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Sensitive and Specific Serodiagnosis of *Leishmania infantum* Infection in Dogs by Using Peptides Selected from Hypothetical Proteins Identified by an Immunoproteomic Approach

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In Brazil, the percentage of infected dogs living in areas where canine visceral leishmaniasis (CVL) is endemic ranges from 10 to 62%; however, the prevalence of infection in dogs is probably higher than figures reported from serological studies. In addition, problems with the occurrence of false-positive or false-negative results in the serodiagnosis of CVL have been reported. The present work analyzed the potential of synthetic peptides mapped from hypothetical proteins for improvement of the serodiagnosis of *Leishmania infantum* infection in dogs. From 26 identified leishmanial proteins, eight were selected, considering that no homologies between these proteins and others from trypanosomatide sequence databases were encountered. The sequences of these proteins were mapped to identify linear B-cell epitopes, and 17 peptides were synthesized and tested in enzyme-linked immunosorbent assays (ELISAs) for the serodiagnosis of *L. infantum* infection in dogs. Of these, three exhibited sensitivity and specificity values higher than 75% and 90%, respectively, to differentiate *L. infantum*-infected animals from *Trypanosoma cruzi*-infected animals and healthy animals. Soluble *Leishmania* antigen (SLA) showed poor sensitivity (4%) and specificity (36%) to differentiate *L. infantum*-infected dogs from healthy and *T. cruzi*-infected dogs. Lastly, the three selected peptides were combined in different mixtures and higher sensitivity and specificity values were obtained, even when sera from *T. cruzi*-infected dogs were used. The study's findings suggest that these three peptides can constitute a potential tool for more sensitive and specific serodiagnosis of *L. infantum* infection in dogs.

The leishmaniases consist of a wide range of diseases present in 88 countries, with 12 million people infected and 350 million at risk of infection (1). Zoonotic visceral leishmaniasis is a severe disease caused by *Leishmania infantum* in the Mediterranean area, the Middle East, Africa, Asian countries, and Latin America (2, 3). The disease also is emergent in dogs living in the United States, Canada, northern Italy, and Germany (4–6). In Brazil, the disease is caused by *Leishmania chagasi* (syn. *L. infantum*), with the parasites being transmitted by *Lutzomyia longipalpis* and *Lutzomyia cruzi* and wild and domestic canids representing the main reservoirs of parasites (7).

Upon infection, dogs develop asymptomatic or symptomatic clinical forms of disease (8–10). Serological tests used for symptomatic canine visceral leishmaniasis (CVL) diagnosis are facilitated by the strong humoral response that generally accompanies the development of acute disease (11, 12). Courtenay et al. (13) showed that a high percentage of asymptomatic dogs developed symptoms after some months and that those dogs were able to infect about 99.6% of sandflies. In this context, in areas in which CVL is endemic, about 10 to 62% of apparently healthy and/or seronegative dogs are positive for *Leishmania* by PCR (14–17). Thus, asymptomatic dogs, which play a role in the transmission of parasites, are not detected by conventional serological tests, such as the indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA) (18). Nonetheless, the detection of asymptomatic CVL might be crucial in controlling epidemics and avoiding the spread of disease among dogs, as well as between dogs and human populations (19, 20).

There are areas of endemicity where transmission of *Leishmania* spp. and *Trypanosoma cruzi* parasites are superposed and, due to the phylogenetic similarity between those parasites, serological cross-reactions and/or false-positive results are quite common (21, 22). As a strategy to develop a more sensitive and specific method for serodiagnosis of CVL, some individual *Leishmania* proteins were used as recombinant antigens (23, 24). However, due to the high variability observed in the humoral responses of infected dogs, efficient diagnosis based on purified antigens might require a mixture of antigens or the use of chimeric antigens con-

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taining several leishmanial proteins (25). One alternative means to identify sensitive and specific antigens for the diagnosis of CVL is through the use of synthetic peptides. These antigens are relatively simpler and cheaper to produce than recombinant proteins. It also has been reported that the use of synthetic peptides (individually or in a mixture format), in comparison with the use of recombinant proteins, is able to increase the sensitivity and/or specificity of immunoassays for the serodiagnosis of parasitic diseases (26), such as canine and human visceral leishmaniasis (27, 28).

In an attempt to identify more-refined antigens for the serodiagnosis of CVL, 26 hypothetical proteins from *L. infantum*, which were identified previously by means of an immunoproteomic approach (29), were evaluated in this study. Of the hypothetical leishmanial proteins investigated, eight were identified as *Leishmania*-specific proteins. Therefore, the present study sought to map B-cell epitopes from these eight antigens and to use their corresponding peptides for improvement of the sensitivity and specificity of the serodiagnosis of *L. infantum* infection in dogs.

MATERIALS AND METHODS

Ethics statement. Experiments were performed in compliance with national guidelines for institutional animal care, and the Committee on the Ethical Handling of Research Animals from the Federal University of Minas Gerais approved this study (protocol number 043/2011). Serum samples were kindly provided by Alexandre Barbosa dos Reis, Maria Norma Melo (Department of Parasitology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Brazil), and Fernando Aécio de Amorim Carvalho.

