Vet Res Commun (2015) 39:87–95
DOI 10.1007/s11259-015-9625-6

ORIGINAL ARTICLE

Ability of immunodiagnostic tests to differentiate between dogs naturally infected with *Leishmania infantum* and Leishmune®-vaccinated dogs

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Received: 9 August 2014 / Accepted: 6 January 2015 / Published online: 11 February 2015

Abstract Visceral leishmaniasis (VL) is a serious chronic disease with a lethality rate of up to 10 % in humans. In urban areas of Brazil, dogs are the main reservoirs of the etiological agent (*Leishmania infantum*) of VL, and the Brazilian Ministry of Health recommends the euthanasia of animals that are seropositive in both the immunochromatographic dual path platform rapid test (DPP®; Bio-Manguinhos) and the enzyme-linked immunosorbent assay (ELISA) with an *L. major*-like antigen (Bio-Manguinhos). Vaccination is an additional tool in the control of canine VL, but the use of Leishmune® (Zoetis Indústria de Produtos Veterinários, São Paulo, SP, Brazil), which contains the fucose mannose ligand (FML) isolated from *L. donovani*, is not currently recommended by the Brazilian Ministry of Health because vaccinated animals may exhibit positive serology and there are reservations regarding the efficacy of the vaccine. The aims of the present study were: (i) to verify the abilities of the fast agglutination screening test (FAST), the direct agglutination test (DAT), the indirect fluorescent-antibody test (IFAT), the DPP rapid test, and ELISA tests with *L. major*-like and FML antigens to differentiate between *L. infantum*-infected and Leishmune®-vaccinated dogs, and (ii) to analyze the sensitivities and specificities of the different methods. The reactivities to these tests of Leishmune®-vaccinated dogs (n=71), asymptomatic (n=20) and symptomatic (n=20) naturally infected dogs, and unvaccinated healthy control dogs (n=5) were compared. None of the Leishmune®-vaccinated dogs tested seropositive in FAST and DAT, although one dog was reactive to DPP and four dogs to ELISA/*L. major*-like and IFAT tests. While 69 (97 %) of vaccinated dogs reacted to ELISA/FML, only one was seropositive in both ELISA/*L. major*-like and IFAT tests. Individually, all immunodiagnostic tests presented high specificities and positive likelihood ratios (LR+), and high specificity values were obtained when the tests were considered in pairs. However, sensitivity and LR- values were low for ELISA/*L. major*-like and IFAT tests individually, and for all pair combinations of tests except for FAST with DPP.

Keywords Canine visceral leishmaniasis · Immunodiagnostic tests · Leishmune® canine vaccine · Seroconversion

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Introduction

Visceral leishmaniasis (VL), also known as kala-azar, is a serious chronic disease, the lethality rate of which can be as

high as 10 % in humans (Werneck et al. 2003; Belo et al. 2014). The etiological agent in the Americas is *Leishmania infantum* (syn. *Leishmania chagasi*) (Maurício et al. 2000). In urban areas of Brazil, dogs are the main reservoirs of the parasite, and one of the strategies adopted for the control of VL is the identification and subsequent euthanasia of infected dogs (Romero and Boelaert 2010). The Visceral Leishmaniasis Control and Surveillance Programme (VLSCP), which is sponsored by the Brazilian Ministry of Health, recommends that diagnosis of canine VL should be based on a dual path platform (DPP®) immunochromatographic test together with an enzyme-linked immunosorbent assay (ELISA) (Ministério da Saúde do Brasil, 2011).

According to mathematical models, vaccination of the canine population represents one of the most effective approaches for reducing the incidence of human and canine VL (Dye 1996). Leishmune® (Zoetis Indústria de Produtos Veterinários, São Paulo, Brazil), the first second-generation vaccine to be developed against canine VL, comprises the fucose mannose ligand (FML) isolated from *L. donovani* plus a saponin adjuvant. This vaccine, which was licensed in Brazil in 2003 (Luz et al. 2013), has proven efficient in blocking the zoonotic VL transmission cycle, but questions have been raised concerning the difficulty of differentiating infected dogs from healthy Leishmune®-vaccinated dogs using the serological assays specified by VLSCP. It has been suggested, therefore, that the vaccination of dogs could make it more difficult to implement the surveillance and control program, and might occasionally result in the euthanasia of healthy vaccinated dogs (Ministério da Saúde do Brasil, 2005; World Health Organization 2010).

The objectives of the present study were: (i) to verify the abilities of the fast agglutination screening test (FAST), the direct agglutination test (DAT), the indirect fluorescent-antibody test (IFAT) test, the DPP-CVL rapid test, and ELISA tests with *L. major*-like and FML antigens (ELISA/*L. major*-like and ELISA/FML, respectively) to differentiate between *L. infantum*-infected and Leishmune®-vaccinated dogs, and (ii) to analyze the sensitivities and specificities of the different tests with the aim of selecting the most appropriate for application in a surveillance and control program in which vaccination with Leishmune® forms an integral part.

Materials and methods

The study was submitted to and approved by the Ethics Committee for Animal Research of the Universidade Federal de São João del Rei under protocol number 35/2010. All procedures involving experimental animals were conducted according to Brazilian regulations and the guidelines issued by the Colégio Brasileiro de Experimentação Animal (COBEA).

