing, anti-dog IgG-HRP conjugate was added and incubated at 37°C for 30 min. After another series of washing, the chromogen was added, generating a color reaction that was reciprocal to the specific antibody bound to the antigen. The absorbance values were quantified in a plate reader.

Negative control serum samples from unexposed non-endemic dogs. Serum samples were obtained from Australian dogs, which were either normal or with clinical conditions such as Cushing’s syndrome, anemia, gastro-enteritis, epilepsy, polyuria, polydipsia, seizure, kidney and bladder infections, diabetes, pancreatitis, chronic seborrhea. Sick dogs were included in the controls to obviate any interfering factors due to non-specific inflammatory reactions. None of these dogs have been exposed to leishmania infection in Australia and therefore served as unexposed controls for this study.

Samples from endemic dogs for evaluating Can-Leish IgG ELISA. Dogs suspected of infection with Leishmania parasites were examined at the Veterinary Teaching Hospital of the University of Trás-os-Montes e Alto Douro (VTH-UTAD), this research work followed the ethical guidelines of the VTH-UTAD, and was carried out after permission was granted from the owners to physically examine and test their dogs. Collection of samples was performed in agreement with the Portuguese legislation (Portaria No 1005/92), which approves the technical norms for protection and humane handling of animals used for scientific purposes. Two sets of serum samples derived from the endemic dogs were used in this study. The first set of (N = 14) samples were parasitologically confirmed positive sera samples that were procured from visceral leishmaniasis cases in Brazil and Italy and used for optimizing the Can-Leish IgG ELISA. A second set of (N = 37) serum samples was collected from the Alto Douro region, endemic for zoonotic leishmaniasis in Portugal. Based on clinical symptoms such as lymphadenopathy, dermatitis, alopecia, skin ulceration, weight loss, onychogryposis, ocular lesions, epistaxis, and cachexia the dogs were divided into two groups—symptomatic (N = 33) and asymptomatic (N = 4).

These samples were used as test samples to evaluate the sensitivity of the Can-Leish ELISA. The tests included (a) parasitological investigation of bone marrow biopsy samples for the presence of amastigotes in Giemsa-stained smears. Biopsy was performed in 21 symptomatic and 4 asymptomatic animals: amastigotes were detected by microscopic examination (1000 fields) of Giemsa-stained preparations in 20 of the former, whereas the latter were parasitologically negative. The bone marrow samples from 12 symptomatic dogs were not examined. Serological investigations included (b) DAT, (c) IFAT, and (d) exo-antigen-based ELISA. Peripheral blood was taken from the cephalic vein and serum stored at −20°C.

Direct agglutination test (DAT). The antigen for DAT was prepared from L. donovani and the test was performed by following the modified version for the canine reservoir. The diluent for serum samples consists of 0.25 (w/v) gelatin supplemented with 0.2 M β-mercaptoethanol in 0.15 M NaCl. Briefly, sera were doubly diluted starting from 1:20 to 1:20,480, using 50 μL diluted sample for each test, and incubated for 1 h at 37°C. Fifty μL of antigen was then added to each well. After incubation for 18 h at room temperature (21°C to 22°C), the titer obtained in each of the serum samples was defined as the reciprocal of the highest dilution at which agglutination was still visible, taking 1:320 as the cut-off for positivity.

Immunofluorescence antibody test (IFAT). The test was carried out using L. infantum promastigotes fixed on multi-spot slides. Two-fold dilutions of the sera samples ranging from 1:20 to 1:320 in phosphate-buffered saline supplemented with 0.05% Tween 20 and 2% skim milk powder (PBS/T/M) were added (10 μL per circle) to the antigen and incubated for 30 min at 37°C in a moist chamber. After two washes with PBS-T and a further one with distilled water, a rabbit anti-dog IgG-FITC conjugate diluted 1:32 in PBS/T/M supplemented with 1:10,000 Evans blue was added. Following further incubation and washings, slides were dried and observed by using an epifluorescence microscope (Leitz, Wetzlar, Germany). Titer were expressed as the reciprocal of the highest serum dilution giving clearly defined promastigote fluorescence and only those of 1:80 or higher were considered positive.

Specificity testing. Serum samples from dogs infected with Hepatozoon canis and Toxoplasma gondii were tested.

Data analyses. The absorbance values were plotted in the form of a graph and histogram and compared with the reactivity of the control samples. A cut-off value (COV) was determined by using the mean plus 3SD reactivity of control sera. The cut-off value was the indicator for determining a test sample as either positive (> cut-off value) or negative (< cut-off value). Once the sera were defined, then the percent of sensitivity was calculated, assuming all endemic sera are from
the infected dogs. An F-test was applied to analyze the data statistically wherever necessary.

