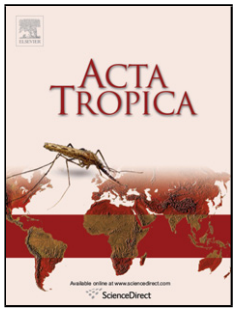

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DIAGNOSTIC ACCURACY OF rKLO8 VERSUS rK26 ELISAs FOR SCREENING OF CANINE VISCERAL LEISHMANIASIS

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Graphical abstract

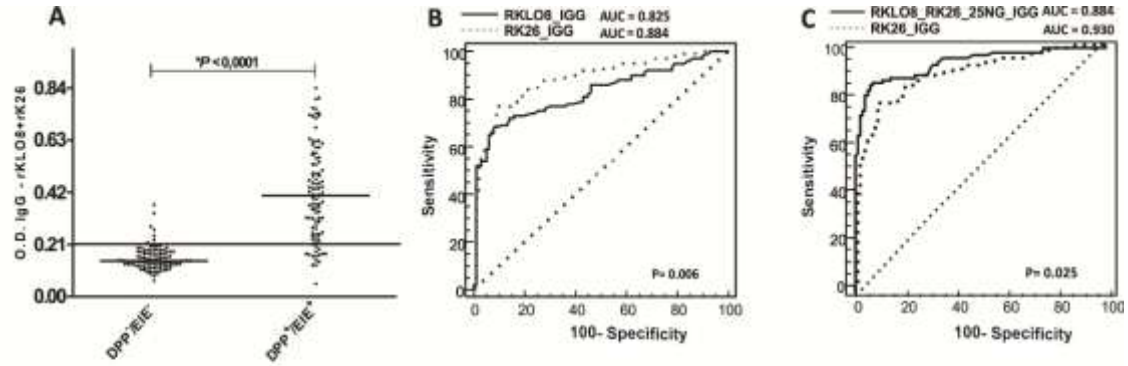

Table 1. Sensitivity and specificity of rKLO8 and rK26 in CVL diagnosis.

Antigen	Sensitivity (%)	Specificity (%)	Area Under the Curve (AUC)
rKLO8	68	92	0.825
rK26	77	91	0.884
rKLO8+rK26	85	93	0.930

ABSTRACT

Canine visceral leishmaniasis (CVL) represents an important public health issue. Despite numerous diagnostic tests available, CVL diagnosis still needs to be improved to achieve a more accurate detection rate. Recently, rKLO8, a new antigenic protein of Sudanese *Leishmania donovani,* was studied for the first time in diagnosis of human visceral leishmaniasis (HVL) and showed good performance. The present study aimed to evaluate serum reactivity to rKL08 and the reference antigen rK26, and to compare both diagnostic proteins with the combined DPP® CVL rapid test and ELISA (EIE-Bio-Manguinhos) confirmatory test, which are both recommended for the diagnosis of CVL in Brazil. Serum samples of dogs were grouped into: (I) DPP®/EIE negative (n=100) and (II) DPP®/EIE positive sera (n=100). Enhanced levels of IgG, mainly IgG2, to both rKLO8 and rK26 were found in group II. Sensitivity was 68% and 77% and specificity was 92% and 91%, for rKLO8 and rK26 antigens, respectively. Moreover, the combination of rKLO8 and rK26 antigens (rKLO8+rK26) exhibited higher sensitivity (85%) and specificity (93%). Thus, our results show that apart from the improved diagnostic power of rKLO8 in HVL, this new antigen is also suitable for the diagnosis of CVL. Further, the combination of rKLO8 and rK26 antigens increases the diagnostic accuracy of CVL.

Key Words: Canine visceral leishmaniasis; serodiagnosis; rKLO8; rK26; DPP rapid test.

Highlights

- The new antigen rKLO8 from an African strain of *L. donovani* was tested for diagnosis of CVL.
- The rKLO8 protein is suitable for diagnosis of CVL, with a similar performance compared to the reference antigen rK26.
- Combination of rKLO8 and rK26 shows improved diagnostic performance of CVL.
- Inclusion of rKLO8 in the DPP® rapid test deserves further attention.

1. Introduction

Visceral leishmaniasis (VL) is an emerging parasitic zoonosis caused by intracellular protozoan parasites of the *Leishmania genus* (WHO, 2015). Infected dogs are a potential source of infection for the phlebotomine vector, thus posing risks for indirect transmission of the parasite to humans (Quinnell and Courtenay, 2009). Canine visceral leishmaniasis (CVL) ranges from subclinical infections with apparently healthy animals, to widespread chronic infections that inevitably lead to the death of the animal if not adequately treated (Solano-Gallego et al., 2011). In the Americas, it is estimated that most cases of CVL are caused by *Leishmania infantum (syn. Leishmania chagasi)* and also by other species of *Leishmania*, such as *Leishmania (V.) braziliensis* (Dantas-Torres, 2009). In Brazil, the seroprevalence rates in dogs vary widely reaching up to 75% in highly endemic foci (Paranhos-Silva et al., 1996; Cortada et al., 2004; Dantas-Torres, 2009). Governador Valadares is an endemic area for leishmaniasis in Minas Gerais, Brazil. The first cases of HVL in Governador Valadares were recorded in the 60s. In a study carried out between 2008 and 2011 in 35 districts of Governador Valadares, 86 indigenous cases of HVL were reported and 4,992 (30.2%) dogs out of 16,529 were seropositive for CVL (Barata et al., 2013).