Animal groups

Four groups of animals were studied. Group I consisted of 71 Leishmune®-vaccinated dogs (39 males and 32 females) belonging to the Military Police of Belo Horizonte, MG, Brazil, and included animals of various breeds (German Sheppard, Belgian Sheppard and Labrador) between 9 months and 9 years of age. Members of this group were tested in October 2010 for anti-*Leishmania* antibodies using ELISA and IFAT diagnostic test kits for canine VL (Instituto de Tecnologia em Imunobiológicos, Bio-Manguinhos) and all were found to be seronegative. Subsequently, the animals received three doses of Leishmune®, with 21-day intervals between each dose, followed by annual booster doses as recommended by the manufacturer. The last booster dose was applied between August and December 2012 and blood sampling was performed at least 45 days after. In 2011, however, one female animal was pregnant and one male dog was absent from the kennel and their booster doses could not be administered. Group II comprised 20 serum samples obtained from naturally infected dogs, clinically classified as asymptomatic (Mancianti et al. 1988), that were positive for polymerase chain reaction (PCR) of *Leishmania* minicircle kinetoplast (k) DNA (kDNA PCR) and for the direct parasitological examination of skin and spleen fragments (using Giemsa stain). Group III comprised 20 serum samples from naturally infected dogs, clinically classified as symptomatic (Mancianti et al. 1988), that were positive for the same tests as described for group II. The animals from groups II and III were mongrel dogs (males and females) of variable ages that had been evaluated in a previous study conducted in Belo Horizonte by Teixeira-Neto et al. (2010). All 40 dogs, except one from group II, were seropositive for ELISA and IFAT, positive for PCR-RFLP (*L. infantum* DNA) and positive for the parasitological test, which confirmed the presence of amastigotes in the tissues. Although the serum samples of groups II and III were from dogs with confirmed infection, they were submitted to the same tests as those from dogs of the other two groups. The control group IV consisted of five unvaccinated healthy animals (males and females) that had been born and raised in breeding kennels at the Universidade Federal de Ouro Preto, Ouro Preto, MG, Brazil. Absence of infection was determined by negative serological, molecular and parasitological tests (Teixeira-Neto et al. 2010).

Immunodiagnostic tests

Samples of blood (2.5 mL) were collected from animals of all groups and transferred to 10 mL sterile tubes containing no anticoagulant. After separation, serum samples were stored in the freezer at - 20 °C until required for analysis.

FAST and DAT tests were carried out according to the methods described by da Silva et al. (2005). DAT was

performed initially using serum samples diluted 1:100 and 1:200, and those presenting a positive reaction were assayed in serial dilution from 1:100 to 1:102400 in V-bottom microplates. IFAT, DPP and ELISA/*L. major*-like assays were performed using IFI-LVC, DPP-CVL rapid test, and EIE-LVC kits, respectively, according to the instructions provided by the manufacturer (Bio-Manguinhos). ELISA/FML assays were performed according to the method described by Cabrera et al. (1999) with minor modifications. Briefly, 96-well polystyrene microplates were coated with FML antigen (2 μg/well) diluted with carbonate bicarbonate buffer (pH 9.6) and incubated at 37 °C for 1 h and then at 4 °C overnight. The microplates were washed four times with phosphate buffered saline containing 1 % skimmed milk and 0.05 % Tween (PBST) using an automatic microplate washer (TP-Washer NM, model 3.4e; Thermoplate, São Paulo, Brazil). Serum samples, along with positive and negative controls, were diluted 1:100 with PBST and aliquots (50 μL/well) were transferred in triplicate to the coated microplates. Incubation was carried out at 37 °C for 1 h, after which the microplates were washed again as described above. An aliquot (100 μL) of peroxidase-conjugated specific anti-IgG was added to each well and the microplates were incubated at 37 °C for an additional 30 min and then washed as described earlier. The substrate supplied in the EIE-LVC kit was added to the wells (100 μL/well) and the plates were left at room temperature for 30 min. The reaction was blocked with 2 M sulfuric acid solution (50 μL/well) and the optical density (OD) determined at 450 nm using a microplate reader (TP Reader NM, model 1.4; Thermoplate, Brazil). The cut-off point was taken as 3 × mean OD value of the negative controls provided in the kit, while the limits of the grey area were considered to be cut-off point±1.2 × cut-off point. Samples were classified as reactive (OD above grey area), non-reactive (OD below grey area) and indeterminate (OD within grey area).

Statistical analyses

Analyses were performed using R Statistical Software (version 2.15.1). The specificities of the individual immunodiagnostic tests were estimated with reference to groups I (Leishmune®-vaccinated dogs; n=71) and IV (control healthy dogs; n=5), while sensitivities were determined by considering groups II and III (asymptomatic and symptomatic infected dogs) taken together as reference (n=40). The confidence intervals of the values obtained were calculated using the Wilson score method with asymptotic variance (Newcombe 1998). Specificities and sensitivities were also determined for pairs of tests when performed in series. The specificity of ELISA/FML was not calculated because the antigen employed was the same as that of the vaccine Leishmune®. Positive and negative likelihood ratios (LR+ and LR-, respectively) and diagnostic odds ratios (DOR) were calculated for all of the immunodiagnostic tests except for ELISA/FML.

Results

Figure 1 shows the results of immunodiagnostic tests applied to all four groups of animals and demonstrates that the probability of a Leishmune®-vaccinated dog testing positive for infection by *L. infantum* is low.

Of the 71 vaccinated dogs investigated, only one presented positive results in the ELISA/*L. major*-like and IFAT assays conjointly. Two of the vaccinated animals were seronegative in all of the tests applied. Overall, 28 (70 %) of the naturally infected dogs and 4 (5.6 %) of the Leishmune®-vaccinated dogs were seropositive in the ELISA/*L. major*-like test. In the ELISA/FML test, 30 (75 %) of the naturally infected dogs and 69 (97 %) of the Leishmune®-vaccinated dogs were seropositive.