RESULTS

Optimization of assay components. Checker-board analyses were performed to determine the optimal requirement for different assay components. Promastigote exo-antigens from \( L. \text{infantum} \), \( L. \text{donovani} \), and \( L. \text{major} \) were quantified and ELISA wells were coated at 50 and 75 \( \mu \text{g/mL} \) concentration. Serum samples from microscopically confirmed infected dogs were tested with serial dilutions (from 1:1,000 to 1:4,000 dilutions). Goat anti-dog IgG-HRP conjugate was used at 1:20,000 to 1:60,000 dilution. The checker-board analyses (data not shown) have indicated the following optimal conditions: (a) exo-antigens at 50 \( \mu \text{g/mL} \) concentration, (b) serum at 1:2,000 dilution, and (c) the conjugate at 1:20,000 dilution. There was clear discrimination between the reactivity of a negative versus a positive sample in this assay.

Evaluation of promastigote exo-antigens. \( L. \text{infantum} \), \( L. \text{donovani} \), and \( L. \text{major} \) exo-antigens were tested in the range of 100 to 5 \( \mu \text{g/mL} \) concentration for their ability to distinguish between negative versus infected serum samples. After immobilization, unbound antigens were removed and wells were blocked. Two negative (Ref-ve) and two \text{Leishmania} positive (Ref+ve) sera samples were tested at 1:2,000 dilution and the conjugate was used at 1:20,000 dilution. Results in Figure 1 show that individual exo-antigens generated a kinetic response similar in pattern and showed the ability to differentiate a negative sample from a positive one. The intensity of reactivity however, varied depending on the level of antibodies present in the sera. Ref+ve1 sera showed significantly elevated reactivity with \( L. \text{infantum} \) and \( L. \text{major} \) antigen and Ref+ve2 sera reacted identically in all three individual exo-antigens. Antibody titer varied depending on the amount of antigen immobilized in the reactant wells. The level of antibody was detectable in wells coated with as low as 5 \( \mu \text{g/mL} \) antigen and absorbance values were significantly different \((P < 0.001)\) between the positive and the negative samples. All three individual exo-antigens generated low-level background absorbance with the negative samples.

Use of individual and cocktail exo-antigens. Because the dogs are infected with different species of \text{Leishmania} in different geographical regions, it is vital to develop an assay suitable for different endemic regions. Exo-antigens from \( L. \text{infantum} \), \( L. \text{donovani} \), and \( L. \text{major} \) as well as a cocktail of the three antigens were tested at 50 \( \mu \text{g/mL} \) concentration and 1:2,000 serum dilution based on earlier checker-board analysis. Conjugate was used at 1:20,000 dilution. In Figure 2, plotted absorbance readings clearly distinguish between the reactivities of positive sera and negative control sera \((P < 0.05)\). The cocktail antigen has generated the highest S/N ratio.

Development of Can-Leish IgG-ELISA. The exo-antigen-based Can-Leish IgG-ELISA (Exo-antigen ELISA) assay has been developed and assessed in different stages as shown below:

Stage 1: Determination of cut-off level. The ELISA wells coated with cocktail exo-antigens of \( L. \text{infantum} \), \( L. \text{donovani} \), and \( L. \text{major} \) were used to test the sera samples. A total of 18 negative control sera samples from Australian dogs

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Comparative reactivity of individual promastigote-secreted antigens in ELISA and their ability to distinguish serum samples from negative (Ref-ve) vs. \text{Leishmania} positive (Ref+ve) dogs.
showing some obvious pathological symptoms but not exposed to \textit{Leishmania} infection were used as the negative controls. A total of 14 samples from parasitologically confirmed \textit{Leishmania}-infected dogs were used as the Ref+ve sera. Both control and Ref+ve samples were diluted to 1:2,000 and reacted in the antigen-coated wells for 1 h at 37°C. After washing the unbound serum components, wells were reacted with the (anti-dog IgG-HRP) conjugate at 1:20,000 dilution for 30 min at 37°C. After removing the unbound conjugate from the wells, the substrate TMB was added and developed color for 30 min at room temperature. The plates were read at 450/620 nm and the data plotted in Figure 3. A cut-off value (OD $= 0.3$) was determined by calculating the mean absorbance plus 3SD (mean + 3SD) reactivity value of 18 Australian negative control sera. Using this cut-off (OD $= 0.3$), all 14 samples from infected dogs from Italy and Brazil were determined to be positive in ELISA. Based on the antibody level, these samples showed a varied level of absorbance.

