Comparative evaluation of the DPP® CVL rapid test for canine serodiagnosis in area of visceral leishmaniasis

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A R T I C L E   I N F O

Article history:
Received 5 March 2014
Received in revised form 6 September 2014
Accepted 7 September 2014

Keywords:
Canine leishmaniasis
Serological diagnosis
Enzyme-linked immunosorbent assay (ELISA)
Indirect fluorescence test (IFA)
DPP® CVL rapid test
Cross-reactivity

A B S T R A C T

We investigated the performance of the DPP® canine visceral leishmaniasis (CVL) rapid test, a novel immunochromatographic assay launched by BioManguinhos [Brazil], which was recently included in the new Brazilian protocol for screening CVL in serological surveys. The present study compared the DPP® with the ELISA and IFA produced by BioManguinhos (Brazil) both with L. major-like antigens and with in-house tests using Leishmania infantum chagasi (in-house ELISA and in-house IFA). We analyzed the sera from clinically symptomatic (n = 47) and asymptomatic (n = 38) infected dogs from an endemic area of CVL, as well as from healthy (n = 18) dogs, in addition to the sera of dogs (n = 81) infected with other pathogens. The DPP® and the in-house ELISA showed a sensitivity of 90.6% and 94.1%, respectively, and specificity of 95.1% and 97.5%, respectively, and both presented cross-reactivity only with the sera of dogs with babesiosis, 44% for the DPP® and 22% for the in-house ELISA. The clinical groups were detected equally by the two assays. The ELISA BioManguinhos, IFA BioManguinhos, and in-house-IFA showed a good sensitivity, 90.6%, 96.5% and 89.4%, respectively, but very low specificity, 77.8%, 69.1% and 65.8%, respectively, due to the high cross-reactivity with the sera from the animals harboring other pathogens. The in-house ELISA provided the highest accuracy (95.8%), followed by the DPP® (92.7%), ELISA BioManguinhos (84.3%), IFA BioManguinhos (83.1%), and in-house IFA (78.0%). The simultaneous use of the DPP® and ELISA BioManguinhos reached a sensitivity of 99.1% and 82.1% when used sequentially. In conclusion, the DPP® performed well as serological test for CVL, and detected both asymptomatic and symptomatic dogs in equal proportions. Although its sensitivity is not ideal yet, discarding the IFA and including the DPP® improved the accuracy of the new Brazilian CVL diagnostic protocol, particularly of detecting truly infected dogs. Moreover, considering the higher specificity of DPP® (95.1% vs 77.8%), positive predictive value (95.1% vs 81.1%) and positive likelihood value (18.3% vs 4.1%) in comparison with the ELISA BioManguinhos, the use of DPP® as a confirmatory test instead of a screening test is suggested.

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http://dx.doi.org/10.1016/j.vetpar.2014.09.002
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Please cite this article in press as: Laurenti, M.D., et al., Comparative evaluation of the DPP® CVL rapid test for canine serodiagnosis in area of visceral leishmaniasis. Vet. Parasitol. (2014), http://dx.doi.org/10.1016/j.vetpar.2014.09.002
1. Introduction

Visceral leishmaniasis (VL) is a zoonotic disease in Brazil caused by the digenetic protozoan *Leishmania infantum chagasi*, which is primarily transmitted by the *Lutzomyia longipalpis* vector. The disease affects humans and wild and domestic animals. The domestic dog presents an important skin parasitism that favors infection of the sand fly and is considered the main reservoir of the parasite (Lainson and Shaw, 1987). From the epidemiological point of view, the disease in canines must be considered because of the high competence of infected dogs, including those that are asymptomatic, to transmit the parasite to the vector (Marzochi et al., 1985; Laurenti et al., 2013).

The Brazilian program for controlling leishmaniasis is based on the early diagnosis and treatment of human cases, the spraying of insecticides in areas where human cases are diagnosed and the elimination of seropositive dogs to reduce the sources of infection for sand flies. However, several studies have questioned the effectiveness of the actions taken against the domestic reservoir; as the serology is not accurate enough to detect the infection in dogs, the impact on human transmission is limited, and canine euthanasia is costly (Paranhos-Silva et al., 1996; Dietze et al., 1997). Therefore, a more accurate diagnosis is important both for preventing the unnecessary culling of uninfected dogs and to reduce the presence of infected animals in the environment (Ferreira et al., 2008). Among the methods most applied for the serological diagnosis, the indirect fluorescence assay (IFA) is used quite frequently in epidemiological studies (Alvar et al., 2004). Its sensitivity and specificity range between 60% and 100% (Almeida et al., 2005; da Silva et al., 2013), but cross-reactivity with the antibodies of dogs infected with other diseases has been reported (Zanette et al., 2014). The enzyme-linked immunosorbent assay (ELISA) is also commonly used for the diagnosis of canine visceral leishmaniasis (CVL). This technique allows the simultaneous analysis of a large number of samples and the use of crude, soluble, purified or recombinant antigens.