Within the group of asymptomatic infected dogs, two animals (10 %) were seronegative and eight (40 %) were seropositive for all immunodiagnostic tests. However, nine dogs (45 %) showed seropositive responses in the FAST, DAT, IFAT, DPP and ELISA/*L. major*-like tests conjointly, while 12 dogs (60 %) presented positive results in the FAST, DAT, IFAT, DPP and ELISA/FML taken together. The dilutions of the seropositive DAT samples in asymptomatic infected animals varied between 1:400 and 1:51200. Within the group of symptomatic infected dogs, 14 (70 %) were seropositive for all immunodiagnostic tests, while one animal was seronegative for DAT, ELISA/*L. major*-like and ELISA/FML, and another was seronegative for DAT and IFAT. The dilutions of the seropositive DAT samples in symptomatic infected animals varied between 1:3200 and 1:10200.

When assessed individually, all of the tests presented high specificities, with the FAST and DPP tests exhibiting the highest sensitivities followed by the DAT, IFAT, ELISA/FML and ELISA/*L. major*-like tests (Table 1). According to the paired analyses, high specificity values were obtained for all combinations of tests, whereas the sensitivity values were low for the majority of combinations (Table 1). The results of the paired analyses are particularly important considering that the Brazilian Ministry of Health recommends that a definite diagnosis of infection should be based on seropositivity in at least two of a series of tests.

The FAST and DAT tests exhibited the highest LR+ and DOR values, followed by the DPP test, whereas ELISA/*L. major*-like presented the lowest values. The FAST and DPP tests exhibited LR- values below the accepted cut-off point (Table 2).

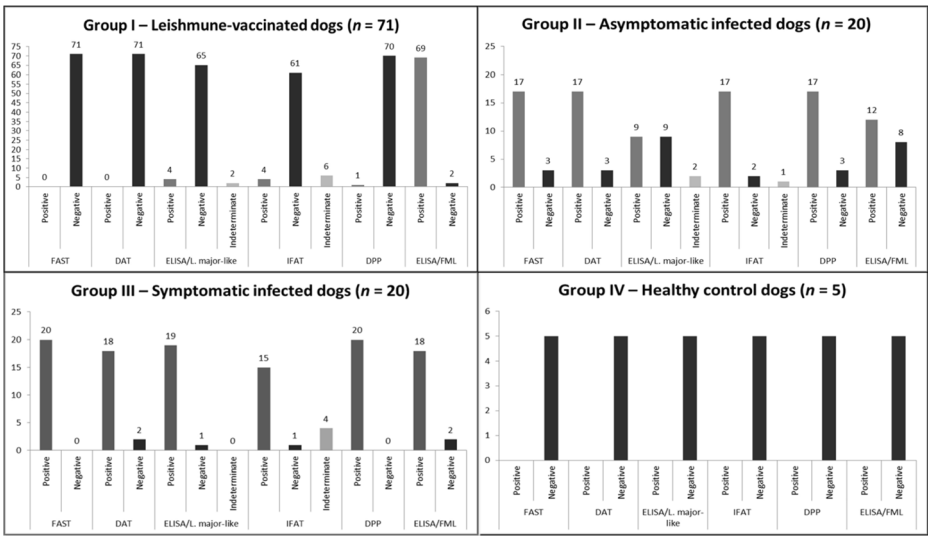

Fig. 1 Results of immunodiagnostic tests applied to Leishmune®-vaccinated dogs, asymptomatic and symptomatic dogs naturally infected with *Leishmania infantum*, and healthy control dogs. FAST - fast agglutination screening test; DAT - direct agglutination test; ELISA/*L. major*-like - enzyme-linked immunosorbent assay with *L. major*-like antigen; IFAT - indirect fluorescent-antibody test; DPP - dual path platform-CVL rapid test; ELISA/FML - enzyme-linked immunosorbent assay with fucose mannose ligand antigen

Discussion

The present study examined aspects related to the ability of immunodiagnostic tests to differentiate between dogs naturally infected with *Leishmania infantum* and Leishmune®-vaccinated dogs, and sought to determine the quality of such tests in a context in which the vaccination of dogs with Leishmune® would be employed as a VL control strategy. The results for all four groups of animals studied revealed that the probability of a Leishmune®-vaccinated dog testing positive for infection by *L. infantum* was low. These data support the hypothesis of Palatnik-de-Sousa *et al*. (2009) that vaccination with Leishmune® should not interfere negatively with the measures proposed in the Brazilian Program of Surveillance and Control of Visceral Leishmaniasis. Moreover, if a campaign of vaccination with Leishmune® was to be implemented, the erroneous euthanasia of a vaccinated animal presenting a false positive result would be essentially prevented since the guidelines of the Brazilian Ministry of Health require positive diagnosis to be based on two sequential tests, i.e., an initial screening using a DPP test followed by confirmation with the ELISA *L. major*- like test (Ministério da Saúde do Brasil, 2011).

Additional evidence in support of the hypothesis has been provided by Barichello (2010), who showed that 89.8 and 97 % of Leishmune®-vaccinated dogs (n=39) were seronegative for *Leishmania* according to ELISA/*L. major*-like and IFAT tests conducted using kits produced by Bio-Manguinhos, and 100 % of the vaccinated dogs tested negative according to tests performed using the ELISA/S7 test kit produced by Biogene. Even though the choice of appropriate tests to avert the unnecessary euthanasia of dogs represents an extremely important issue, discussion in the literature of this aspect is not particularly enlightening. In the present study, all combinations of tests presented high specificity but most showed low sensitivity. The combination offering the highest values of specificity and sensitivity was FAST+DPP, whereas the combination with the lowest values was ELISA/*L. major*-like+IFAT.

