Evaluation of three recombinant *Leishmania infantum* antigens in human and canine visceral leishmaniasis diagnosis

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**A B S T R A C T**

Visceral leishmaniasis (VL) is a neglected disease and is fatal if untreated. Dogs serve as reservoirs for *Leishmania infantum* (syn. *L. chagasi*) due to their susceptibility to infection and high skin parasitism. Therefore, VL control in Brazil involves the elimination of seropositive dogs, among other actions. However, the most frequently used serological tests have limitations regarding sensitivity and specificity. In this study, we have selected three *Leishmania* antigens (C1, C8 and C9) and have produced them as recombinant proteins using PET-28a-TEV vector and *Escherichia coli* BL-21 as expression system. When tested in ELISA with human samples, the C9 antigen was the one showing the most promising results, with 68% sensitivity and 78% specificity. When testing canine samples, the C1, C8 and C9 antigens showed a sensitivity range from 70% to 80% and specificity range from 60% to 90%. The C1 antigen presented higher sensitivity (80%) and the C8 antigen presented higher specificity (90%). Due to it, we decided to mix and test C1 and C8 antigens together, resulting in the C18 antigen. The mix also yielded high percentages of detected symptomatic and asymptomatic dogs however it did not improve the performance of the diagnostic. Comparison of our tests with the tests recommended by the Brazilian Ministry of Health revealed that our antigens’ sensitivities and the percentage of detected asymptomatic dogs were much higher. Our results suggest that the C1, C8, C18 and C9 recombinant proteins are good antigens to diagnose canine visceral leishmaniasis and could potentially be used in screening tests. To diagnose human visceral leishmaniasis, the C9 antigen presented reasonable results, but more optimization must be performed for this antigen to provide better performance.

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1. Introduction

Leishmaniasis is a serious public health problem that affects approximately 98 countries worldwide (Alvar et al., 2012). Visceral leishmaniasis (VL), also known as kala-azar, is characterized by high fever, substantial weight loss, splenomegaly, hepatomegaly and anemia (Desjeux, 1996). In Brazil, where VL is caused by *Leishmania infantum* (syn. *L. chagasi*), the disease has expanded from rural to urban areas (Margonari et al., 2006; Mauricio et al., 2000). Serological diagnosis is widely and frequently used to diagnose humans and dogs, the latter being identified as *L. infantum* reservoirs (Alvar et al., 2004; Deane, 1961). Although specific humoral responses in human (Galvão-Castro et al., 1984) and canine visceral leishmaniasis are generally very intense, with high levels of specific immunoglobulins, the rate of *Leishmania* infection is underestimated in dogs from endemic areas (Alvar et al., 2004).

According to the Brazilian Ministry of Health, canine visceral leishmaniasis (CVL) surveillance must be determined based on the DPP® CVL rapid test and the enzyme linked immunosorbent assay (ELISA) produced by Bio-Manguinhos/FIOCRUZ, Brazil (Brasil, 2011). However, these tests have limitations such as low sensitivity, mainly in asymptomatic dogs (Grimaldi et al., 2012; Faria et al., 2011), and cross-reactivity (Porrogetti et al., 2007). Thus, new antigens have been studied to provide better assays. Recombinant
proteins usually improve test performances and represent an open field of research (Gomes et al., 2008; Maia and Campino, 2008). Some of these antigens, such as heat shock proteins, enzymes and hypothetical proteins, have been extensively investigated (Srividy et al., 2012).

In human visceral leishmaniasis (HVL), the Brazilian Ministry of Health recommends the immunofluorescence immunoassay (IFI) and ELISA to diagnose the disease. The most commonly used antigen in ELISA is a crude soluble antigen extracted from promastigotes (Pedras et al., 2008), but many proteins have been investigated to be used in this reaction (Boarino et al., 2005; Kumar et al., 2001; Oliveira et al., 2011). However, there are no commercially available ELISA kits in Brazil and only one commercial IFI kit, which is produced by Bio-Manguinhos/FIOCRUZ, Brazil. Immunochromatographic tests are also used in HVL diagnosis, as well as in-house polymerase chain reactions (Cruz et al., 2013; de Assis et al., 2011). Thus, there is a need to develop good antigens to be used mainly in ELISA to diagnose humans because there is no commercially available test in our country.