Despite the wide variety of diagnostic methods available in Brazil, the ELISA (screening assay) and the IFA (confirmatory assay), both of which with *L. major*-like antigens and produced by BioManguinhos (Fiocruz, Rio de Janeiro, Brazil), were recommended by the Ministry of Health to assess the canine seroprevalence until 2011. However, aiming to enhance the accuracy of the serodiagnosis, the government published a Technical Note introducing the DPP® CVL rapid test, a new rapid assay launched by BioManguinhos (Fiocruz, Rio de Janeiro, Brazil) that uses the rK26/rK39 fusion protein as an antigen, recommending the DPP® as a screening test and the ELISA-L. major-like as a confirmatory assay for diagnosing CVL (Grimaldi et al., 2012; Coura-Vital et al., 2013).

There are few studies comparing the performance of DPP® with other serological tests, considering the CVL clinical groups and the dogs with potentially cross-reactive infections. Thus, the purpose of this study was to evaluate the performance of the DPP® in the CVL diagnosis through its comparison with the ELISA and the IFA BioManguinhos and also with the in-house ELISA and IFA using a homologous antigen. The cross-reactivity was tested using the sera of dogs infected with *Babesia canis vogeli*, *Dirofilaria immitis*, *Trypanosoma cruzi*, *Ehrlichia canis*, *Neospora caninum*, *Toxoplasma gondii* and *Toxocara canis*. In addition, the impact of the introduction of the DPP® into the new Brazilian CVL diagnostic protocol was also investigated.

2. Materials and methods

Population of the study: Mongrel dogs referred to the Center for Zoonosis Control of Araçatuba (21°12′32″S and 50°25′58″W), Northwestern São Paulo state, a municipality highly endemic for visceral leishmaniasis, with different ages and gender, destined for euthanasia were enrolled in this study. Eighty-five dogs with a positive parasitological diagnosis by immunohistochemistry were divided into two groups, one composed of 47 dogs presenting clinical signs of leishmaniasis, such as weight loss, lymphadenomegaly, hepatosplenomegaly, pale mucous membranes, skin lesions, onychogryphosis, epistaxis and keratoconjunctivitis, designated the symptomatic group, and another group composed of 38 dogs without external clinical signs, with serum protein >8.5 mg/dL and serum creatinine within normal limits, according to the International Renal Interest Society (IRIS, 2006), designated the asymptomatic group.

For the specificity evaluation, 81 sera from dogs born in a VL-free area of Brazil were tested: 18 from healthy animals (negative controls) and 63 (cross-reaction controls) from 9 dogs naturally infected with *B. canis vogeli* presenting pale mucous membranes, anemia, intraerythrocytic inclusions of *Babesia* sp. and anti-*Babesia* antibodies determined by IFA, with titers ranging from 400 to 800; 17 dogs naturally infected with *Ehrlichia* presenting thrombocytopenia, *Ehrlichia* morulae within the macrophages on capillary blood smears and antibodies detected by a commercially available kit (SNAP®-3DX®, IDEXX Laboratories Inc., Westbrook, ME, USA); 9 dogs naturally infected with *T. gondii* presenting clinical signs of the disease and anti-*T. gondii* antibodies detected by IFA, with titers ranging from 1:128 to 1:1024; 6 dogs presenting clinical signs of *N. caninum* infection, with anti-*N. caninum* antibodies with titers ranging from 400 to 800 by IFA; 4 dogs co-infected with *T. gondii* and *N. caninum* with compatible clinical signs and serological confirmation; 3 dogs infected with *D. immitis* presenting with parasites on thick blood smears; 9 dogs infected with *T. canis* confirmed by PCR and 6 dogs experimentally infected with *T. cruzi* (Colombian strain) with chronic cardiomyopathy and anti-*T. cruzi* IgG antibodies determined by IFA, with titers ranging from 160 to 640.