The results obtained with ELISA/FML demonstrate that the production of specific anti-FML antibodies had been induced by natural *Leishmania* infection and through vaccination. Although the 36 kDa glycoprotein nucleoside hydrolase (NH36) is the main epitope of the FML antigen complex (Santana et al. 2002), detection of significant amounts of NH36 antigens in the other immunodiagnostic tests employed would not be expected since the glycoprotein represents a very small fraction of the parasite proteome. Indeed, NH36 is not considered an important antigen as determined by proteomic analysis of *Leishmania* (Dea-Ayuela et al. 2006; Forgber et al. 2006). Thus, differences between the results obtained with ELISA/FML and the other tests within the group of Leishmune®-vaccinated dogs reflect the differential affinities of the antigens (Palatnik-de-Sousa et al. 2009).

The variation in the results of the asymptomatic group was greater than that observed in the symptomatic group and this may be explained by the enhanced cell-mediated immunity and the reduced humoral immune response presented by asymptomatic animals, which could be expressed as a diminished sensitivity to the serological tests (Bourdoiseau et al. 1997; Cabral et al. 1998). Other researchers have reported a reduced proportion of seropositivity in serological tests carried out with asymptomatic animals (Dye et al. 1993; Quaresma 2007), while Reis (2001) observed a variation in antibody titers in both the IFAT and the ELISA/extract tests in asymptomatic animals.

Consistent results within the four groups of animals assessed in the present study were obtained using the FAST, DAT and DPP tests, while those produced by the ELISA/*L. major*-like and IFAT tests showed the highest variabilities.

Table 1 Sensitivities and specificities of individual and paired immunodiagnostic tests

Test	Sensitivity (confidence interval)	Specificity (confidence interval)
Analyzed individually		
FAST[a]	0.92 (0.80–0.97)	1 (0.95–1)
DAT[b]	0.87 (0.74–0.95)	1 (0.95–1)
ELISA/*L. major*-like[c]	0.70 (0.55–0.82)	0.95 (0.87–0.98)
IFAT[d]	0.80 (0.65–0.89)	0.95 (0.87–0.98)
DPP[e]	0.92 (0.80–0.97)	0.99 (0.93–1)
ELISA/FML[f]	0.75 (0.60–0.86)	Not applicable
Analyzed pairwise		
FAST+DAT	0.81 (0.59–0.92)	1 (0.99–1)
FAST+ELISA/*L. major*-like	0.65 (0.44–0.80)	1 (0.99–1)
FAST+IFAT	0.74 (0.52–0.86)	1 (0.99–1)
FAST+DPP	0.86 (0.64–0.94)	1 (0.99–1)
FAST+ELISA/FML	0.69 (0.60–0.83)	Not applicable
DAT+ELISA/*L. major*-like	0.61 (0.41–0.80)	1 (0.99–1)
DAT+IFAT	0.70 (0.48–0.85)	1 (0.99–1)
DAT+DPP	0.81 (0.59–0.92)	1 (0.99–1)
DAT+ELISA/FML	0.65 (0.44–0.82)	NA
ELISA/*L. major*-like+IFAT	0.56 (0.36–0.73)	1 (0.98–1)
ELISA/*L. major*-like+DPP	0.65 (0.44–0.80)	1 (0.99–1)
ELISA/*L. major*-like+ELISA/FML	0.52 (0.33–0.71)	Not applicable
IFAT+DPP	0.74 (0.52–0.86)	1 (0.99–1)
IFAT+ELISA/FML	0.60 (0.39–0.77)	Not applicable
DPP+ELISA/FML	0.69 (0.48–0.83)	Not applicable

[a] Fast agglutination screening test

[b] Direct agglutination test

[c] Enzyme-linked immunosorbent assay with *L. major*-like *antigen*

[d] Indirect fluorescent-antibody test

[e] Dual path platform CVL rapid test

[f] Enzyme-linked immunosorbent assay with fucose mannose ligand antigen

This disparity may be explained by the sources of the antigens used in the tests, since the *L. donovani* promastigotes employed in FAST and DAT, and the *L. infantum* recombinant proteins employed in DPP, originated from species of the *L. donovani* complex, while the antigens in the IFAT and ELISA/*L. major*-like test kits produced by Bio-Manguinhos originated from the *L. major* complex. According to Barbosa-de-Deus et al. (2002), detection of heterologous antigens by VL-induced antibodies may occur, although the reaction is less intense and less specific than that observed with homologous antigens (Rajasekariah et al. 2008).

Since the specificities of the tests employed in this study were relatively high, the positive and indeterminate results obtained with IFAT and ELISA *L. major*-like assays for several of the Leishmune®-vaccinated dogs may indicate potential infection. Indeed, the possibility that some group I dogs might be infected by *Leishmania* must not be neglected because the protection conferred by the vaccine varies between 95 and 98 % (Borja-Cabrera et al. 2002, 2008). Alternatively, since no diagnostic tests for canine VL are 100 % specific (Gontijo and Melo 2004; Ministério da Saúde do Brasil, 2011), the occasional occurrence of false-positive reactions must be expected (Ferreira et al. 2007; Oliveira et al. 2008). In this context, it is important to emphasize that all group I animals presented seronegativity in the more specific tests, namely FAST, DAT and DPP, with the exception of a single animal in the DPP test.

Regarding the low sensitivity values established for some of the immunodiagnostic tests, it is possible that some infected dogs could have been in the initial stages of infection in which parasites were present in the skin but seroconversion had yet to occur. These findings are in agreement with de Queiroz et al. (2010) who reported that 87.5 % of asymptomatic dogs showed seronegativity in two different tests, while PCR of skin samples revealed 100 % positive results.

