Article

rMELEISH:A Novel Recombinant Multiepitope-Based Protein Applied to The Serodiagnosis of Both Canine and Human Visceral Leishmaniasis

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Abstract: Background: Visceral leishmaniasis (VL) is s critical public health problem in over ninety countries. The control measures adopted in Brazil have been insufficient when it comes to preventing the spread of this neglected disease. In this context, a precise diagnosis of VL in dogs and humans could help to reduce the number of cases of this disease. Distinct studies for the diagnosis of VL have used single recombinant proteins in serological assays; however, results have been variable, mainly in the sensitivity of the antigens. The development of multiepitope-based proteins could be relevant in solving such a problem. **Methods:** A chimeric protein (rMELEISH) was constructed based on amino acid sequences from kinesin 39 (k39), alpha-tubulin, and heat shock proteins HSP70 and HSP 83.1, and tested in ELISA for the detection of *L. infantum* infection in humans and dogs. **Results:** rMELEISH was able to discriminate between VL cases and cross-reactive diseases and healthy samples, with sensitivity and specificity values of 100% as compared to the use of a soluble Leishmania antigenic extract (SLA). **Conclusions:** Preliminary data suggest that the rMELEISH protein presents a potential to be tested in future studies against a larger serological panel for the diagnosis of canine and human VL.

Keywords: Leishmaniasis; recombinant chimeric protein; serodiagnosis; visceral leishmaniasis; humans; dogs

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1. Introduction

Leishmaniasis is a neglected disease caused by protozoan parasites of the genus Leishmania, which is endemic in 99 countries [1]. There are about 20 parasite species capable of causing disease in humans, with an estimated annual incidence of 0.2 to 0.4 million cases of visceral leishmaniasis (VL) and 0.7 to 1.2 million cases of tegumentary leishmaniasis (TL) [2]. Over 90% of VL cases are registered in Bangladesh, Ethiopia, Brazil, India, Sudan, and South Sudan. TL is more widespread and occurs in countries such as Afghanistan, Algeria, Brazil, Iran, Peru, Ethiopia, North Sudan, Costa Rica, Colombia, and Syria [3]. In the Americas, Brazil is responsible by more than 90% of documented VL cases, and the Leishmania infantum species is largely responsible for the disease in dogs and humans [2]. The human (HVL) and canine VL (CVL) represent serious public health risks because of asymptomatic infections in both hosts and by the fact that domestic dogs are important reservoirs of the parasites and a source of infection to vectors and humans [4,5].

The strategy adopted by the Brazilian Ministry of Health to control VL control is based on early diagnosis, treatment of human cases, monitoring of seroreactive dogs, and vector control. However, such measures have been shown to have little effect [3,6,7]. The severity of the disease and the role of dogs as reservoirs emphasize the importance of monitoring and surveying *L. infantum* infections to prevent VL from spreading [8–10]. Thus, improving the diagnosis for both CVL and HVL is relevant to disease control and to formulating more effective public health policies.

A variety of laboratory methods have been used to diagnosis VL in humans and dogs, such as indirect fluorescence antibody test, enzyme-linked immunosorbent assay (ELISA), dot-ELISA, direct agglutination test, western-blotting, and immunochromatographic assays [11–13]. There are currently six diagnostic HVL kits registered for sale in Brazil, most of which are imported. A recent study showed variable sensitivity and specificity in the performance of these tests, which was more pronounced in patients co-infected with HIV (Human Immunodeficiency Virus) [14]. Moreover, the cost-effectiveness of these tests varied significantly [15]. The Ministry of Health recommends CVL screening by the Dual-Path Platform (DPP; Bio-Manguinhos/Fiocruz, Rio de Janeiro, Brazil) and confirmation of CVL cases using an ELISA kit (EIE-LVC kit; Bio-Manguinhos/Fiocruz, Rio de Janeiro, Brazil) [16,17]. However, the diagnosis of CVL cases may be underestimated, since the standard diagnosis for dogs in endemic areas presents low accuracy, considering that one in five seronegative dogs is infected [18]. Furthermore, when analyzing the available serological tests and qPCR (quantitative polymerase chain reaction), the level of agreement between the tests ranged from poor to moderate [19]. In this sense, the currently available diagnostics kits lack sufficient sensitivity and/or specificity, impairing the action of the country's health policy programs.