This study was submitted and approved by the Ethical Committee for the use of Animals of the Medical School of the University of São Paulo, under protocol 296/10. All of the procedures were conducted according to the guidelines of the COBEA – Brazilian College of Experimentation in Animals.

Parasitological diagnosis: Immunohistochemistry was used as the gold standard to define canine infection by *Leishmania* spp. All the dogs with parasites in both the...
spleen and the lymph node were enrolled in this study. Immunohistochemical reactions were performed according to Moreira et al. (2007), using the total serum of mice chronically infected with *Leishmania* as a primary antibody and the LSAB kit (DakoCytomation, USA) for the signal amplification and detection of parasites in situ.

**Serological tests**: A total of 166 sera were analyzed using five tests, as described below.

**DPP®**: A chromatographic immunoassay based on Dual Path Platform technology (DPP®-CVL rapid test, BioManguinhos, Rio de Janeiro, Brazil), performed according to the manufacturer’s recommendations.

**ELISA BioManguinhos**: An enzyme-linked immunosorbent assay (EIE® BioManguinhos, Rio de Janeiro, Brazil), performed according to the manufacturer’s recommendations.

**IFA BioManguinhos**: An indirect Fluorescence Antibody Test (IFİ® BioManguinhos, Rio de Janeiro, Brazil), performed according to the manufacturer’s recommendations.

**In-house ELISA**: A microtiter of 96-well flat bottoms were sensitized with *L. infantum* chagasi crude antigen (10 μg protein/mL in 0.1 M carbonate–bicarbonate buffer pH 9.5, using a volume of 100 μL/well, and incubated in a humid chamber overnight at 4 °C. Then, the microtiter was washed with 0.15 M phosphate buffer pH 7.2 containing 0.05% Tween-20 (PBS-T). The plates were blocked with a solution of 10% skimmed milk powder in PBS-T incubated in a humid chamber for 2 h at 37 °C. The plates were washed three times with PBS-T. Samples of the test sera, as well as the positive and negative controls, were added in duplicate at a dilution of 1:400 in PBS-T and incubated at 37 °C for 1 h in a humid chamber. The plates were washed three times with PBS-T. Anti-canine IgG (A40 – 123AP) conjugated to alkaline phosphatase (Bethyl, USA) was added at a 1:2000 dilution in PBS-T and incubated at 37 °C for 45 min in a humid chamber. The plates were washed three times with PBS-T. The development of the color reaction was performed with a chromogenic substrate (1.0 mg/mL pNPP, Sigma, USA) in a 0.1 M carbonate–bicarbonate buffer pH 9.5 with incubation at room temperature for 30 min. The reaction was stopped with 3 M NaOH, and the absorbance was read with a 405 nm filter. The reaction cut-off was determined by using the mean absorbance of the negative sera plus three times the standard deviation.

**In-house IFA**: Immunofluorescence glass slides were prepared with *L. infantum* chagasi promastigotes fixed with 2% formaldehyde and stored at −20 °C. Dilutions of 1:40 of the test sera and the positive and negative controls were added, followed by incubation for 30 min at 37 °C. Subsequently, the slides were washed three times with PBS pH 7.2 for 5 min each, followed by incubation with anti-dog IgG fluorescent conjugate (anti-dog IgG (whole molecule) FITC conjugate, F-7884, Sigma, USA) at a dilution of 1:100 in Evans blue for 30 min at 37 °C. After washing three times with PBS pH 7.2 for 5 min each, the slides were mounted in buffered glycerol and coverslips and immediately observed in fluorescence microscope at a 40× objective.

### 2.1. Combined testing in parallel and sequentially

The combined sensitivity and specificity for the simultaneous (in parallel) or the sequential (in series) testing were estimated as follows: The sensitivity for tests A and B in parallel = Sensitivity of A + Sensitivity of B – (Sensitivity of A × Sensitivity of B). The specificity for tests A and B in parallel = Specificity of A × Specificity of B. The sensitivity for tests A and B in series = Sensitivity of A + Sensitivity of B. The specificity for tests A and B in series = Specificity of A + Specificity of B – (Specificity of A × Specificity of B).