**Stage 2: Testing canine samples from the endemic region.** A total of 37 dog samples were obtained through the veterinary hospital in Alto Douro, the endemic region of Portugal. These samples were categorized as asymptomatic ($N = 4$) and symptomatic ($N = 33$) based on the clinical symptoms. Three and $N = 21$ biopsy samples from asymptomatic and symptomatic dogs respectively were tested parasitologically and results are shown in Table 1 ($N = 12$ samples from symp-
amonic dogs were not tested parasitologically). All $N = 3$ asymptomatic and one sample from the symptomatic group were found to be negative parasitologically. All $N = 33$ samples were tested in DAT and in the exo-antigen-based Can-Leish IgG ELISA. Based on a 0.3 cut-off level, one sample (PT-96) of four asymptomatic and all ($N = 33$) samples from symptomatic group were found to be positive in the exo-antigen ELISA (Table 1). The PT-96 sample had previously tested negative in DAT and IFAT. To prove whether the ELISA results was a false positive or not, this sample was re-tested by IFAT. The PT-96 was found to be positive at 1:160 dilution by IFAT.

Stage 3: Comparative serological evaluation. Results of Portuguese samples that were tested in DAT, IFAT, and in exo-antigen ELISA were compared. Although three different tests were used, not all samples were tested in all three tests and therefore the total number of samples tested in each test differed as shown in Table 1. In DAT, any sample showing $> 1:320$ titer and in IFAT $> 1:80$ dilution is considered to be positive. Any sample showing OD $> 0.3$ is considered to be positive in the exo-antigen ELISA. The ELISA results showed a kinetic response with absorbances ranging from 0.126 to 2.999 and were therefore graded for easy visualization and compared with the titer of DAT (Figure 4A) and IFAT (Figure 4B). Results are presented in five panels: Panel A: absorbance values of blank and negative control samples. Low absorbance values in ELISA agree with negative results of DAT and IFAT; Panel B: asymptomatic $N = 4$ samples, were negative by DAT and IFAT. However, one sample (PT-96) was found to be positive in the exo-antigen ELISA and also in IFAT by repeat testing. Samples from 33 symptomatic dogs were further sub-grouped: Panel C: $N = 12$ symptomatic samples, parasitology not done. However, these samples were positive in DAT, IFAT, and exo-antigen ELISA (Figures 4A and 4B). Panel D: One symptomatic sample with negative biopsy (parasitology) results was also found to be negative by DAT but positive in exo-antigen ELISA.; Panel E: A total of $N = 20$ symptomatic samples were available for testing and found to be positive by parasitology and by exo-antigen ELISA. However, Sample PT-77 could not be tested in IFAT. The remaining samples were found to be positive in DAT and IFAT.

For calculating sensitivity of the three serology assays, only $N = 33$ symptomatic and $N = 1$ asymptomatic sero-positive (PT-96) samples were taken into consideration. Biopsy testing on $N = 22$ samples revealed 20 samples positive (91% sensitivity), DAT on 34 samples generated 32 positive (94% sensitivity) (Figure 4A), IFAT on 33 samples produced 32 positive (97% sensitivity) (Figure 4B) as compared with 100% sensitivity (34 + ve out of 34 tested) in exo-antigen
ELISA (Figure 4A + B and Table 1). In Figure 4A, roughly \( N = 15 \) samples showed a maximum DAT titer of 1:20,480. There were at least 6 samples with the minimum DAT titer generated positive absorbance values (OD > 0.3) in ELISA. In Figure 4B when IFAT results were compared with ELISA, 6 sera samples showed the maximum titer of 1:320 dilution. Of 36 samples tested in IFAT, 32 were positive in IFAT (Figure 4B and Table 1). The ELISA generated the highest percentage of sensitivity (100%) as compared with IFAT (97%) and DAT (94%) (Table 1). Further, data in Table 1 show that the ELISA has detected all samples (100%) from symptomatic dogs. In addition, the ELISA detected one sample in the asymptomatic group suggesting the possibility of exposure to Leishmania infection. The same sample was found to be negative by parasitological examination as well as in DAT. On the whole, a good correlation was seen between the ELISA serology and the clinical symptoms.