The ELISA assay is the candidate of choice for a rapid and reliable Leishmania-infection diagnostic test since it is practical, standardizable, and suitable for mass screening [20,21]. However, its specificity and sensitivity depend on the antigen type and quality, and it could be improved by using recombinant multiepitope-based proteins since the variability in the humoral response found in humans and dogs is high. In this context, the combination of distinct antigens in a unique product could potentially improve diagnostic efficacy. Therefore, the selection of parasite antigenic proteins followed by the identification of B-cell epitopes and resulting in the construction of chimeric proteins could account for a more reliable diagnosis of CVL and HVL [5,22,23].

Such an experimental strategy has been shown to be a valuable platform for serological diagnosis of diseases [5,23–28] and vaccines [29–31], where satisfactory results were obtained as compared to the use of soluble and/or crude antigenic extracts or isolated recombinant proteins.

In this study, a recombinant multiepitope-based protein (called rMELEISH) was constructed based on amino acid sequences from kinesin 39 (k39), alpha-tubulin, and heat shock proteins (HSP70) and HSP 83.1 and the resultant chimeric protein was used for the detection of *L. infantum* infection in humans and dogs. Results further showed that the

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recombinant protein was able to discriminate between VL cases and cross-reactive diseases. In addition, testing human samples, rMELEISH identified VL patients, but was not recognized by antibodies in sera from endemic controls or those presenting cross-reactive diseases.

2. Materials and Methods

2.1. Parasites

A *L. infantum* (MHOM/BR/1970/BH46) strain was used. Stationary promastigotes were grown at 24°C in Schneider's medium (Sigma, St. Louis, MO, USA), which was supplemented with 20% inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at pH 7.4. The soluble Leishmania antigen extract (SLA) was prepared as described Coelho et al. 2003 [32]. Briefly, 109 stationary-phase promastigotes per mL of *L. infantum* were washed three times in 5 mL of cold sterile phosphate-buffered saline (PBS). After seven cycles of freezing (-196°C) and thawing (+37°C), the suspension was centrifuged at 8,000 x g for 20 min at 4°C. The supernatant containing SLA was collected in 500 mL aliquots and then stored at -80°C until used. The protein concentration was estimated by the Bradford method [33].

2.2. Canine sera

Sera samples from asymptomatic (n=20) or symptomatic (n=25) VL dogs were used. Symptomatic animals showed clinical signs of disease, such as emaciation, alopecia, anemia, conjunctivitis, dehydration, dermatitis, erosion, ulcerations, lymphadenopathy, and onychogryposis, while asymptomatic dogs were free of any sign of VL. A PCR technique in the spleen or bone marrow aspirates was used to identify *L. infantum* kDNA. Samples were also obtained from healthy dogs living in endemic (n=30, Belo Horizonte) and non-endemic (n=20; Poços de Caldas, Minas Gerais, Brazil) areas. These animals showed no clinical sign of disease and presented negative serological results. Sera from Leish-Tec®-vaccinated dogs (n=20) and from those experimentally infected with *Ehrlichia canis* (n=15) or *Babesia canis* (n=10), all of which were maintained in kennels to prevent their contact with transmitting vectors of leishmaniasis, were also used. The study was approved by the Ethical Committee on the Use of Animals in Research of the Federal University of Minas Gerais (UFMG), with the protocol number 244/2018.

2.3. Human samples

The study was also approved by the Human Research Ethics Committee of UFMG, with protocol number CAAE–32343114.9.0000.5149. All blood samples were collected in vacutainer tubes without anticoagulant (BD Biosciences), and then processed by centrifugation (1,000 x g for 10 min at room temperature), inactivated by heating for 30 min at 56°C, and centrifuged for 5 min at 1,000 x g at 4°C. Samples were collected from VL patients (n=35) who were diagnosed by clinical evaluation and PCR to identify *L. infantum* kDNA in organic aspirates. Sera collected from healthy subjects living in endemic areas of the disease (n=30) who did not present any clinical sign of VL and showed negative serological results, were also used, as were samples from Chagas Disease (n=25), leprosy (n=10), tuberculosis (n=10), malaria (n=10), and HIV-infected (n=25) patients.