In previous works from our group, several Leishmania proteins of unknown function were identified as immunogenic to canine sera using an immunoproteomic approach (Costa et al., 2011; Faria et al., 2011). Some of these proteins were selected for production in Escherichia coli and for subsequent purification and use in ELISA tests. Thus, this paper aims to contribute to advances in VL diagnosis through the production and testing of three different Leishmania antigens.

2. Material and methods

2.1. Antigens selection

Three Leishmania proteins were selected based on four different criteria. First, they should not have been previously described in the literature except in the automatic annotation of sequenced trypanosomatid genomes and our research (Costa et al., 2011). Second, they must be conserved proteins in all Leishmania species with annotated genomes. Third, they must present B cell epitopes based on predictions made by two independent software (BCPpreds and ABCPreds) (Faria et al., 2011). Fourth, their mapped epitopes cannot react with Trypanosoma cruzi-infected canine sera nor with healthy canine sera in immunoblottings; additionally, their epitopes must be highly immunogenic when tested with L. infantum-infected canine sera (Faria et al., 2011). It is worth mentioning that C1 antigen, in addition to the four criteria mentioned above, has also generated peptides that showed good reactivity in ELISA to CVL positive samples, as shown by our group (Faria et al., 2011). The proteins studied in this paper were named C1, C8 and C9.

2.2. Recombinant protein production

Nucleotide and amino acid sequences used in this study were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Initially, genomic DNA was extracted from L. infantum (MHOM/BR/1972/BH46) using the QIAamp DNA Mini Kit (QIAGEN) and used as template in PCR reactions. Forward and reverse primers, shown in Table 1, were designed using Oligo Express 1.4 software (www.genelink.com/tools/gl-oe.asp) and Primer Premier 5.0 (www.premierbiosoft.com/primerdesign/). The amplification reaction was carried out using the following conditions: primary denaturation at 94 °C for 5 min followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C (C1 and C8) or 62 °C (C9) for 1 min and extension at 72 °C for 1 min, and a final extension for 5 min.

<table>
<thead>
<tr>
<th>Name</th>
<th>GenBank Identity</th>
<th>Forward and reverse primers sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>XP_001467184.1</td>
<td>5′-GCTAGCAGTATGATACTACAGGGTAATAC3′-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-AGGCTTCTAGATCGGCTTTGGTCTG3′-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-GGCTCATGCGCCACGCTCGG-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-GGATCCATTATTTTCTCACTTCTTCGTCGTGG-3′</td>
</tr>
</tbody>
</table>

The PCR product was cloned into the Nhel/HindIII (for C1 and C8 proteins) or Ndel/BamHI (for C9 protein) restriction sites of the pET-28a-TEV vector, which enabled a 6 residue histidine tag to be fused to the protein. Next, E. coli (BL21 strain) electrocompetent cells were transformed with the recombinant plasmids. To achieve protein expression, a single colony was grown in 2× YT medium (1.6% Bactotryptone, 1% yeast extract, 0.5% NaCl) containing 0.05 mg/ml kanamycin for 16 h. This culture was inoculated into 1 L of fresh 2× YT with kanamycin, maintained under the same conditions as above and induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) when the culture reached an optical density of 0.6 at 600 nm. Expression was carried out for 4 h at 37 °C, and cells were then lysed by five passages through a homogenizer (EmulsiflexTM C3). In SDS-PAGE, it was observed that the recombinant proteins remained in insoluble fraction. Then, proteins were purified using a 5 mL HIS-Trap column (GE Healthcare Life Science) attached to an AKTA Prime chromatography system (GE Healthcare Life Science). The antigens were eluted with 0.5 M imidazole [20 mM sodium phosphate; 0.5 M NaCl; 0.5 M imidazole; 8 M urea] and stored in this buffer.

2.3. Sera samples

The antigenicity of the antigens was evaluated using canine and human sera samples. Canine samples from healthy dogs (n = 10) were used as controls, and samples from dogs naturally infected with L. infantum (n = 31) were also tested. Sera samples from L. infantum-infected dogs with well-defined clinical status were also evaluated. Those dogs were classified as asymptomatic (n = 14) and symptomatic (n = 12) after being scored for 6 typical signs of CVL (Quinnell et al., 2001). All dogs were diagnosed based on parasitological (amastigotes in stained bone marrow) and serological (indirect immunofluorescence test – IFAT) results.