For the diagnosis of CVL, several strategies have been established, based on parasitological methods, immunological and serological tests, and molecular techniques in association with clinical and epidemiological parameters (Miro et al., 2008). The clinical diagnosis of CVL is difficult to determine because of the large percentage of existing asymptomatic dogs, and

similarity to other infectious diseases affecting dogs (Alves et al., 2012). Serological tests are commonly used for the diagnosis of CVL (Gomes et al., 2008), including the direct agglutination test (DAT), the enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IFI). Currently, ELISA is the method of choice in population-based surveys (Morales-Yuste et al., 2012), because of the suitability to test large number of samples in a short period of time, and the versatility to use several types of antigens, such as purified antigens, synthetic peptides and recombinant proteins (Maia and Campino, 2008). However, it presents important limitations for the diagnosis of CVL, including lack of sensitivity and specificity, which are strongly influenced by the type of antigen used (Sundar and Rai, 2002).

The recombinant antigens rK26, a hydrophilic protein of 247 amino acids specifically expressed in *L. donovani* and *L. infantum*, and rK39 of *L. infantum* have been shown to be suitable for CVL diagnosis (Bhatia et al., 1999; Da Costa et al., 2003; Mettler et al., 2005). The rK39 antigen is more sensitive for the diagnosis of symptomatic cases of CVL (100%) compared to asymptomatic CVL (66%) (Porrozzi et al., 2007). Recently, rK28, a multi-epitope recombinant chimeric protein obtained by fusing *L. infantum* k9 gene with single repeat units of k39 and k26 genes (Boarino et al., 2005), was used to develop an immunochromatographic rapid test, the Dual-Path Platform (DPP; Bio-Manguinhos/Fiocruz, Rio de Janeiro, Brazil), which was recommended for the screening of infected dogs (Grimaldi et al., 2012; Coura-Vital et al., 2014).

Although DPP demonstrates excellent sensitivity for identifying symptomatic dogs (98%), it showed lower efficacy for the diagnosis of asymptomatic animals (47%) (Grimaldi et al., 2012). Since 2011, the Brazilian

Department of Communicable Disease Control, Ministry of Health, recommended the use of DPP in combination with ELISA-BioManguinhos (EIE) as screening method in CVL surveys. EIE employs total antigens of *L. major* and thus confirms positive results (Grimaldi et al., 2012; Coura-Vital et al., 2014).

Despite numerous diagnostic tests available for CVL, the issue continues to represent a challenge. There is no single method to obtain maximum sensitivity and specificity in order to allow an accurate diagnosis of the disease (Coura-Vital et al., 2014), and since asymptomatic dogs can participate in the natural transmission cycle of VL, the importance of vector and host surveillance with an early, reliable and rapid diagnostic test requires the refinement of current methods to improve CVL diagnosis (Quinnell et al., 2013). Recently, a new kinesin-homolog, rKLO8, from a Sudanese *strain of L. donovani* has been cloned and tested for its diagnostic value in VL patients. By ELISA it was demonstrated that rKLO8 had a higher sensitivity (98.1% vs 96.2%) and specificity (96.1% vs 94.8%) in East African VL patients as compared to the currently used rK39 from *L. infantum*, respectively (Abass et al., 2013). In the present study, we evaluated for the first time canine serum antibody reactivity towards rKL08 in comparison to rK26. Receiver Operator Characteristic Curve (ROC) analysis was applied to study differences between the rKLO8 and rK26 in the diagnosis of CVL using serum samples from dogs tested negative (DPP^- EIE^-) and positive (DPP^+EIE^+) for CVL.

2. Materials and methods

2.1 Serum samples

A total of 200 sera collected from dogs were obtained from the Zoonosis Control Center (ZCC) serum repository, Municipal Health Secretariat, Governador Valadares (GV), Minas Gerais, an area endemic for visceral and tegumentar leishmaniasis in Brazil. The serum samples were first tested, in a pre-evaluation trial conducted by technicians from the ZCC-GV, with the qualitative rapid test “Dual Path Platform” (TR DPP® CVL – Bio-Manguinhos) (DPP), and ELISA Bio-Manguinhos (EIE-CVL, FIOCRUZ, Rio de Janeiro, Brazil). DPP, based on the use of rK28 protein, and EIE Bio-Manguinhos, which employs total *L. major* antigens, are tests currently recommended by the Brazilian Ministry of Health for screening CVL (Grimaldi et al., 2012). Based on data from the manufacturer’s, the DPP test presents a sensitivity of 100% and specificity of 87.5 - 91.7%, (62 dogs tested), and the EIE presents 94.54% sensitivity and 91.76% specificity. Serum samples were grouped as follows: 1) DPP-/EIE- group (n=100), with sera from dogs tested negative for DPP and negative for EIE (considered the seronegative control), and 2) DPP+/EIE+ group (n=100), with sera from dogs tested positive for DPP and positive for EIE (considered the seropositive control). All samples were stored at -20°C until testing in rKLO8-ELISA. The Ethics Committee on Animal Experimentation of the Federal University of Juiz de Fora approved the study protocol (no. 016/2015).