2.4. Design of the synthetic gene, cloning, and expression

The amino acid sequence of the rMELEISH protein was submitted to the Protein Homology Recognition Engine V 2.0 server (Phyre2) [34] for modeling in intensive mode. After modeling, the protein structure was visualized using the UCSF Chimera software (version 1.11.2) [35]. The synthetic gene was custom synthesized by Epoch Biosciences with codon usage for *E. coli* and cloned as *NdeI*/*XhoI* fragment into pET21a in-frame with a C-terminal histidine tag to allow protein purification by affinity chromatography. The

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resulting plasmid was used to transform *E. coli* BL21 (DE3) *plysS* competent cells and selection was performed on LB agar plates containing 100 μg/mL ampicillin. The DNA and amino acid sequences for the entire synthetic gene construct are proprietary (under Brazilian patent No. BR1020140313311) and is not available to be shared at this stage. An individual colony was inoculated in 5 mL LB (10 g/L Casein Peptone, 5 g/L Yeast extract, 10 g/L NaCl, pH 7.2) containing 100 μg/mL ampicillin and allowed to grow overnight at 37°C under agitation (200 rpm). One point twenty-five mL of the pre-culture was transferred to 25 mL LB in a 250 mL erlenmeyer. The culture was grown in the aforementioned conditions until an OD_{600} of 0.6 at which point 1 mM IPTG was added. Aliquots were withdrawn 0.5, 1.5, and 2.5 h after induction. The induced culture was harvested by centrifugation at 6000 ×g for 15 min at 4°C and the pellet was stored at −80°C.

2.5. Purification of rMELEISH protein

The frozen pellet was resuspended in 1 mL lysis buffer (8 M urea, 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) following incubation at 4°C for 16 h. Cell suspension was then sonicated (5 pulses of 10 sec with 1-min intervals) using a Vibra Cell sonicator (Sonics & Materials, Inc.) and incubated on ice for 2 h following centrifugation at 6000 ×g for 15 min at 4°C. The supernatant was added to 0.5 mL Ni-Sepharose 6 Fast Flow resin (Sigma-Aldrich) (resuspended in lysis buffer), which was then incubated at 4°C for 90 min on a vertical disc rotator. Next, the resin was sedimented and washed four times with 1 mL washing buffer (8 M urea, 50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, pH 8.0). The protein was eluted in three fractions using 0.5 mL elution buffer (8 M urea, 50 mM NaH_2PO_4, 300 mM NaCl, 50, 100 and 200 mM imidazole, pH 8.0).

2.6. Gel electrophoresis and western blotting

Protein integrity and molecular mass calculation were evaluated by running samples on 12% SDS-PAGE. Proteins were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich). Following electrophoresis, the proteins were transferred electrophoretically to a Polyvinylidene difluoride (PVDF) membrane for western blotting. The membrane was blocked with 5% skim milk powder in phosphate-buffered saline (PBS) for 2 h at room temperature. It was then washed three times with PBS containing 0.1% tween 20 and incubated with monoclonal mouse anti-His AP (Alkaline Phosphatase, Sigma), diluted 1:1,000 in PBS for 2 h at room temperature. Following three washes with PBST, the specific protein band was visualized by the nitroblue tetrazolium/5-bromo-4chloro-3'-indolylphosphate (NBT/BCIP) detection method.