**Data analysis**: To calculate the sensitivity, the sera of dogs with parasitological diagnoses for visceral leishmaniasis were used, and for specificity, the sera of healthy dogs (negative controls) and from those infected with other pathogens (cross reaction controls) were used. The accuracy, positive/negative predictive values, and positive/negative likelihood ratios were also determined for all tests. The confidence intervals were estimated at 95%. The statistical analysis was performed using Stata software (version 11.0), and a P-value of <0.05 was considered statistically significant.
3. Results

DPP® - Dual Path Platform: The DPP® rapid test for canine leishmaniasis detected 77/85 of infected dogs, resulting in a sensitivity of 90.6%. The results by the clinical group indicated reactivity with 42/47 (89.4%) sera of the symptomatic animals and 35/38 (92.1%) of the sera of asymptomatic dogs ($P > 0.05$). No positive reaction was observed with the negative control sera ($n = 18$), and the test was positive with only 4/9 (44%) of the sera from dogs with babesiosis, reflecting a specificity of 95.1% (Tables 1 and 2). The DPP® rapid test demonstrated good accuracy, 92.7%. The positive/negative predictive values and the positive/negative likelihood values are presented in Table 1.

ELISA BioManguinhos: The ELISA BioManguinhos was positive in 77/85 (90.6%) of the infected dogs from the VL endemic area. The test detected 43/47 (91.5%) of the symptomatic dogs and 34/38 (89.5%) of the asymptomatic dogs ($P > 0.05$). Among the 81 canine sera tested to estimate the specificity, 11% (2/18) of those from the healthy controls were reactive. For the dogs with other diseases, the sera of 67% (4/6) of the dogs with Chagas’ disease, 50% (3/6) of those with neosporosis, 35% (6/17) of those with ehrlichiosis, and 33% (3/9) of those with babesiosis showed positive results, reflecting a specificity of 77.8% (Tables 1 and 2). The ELISA BioManguinhos showed an accuracy of 84.3%. The positive/negative predictive values and the positive/negative likelihood values are presented in Table 1.

IFA BioManguinhos: The commercial IFA BioManguinhos tested positive in 82/85 (96.5%) of the sera of the dogs with a positive parasitological diagnosis. Considering the clinical groups, the assay was positive in 44/47 (93.6%) of the symptomatic dogs and in 38/38 (100%) of the asymptomatic dogs ($P > 0.05$). One (6%) of the 18 negative sera was reactive, in addition to 10/17 (59%) from the dogs with ehrlichiosis, 5/9 (56%) from the dogs with babesiosis, 3/6 (50%) from the dogs with Chagas’ disease, 3/9 (33%) from the dogs with toxoplasmosis, 2/6 (33%) from the dogs with neosporosis, and 1/3 from the dogs with $D. immitis$ (33%). These results reflected a specificity of 69.1% (Tables 1 and 2). The IFA BioManguinhos showed an accuracy of 83.1%. The positive/negative predictive values and the positive/negative likelihood values are presented in Table 1.

In-house ELISA: The test, using the crude $L. (L.)$ i. chagasi antigen, detected 80/85 (94.1%) dogs with a positive parasitological diagnosis. When assessing the results according to the clinical group, the in-house ELISA was positive in 44/47 (93.6%) symptomatic dogs and in 36/38 (94.7%) asymptomatic dogs ($P > 0.05$). All of the control sera ($n = 18$) were negative, and 2/9 (22%) of the sera from the dogs with babesiosis tested positive, giving an overall. The in-house ELISA showed the best accuracy, at 95.8%. The positive/negative predictive values and the positive/negative likelihood values are presented in Table 1.

In-house-IFA: The assay tested positive for 76/85 (89.4%) of the infected dogs. Considering the clinical forms, 44/47 (93.6%) of the symptomatic and 32/38 (84.2%) of the asymptomatic dogs were reactive ($P > 0.05$). In the negative healthy control sera, reactivity was observed in 8/17 (47%); reactivity was also observed in 5/5 (100%) of the sera of dogs with neosporosis, 4/4 (100%) of the sera of dogs co-infected with neosporosis/toxoplasmosis, 4/6 of the sera of dogs with Chagas’ disease (67%), 3/9 of the sera of dogs with toxoplasmosis (33%) and in 3/17 of the sera of dogs with ehrlichiosis (18%), yielding a specificity of 65.8% (Tables 1 and 2). The in house-IFA showed an accuracy of 78%. The positive/negative predictive values and the positive/negative likelihood values are presented in Table 1.

3.1. Combining results

The parallel testing of the ELISA BioManguinhos and IFA BioManguinhos yielded a sensitivity of 93.1% and a specificity of 53.8%. For DPP® and ELISA BioManguinhos, the respective values were of 99.1% and 73.9%. The sequential testing of the ELISA BioManguinhos and IFA BioManguinhos yielded a sensitivity of 87.4% and a specificity of 93.1%. When tested in series, the respective values for DPP® and ELISA BioManguinhos were 82.1% and 98.9% (Table 3).