2.2 Antigens

The recombinant protein rKLO8 were produced and quality controlled by Philipps University of Marburg using *Escherichia coli* M15 transformed with the pQE41/KLO8 plasmid. Briefly, genes were amplified by PCR and cloned into

the bacterial expression vector pQ41 (Qiagen GmbH, Germany), containing an N-terminal histidine tag. The proteins were over-expressed in *E. coli* M15 (Qiagen GmbH, Germany) and purified, as described previously (Abass et al., 2013). Purity and size were checked by gel electrophoresis and western blotting with anti-His antibodies and sera of VL patients. Recombinant antigens were freeze-dried, shipped to Juiz de Fora/Brazil, reconstituted in PBS and kept at -80ºC until testing in ELISA. The recombinant protein of *L. infantum* rK26 was kindly provided by the Infectious Disease Research Institute (IDRI), Seattle, Washingnton, USA. Both rKLO8 and rK26 proteins are specific to *L. donovani complex.*

2.3 ELISA

Ninety-six well-plates (Plast-Bio, Curitiba, Brazil) were coated overnight with 100 µL/well rKLO8 and rK26 individually (0.5 µg/mL) or in combination (0.25 µg/mL of each) diluted in 0.1 M $NaCO_3$ buffer (pH 9.6) solution. Plates were then washed with phosphate buffered saline (PBS) containing 0.05% (v/v) Tween-20 (PBS-T) and blocked with 2% (w/v) BSA in PBS-T for 1 hour at room temperature. After washing with PBS-T, 50 µl of diluted serum samples (1:2000 for IgG; 1:1000 for both IgG1 and IgG2) were added to each well and the plates incubated for 1 hour at room temperature. After washing with PBS-T, 50 µl of either peroxidase-conjugated rabbit anti-dog IgG (1:10,000 dilution) (Sigma, St. Louis, MO), peroxidase-conjugated goat polyclonal anti-dog IgG1 (1:10,000 dilution) (AbD Serotec, Bio-Rad Company, USA), or peroxidase-conjugated polyclonal sheep anti-dog IgG2 (1:5,000 dilution) (AbD Serotec) were added. Plates were incubated at room temperature for 1h, washed, and a substrate

solution containing tetramethylbenzidine (TMB) and H_2O_2 (BD, Sao Paulo-Brazil) was used. The reaction was stopped with 2N H_2SO_4 and the optical density (OD) measured at 450 nm (Spectramax-190, Molecular Devices, Sunnyvale, CA, USA). Each sample was tested in duplicates and the mean OD was determined.

2.4 Statistical analysis

Two strategies were used to study differences between the rKLO8 and rK26, using group I (DPP-/EIE-) and group II (DPP+/EIE+) as seropositive and seronegative controls: (i) Median differences between optical densities and (ii) comparison of ROC curves. Initially, a comparison between median values of the optical densities (ODs) for total IgG, using the nonparametric Kruskal-Wallis test, followed by the non-parametric Mann Whitney was performed with GraphPad Prism 5.0 (GraphPad software). The second strategy was the comparison of pairs of ROC curves, comparing rKL08 with rK26, and also rKL26 and rKL08 each one against the combination of rKL08 and rK26 (rK26 + rKL08). The parameters of ROC curve include the global accuracy of the test, represented by the area under the curve (AUC), a widely accepted metric for evaluating diagnostic accuracy (McFall and Treat, 1999). The greater the AUC, the better the accuracy of the diagnostic test, and an AUC of 1 represents perfect accuracy (Langlotz, 2003). The difference between the two areas was evaluated by the nonparametric Wilcoxon Test, performed using MEDCALC. The parameters of the curve, sensitivity, specificity, positive and negative likelyhood ratios were extracted at the best cutoff point indicated by each curve and also analyzed and discussed.

3. Results

3.1 IgG, IgG1 and IgG2 antibody reactivity against rKLO8 and rK26

Total IgG reactivity against rKLO8 and rK26 recombinant proteins was evaluated in sera from dogs of the city of Governador Valadares, an endemic area of leishmaniasis in Brazil. Figure 1 shows the distributions of optical densities of both proteins at the cut-off of 0.17, established by ROC curve. Figure 1 shows that seropositive controls (group II, DPP^+EIE^+) exhibited higher ($p < 0.001$) IgG reactivity to both, rKLO8 and rK26 with more dispersion around the median value, as compared to group I (DPP^-EIE^-), indicating that IgG reactivity against rKLO8 was very similar to rK26, with mean OD_{450nm} values of 0.069 ± 0.546 for rKLO8 and 0.067 ± 0.617 for rK26.

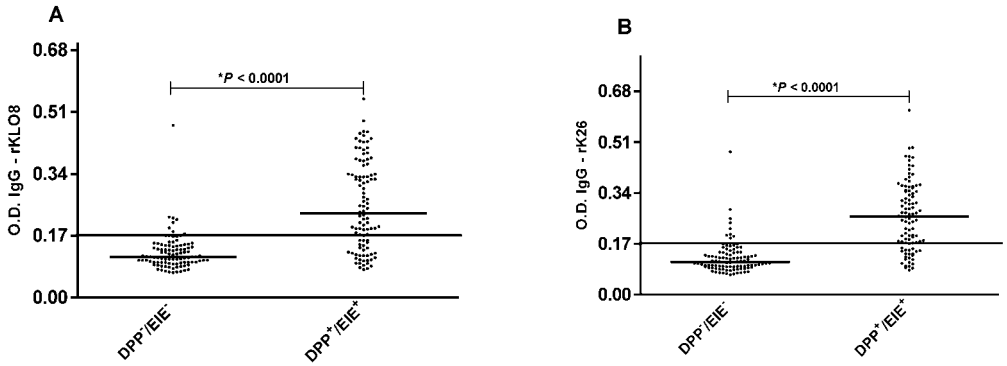

Figure 1. Serum IgG levels against rKLO8 (A) and rK26 (B) antigens in dogs seronegative (DPP^-/EIE^-; n=100) and seropositive (DPP^+/EIE^+; n=100) for CVL. The cut off (0.17) was established by ROC curve. Bars represent median. O.D. = optical density determined by ELISA.