2.7. ELISA assay

Titration curves were plotted to determine the most appropriate antigen concentration and antibody dilution to be used. The wells of polystyrene plates (Sarsted) were sensitized with 35 ng purified rMELEISH protein, which was diluted in 100 μL 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6). After incubation at 4°C for 16 h, the coated wells were washed with PBST (PBS supplemented with 0.2% tween 20, pH 7.2) and blocked for 2 h at 37°C with PBS containing 5% (w/v) dried skim milk powder and washed again with PBST. Subsequently, 100 μL of a dilution [100 μL PBST, 5% (w/v) dried skim milk powder, and 5 μL serum] was placed into the wells resulting in a final dilution of approximately 1/20. After incubation for 1 h at 37°C, the wells were washed with PBST and 100 μL of peroxidase-labeled goat anti-dog IgG conjugate diluted at 1:25,000 in PBS buffer (pH 7.2), containing 5% (w/v) dried skim milk powder, was added following incubation for 1 h at 37°C. The wells were again washed with 100 μL TMB by incubating for 30 min at room temperature. The optical density (OD) values were read at 450 nm.

2.8. Fluorescence and circular dichroism spectroscopy assays

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Conformational changes in the chimeric protein were evaluated by Fluorescence spectroscopy using the Jasco FP-6500 Spectrofluorimeter (Jasco Analytical Instruments, Tokio, Japan) coupled to a Jasco ETC-273T Peltier system (Jasco Analytical Instruments) with water circulation. The rMELEISH protein (0.05 mg/mL) was diluted in 10 mM sodium acetate buffer at pH 4.0 containing 10 mM Tris-HCl, pH 7.0 and 9.0, at 25°C. The excitation and emission slits were fitted at 5.0 and 10.0 nm, respectively. The excitation wavelength was 295 nm and emission spectra were recorded from 300 – 400 nm. The Circular Dichroism (CD) assays were performed using Jasco J-815 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Peltier type temperature controller and thermostatized cuvette cell linked to a thermostatic bath. The far-ultraviolet (UV) CD spectra of the rMELEISH (0.1 mg/mL) in 2 mM acetate, pH 4.0, and 2 mM Tris HCl, pH 7.0 and 9.0, were recorded using a 0.1 cm path length quartz cuvette. After five consecutive measurements, the mean spectrum for each pH was recorded and the buffer contribution spectrum was subtracted. The ellipticities were converted into molar ellipticity [θ] based on molecular mass of 115 Da per residue [36].

2.9. Statistical analysis

Results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed using GraphPad Prism™ (version 6.0 for Windows). Receiver-operator characteristic (ROC) curves were constructed using the OD values from VL sera versus those from negative or cross-reactive samples. The diagnostic performance of the antigens was evaluated by calculating the sensitivity (Se), specificity (Sp), area under the curve (AUC), and Youden index (J). Confidence intervals (CI) were defined using 95% confidence level (95%CI). Differences were considered significant with $P < 0.05$.

3. Results

3.1. Characterization and purification of rMELEISH protein

In order to design a multiepitope protein that could be of diagnostic use, linear and conserved B-cell epitopes, which were shown to be antigenic for VL and present specific antibodies against them, were selected, grouped in tandem, and connected by flexible glycine-serine linkers. This would allow the epitopes to be freely available for interaction with their cognate antibodies, thus contributing to the overall sensitivity and specificity of the diagnostic test. Some molecules were randomly repeated to increase epitope density, resulting in a chimeric protein called rMELEISH. After purification, the recombinant protein had a molecular weight of ~25.0 kDa (Fig. 1d), and a western blotting experiment was conducted using an anti-histidine antibody to confirm the presence of rMELEISH protein (Fig 1b). In addition, an SDS-PAGE 12% gel is shown (Fig 1a).