In the present study, FAST exhibited values for sensitivity and specificity (92 and 100 %, respectively) that were higher than those of the other tests and well within the range (80 to

Table 2 Likelihood ratios (LR) and diagnostic odds ratios (DOR) of immunodiagnostic tests

Test	LR+ (confidence interval)	LR- (confidence interval)	DOR (confidence interval)
FAST[a]	140.85 (8.87–2235.22)	0.09 (0.03–0.23)	1639.28 (82.53–32560.34)
DAT[b]	133.34 (8.39–2118.22)	0.14 (0.06–0.29)	1551.86 (78.05–30853.56)
ELISA/*L. major*-like[c]	13.30 (5.02–35.27)	0.32 (0.20–0.51)	42.00 (12.49–141.25)
IFAT[d]	15.20 (5.78–39.95)	0.21 (0.11–0.39)	72.00 (20.21–256.49)
DPP[e]	70.30 (10.01–493.63)	0.08 (0.03–0.23)	925.00 (93.00–9200.04)

[a] Fast agglutination screening test

[b] Direct agglutination test

[c] Enzyme-linked immunosorbent assay with *L. major*-like *antigen*

[d] Indirect fluorescent-antibody test

[e] Dual path platform CVL rapid test

100 %) reported in previous studies (Hailu et al. 2006; Schallig et al. 2002). In contrast, the observed sensitivity of DAT (87 %) was below literature values, which range from 93 to 100 %, while the specificity of the test (100 %) exceeded the previously reported values of 91 % (Silva et al. 2006) and 95 % (Ferreira et al. 2007; Hailu et al. 2006; Veeken et al. 2003).

The sensitivity of the ELISA/*L. major*-like test was estimated at 70 %, a value that is low in comparison with the figure of 94.54 % declared by the producers of the EIE kit (Bio-Manguinhos). In contrast, the specificity of the test was 95 %, a value that is higher than that (91.76 %) claimed by the manufacturer. In a similar manner, IFAT exhibited a sensitivity of 80 %, a figure that is lower than the 90 % quoted by the producers of the IFI kit (Bio-Manguinhos), and a specificity of 95 %, a value that is higher than the 80 % stated by the manufacturer. With respect to the DPP-CVL rapid test, the determined sensitivity of 92 % was within the range (89.7–93.1 %) stated by the producers (Bio-Manguinhos) of the kit, whereas the specificity of 99 % was above the declared range of 93.8–97.9 %).

The sensitivity of ELISA/FML determined in the present study was lower than that established by Cabrera et al. (1999) who recorded values of 100 % for both sensitivity and specificity in symptomatic and asymptomatic dogs. In contrast, Cândido et al. (2008) reported sensitivity and specificity values for ELISA/FML of 90 and 93.3 %, respectively, for oligosymptomatic dogs, and of 86.7 and 96.7 % for symptomatic animals. These authors suggested that the discrepancy between the two studies arose because they had employed an anti-dog IgG conjugated to peroxidase for the detection of antibodies while Cabrera et al. (1999) had used protein A conjugated to peroxidase. It appears that the application of peroxidase-conjugated protein A presents advantages because it not only reacts with all classes of IgG but also partially reacts with IgA and IgM (Surolia et al. 1982). Additionally, peroxidase-conjugated protein A increases the difference in the absorbance of the serum from infected and healthy dogs, thus facilitating the discrimination of seropositive animals and improving the sensitivity of the assay (Lima et al. 2005). This explanation is applicable to the present study since the ELISA/FML test employed peroxidase-conjugated anti-IgG and the established sensitivity of 75 % was well below the value reported by Cabrera et al. (1999). In this context, it is noteworthy that ELISA/FML yielded negative results with serum samples from two Leishmune®-vaccinated dogs that presented seronegativity in all of the other tests.

The differences in sensitivity and specificity observed in the present study may be attributed to modifications in the technical procedures and variations in the batches of serological kits employed. Additionally, the symptomatic and asymptomatic dogs studied were considered positive for *Leishmania* on the basis of assessments made using molecular and/or parasitological assays, and these are able to identify infection earlier than serological tests. According to Reithinger et al. (2002), the type of antigen used (evolutionary form or species) and possible changes in standard methods, such as incubation time and microtiter plates employed, may influence the sensitivity and specificity values of immunodiagnostic tests. Moreover, depending on the antigen used, the sensitivity of immunodiagnostic tests may vary from 95 to 99.5 % while the specificity may fluctuate from 97.1 to 100 % (Badaró and Duarte 1996). It is important to emphasize that, in order to make meaningful comparisons between different studies, the confidence intervals of the sensitivity and specificity values must be reported, although such information is frequently missing from publications. Moreover, the percentages of sensitivity and specificity normally reported in the literature typically represent approximations of the true values, which will vary according to the number of dogs studied and random variables associated with the study.

The low values for sensitivity and LR- obtained in the present study indicate that dogs that are truly infected with *Leishmania* may not be identified by immunodiagnostic tests. This issue is as relevant as the finding that Leishmune®-vaccinated dogs could be seropositive for the parasite, since every infected dog that is not diagnosed as such increases the probability of transmission of the infection to humans and to other dogs and, thereby, reduces the effectiveness of the surveillance and control measures. The guideline requiring that *Leishmania* infection should be confirmed through two positive serological tests tends to aggravate this problem and should be reevaluated. Indeed, if the combination of serological tests recommended by the Brazilian Ministry of Health were applied, 35 % of truly infected dogs would be recorded as negative. It is important to stress that the low sensitivity of a test is an intrinsic characteristic of the method employed and is not associated with vaccination status.