We next tested rKLO8 and rK26 antigens for the reaction with different IgG subclasses. Figure 2 shows that in both groups (DPP^-/EIE^- and DPP^+/EIE^+), the amounts of IgG2 antibodies are higher than IgG1. In addition, levels of IgG2 reacting with both, rKL08 and rK26 were markedly increased in seropositive

dogs (DPP^+/EIE^+). In contrast, IgG1 levels were comparable between DPP^-/EIE^- and DPP^+/EIE^+ dogs.

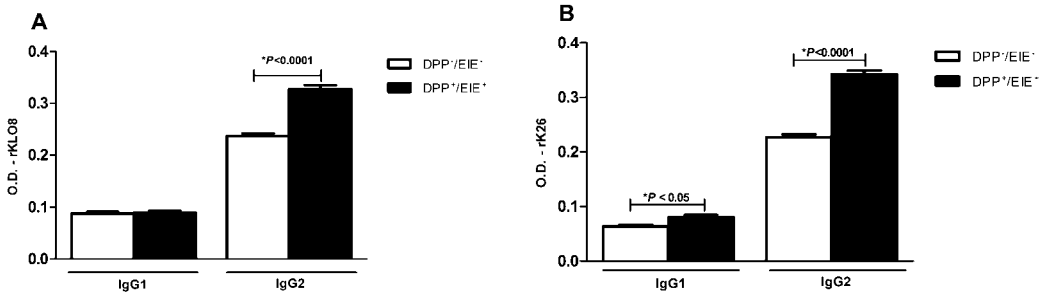

Figure 2. IgG1 and IgG2 serum reactivity against rKLO8 (A) and rK26 (B) in dogs seronegative (DPP^-/EIE^-, n=100), and seropositive (DPP^+/EIE^+, n=100) for CVL. Bars represent mean±SEM. O.D. = optical density determined by ELISA.

3.3 Receiver operator characteristic (ROC) curve analysis

ROC curve analysis was performed to evaluate the accuracy of rKLO8 and rK26 in the diagnosis of CVL. Figure 3 shows the comparison of ROC curves obtained for rKLO8-ELISA and rK26-ELISA with respect to total IgG (3A), IgG1 (3B) and IgG2 (3C). The area under the curve (AUC) for total IgG of the rK26 antigen was 0.884 (CI 95% = 0.831–0.925) close to that observed for rKLO8 (AUC_{rKLO8} = 0.825, CI 95% = 0.765–0.875), however reaching significance ($p < 0.05$). In accordance, the AUC for IgG2 detection (Figure 3C) showed that rK26 had a slightly better (p <0.05) value (AUC_{rK26} = 0.898; CI 95%: 0.847-0.936) in comparison to rKLO8 (AUC_{rKLO8} = 0.822; CI 95%: 0.762-0.873). In addition, AUC values for IgG1 were similar (p = 0.075) between rKLO8 (AUC_{rKLO8} = 0.547, CI 95%: 0.476-0.618) and rK26 (AUC_{rK26} = 0.640, CI 95%: 0.569-0.706) (Figure 3B). Thus, rK26 had higher AUC values than rKLO8, however the statistically significant difference appears to be biologically irrelevant.

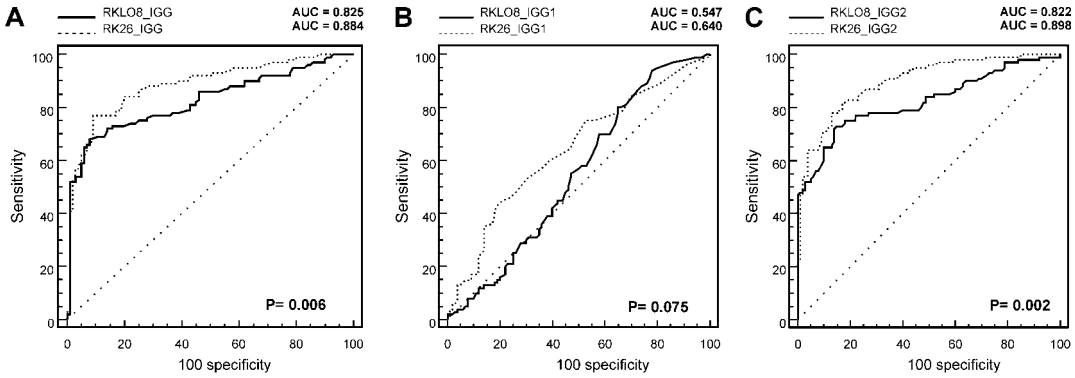

Figure 3. Comparison of rKLO8 and rK26 antigens by ROC curve. ROC curves were generated from the ELISA values in the detection of IgG (A), IgG1 (B) and IgG2 (C) in the diagnosis of CVL.

3.4 Combination of rKLO8 and rK26

In an attempt to improve the diagnostic accuracy, a combination of the two recombinant proteins, rKLO8 and rK26, was used and compared in ELISA. Figure 4 shows that the amounts of total IgG against rKLO8 + rK26 were higher (p<0.0001) in the DPP^+EIE^+ group in comparison to the healthy control group (DPP^-EIE^-).

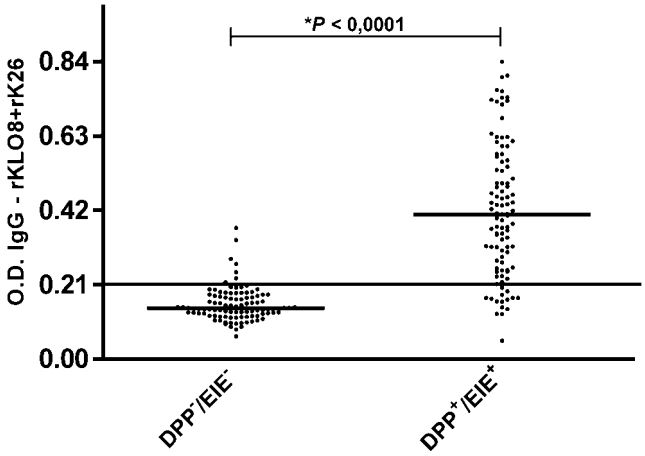

Figure 4. IgG serum reactivity against rKLO8 + rK26 in dogs seronegative (DPP^-/EIE^-, n=100), and seropositive (DPP^+/EIE^+, n=100) for CVL. The cut off (0.21) was established by ROC curve. Bars represent median. O.D. = optical density determined by ELISA.