Human sera samples from healthy (n = 10) and L. infantum-infected donors (n = 80), with parasitological (amastigotes in stained bone marrow) and serological (IFAT) confirmatory results, were also used. These samples were from a serum bank and were kindly provided by Dr. Eliana Furtado (Fundação Ezequiel Dias – FUNED) and Dr. Elenice Moreira Lemos (Universidade Federal do Espírito Santo – UFES). The study was approved by the appropriate Ethics Committee.

2.4. ELISA for immunodiagnosis

A titration curve was performed to determine the best protein concentration as well as the dilution of the sera and of the enzyme-conjugated antibodies. Plates (BD FalconTM) were sensitized with recombinant proteins (1.0 µg/mL in Carbonate–Bicarbonate Buffer, pH 9.6) for 16–18 h at 4 °C. A mixture of proteins was also tested. The C18 protein was a mixture of equal parts of the C1 and C8 antigens. Binding sites were blocked with PBS (0.7 M Na2HPO4; 0.3 M NaH2PO4; 0.15 M NaCl; pH 7.4) supplemented with 0.05% Tween 20 and 5% powdered skim milk for 1 h at 37 °C. Serum samples were diluted in PBS/T with 0.5% powdered skim milk, added to the reaction and incubated at 37 °C for 1 h. The optimal serum dilution varied as described: 1:100 (canine sera tested with recombinant
proteins C1 and C9) and 1:200 (canine sera tested with recombinant protein C8 and C18 and human sera tested with all proteins). Plates were washed three times with PBS/T and then incubated with peroxidase-conjugated immunoglobulin G (Sigma–Aldrich) in PBS/T with 0.5% powdered skim milk at 37 °C for 1 h. Anti-dog immunoglobulin was diluted 1:2500 and anti-human was diluted 1:1000. After washing three times with PBS/T, the reactions were developed with FAST OPD (Sigma–Aldrich), and plates were incubated for 30 min in the dark. Reactions were stopped with 2 M H2SO4, and the plates were read at 492 nm in a Multiskan Go Reader (ThermoScientific).

A cut off point for optimal sensitivity and specificity was determined using ROC analysis [Greiner et al., 2000], and the area under the curve (AUC) was calculated to assess the performance of the tests. All of the statistical analyses were performed using GraphPad Prism (version 5.0) and MedCalc (version 7.3).

2.5. Comparison with Dpp® rapid test and ELISA Bio-Manguinhos

The results of the ELISA using C1, C8, C9 and C18 proteins for CVL diagnosis were compared with those obtained with the Dpp® rapid test and EIE–LVC Bio-Manguinhos kit, which are currently recommended by the Brazilian Ministry of Health for dog screening (Brasil, 2011). While the Dpp® rapid test is based in the use of two different recombinant proteins, the EIE–LVC Bio-Manguinhos kit employs Leishmania major crude antigen. To do this comparison, the same serum samples were tested using both assays according to the manufacturer’s instructions.

Agreement beyond chance between the tests was assessed using the Cohen Kappa (k) coefficient (Cohen, 1968) and interpreted according to the following scale: 0.00–0.20, negligible; 0.21–0.40, weak; 0.41–0.60, moderate; 0.61–0.80, good and 0.81–1.00, excellent (Landis and Koch, 1977).

3. Results

3.1. Recombinant protein production

The C1, C8 and C9 antigens were produced using recombinant DNA technology. Initially, after PCR steps, different amplicons were obtained in three different reactions. A 1077-bp amplicon was obtained, as well as a 597-bp and a 591-bp, which were referred as C1, C8 and C9, respectively (data not shown).

After digestion of the PCR products with the appropriate restriction enzymes, cloning into the expression vector was performed and proteins expression was successfully achieved. Bacterial culture, analyzed in SDS-PAGE after induction, showed highly expressed bands for each of the recombinant proteins. In Fig. 1 it is shown that recombinant proteins remained mostly in the insoluble portion of that bacterial culture and that purification process was successful. Bands of the correct mass (39.8 kDa, 22.1 kDa and 24.4 kDa for C1, C8 and C9, respectively) were observed.

Protein yield was estimated at an average of 7 mg/L bacterial culture for the C9 protein and 97.7 mg/L for C1. A larger yield of 509 mg/L was obtained for the C8 protein, what makes these antigens very interesting to be used in large-scale diagnosis campaigns. The proteins after the purification in the His-Trap column are shown in Fig. 1.