Currently, there is considerable controversy regarding the distinction between Leishmune®-vaccinated and infected dogs through the application of immunodiagnostic tests. Some researchers have claimed that it is not possible to differentiate between these two groups of animals using indirect ELISA (de Amorim et al. 2010; Fernandes 2013). Furthermore, Marcondes et al. (2011) stated that commercial polyclonal antibodies for total IgG, IgG1 and IgG2 are not able to distinguish infected and vaccinated dogs. The same group (Marcondes et al. 2013) has recently reported that an in-house ELISA, as well as the official serological tests adopted by the Brazilian Ministry of Health, exhibit cross-reactivity up to 6 months after the first dose of vaccine. Cross-reactivity in Leishmune®-vaccinated dogs, as revealed by official serological tests, has also been recorded by Araújo (2006) and Andrade et al. (2009). According to these researchers, seropositivity is typically detected in assessments performed near to the time of vaccination, but such reactivity

tends to diminish with time (Araújo 2006; Andrade et al. 2009; Marcondes et al. 2011, 2013). In the light of this evidence, it is important to monitor vaccinated dogs for an extended period. In the present study, for example, Leishmune®-vaccinated dogs became seronegative in various tests after 3 years of vaccination even when receiving annual boosters. However, these dogs were not submitted to molecular or parasitological tests before the study to verify if they were parasite-free. The two animals that were seronegative in all tests received the last booster dose at the same time, indicating that the interval between booster injections did not interfere with the results. The explanation for this lack of response is probably that these dogs were highly resistant and had a cell-mediated immune response together with an absence of antibody response. It is important to emphasize that the Leishmune®-vaccinated dogs belonged to the Military Police and received high quality care. However, since these dogs lived in a *Leishmania*-endemic area, it is not possible to state with total certainty that they were risk-free, although potential exposure to infection would have been minimal since the animals spent most of the time in kennels rather than roaming free.

The results presented herein verify that FAST, DAT and DPP assays provide higher values for sensitivity, LR+ and DOR compared with ELISA/*L. major*-like and IFAT tests when applied to symptomatic and asymptomatic infected dogs. Furthermore, our study demonstrates that the probability of Leishmune®-vaccinated dogs being assessed as seropositive in these tests is minimal. Hence, the DPP method presently recommended by the Brazilian Ministry of Health is a suitable diagnostic test for canine VL and should continue to be applied if a campaign of immunization with Leishmune® is authorized.

The present study has also shown that ELISA/*L. major*-like could be replaced by either FAST or DAT in the future, since the latter exhibit higher LR+, DOR and specificity values in Leishmune®-vaccinated dogs and higher sensitivity values in naturally infected dogs. Although the antigens used by FAST and DAT are imported, and hence more costly, the tests are simple and rapid and, since refrigerated storage facilities and sophisticated equipment are not required in their application, they could be used most effectively in areas with poor infrastructure (El-Harith et al. 1988; Silva et al. 2006; Organización Panamericana de la Salud, 2006; Sundar and Rai 2002). Naturally, the cost-benefit of these methods must be evaluated by the health authorities along with the applicability of the tests in areas that are endemic for *Leishmania* and other infections. Finally, FAST and DAT may be used as further evidence in cases where Leishmune®-vaccinated dogs have shown seropositivity in other tests. It should be noted that, while ELISA/FML may demonstrate the occurrence of seroconversion against the FML antigen, this must not be understood as evidence of vaccine-induced protection. The significant originality of the present study resides in the detailed analyses of the performance of different serological tests in Leishmune®-vaccinated dogs, the results of which represent a novel contribution to knowledge regarding the sensitivity and specificity of immunodiagnostic methods.

Acknowledgments The authors wish to thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) for financial support. The authors are grateful to the Companhia Independente de Policiamento com Cães da Polícia Militar de Belo Horizonte for provision of vaccinated dogs.

Conflict of Interest The authors declare that they have no conflict of interest.

References

Andrade RA, Araújo MSS, Reis AB, Gontijo CMF, Vianna LR, Mayrink W, Martins-Filho AO (2009) Advances in flow cytometric serology for canine visceral leishmaniasis: diagnostic applications when distinct clinical forms, vaccination and other canine pathogens become a challenge. Vet Immunol Immunopathol 128:79–86

Araújo MSS (2006) Alterações imunológicas no sangue periférico de cães submetidos à imunoprofilaxia para leishmaniose visceral canina. Doctorate Thesis, Universidade Federal de Minas Gerais, Belo Horizonte

Badaró R, Duarte MIS (1996) Leishmaniose visceral (Calazar). In: Veronese R, Focacci R (eds) Tratado de Infectologia, vol 2. Atheneu, São Paulo, pp 1234–1259

Barbosa-de-Deus R, Mares-Guia ML, Nunes AZ, Costa KM, Junqueira RG, Mayrink W, Genaro O, Tavares CAP (2002) *Leishmania major*-like antigen for specific and sensitive serodiagnosis of human and canine visceral leishmaniasis. Clin Vaccine Immunol 9:1361–1366

Barichello FFG (2010) Avaliação da resposta imunológica de cães vacinados com a vacina FML (Leishmune®) e cães naturalmente infectados com leishmaniose visceral canina por meio de dois métodos sorológicos: ELISA e RIFI. MSc Dissertation, Universidade Estadual de Campinas, Campinas

Belo VS, Struchiner CJ, Barbosa DS, Nascimento BWL, Horta MAP, da Silva ES, Werneck GL (2014) Risk factors for adverse prognosis and death in American visceral leishmaniasis: a meta-analysis. PLoS Negl Trop Dis 8:e2982

Borja-Cabrera GP, Correia Pontes NN, Silva VO, Paraguai-de-Souza E, Santos WR, Gomes EM, Luz KG, Palatnik M, Palatnik-de-Sousa CB (2002) Long lasting protection against canine kala-azar using the FML-QuilA saponin vaccine in an endemic area of Brazil (São Gonçalo do Amarante). Vaccine 20:3277–3284