Figure 5 shows that the combination of rKLO8 + rK26 had a higher AUC ($AUC_{rKLO8+rK26}$) with a value of 0.930 (CI 95%: 0.885-0.961) in comparison to rKLO8-ELISA (AUC_{rKLO8} = 0.825, CI 95%: 0.765 – 0.875) and rK26-ELISA (AUC_{rK26} = 0.884, CI 95%: 0.831 – 0.925) (p<0.05) tested separately.

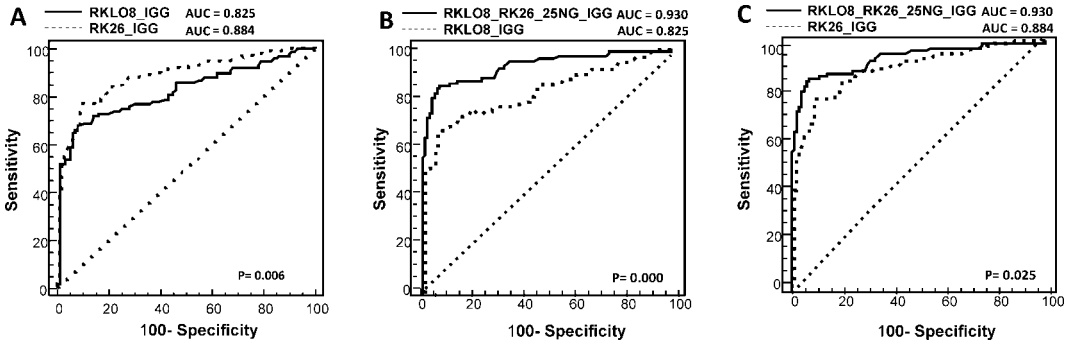

Figure 5. ROC curves generated from the ELISA values of sera to compare the performance of rKLO8 versus rK26 (A); rKLO8+rK26 versus rKLO8 (B); rKLO8+rK26 versus rK26 (C) in the diagnosis of CVL.

Other parameters of the ROC curve include the overall accuracy of the test, represented by the values of sensitivity, specificity and likelihood ratios (LR) in the best cut-off point of each curve. These parameters were determined by ROC analysis of total IgG against rKLO8 and rK26. A sensitivity of 68% (CI 95%: 57.9-77.0) and specificity of 92% (CI 95%: 84.8-96.5) for rKLO8, and 77% sensitivity (CI 95%: 67.5-84.8) and 91% specificity (CI 95%: 83.6-95.8) for rK26 were observed. Overlapping confidence intervals for both antigens in relation to sensitivity and specificity, indicate similar diagnostic accuracy of these tests (Table 1).

ROC curve analysis was also performed for the combination of rKLO8 and rK26 antigens to test sensitivity and specificity in order to evaluate their potential diagnostic power of CVL. The ELISA using rKLO8 and rK26 antigens in combination (rKLO8+rK26-ELISA) showed a sensitivity of 85% (CI 95%:

76.5-91.3), and specificity of 93% (CI 95%: 86.1-97.1). In addition, the value for positive LR was 12.14 and for negative LR was 0.16 (Table 1). These results indicate that the combination of rKLO8 and rK26 antigens generates better accuracy in the diagnosis of CVL than rKLO8 or rK26 alone.

Table 1. Sensitivity and specificity of the rKLO8 and rK26 antigens in the serodiagnosis of canine visceral leishmaniasis.

Antigen	Cutoff	Sensitivity (%)	95% Confidence Interval	Specificity (%)	95% Confidence Interval	LR+	LR-	AUC
rKLO8	0.17	68	57.9-77.0	92	84.8-96.5	8.50	0.35	0.825
rK26	0.17	77	67.5-84.8	91	83.6-95.8	8.56	0.25	0.884
rKLO8+ rK26	0.21	85	76.5-91.3	93	86.1-97.1	12.14	0.16	0.930

The cut off, sensitivity, specificity, positive (LR+) and negative (LR-) likelihood values were determined based on the analysis of ROC curves. AUC = area under the curve.

4. Discussion

An accurate diagnosis is necessary to control CVL. Clinical signs of CVL are quite variable and identification of canine infection which is mainly based on serological tests is sometimes difficult. Thus, there is still a need to improve the current diagnostic tools. The major findings of the present study are: i) IgG reactivity to rKLO8 was higher in dogs tested positive for CVL (DPP^+EIE^+), with a predominance of IgG2 isotype; ii) ROC analysis revealed that rKLO8 and rK26 has a similar performance, with a slightly better AUC value for rK26; and iii) combination of rKLO8 and rK26 antigens gives a better diagnostic accuracy of CVL than single rKLO8 or rK26.