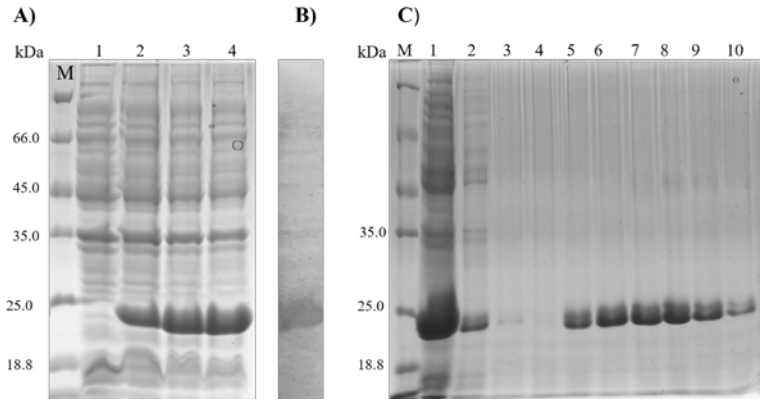

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Figure 1. SDS–PAGE analysis of the rMELEISH. **A)** 12% SDS–PAGE of the accumulation of the rMELEISH post-induction with 1 mM IPTG. Lane M: Unstained Protein Weight Marker Standards (Thermo Scientific) Lanes 1-4; 0, 0.5, 1.5 and 2.5 for induce time (h), respectively. **B)** Western blotting analysis of crude extract after 2.5 hours of induction. **C)** Protein was eluted in five fractions using 0.5mL elution buffer contains different concentrations of imidazole. M: Unstained Protein Weight Marker Standards (Thermo Scientific), Lane 1: flow through (FT); lane 2-4: wash; lanes 5-6: elutions with 50 mM imidazole; lanes 7-8: elutions with 100 mM imidazole, which is considered the best purification condition and lanes 9-10: elutions with 200 mM imidazole.

3.2. Tertiary and secondary structure of rMELEISH protein

Fluorescence spectra of rMELEISH showed typical emission bands of solvent exposed tryptophan residues (Fig. 2a). The intensity of the emission bands was similar in all pH ranges; however, a red shift of 2 nm (340 to 342 nm) was observed at pH 4.0 and 9.0, relative to the emission band at pH 7.0. Far-UV CD spectra of rMELEISH exhibit a dichroic signal close to 0 at pH 4.0 and 9.0 (Fig. 2b), without characteristic secondary structure bands, and a very low signal appears at pH 7.0.

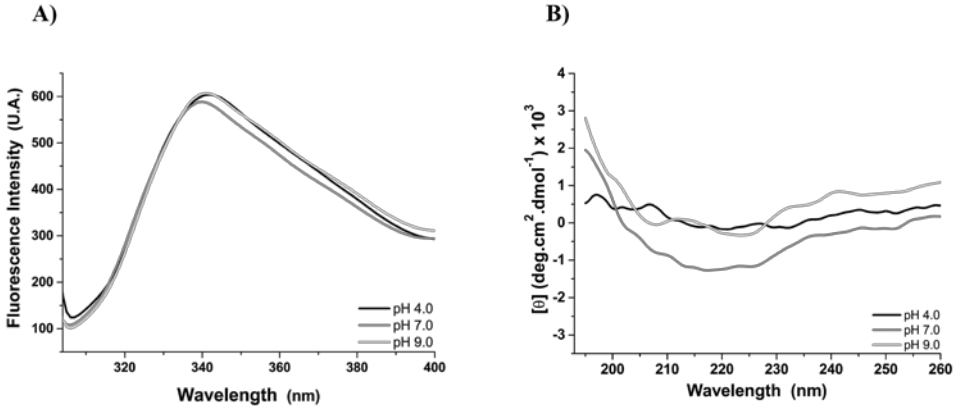

Figure 2. Structural analysis of rMELEISH by fluorescence and Circular Dichroism. **A)** Fluorescence spectra of the rMELEISH as a function of pH 4.0, 7.0 and 9.0 at 25°C. The fluorescence emission bands were red shifted by 2 nm from 340 nm (pH 7.0) to 342 nm (pH 4.0 and 9.0). **B)** Far-UV CD spectra of the rMELEISH at pH 4.0, 7.0 and 9.0 and 25°C. The typical CD bands of secondary structure were not observed at pH 4.0 and 9.0 and a very low signal appears at pH 7.0.

3.3. Diagnostic evaluation of rMELEISH for visceral leishmaniasis

Initially, the diagnostic efficacy of rMELEISH protein was evaluated in ELISA for human VL. L. infantum SLA was used as a comparative antigen. Results showed that the chimeric protein was recognized by antibodies in VL patients' sera, presenting low reaction with healthy or cross-reactive sera (Fig. 3). ROC curves were constructed with the individual OD values for each antigen, with the results showing 100% sensitivity and specificity values for rMELEISH, while the values using SLA were 91.4% and 76.4%, respectively (Table 1).