3.2. Antigenicity of C1, C8, C9 and C18 proteins

3.2.1. Human ELISA

When testing human sera samples by ELISA, the C1 and C8 proteins showed similar performance, with poor discrimination between infected and control groups (p < 0.05), as well low accuracy. The results obtained with the C9 protein indicated that some sera samples from infected patients were able to recognize this recombinant antigen, providing a sensitivity of 68% and specificity of 78%. There was a significant difference between the control and infected groups (p = 0.003), and the test was considered moderately accurate (AUC = 0.79). In Fig. 2, the absorbance distribution of C1, C8, C9 ELISA in human samples is shown as well as the cut off point for optimal sensitivity and specificity. The sensitivities and specificities obtained with the three antigens with human sera are shown in Table 2.

3.2.2. Canine ELISA

Canine sera samples were individually tested against the C1, C8, and C18 (a mixture of C1 and C8) antigens. The C18 mixture was proposed due to the good results obtained with C1 and C8 individually, as detailed next.

In Fig. 3, the absorbance distribution of C1, C8, C9 and C18-ELISA in canine samples is shown as well as the cut off point for optimal sensitivity and specificity. Promising results for canine sera samples were obtained with the C1 and C8 antigens, which provided sensitivities of 80% and 75%, respectively. According to Swets (1988), these antigens provided tests with moderate accuracy (AUC = 0.76 for C1 and AUC = 0.86 for C8). The C1 and C8 antigens were able to significantly differentiate between control and infected groups (p < 0.005; Table 3). Additionally, all sera samples from asymptomatic dogs were detected by the C1 protein, while 58% of sera from symptomatic dogs were detected by C8. Analyzing the results obtained to C1 and C8 antigens, we verified that the most sensitive one (C1: 80%) along with the most specific one (C8: 90%) could result in a promising mixture. Furthermore, the detection of all asymptomatic dogs of C1 and also the high accuracy of C8 encouraged the tests with C18, in ELISA.

The C18 antigen presented a good performance, with a slight improvement in sensitivity (85%), comparing to C1 (80%) and C8 (75%). The test showed accuracy of 0.79 and specificity of 60%. All the asymptomatic dogs were detected in this test, but a lower percentage of symptomatic dogs (33%) was considered positive. Taken together, the mix of C1 and C8 (=C18) does not improve substantially the performance of the test.

The C9 protein, as well as the other antigens, provided a moderately accurate test. The sensitivity (71%) was lower than for the

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Diagnostic performance of C1, C8 and C9 recombinant proteins in serum samples from L. infantum-infected patients.</th>
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</thead>
<tbody>
<tr>
<td>Antigen</td>
<td>Se (%)</td>
</tr>
<tr>
<td>C1</td>
<td>66.2</td>
</tr>
<tr>
<td>C8</td>
<td>58.2</td>
</tr>
<tr>
<td>C9</td>
<td>67.9</td>
</tr>
</tbody>
</table>

* Se: sensitivity.  
Sp: specificity.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Diagnostic performance of C1, C8, C9 and C18 recombinant proteins, Dpp® and EIE-LVC Bio-Manguinhos tests in serum samples from L. infantum-infected dogs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen</td>
<td>Se (%)</td>
</tr>
<tr>
<td>C1</td>
<td>80.0</td>
</tr>
<tr>
<td>C8</td>
<td>75.0</td>
</tr>
<tr>
<td>C18</td>
<td>85.4</td>
</tr>
<tr>
<td>C9</td>
<td>70.6</td>
</tr>
<tr>
<td>Dpp®</td>
<td>72.9</td>
</tr>
<tr>
<td>EIE-LVC</td>
<td>64.5</td>
</tr>
</tbody>
</table>

NA: not applicable; EIE-LVC: EIE-LVC Bio-Manguinhos.

* Se: sensitivity.  
Sp: specificity.

Sym percentage of symptomatic dogs diagnosed as positive.

Asym: number of asymptomatic dogs diagnosed as positive.
other antigens, and the percentage of detected symptomatic and asymptomatic dogs was in between the values for the C1 and C8 antigens (Table 3).