In the present study, rKLO8, a novel antigenic protein of *L. donovani*, was studied for the first time in CVL diagnosis and showed good performance, in accordance with the high specificity (96.1%) that has been reported for

rKLO8 in the diagnosis of human VL in Sudan and India (Abass et al., 2013; Abass et al., 2015). The high serum IgG levels against both rKLO8 and rK26 in DPP^{+}/EIE^{+} seropositive group is in agreement with other studies that also detected enhanced serum antigen-specific IgG levels in dogs with visceral leishmaniasis (Rodriguez et al., 2006; Porrozzi et al., 2007). However, some serum samples from the negative (DPP^{-}/EIE^{-}) control group also expressed augmented levels of IgG antibodies to both rKLO8 and rK26 antigens. This discrepancy may be explained by differences and particularities between the techniques used in the diagnosis of CVL, which may result in false negative test results. Accordingly, it was reported that VL patients from Sudan with low antibody levels tend to give false negative results in rapid tests (Abass et al., 2013). Recently, other authors have observed differences between the tests used in the diagnosis of CVL. Castro Júnior *et al*. (2014) reported that from twelve dogs diagnosed as positive by DPP, only nine were tested positive by conventional or real-time PCR technique which is considered a more sensitive and specific diagnostic method (Castro-Júnior et al., 2014). Other studies have shown that drawbacks of the DPP test may be related to parasite load, suggesting that DPP test positivity is related to dogs with potential to transmit the parasite to the vector (Cavalcanti et al., 2015).

It is well known that T cells and cytokines may affect isotype production in B cells. Accordingly, IgG subclass responses in CVL may reflect distinct patterns of Th1 or Th2 immune responses in symptomatic versus asymptomatic dogs (Asl et al., 2013). Several studies in CVL have reported that there is a correlation between clinical status and levels of IgG subclasses, with high levels of IgG1 to be associated with clinical leishmaniasis (Deplazes et al., 1995;

Iniesta et al., 2005; Asl et al., 2013). On the other hand, Th1 responses characterized by a predominant IgG2 antibody response and high IFN-γ production appears to be associated with the control of leishmaniasis and maintenance of asymptomatic infection (Asl et al., 2013; Cruz-Chan et al., 2014). In addition, high levels of IgG2 in dogs vaccinated with the fucose-mannose ligand (FML) of *L. donovani* correlated with vaccine induced protection (De Oliveira Mendes et al., 2003). In the present study, high IgG2 and low IgG1 levels against both rKLO8 and rK26 antigens were detected in DPP^+/EIE^+ dogs. The samples studied were from a serum repository of randomly collected dog sera in the city of Governador Valadares, an endemic area of visceral leishmaniasis. The elevated levels of IgG2 and low IgG1 in the DPP^+/EIE^+ studied group suggest the occurrence of a large number of subclinical infection, despite lack of clinical data (Deplazes et al., 1995; Asl et al., 2013).

ROC curves were used to interpret accuracy of antigens used in this study. The sensitivity and specificity values for rKLO8 were 68% and 92% and for rK26 77% and 91%, respectively, demonstrating a similar performance of both antigens in the diagnosis of CVL, although rK26 displayed a slightly better AUC value for both, total IgG and IgG2 in comparison to rKLO8. A study performed in a HVL endemic area in northwest Brazil has shown that the rK26 antigen has a high sensitivity (94%) in symptomatic dogs but low sensitivity (64%) in asymptomatic dogs (Porrozzi et al., 2007), findings which are in accordance with our results. Of note, sera of HVL patients from India demonstrated significantly higher antibody responses to rKLO8 and rK39 compared to sera from Sudanese and French patients (Abass et al., 2015).

These differences may be explained by the existence of antigen heterogeneity of *Leishmania* parasites in different endemic regions, or, alternatively, may reflect different immune responses of infected hosts.

ROC curve analysis demonstrated that combined rKLO8 and rK26 antigens increased sensitivity (85%) and specificity (93%) of the diagnostic ELISA. Thus, a combination of rKLO8 and rK26 yields a better accuracy to CVL diagnosis than the rKLO8 and rK26 antigens used separately. The straightforward strategy of combining different antigens in a single test is a new approach in the diagnosis of CVL, to increase the diagnostic sensitivity (Boarino et al., 2005; Fraga et al., 2014). However, it is known that the sensitivity of the DPP assay depends whether dogs have clinical symptoms; it was higher in symptomatic (98%) than in asymptomatic dogs (47%) (Grimaldi et al., 2012). Thus, new efforts to increase the diagnostic sensitivity, especially in asymptomatic dogs, should be encouraged, as it determines whether dogs will be euthanized or not (Marcondes et al., 2013; Coura-Vital et al., 2014). On the other hand, false negative results are unacceptable, since it favors the maintenance of the domestic transmission.

In conclusion, the results indicate improved diagnostic performance of CVL when rK26 was combined with rKLO8, especially in dogs with asymptomatic leishmanial infection. Thus, inclusion of rKLO8 in a future version of the DPP rapid test for the serodiagnosis of CVL deserves further attention.

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REFERENCES

Abass, E., Bollig, N., Reinhard, K., Camara, B., Mansour, D., Visekruna, A., Lohoff, M., Steinhoff, U., 2013. rKLO8, a Novel Leishmania donovani - derived recombinant immunodominant protein for sensitive detection of visceral leishmaniasis in Sudan. PLoS Neglected Trop. Dis. 7. doi: 10.1371/journal.pntd.0002322

Abass, E., Kang, C., Martinkovic, F., Semião-Santos, S., Sundar, S., Walden, P., Piarroux, R., el Harith, A., Lohoff, M., Steinhoff, U., 2015. Heterogeneity of Leishmania donovani parasites complicates diagnosis of visceral leishmaniasis: comparison of different serological tests in three endemic regions. PLoS One 10, e0116408. doi: 10.1371/journal.pone.0116408