4. Discussion

The nonspecific and varied clinical manifestations of CVL that can overlap symptomatically with other canine infections and the lack of signs in asymptomatic animals make the diagnosis of leishmaniasis one of the most challenging problems concerning the disease. Therefore, an
intensive search was undertaken for more suitable, reliable and rapid methods, as well as for antigens with the potential to promote high sensitivity and specificity in the diagnosis of CVL. Among the potential antigens, the recombinants rK39, rk26 and rA2 are viewed as promising (da Costa et al., 2003; Porrozzi et al., 2007; Lemos et al., 2008; Zanette et al., 2014).

In Brazil, where VL is a real public health problem, the canine diagnosis is critical, especially because the elimination of seropositive dogs is an adopted measure to control the spread of human and canine VL. To enhance the accuracy of the CVL diagnosis, the Brazilian Ministry of Health introduced a rapid immunochromatographic test based on the Dual Path Platform – DPP®, manufactured by BioManguinhos (Fiocruz, Rio de Janeiro, Brazil) and containing a fusion of two recombinant antigens (rK39, rk26) in the new Brazilian protocol to be used as a screening test. To confirm the positive results of the rapid test, an ELISA with soluble L. major-like antigens produced by BioManguinhos is recommended (Grimaldi et al., 2012).

Very few comparative studies on DPP® have been performed previously, especially focusing on the clinical groups of VL and the possible cross-reactivity with the sera of dogs with diseases caused by other common canine pathogens. For this reason, we analyzed the performance of the DPP®, comparing it with the official tests used in the Brazilian VL control program by the end of 2011, the ELISA (EIE® LVC kit, BioManguinhos, Brazil) and Indirect Fluorescence Assay (IFI® LVC kit, BioManguinhos, Brazil), which both employ promastigotes of the L. major-like antigen. The in-house ELISA and IFA tests that were developed with specific antigens were also used for the DPP® comparison. In addition, the impact of introducing DPP® into the new Brazilian protocol on the diagnosis of CVL was evaluated.

The present study demonstrated good performance of the DPP® that showed 90.6% sensitivity and 95.1% specificity (Table 1). Of note is that DPP® was equally good at detecting anti-Leishmania antibodies in both symptomatic (89.4%) and asymptomatic (92.1%) dogs (Table 1), differing from a previous report that found a low sensitivity (47%) in identifying parasite-positive dogs without signs of CVL and very high sensitivity (96%) for symptomatic dogs (Grimaldi et al., 2012). It appears that our asymptomatic dogs may have been at an active stage of infection, progressing toward overt disease (Oliva et al., 2006; Foglia Manzillo et al., 2013), in contrast to the other casuistic of asymptomatic animals. This finding suggests that the performance of DPP® depends on the infection stage of the asymptomatic dogs. Other studies using either rk26 or rK39 in ELISA or rapid assay detected from 46% to 98% of the asymptomatic dogs (Rosário et al., 2005; Porrozzi et al., 2007; Quinnell et al., 2013).

Regarding specificity, DPP® reacted with 44% of the sera from the dogs infected with B. canis vogeli (Table 2), which is not a negligible percentage, but in comparison with the other tests except for the in-house ELISA, the test showed the lowest cross-reactivity. Another commercial rapid immunochromatographic assay, the Kalazar Detect Test, which employs the recombinant antigen k39, showed cross-reactions with the sera of dogs with echinococcosis (7.7%), ehrlichiosis/babesiosis co-infection (50%), toxoplasmosis (10%), neosporosis (12.5%) and neosporosis/toxoplasmosis co-infection (23%) (Zanette et al., 2014), suggesting that the DPP® that uses rk39 in addition to rk26 has the potential to provide better results.

It is known that a screening test is selected because of its high sensitivity to detect potential disease, and a confirmatory diagnostic test is selected for its high specificity, giving more weight to the accuracy of the assay. Moreover, the screening test is usually cheaper than the confirmatory assay. The two sequential tests that have been selected for current use in Brazil, the DPP® (screening) and ELISA BioManguinhos (confirmatory) were equally sensitive (90.6%), but the specificity was 95.1% and 84.3%, respectively, which resulted in a higher LR+ for the quick test (18.3% vs 4.1%), higher PV+ (95.1% vs 81.1%) and higher accuracy (92.7% vs 84.3%) (Table 1), demonstrating that the DPP® performed better in assuring results that were truly positive, which suggests its potential to be used as a confirmatory rather than a screening test. Coura-Vital et al. (2013) also proposed this use for DPP®, particularly in areas with a high demand for testing, since this allows easier performance and reduced cost.