### 3.3. Comparison between different tests

Great differences were found when comparing different CVL diagnostic tests. When the EIE-LVC Bio-Manguinhos kit was cross-tabulated with recombinant antigens, the agreement was very poor. The index ranged between 0.160 (compared to C18) and 0.257 (compared to C8). All of these correlations indicate a negligible or weak agreement between these tests. Sensitivity of EIE-LVC Bio-Manguinhos was 65% and specificity was 100%. Similarly, the DPP® rapid test also showed a negligible agreement (between 0.052 and 0.142) when compared to our recombinant antigens, while sensitivity and specificity were 73% and 90% respectively. Interestingly, agreement between the DPP® rapid test and the EIE-LVC Bio-Manguinhos kit was also weak (0.252).

We found an encouraging result regarding the detection of asymptomatic sera. While our recombinant antigens detected approximately 78–100% of the asymptomatic sera according to the antigen, the DPP® rapid test detected only 10% of these sera.
Additionally, the EIE-LVC Bio-Manguinhos kit could not detect any asymptomatic samples, which means that all asymptomatic sera were classified as negative by this kit.

4. Discussion

Serological diagnosis plays a critical role in the confirmation of VL. However, a method with satisfactory efficiency is not yet available for dogs or humans (Faria and Andrade, 2012; Srivastava et al., 2011). Several *Leishmania* antigens have been characterized, and recombinant technology has been used for the development of recombinant antigens. In this work, three different proteins from *L. infantum* were investigated with regards to VL diagnosis. These proteins are very interesting because, despite the fact that they are not described in the literature (except by our research group in Costa et al., 2011), all of them predicted to contain B cell epitopes, and one of them (C1) generated a synthetic peptide able to recognize specific antibodies in an ELISA for CVL diagnosis (Faria et al., 2011).

We believe that B cell epitope prediction showed great efficiency because all of the antigens were reactive in both types of serum samples (canine and human). However, the prediction is not specific to a *Leishmania* response. The antigens were reactive with samples from infected as well as non-infected subjects, mainly within the human sera. Among the tested antigens, only one of them demonstrated reasonable performance for human LV diagnosis, while all of them were good for canine diagnosis. The results suggest that the humoral immune response in infected humans and dogs is different. Additionally, it can also be explained by the selection of our proteins. As published by our group, the proteins tested in this study were selected from immunosassays (western blotting and immunoblotting on cellulose membranes) in which canine sera were employed (Costa et al., 2011; Faria et al., 2011). Therefore, it was expected that the performance of the *Leishmania* antigens would be better in CVL diagnosis than in human diagnosis.

Recombinant antigens for HVL diagnosis employed in ELISA are not frequently found in literature. In our study, the C9 antigen presented an accuracy of 0.79, almost 80% specificity and about 70% sensitivity. Oliveira et al. (2011) showed that a hypothetical protein selected from a genomic *L. infantum* library with pooled sera had a low sensitivity value of 36.4%. Interestingly, this same protein performed very well in CVL diagnosis, which also occurred with the antigens tested in this study.

Still regarding the diagnosis of HVL, other authors showed better results than ours. Among the group of enzymes, rLACK (*Leishmania* homolog of receptors for activated C-kinase) presented 97% sensitivity and 84% specificity in an HVL ELISA (Maale et al., 2003). Among the heat shock proteins, different members of this family were found to be potential antigens for human diagnosis. Sera from patients with HVL were recognized by recombinant Leishmania Hsp90 and Hsp70, while sera from patients with Chagas disease were not, making them useful in differential diagnosis (de Andrade et al., 1992). Larreta et al. (2002) investigated the *L. infantum* GRP94 protein, a member of the HSP83/90 family. This protein was recognized by 78% of sera from VL patients. Investigating the use of purified parasite antigens, Lakhal et al. (2012) tested the use of crude histone proteins in sera from Mediterranean VL patients. The crude proteins showed a good ability to discriminate HVL cases and healthy controls (97.6% sensitivity and 100% specificity), but the occurrence of cross-reactions was not evaluated.