Alves, A.S., Mouta-Confort, E., Figueiredo, F.B., Oliveira, R.V.C., Schubach, A.O., Madeira, M.F., 2012. Evaluation of serological cross-reactivity between canine visceral leishmaniasis and natural infection by Trypanosoma caninum. Res Vet Sci, v. 93, n. 3, p. 1329-33. doi: 10.1016/j.rvsc.2012.07.006

Asl, A.S., Sharifi, M., Mohebali, M., Zarei, Z., Babaloo, Z., Akhoundi, B., 2013. Evaluation of canine anti-Leishmania IgG subclasses and their relation with skin signs in naturally infected dogs in the northwest of iran. Turk. J. Vet. Anim. Sci. 37, 512-515. doi:10.3906/vet-1112-24

Barata, R.A., Peixoto, J.C., Tanure, A., Gomes, M.E., Apolinário, E.C., Bodevan, E.C., De Araújo, H.S., Dias, E.S., Pinheiro, A.D.C., 2013. Epidemiology of visceral leishmaniasis in a reemerging focus of intense transmission in Minas Gerais State, Brazil. BioMed Res. Int. 2013. doi: 10.1155/2013/405083

Bhatia, A., Daifalla, N.S., Jen, S., Badaro, R., Reed, S.G., Skeiky, Y.A.W., 1999. Cloning, characterization and serological evaluation of K9 and K26: two related hydrophilic antigens of Leishmania chagasi. Mol. Biochem. Parasitol. 102, 249-261. doi:10.1016/S0166-6851(99)00098-5

Boarino, A., Scalone, A., Gradoni, L., Ferroglio, E., Vitale, F., Zanatta, R., Giuffrida, M.G., Rosati, S., 2005. Development of recombinant chimeric antigen expressing immunodominant B epitopes of Leishmania infantum for serodiagnosis of visceral leishmaniasis. Clin. Diagn. Lab. Immunol. 12, 647-653. doi: 10.1128/CDLI.12.5.647-653.2005

Castro-Júnior, J.G., Freire, M.L., Campos, S.P.S., Scopel, K.K.G., Porrozzi, R., Da Silva, E.D., Colombo, F.a., Silveira, R.D.C.V.D., Marques, M.J., Coimbra, E.S., 2014. Evidence of Leishmania (Leishmania) infantum infection in dogs from Juiz de Fora, Minas Gerais state, Brazil, based on immunochromatographic dual-path platform (DPP®) and PCR assays. Rev. Inst. Med. Trop. São Paulo 56, 225-229. doi: 10.1590/S0036-46652014000300008

Cavalcanti, A.S., Ribeiro-Alves, M., Pereira Lde, O., Mestre, G.L., Ferreira, A.B., Morgado, F.N., Boite, M.C., Cupolillo, E., Moraes, M.O., Porrozzi, R., 2015. Parasite load induces progressive spleen architecture breakage and impairs cytokine mRNA expression in Leishmania infantum-naturally infected dogs. PLoS One 10, e0123009. doi: 10.1371/journal.pone.0123009

Cortada, V.M.C.L., Doval, M.E.C., Souza Lima, M.A.A., Oshiro, E.T., Meneses, C.R.V., Abreu-Silva, A.L., Cupolilo, E., Souza, C.S.F., Cardoso, F.O., Zaverucha do Valle, T., Brazil, R.P., Calabrese, K.S., Gonçalves da Costa, S.C., 2004. Canine visceral leishmaniosis in Anastácio, Mato Grosso do Sul

State, Brazil. Vet. Res. Commun. 28, 365-374. doi:10.1023/B:VERC.0000035014.80785.b7

Coura-Vital, W., Ker, H.G., Roatt, B.M., Aguiar-Soares, R.D.O., Leal, G.G.D.A., Moreira, N.D.D., Oliveira, L.A.M., Machado, E.M.D.M., Morais, M.H.F., Corrêa-Oliveira, R., Carneiro, M., Reis, A.B., 2014. Evaluation of change in canine diagnosis protocol adopted by the visceral leishmaniasis control program in Brazil and a new proposal for diagnosis. PLoS ONE 9, 1-6. doi: 10.1371/journal.pone.0091009.

Cruz-Chan, J.V., Aguilar-Cetina, A., Villanueva-Lizama, L.E., Martínez-Vega, P.P., Ramírez-Sierra, M.J., Rosado-Vallado, M.E., Guillermo-Cordero, J.L., Dumonteil, E., 2014. A canine model of experimental infection with Leishmania (L.) mexicana. Parasit. Vectors 7, 361. doi: 10.1186/1756-3305-7-361

Da Costa, R.T., França, J.C., Mayrink, W., Nascimento, E., Genaro, O., Campos-Neto, A., 2003. Standardization of a rapid immunochromatographic test with the recombinant antigens K39 and K26 for the diagnosis of canine visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 97, 678-682. doi: 10.1016/s0035-9203(03)80102-5

Dantas-Torres, F., 2009. Canine leishmaniosis in South America. Parasites Vectors 2 Suppl 1, S1. doi: 10.1186/1756-3305-2-S1-S1

De Oliveira Mendes, C., Paraguai De Souza, E., Borja-Cabrera, G.P., Melo Batista, L.M., Aparecida Dos Santos, M., Parra, L.E., Menz, I., Palatnik, M., Palatnik De Sousa, C.B., 2003. IgG1/IgG2 antibody dichotomy in sera of vaccinated or naturally infected dogs with visceral leishmaniosis. Vaccine 21, 2589-2597. doi: 10.1016/S0264-410X(03)00046-X

Deplazes, P., Smith, N.C., Arnold, P., Lutz, H., Eckert, J., 1995. Specific IgG1 and IgG2 antibody responses of dogs to Leishmania infantum and other parasites. Parasite Immunol. 17, 451-458. doi: 10.1111/j.1365-3024.1995.tb00914.x