Table 2. Diagnostic performance of the antigens for visceral leishmaniasis. Sera samples were used in ELISA experiments against rMELEISH and L. infantum SLA, in order to obtain the individual optical density values. ROC curves were constructed and the diagnostic efficacy of was evaluated by calculating the sensitivity (95%CI), specificity (95%CI), area under the curve (AUC), and Youden index (J).

	Canine sera							
Antigen	**AUC**	**p-value**	**Cut-off**	**Se**	**95%CI**	**Sp**	95%CI	J
rMELEISH	1.0	<0.0001	>0.2130	100	92.13-100	100	96.19-100	1.0
SLA	0.63	0.017	>0.5480	51.11	35.77-66.30	88.42	80.23-94.08	0.39
	Human sera							

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Antigen	AUC	p-value	Cut-off	Se	95%CI	Sp	95%CI	J
rMELEISH	1.0	<0.0001	>0.2712	100	90.00-100	100	96.70-100	1.0
SLA	0.86	<0.0001	>0.4493	91.43	76.94-98.20	76.36	67.32-83.94	0.68

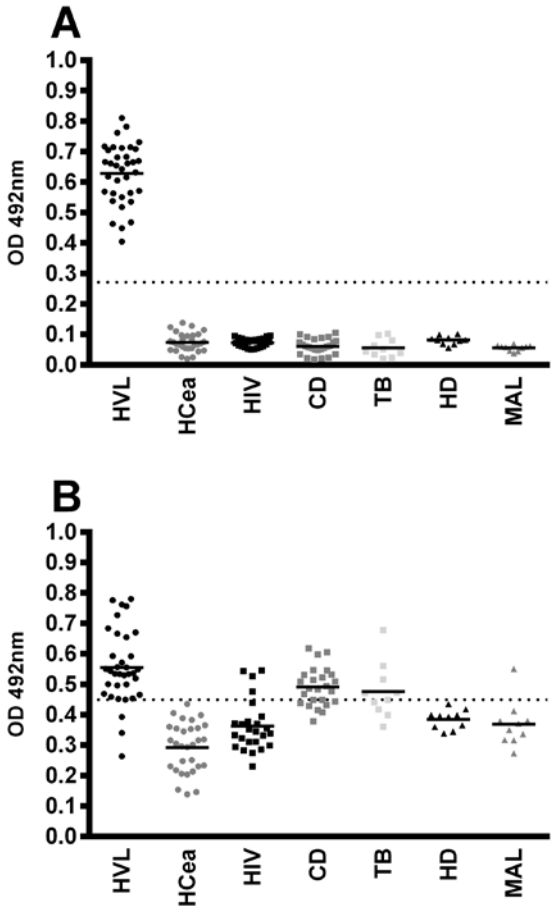

Figure 3. ELISA reactivity for diagnosis of human visceral leishmaniasis. ELISA assays were performed using sera samples from visceral leishmaniasis patients (HVL; n=35), sera from healthy subjects living in endemic region of disease (HCea; n=30); as well as from patients with Chagas Disease (CD, n=25), leprosy (HD, n=10), tuberculosis (TUB, n=10), malaria (MAL, n=10), and HIV-infected (HIV; n=25). ROC curves were constructed with the individual OD values for each serum sample against rMELEISH (panel **A**) or L. infantum SLA (panel **B**), and data are shown. The dotted lines represent the cut-off value obtained by the ROC curves, which were used to obtain sensitivity, specificity, and AUC of the antigens. The mean of each group is also shown.

The diagnostic efficacy of rMELEISH was then evaluated for canine VL. Results showed that the chimera was recognized by all asymptomatic and symptomatic VL dog sera but not by healthy or cross-reactive sera (Fig. 4). SLA was poorly identified by asymptomatic sera and presented higher cross-reactivity against other samples. ROC curves were also constructed and the results showed sensitivity and specificity values of 100% and 98.6%, respectively, for rMELEISH, and 51.11% and 88.42%, respectively, for SLA (Table 1).