For CVL diagnosis, we tested the three *Leishmania* proteins (C1, C8 and C9), and the mixture of two of them (C1 + C8, named C18). All antigens were able to discriminate infected from non-infected animals (*p* < 0.003), and the accuracy values were very similar, ranging from 0.76 to 0.86 (moderate accuracy according to Swets, 1998). The higher sensitivity (85%) was found using the C18 antigen (C1 + C8). However, when we tested C18 antigen, test performance remained very similar to the obtained with C1 and C8 separately. Some authors have described that when different epitopes are joined in a single solution or in a chimeric molecule, the combined new antigen do not increase test performance. Faria et al. (2011) described some peptides with sensitivities between 70.9% and 88.7%, and also specificities between 55.0 and 95.0% in ELISA anti-CVL. When these peptides were tested together, mixed into a single solution, test performance did not improve, with sensitivity 75.0% and specificity of 95.0%. Likewise, Boarino et al. (2005) have generated a chimeric antigen, which presented similar results compared to the antigens that generated the chimera. While K9, K26 and K39 presented sensitivities around 95.0–100% in ELISA anti-CVL (Rosati et al., 2003), chimeric antigen constructed with these three epitopes presented sensitivity of 96.0%. Regarding specificity, it was 100% when the epitopes were tested separately; however, it was a little lower, 99%, for the chimeric antigen. Interestingly, C1 was able to detect 100% of asymptomatic dogs. Within the sequence of this protein, there is a sequence of a peptide that was synthetically produced and tested in an ELISA with canine sera. The peptide was named PSLc4 and could identify 92.3% of asymptomatic dogs, as published by our group in Faria et al. (2011). When we compared sensitivities and specificities of the synthetic peptide (PSLc4) and the protein that generated it (C1), we observed a protein sensitivity of 80% and a synthetic peptide sensitivity of 76%. Regarding specificity, the synthetic peptide presented a slightly higher value (70%) than the C1 protein (60%). We observed that the diagnostic performance of the synthetic peptide (PSLc4) was similar to that obtained with the protein (C1) that generated the sequence. This is relevant information when deciding between antigens. Each laboratory usually possesses different features regarding the time, personal training and cost effectiveness for recombinant protein or synthetic peptides production. Additionally, the storage conditions and stability of each antigen must be considered.

Our findings corroborate other literature data on CVL diagnosis. Martins et al. (2013) obtained a specificity of 100% and a sensitivity of 95% when testing sera from asymptomatic dogs (*n* = 19) in an ELISA with a recombinant hypothetical protein. Of note, we tested a larger panel of sera (*n* = 57), but within the group of asymptomatic dogs (*n* = 14), our recombinant proteins also provided great sensitivities, ranging from 78% to 100%. de Souza et al. (2013) obtained great results in a CVL ELISA with a *Leishmania* enzyme, ATP diphosphohydrolase, and IFI-positive sera samples from 48 naturally infected dogs. This antigen was able to detect all of the infected dogs, and the observed specificity was 100%.

When tests with the recombinant *Leishmania* proteins were cross-tabulated with the EIE-LVC Bio-Manguinhos kit and with the DPP® rapid test, both tests presented high specificity (100% and 90% respectively) but not high sensitivity (64% and 73% respectively), and the agreement was poor in both situations. This result could be explained mainly by the different antigens employed in the tests. Our tests appeared to be more sensitive and accurate because they use recombinant proteins, while the EIE-LVC Bio-Manguinhos kit employs crude antigen. In contrast, the DPP® rapid test employs recombinant proteins just like our tests. While our recombinant proteins detected approximately 78–100% of the asymptomatic sera depending on the antigen, the DPP® rapid test detected only 10% of these sera. It has been demonstrated that the diagnostic kit displayed high specificity (96%) but low sensitivity (47%) in identifying parasite-positive dogs without signs of CVL (Grimaldi et al., 2012). However, intrinsic properties of the protein and the platform employed in the test influences the performance and could be responsible for the observed differences.
5. Conclusion

In summary, the recombinant proteins evaluated in this work showed different efficiencies regarding reactivity with antibodies from *L. infantum*-infected dogs and humans. The best results were obtained for CVL diagnosis, which indicates that these proteins are potential antigens and could help in leishmaniasis control. The sensitivity of our tests was higher than the sensitivity of the currently applied tests for CVL diagnosis. The results suggest that best antigens must be used in screening tests. Additionally, for HVL diagnosis, C9 presented a reasonable diagnostic performance. As we cannot find many studies on HVL diagnosis using recombinant proteins, C9 remains a potential target for optimizations. Further validation and cross-reactivity investigations are needed to fully characterize these proteins.

Authors’ contributions

Conceived and designed the experiments: HMA, RAPN, DCB. Performed the experiments: AMF, FTGR, JLRC, RDMM. Analyzed the data: AMF, HMA. Contributed reagents/materials/analysis tools: HMA, RAPN, DCB. Wrote the paper: ARF, HMA.

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