Specificity of cocktail exo-antigen-based ELISA. Dogs infected with closely related protozoan parasites (Hepatazoon canis and Toxoplasma gondii) were tested in the promastigote exo-antigen-based Can-Leish IgG ELISA to establish the specificity. The serum sample proven positive for H. canis generated 0.128 absorbance, which is negative in Can-Leish ELISA. Of 8 dog samples positive for T. gondii IgG and IgM antibodies that were tested in Can-Leish ELISA, 7 samples showed absorbance values (OD range 0.132 to 0.296; average OD = 0.221) below the ELISA cut-off level. However, one sample was reactive (OD = 0.714).
DISCUSSION

In most countries endemic for human visceral leishmaniasis (HVL) there is at least one animal species reservoir for *Leishmania* parasites. Dogs are the main reservoir of *L. infantum* in southern Europe, the Middle East, Asia, and northern Africa; and of *L. chagasi* in Central and South America.2,5,8,9,11,12,17 Traditionally visceral leishmaniasis caused by *L. infantum* has been seen as a rural disease. However, in recent years this disease is increasingly seen in semi-urban or urban settings at the fringe of large cities. It would appear that both the parasite and the sand fly vector responsible for transmission have adapted to urban settings. The incidence of canine leishmaniasis infection is rising both in domesticated and stray dogs in these semi-urban settings and this poses a considerable health risk to both the human and canine population. The infected dogs thereby play an important role in dissemination of HVL in the region18 and are also an important veterinary problem. There have been several cases of imported dogs bringing *Leishmania* infection into non-endemic countries such as the United States, Canada,19 Europe,20,21 and Australia highlighting the need for a reliable screening test. Serology as a means of field diagnosis for screening dogs for *Leishmania* infection has been examined earlier by several investigators. However, the inherent problems are in the field-oriented parasitological techniques. Because of lack of uniformity and sensitivity with the current assays there is an urgent need for new diagnostic approaches. PCR for increasing sensitivity and specificity has recently shown to be promising19 in detecting *Leishmania* kDNA in blood samples. However, PCR has limited applicability in field applications. In this study, we have demonstrated that by incorporating exo-antigens in an ELISA system, both specificity and sensitivity have been greatly improved.

A sensitive ELISA technique would be a valuable addition for mass screening because of its high throughput and ease in demonstrating sero-conversion under field conditions. If ELISA techniques are made more efficient in terms of sensitivity (by using *Leishmania*-specific antigen) then such a technique would be invaluable for field investigations. Use of exo-antigens in ELISA is a case in point because these antigens are shown to be genus specific. In addition, we developed a ELISA for use in different endemic areas and therefore three different isolates were used for producing exo-antigens, which were used in cocktail format. By using a cocktail of exo-antigens, the Can-Leish ELISA was made a highly sensitive and specific tool and thereby useful for detecting antibody responses against all species of *Leishmania*. Moreover, we demonstrated the value of using high dilutions of serum samples (i.e., 1:2,000 dilution) which gives the added advantage of eliminating the non-specific reactions. Overall, the Can-Leish ELISA demonstrated a 100% correlation with the clinical signs and may also have value in detecting silent carriers of infection. Reliable diagnostic devices are required for tracking down the reservoir dogs that carry *Leishmania* infections. Certain assay parameters were investigated in this study: (a) Optimum level of exo-antigen for differentiating a positive from a negative sample. A cocktail exo-antigen-based assay generated a very high S/N ratio. Higher S/N ratio provides a high level of differentiation between the samples. Cocktail exo-antigens provided a high level of specificity to ELISA, unlike other ELISAs that have been described in the literature, which are based essentially on lysate antigens.4,6 (b) Use of non-endemic serum samples for baseline determination and for calculating the cut-off value. We tested the non-endemic sera from Australian dogs showing a variety of inflammatory reactions. The main objective of using serum samples from these sick dogs is to absolve any interfering factors. These samples provided a basis for developing a cut-off. (c) Other assay parameters such as conjugate and sensitive substrate were incorporated to enhance the quality of the ELISA. The ELISA test is found to be useful in detecting *Leishmania*-specific antibodies in serum samples of dogs derived from different geographical regions such as Italy, Brazil, and Portugal.

Although high-level sensitivity has been demonstrated, one must ascertain the assay specificity. Tests so far have been done with samples obtained from *H. canis* and *T. gondii*-infected dogs showed some promising results on specificity of this assay. However, one sample from *T. gondii* infected dogs showed OD readings higher than the cut off. This may suggest the possible exposure of this dog to leishmaniasis infection. Additional studies are needed on the possible cross reactivity with other protozoan infections. Further this observation has complemented previous reports made with human sera where exo-antigen-based ELISA remained specific. This confirmed high specificity of exo-antigen-based ELISA for serological investigations.13–15 In other words, Can-Leish ELISA is shown to be specific for detection of infection in dogs.

The performance of Can-Leish ELISA was compared with IFAT and DAT by using a panel of matched samples. The ELISA has generated a comparatively very high level of sensitivity than that seen with DAT and IFAT (Table 1). The ELISA was shown to be useful for screening asymptomatic as well as symptomatic dogs. Overall reliability of ELISA was higher than parasitological bone marrow biopsy, DAT, and IFAT. Because the reservoir dogs are the silent carriers of infection, the exo-antigen-based ELISA could be used as a valuable epidemiological tool.

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