Borja-Cabrera GP, Santos FN, Bauer FS, Parra LE, Menz I, Morgado AA, Soares IS, Batista LMM, Palatnik-de-Sousa CB (2008) Immunogenicity assay of the Leishmune® vaccine against canine visceral leishmaniasis in Brazil. Vaccine 26:4991–4997

Bourdoiseau G, Bonnefont C, Hoareau E, Boehringer C, Stolle T, Chabanne L (1997) Specific IgG1 and IgG2 antibody and lymphocyte subset levels in naturally *Leishmania infantum*-infected treated and untreated dogs. Vet Immunol Immunopathol 59:21–30

Cabral M, O'Grady JE, Gomes S, Souza JC, Thompson H, Alexander J (1998) The immunology of canine leishmaniasis: strong evidence for a developing disease spectrum from asymptomatic dogs. Vet Parasitol 76:173–180

Cabrera GP, da Silva VO, da Costa RT, Reis AB, Mayrink W, Genaro O, Palatnik-de-Sousa CB (1999) The fucose-mannose-ligand-ELISA in the diagnosis and prognosis of canine visceral leishmaniasis in Brazil. Am J Trop Med Hyg 61:296–301

Cândido TC, Perri SHV, Gerzoschkwitz TO, Luvizotto MCR, de Lima VMF (2008) Comparative evaluation of enzyme-linked immunosorbent assay based on crude and purified antigen in the diagnosis of canine visceral leishmaniasis in symptomatic and oligosymptomatic dogs. Vet Parasitol 157:175–181

de Amorim IFG, Freitas E, Alves CF, Tafuri WL, Melo MN, Michalick MSM, da Costa-Val AP (2010) Humoral immunological profile and parasitological statuses of Leishmune-vaccinated and visceral leishmaniasis infected dogs from an endemic area. Vet Parasitol 173:55–63

de Queiroz NMGP, de Assis J, Oliveira TMFS, Machado RZ, Nunes CM, Starke-Buzetti WA (2010) Diagnóstico da leishmaniose visceral canina pelas técnicas de imunohistoquímica e PCR em tecidos cutâneos em associação com a RIFI e ELISA-teste. Rev Bras Parasitol Vet 19:32–38

Dea-Ayuela MA, Rama-Iñiguez S, Bolás-Fernández F (2006) Proteomic analysis of antigens from *Leishmania infantum* promastigotes. Proteomics 6:4187–4194

Dye C (1996) The logic of visceral leishmaniasis control. Am J Trop Med Hyg 55:125–130

Dye C, Vidor E, Dereure J (1993) Serological diagnosis of leishmaniasis: on detecting infection as well as disease. Epidemiol Infect 110:647–656

El-Harith A, Kolk AH, Leewenburg J, Muigai R, Huigen E, Jelsma T, Kager PA (1988) Improvement of a direct agglutination test for field studies of visceral leishmaniasis. J Clin Microbiol 26:1321–1325

Fernandes CB (2013) Comparação entre duas vacinas comerciais contra leishmaniose visceral em cães de áreas endêmicas: IgG e subclasses, parasitismo e transmissão parasitária por xenodiagnóstico. MSc Dissertation, Universidade Federal da Bahia, Salvador

Ferreira EC, de Lana M, Carneiro M, Reis AB, Paes DV, da Silva ES, Schallig H, Gontijo CMF (2007) Comparison of serological assays for the diagnosis of canine visceral leishmaniasis in animals presenting different clinical manifestations. Vet Parasitol 146:235–241

Forgber M, Basu R, Roychoudhury K, Theinert S, Roy S, Sundar S, Walden P (2006) Mapping the antigenicity of the parasites in *Leishmania donovani* infection by proteome serology. PLoS One 1:e40

Gontijo CMF, Melo MN (2004) Leishmaniose visceral no Brasil: quadro atual, desafios e perspectivas. Rev Bras Epidemiol 7:338–349

Hailu A, Schoone GJ, Diro E, Tesfaye A, Techane Y, Tefera T, Assefa E, Genetu A, Kebede Y, Kebede T, Schallig HD (2006) Field evaluation of a fast anti-Leishmania antibody detection assay in Ethiopia. Trans R Soc Trop Med Hyg 100:48–52

Lima VMF, Biazzono L, Silva AC, Correa APFL, Luvizotto MCR (2005) Serological diagnosis of visceral leishmaniasis by an enzyme immunoassay using protein A in naturally infected dogs. Pesq Vet Bras 25:215–218

Luz KG, Ribeiro VM, Luvizotto MCR, Palatnik-de-Sousa CB, Menz I, Parra L, Sbardelini E, Papa T (2013) Leishmune Manual Técnico Leishmaniose Visceral Canina. Fort Dodge Saúde Animal. Available at: http://www.leishmune.com.br/download/manual/manual.leishmune.pdf Accessed 17 March 2014

Mancianti F, Grammiccia M, Gradoni L, Pieri S (1988) Studies on canine leishmaniasis control. 1. Evolution of infection on different clinical forms of canine leishmaniasis following antimonial treatment. Trans R Soc Trop Med Hyg 82:566–567

Marcondes M, Ikeda FA, Vieira RF, Day MJ, Lima VM, Rossi CN, Perri SH, Biondo AW (2011) Temporal IgG subclasses response in dogs following vaccination against Leishmania with Leishmune. Vet Parasitol 181:153–159

Marcondes M, de Lima VMF, de Araújo MDL, Hiramoto RM, Tolezano JE, Vieira RFC, Biondo AW (2013) Longitudinal analysis of serological tests officially adopted by the Brazilian Ministry of Health for the diagnosis of canine visceral leishmaniasis in dogs vaccinated with Leishmune®. Vet Parasitol 197:649–652

Maurício IL, Stothard JR, Miles MA (2000) The strange case of Leishmania chagasi. Parasitol Today 16:188–189