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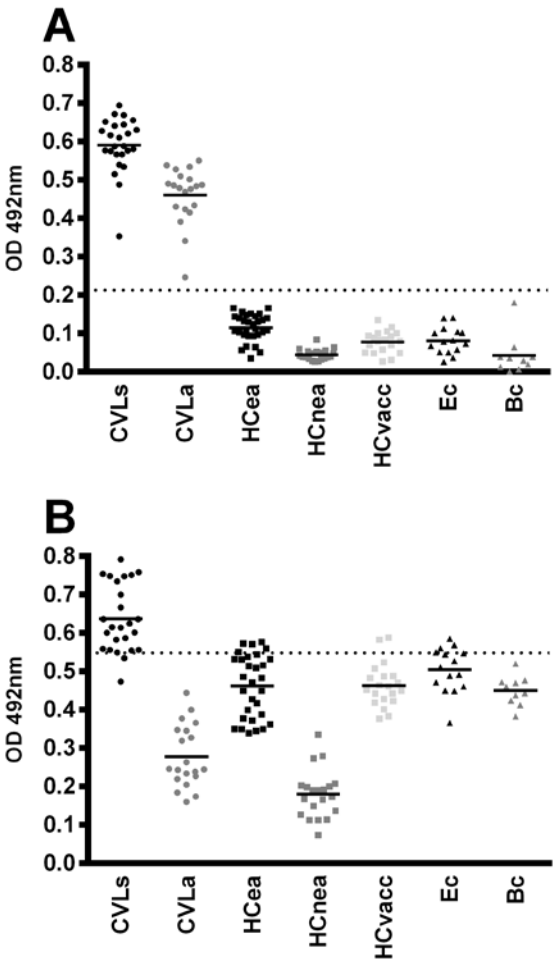

Figure 4. ELISA reactivity for diagnosis of human visceral leishmaniasis. ELISA assays were performed using sera samples from visceral leishmaniasis patients (HVL; n=35), sera from healthy subjects living in endemic region of disease (HCea; n=30); as well as from patients with Chagas Disease (CD, n=25), leprosy (HD, n=10), tuberculosis (TUB, n=10), malaria (MAL, n=10), and HIV-infected (HIV; n=25). ROC curves were constructed with the individual OD values for each serum sample against rMELEISH (panel **A**) or L. infantum SLA (panel **B**), and data are shown. The dotted lines represent the cut-off value obtained by the ROC curves, which were used to obtain sensitivity, specificity, and AUC of the antigens. The mean of each group is also shown.

4. Discussion

VL is a major public health problem that can be fatal if acute and left untreated [37]. In Brazil, the disease is an endemic zoonosis and, although the Ministry of Health has proposed several control measures to prevent its spread, they present variable efficacy and, as a result, the number of cases in dogs and humans has increased in recent years [38]. An accurate early diagnosis is necessary for a more adequate management and control of VL as a late diagnosis is associated with disease progression and death [39,40]. However, available diagnostic techniques present variable sensitive and/or specificity, mainly due to such factors as degree of infection, type and quality of antigen, and populations evaluated [18].

Among the serological tests employed for the VL diagnosis, ELISA is commonly used for the detection of symptomatic cases; however, its sensitivity and specificity vary when it comes to detecting asymptomatic cases. In addition, the use of crude or soluble promastigote and amastigote antigens present cross-reactions that limit their use. Single proteins applied in recombinant format have improved the specificity of the tests, although the sensitivity remains variable. A promising solution to this problem could be

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based on the use of multiepitope-based proteins, which could maximize the sensitivity and specificity in a single product, thus reducing production costs [41]. The construction of such recombinant proteins containing high density epitopes has been an alternative propensity for the diagnosis of diseases given that these proteins exhibit a broad ability to expose their epitopes more efficiently, resulting in improved sensitivity and specificity [42].