The in-house ELISA using the L. (L.) i. chagasi specific antigen demonstrated the best accuracy (Table 1), in agreement with another report that compared five serological tests (da Silva et al., 2013). The higher sensitivity of the in-house ELISA (94.1%) in relation to the DPP® (90.6%) may be attributable to the faster and earlier development of antibodies against crude antigens than against recombinant antigens (Quinnell et al., 2013). The in-house ELISA also demonstrated the lowest cross-reactivity, in contrast with the IFA tests (in-house and BioManguinhos) that reacted with the antibodies of almost all the dogs affected with other pathogens (Table 2), which is of concern because a positive result will result in euthanasia for the dog, whether the result is false or not. Despite the low specificity that we verified, higher specificities have been documented in other studies using IFA; however, they included cross-reaction control sera in a lower number and/or variety than we used in our study (Mettler et al., 2005; Lira et al., 2006; da Silva et al., 2013).

Considering both the new and the old Brazilian protocols for CVL diagnosis and the results obtained in the
present study, the decision to discard the IFA and to include DPP® in the new protocol results in more accurate canine diagnosis. Coura-Vital et al. (2013) also demonstrated a lower accuracy in estimating the magnitude of the infection in endemic areas using the previous protocol compared with the new protocol that includes DPP®.

It is noteworthy that both the DPP® and the ELISA BioManguinhos failed to detect 9.4% of the infected dogs when used individually (Table 1) and 17.9% of the infected dogs when performed sequentially (Table 3). This is of concern because the presence of these dogs in endemic areas contributes to the perpetuation of the infection. The failure to detect a more accurate number of infected dogs indicates that we need to design tests that have an even better performance. Grimaldi et al. (2012) suggest the simultaneous use of multiple recombinant antigens with complementary immunoreactivities to increase the sensitivity once the antigens are differentially recognized at different stages of the disease. Very recently, two new recombinant antigens derived from L. infantum chagasi were tested in a dual path platform (DPP) format (Fraga et al., 2014). When used individually, rLC1A was able to detect 66% of the infected dogs, and rLC2E was able to detect 81.1% of them. The use of both antigens in the same assay increased the sensitivity to 87%, and its simultaneous use with DPP® BioManguinhos increased the sensitivity to 93.5%, reinforcing the use of multiple antigens that cover a broad spectrum of immunoreactivities as an ideal strategy for achieving the maximum sensitivity in diagnostic CVL tests. Our results indicate that the simultaneous use of the DPP® and ELISA BioManguinhos can improve the sensitivity, enabling an estimate detection of 99.1% of the infected animals (Table 3).

In summary, the DPP® performed well as serological test for CVL and detected both asymptomatic and symptomatic dogs in equal proportions. Although the sensitivity of DPP® is not yet ideal, the comparison between the previous (ELISA and IFA BioManguinhos) and the new (DPP® and ELISA BioManguinhos) Brazilian CVL diagnostic protocol showed that discarding the IFA and including the DPP® increased the accuracy, especially for detecting truly infected dogs in endemic areas, tending to diminish canine euthanasia due to false positive results in the years ahead. In addition, considering its higher specificity and positive predictive and positive likelihood values, the use of DPP® as a confirmatory test rather than the ELISA BioManguinhos is also suggested.

Conflict of interest

The authors declare there are no conflicts of interest.

Acknowledgments

The authors thank BioManguinhos for kindly supplying the ELISA, IFA and DPP® kits for the development of this project. We also thank Prof(s). Dr(s). Solange Genari for kindly providing the sera of dogs with neoprosis, toxoplasmosis and neoprosis and toxoplasmosis co-infection, Mitika Hagiwara for the sera from dogs with ehrlichiosis, Eufrósina Umezawa for the sera from dogs with Chaga’s disease, Fabio Scott for the sera from dogs with diphtheriosis and babesiosis and Edythe Yamashiro for the sera from dogs with toxocariasis. This project was supported by grants #2004/07965-2 and #2009/54533-4 from the São Paulo Research Foundation and LIM50 HC-FMUSP. Márcia D. Laurenti and Mary Marcondes are Research Fellows from CNPq, Brazil.

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