Ministério da Saúde do Brasil (2005) Nota técnica vacina anti-leishmaniose visceral canina Leishimune®. Secretaria de Vigilância em Saúde. Departamento de Vigilância das Doenças Transmissíveis, Brasília. Available at: http://portal.saude.gov.br/portal/arquivos/pdf/leishimune_nota_tecnica.pdf Accessed 10 March 2014

Ministério da Saúde do Brasil (2011) Nota Técnica Conjunta N° 01/2011 – CGDT-CGLAB/DEVIT/SVS/MS. Assunto: Esclarecimentos sobre substituição do protocolo diagnóstico da leishmaniose visceral canina (LVC). Secretaria de Vigilância em Saúde. Departamento de Vigilância das Doenças Transmissíveis, Brasília. Available at: http://www.saude.ms.gov.br/controle/ShowFile.php?id=113355 Accessed 10 March 2014

Newcombe RG (1998) Two-sided confidence intervals for the single proportion: comparison of seven methods. Stat Med 17:857–872

Oliveira TMFS, Furuta PI, de Carvalho D, Machado RZ (2008) A study of cross-reactivity in serum samples from dogs positive for Leishmania sp., Babesia canis and Ehrlichia canis in enzyme-linked immunosorbent assay and indirect fluorescent antibody test. Rev Bras Parasitol Vet 17:7–11

Organización Panamericana de la Salud, 2006. Informe final de la reunión de expertos OPS/OMS sobre leishmaniasis visceral en las Américas. PANAFTOSA, Rio de Janeiro. Available at: http://www.paho.org/hq/index.php?option=com_docman&task=doc_view&gid=16960&Itemid Accessed 17 March 2014

Palatnik-de-Sousa CB, Silva-Antunes I, Morgado AA, Menz I, Palatnik M, Lavor C (2009) Decrease of the incidence of human and canine visceral leishmaniasis after dog vaccination with Leishmune in Brazilian endemic areas. Vaccine 27:3505–3512

Quaresma PF (2007) Diagnóstico molecular da leishmaniose visceral canina e quantificação da carga parasitária através da PCR. MSc Dissertation, Fundação Oswaldo Cruz, Belo Horizonte

Rajasekariah GH, Cardoso L, Dogcio DA, Martin SK, Smithyman AM (2008) A novel exo-antigen-based ELISA for the detection of canine leishmaniasis. Am J Trop Med Hyg 78:616–623

Reis AB (2001) Avaliação de parâmetros laboratoriais e imunológicos de cães naturalmente infectados com Leishmania (Leishmania) chagasi portadores de diferentes formas clínicas de infecção. Doctorate Thesis, Universidade Federal de Minas Gerais, Belo Horizonte

Reithinger R, Quinnell RJ, Alexander B, Davies CR (2002) Rapid detection of Leishmania infantum infection in dogs: comparative study using an immunochromatographic dipstick test, enzyme-linked immunosorbent assay, and PCR. J Clin Microbiol 40:2352–2356

Romero GA, Boelaert M (2010) Control of visceral leishmaniasis in Latin America - a systematic review. PLoS Negl Trop Dis 4:e584

Santana DM, Borja-Cabrera GP, Paraguai-de-Souza EP, Sturm NR, Palatnik-de-Sousa CB, Campbell DA (2002) Nucleoside hydrolase from Leishmania (L.) *donovani* is an antigen diagnostic for visceral leishmaniasis. Mol Biochem Parasitol 120:315–319

Schallig HDFH, Canto-Cavalheiro M, da Silva ES (2002) Evaluation of the direct agglutination test and the rK39 dipstick test for the sero-diagnosis of visceral leishmaniasis. Mem Inst Oswaldo Cruz 97:1015–1018

Silva ES, Schoone GJ, Gontijo CMF, Brazil RP, Pacheco RS, Schallig HDFH (2005) Application of direct agglutination test (DAT) and fast agglutination screening test (FAST) for sero-diagnosis of

visceral leishmaniasis in endemic area of Minas Gerais, Brazil. Kinetoplastid Biol Dis 4:4

Silva ES, van der Meide WF, Schoone GJ, Gontijo CMF, Schallig HDFH, Brazil RP (2006) Diagnosis of canine leishmaniasis in the endemic area of Belo Horizonte, Minas Gerais, Brazil by parasite, antibody and DNA detection assays. Vet Res Commun 30: 637–643

Sundar S, Rai M (2002) Laboratory diagnosis of visceral leishmaniasis. Clin Diagn Lab Immunol 9:951–958

Surolia A, Pain D, Khan IM (1982) Protein a: nature's universal anti-antibody. Trends Biochem Sci 7:74–76

Teixeira-Neto RG, Giunchetti RC, Carneiro CM, Vitor RWAC, Coura-Vital W, Quaresma PF, Ker HG, de Melo LA, Gontijo CMF, Reis AB (2010) Relationship of *Leishmania*-specific IgG levels and IgG avidity with parasite density and clinical signs in canine leishmaniasis. Vet Parasitol 169:248–257

Veeken H, Ritmeijer K, Seaman J, Davidson R (2003) Comparison of an rK39 dipstick rapid test with direct agglutination test and splenic aspiration for the diagnosis of kala-azar in Sudan. Trop Med Int Health 8:164–167

Werneck GL, Batista MS, Gomes JR, Costa DL, Costa CH (2003) Prognostic factors for death from visceral leishmaniasis in Teresina, Brasil. Infection 31:74–77

World Health Organization (2010) Control of the Leishmaniasis. Report of a meeting of the WHO expert committee on the control of leishmaniasis. WHO Technical Report Series 949, Geneva. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_949_eng.pdf Accessed 17 March 2014