In this work, a chimeric protein was developed and evaluated as a strategy for the diagnosis of VL in dogs and humans, focusing on three evolutionarily conserved protein families to construct the gene encoding rMELEISH protein: kinesins, heat shock proteins, and tubulins. Alpha-tubulin is well-known for its participation in the immune response of mammals [43]. The K39 antigen is a 39-amino acid-repetitive immunodominant protein that has also been also shown to present antigenicity in mammals and capable of detecting human VL and symptomatic and asymptomatic cases of canine VL [44–50], while heat shock proteins, such as HSP70 and HSP83.1, have also been shown to be involved in the humoral response from infected patients [51–57]. Such antigens were evaluated using information available in the literature, with the main B-cell epitopes being selected based on the following criteria: they had to be (i) immunodominant, (ii) specific for anti-VL antibodies, and (iii) phylogenetically conserved in distinct *Leishmania* species.

There are few studies in the literature using recombinant multiepitope-based proteins to diagnose canine and human VL. A search conducted at Pubmed using the keywords: "Multiepitope protein" and "diagnosis" identified 268 published articles. Out of these, only three were related to the diagnosis of canine and human VL [5,23,25]. Faria et al. 2015 [5] described the use of two multiepitope-based proteins, PQ10 and PQ20, and their diagnostic potential to detect asymptomatic dogs (80%) infected with *L. infantum*, which were more effective than the EIE-LVC Kit. The ELISA sensitivity of PQ10 and PQ20 proteins was 88.8% and 84.9%, respectively, with specificity of 80% and 65%, respectively. Dalimi et al. 2020 [25] reported a multiepitope-based protein, PQ10, which was able to distinguish asymptomatic from symptomatic dogs infected with *L. infantum* from other groups, with sensitivity and specificity values of 94% and 86%, respectively. Yaghoubi et al. 2021 [23] studied the use of a multiepitope-based protein, P1P2P3, for the diagnosis of VL. The authors demonstrated that the protein presented 98% and 95.3% of sensitivity and specificity, respectively. No cross-reactivity was found in this study with healthy samples or those from cross-reactive dogs and humans. In addition, the protein used in this study was able to identify both asymptomatic and symptomatic dogs, with 100% values, as well as diagnose the disease in humans with the same value.

The fluorescence spectra of the rMELEISH in all analyzed pH values were typical of tryptophan completely exposed to a polar solvent [58]. The similar intensity and the 2 nm red shift of the emission bands indicated very small conformational changes in the protein as a function of pH. In addition, the far-UV CD spectra demonstrated a typical unstructured protein at pH 4.0 and 9.0 and a very low profile of structured protein at pH 7.0. The results were compatible with the recombinant multiepitope protein that was constructed to form an unstructured protein [28,59–62]. Additionally, these results are in agreement with the structural arrangement predicted for the multiepitope protein (data not shown), which is fundamental for the leishmaniosis diagnostic method [28,59–62].

Limitations of the study include the absence of a comparative ELISA assay with commercial antigens or diagnostic kits, as well as the small sample size. Therefore, one could speculate about the necessity to test rMELEISH on a wide range of sample obtained from naturally infected dogs and humans. The preliminary data presented here could be considered as a proof of concept of the development and use of a novel recombinant chimeric protein for the diagnosis of canine and human VL.

5. Patents

One patent resulting from the work reported in this manuscript be under protection at Instituto Nacional de Propriedade Industial (INPI): Brazilian patent No. BR1020140313311.

Author Contributions: DSD: JMM, LMN, AAMG, IBG, FSC, MS, JOS, MCF, AAS, RCG, SMF, EAFC, ASG wrote the manuscript; DSD, JMM and ASG prepared the figures. DSD , JMM, PAFR, ASM, FFR, LMN, AAMG, LSR, IBG, FSC, MS, JOS, MACF, RGTN, ATC, MCP, AAS, RCG, SMF, SL, DFMS, JAGS carried out experiments. JMM, MACF, RCG, EAFC and ASG revised and edited the manuscript; ASG conceptualized, wrote and revised the manuscript. DSD and JMM contributed equally to this work**.**

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