Fraga, D.B.M., da Silva, E.D., Pacheco, L.V., Borja, L.S., de Oliveira, I.Q., Coura-Vital, W., Monteiro, G.R., Oliveira, G.G.D.S., Jerônimo, S.M.B., Reis, A.B., Veras, P.S.T., 2014. A multicentric evaluation of the recombinant Leishmania infantum antigen-based immunochromatographic assay for the serodiagnosis of canine visceral leishmaniasis. Parasit. Vectors 7, 136. doi: 10.1186/1756-3305-7-136

Gomes, Y.M., Paiva Cavalcanti, M., Lira, R.A., Abath, F.G.C., Alves, L.C., 2008. Diagnosis of canine visceral leishmaniasis: Biotechnological advances. Vet. J. 175, 45-52. doi: 10.1016/j.tvjl.2006.10.019

Grimaldi, G., Teva, A., Ferreira, A.L., dos Santos, C.B., Pinto, I.D.S., de-Azevedo, C.T., Falqueto, A., 2012. Evaluation of a novel chromatographic immunoassay based on Dual-Path Platform technology (DPP ® CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 106, 54-59. doi: 10.1016/j.trstmh.2011.10.001

Iniesta, L., Gallego, M., Portus, M., 2005. Immunoglobulin G and E responses in various stages of canine leishmaniosis. Vet. Immunol. Immunopathol. 103, 77-81. doi: 10.1016/j.vetimm.2004.08.011

Langlotz, C.P., 2003. Fundamental measures of diagnostic examination performance: usefulness for clinical decision making and research. Radiology 228, 3-9. doi: 10.1148/radiol.2281011106

Maia, C., Campino, L., 2008. Methods for diagnosis of canine leishmaniasis and immune response to infection. Vet. Parasitol. 158, 274-287. doi: 10.1016/j.vetpar.2008.07.028

Marcondes, M., de Lima, V.M.F., de Araújo, M.D.F.L., Hiramoto, R.M., Tolezano, J.E., Vieira, R.F.C., Biondo, A.W., 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. doi: 10.1016/j.vetpar.2013.07.013

McFall, R., Treat, T., 1999. Quantifying the information value of clinical assessments with signal detection theory. Annu. Rev. Psychol. 50, 215-241. doi: 10.1146/annurev.psych.50.1.215

Mettler, M., Grimm, F., Capelli, G., Camp, H., Deplazes, P., 2005. Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic Leishmania infections in dogs. J. Clin. Microbiol. 43. doi: 10.1128/JCM.43.11.5515-5519.2005

Miro, G., Cardoso, L., Pennisi, M.G., Oliva, G., Baneth, G., 2008. Canine leishmaniosis--new concepts and insights on an expanding zoonosis: part two. Trends Parasitol. 24. doi: 10.1016/j.pt.2008.05.003.

Morales-Yuste, M., Morillas-Márquez, F., Díaz-Sáez, V., Barón-López, S., Acedo-Sánchez, C., Martín-Sánchez, J., 2012. Epidemiological implications of the use of various methods for the diagnosis of canine leishmaniasis in dogs with different characteristics and in differing prevalence scenarios. Parasitol. Res. 111, 155-164. doi: 10.1007/s00436-011-2812-7

Paranhos-Silva, M., Freitas, L.A., Santos, W.C., Grimaldi, G.J., Pontes-de-Carvalho, L.C., Oliveira-dos-Santos, A.J., 1996. A cross-sectional serodiagnostic survey of canine leishmaniasis due to Leishmania chagasi. Am. J. Trop. Med. Hyg. 55, 39-44. PMID: 8702020

Porrozzi, R., Santos da Costa, M.V., Teva, A., Falqueto, A., Ferreira, A.L., dos Santos, C.D., Fernandes, A.P., Gazzinelli, R.T., Campos-Neto, A., Grimaldi, G., Jr., 2007. Comparative evaluation of enzyme-linked immunosorbent assays based on crude and recombinant leishmanial antigens for serodiagnosis of symptomatic and asymptomatic Leishmania infantum visceral infections in dogs. Clin. Vaccine Immunol. 14, 544-548. doi: 10.1128/CVI.00420-06

Quinnell, R.J., Carson, C., Reithinger, R., Garcez, L.M., Courtenay, O., 2013. Evaluation of rK39 rapid diagnostic tests for canine visceral leishmaniasis: longitudinal study and meta-analysis. PLoS Neglected Trop. Dis. 7. doi: 10.1371/journal.pntd.0001992

Quinnell, R.J., Courtenay, O., 2009. Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology 136, 1915-1934. doi: 10.1017/S0031182009991156.

Rodriguez, A., Solano-Gallego, L., Ojeda, A., Quintana, J., Riera, C., Gallego, M., Portus, M., Alberola, J., 2006. Dynamics of Leishmania-specific immunoglobulin isotypes in dogs with clinical leishmaniasis before and after treatment. J. Vet. Intern. Med. 20, 495-498. doi: 10.1111/j.1939-1676.2006.tb02887.x

Solano-Gallego, L., Miró, G., Koutinas, A., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., 2011. LeishVet guidelines for the

practical management of canine leishmaniosis. Parasit. Vectors 4, 1-16. doi: 10.1186/1756-3305-4-86

Sundar, S., Rai, M., 2002. Laboratory diagnosis of visceral leishmaniasis. Clin. Diagn. Lab. Immunol. 9, 951-958. doi: 10.1128/CDLI.9.5.951-958.2002

WHO, 2015. Leishmaniasis.