Parasites. *Leishmania infantum* (strain MOM/BR/1970/BH46) was grown at 24°C in Schneider's medium (Sigma, St. Louis, MO) supplemented with 20% heat-inactivated fetal bovine serum (Sigma), 20 mM L-glutamine, 200 U/ml penicillin, and 100 μg/ml streptomycin, at pH 7.4. Parasites were provided by Maria Norma Melo.

Antigen preparation. Soluble *Leishmania* antigen (SLA) extract was prepared from stationary-phase promastigotes of *L. infantum*, as described previously (30). Briefly, 1×10^{10} parasites were washed three times in cold sterile phosphate-buffered saline (PBS). After seven cycles of freezing (−196°C) and thawing (37°C), followed by ultrasonication (Ultrasonic processor, GEX600) with five cycles of 30 s at 38 MHz, the suspension was centrifuged at 8,000 × *g* for 30 min at 4°C, and the supernatant containing SLA was collected. The protein concentration was estimated by the Bradford method (31), and aliquots were stored at −80°C until use.

Serum samples. Serum samples used in this study were obtained from the area of Belo Horizonte, Minas Gerais, Brazil, in which CVL is endemic. Sera of dogs with CVL were selected on the basis of two serological tests (IFAT [Bio-Manguinhos IFAT-LVC kit] and ELISA [Bio-Manguinhos EIE-LVC kit], both from Bio-Manguinhos, Fiocruz, Brazil) for *Leishmania* spp. Dogs with IFAT titers of less than 1:40 or ELISA reactivity below the cutoff value indicated by the manufacturer were considered to be seronegative. Animals with IFAT titers of more than 1:40 and ELISA values over the cutoff were considered to be seropositive and infected with *Leishmania* spp. Thus, symptomatic dogs were those positive by IFAT and ELISA and also parasite positive by PCR-restriction fragment length polymorphism (RFLP) testing in blood samples and presenting more than three clinical symptoms (weight loss, alopecia, adenopathy, onychogryphosis, hepatomegaly, conjunctivitis, and exfoliative dermatitis on the nose, tail, and ear tips). The asymptomatic dogs were seronegative by IFAT and ELISA but were positive by PCR-RFLP assay in blood samples. Healthy dogs were selected from an area of Belo Horizonte in which CVL is endemic, were considered negative based on molecular (PCR assay in blood samples) and serological (IFAT and ELISA) testing, and were clinically free of symptoms. Regarding the *T. cruzi*-infected animals, 19 animals selected in an area with endemic leishmaniasis (Minas Gerais, Brazil) were inoculated intraperitoneally with metacyclic trypomastigote forms of *T. cruzi* strain Be-78 (2,000 trypomastigotes per kg body weight). Following inoculation, samples of blood were collected daily (from day 1 to day 42) by venipuncture of the ear veins of infected dogs. The numbers of parasites in the blood samples were determined under an optical microscope, according to the method described by Brenner (32), and parasitemia curves were plotted using the daily mean numbers of parasites. The production of *T. cruzi*-specific IgG antibodies in the infected animals was determined by ELISA using total *T. cruzi* antigens. Sera of *T. cruzi*-infected dogs were obtained from previous projects that evaluated the immune responses in these infected animals (33, 34).

Sequence analysis and mapping of B-cell epitopes. The process of *in silico* analysis of *L. infantum* hypothetical proteins consisted of two steps, (i) the search for similarity among sequences deposited in nonredundant protein databases and (ii) comparison with the genomes of other trypanosomatids whose genomes have been sequenced completely or are in the phase of annotation, i.e., *Leishmania major*, *Leishmania mexicana*, *Leishmania braziliensis*, *Trypanosoma cruzi*, and *Trypanosoma brucei* (all available at www.genedb.org). The sequences of the 26 hypothetical proteins were mapped using the BepiPred program (http://www.cbs.dtu.dk/services/BepiPred) (35) and the algorithm described by Kolaskar and Tongaonkar to identify antigenic determinants in proteins (http://tools.immuneepitope.org/tools/bcell/iedb_input) (36). B-cell peptides that were matched by the 2 programs simultaneously were selected and synthesized by GenScript. The lyophilized peptides were diluted in Milli-Q water (Millipore) for use in the experiments.

ELISA. Previous titration curves were performed to determine the most appropriate antigen concentration and antibody dilution to be used. Falcon flexible microtiter immunoassay plates (Becton, Dickinson) were coated with individual peptides (each at 1.0 μg/well) in 50 μl of Milli-Q water for 18 h at 37°C or SLA (2.0 μg/well) in 100 μl of coating buffer (pH 9.6) for 18 h at 4°C. After sensitization, free binding sites were blocked for 1 h at 37°C using 200 μl of a solution composed of PBS and 0.05% Tween 20 (PBST) and containing 5% casein. After the plates had been washed five times using PBST, they were incubated with 100 μl of canine serum for 1 h at 37°C. Samples were diluted 1:100 (for peptide ELISA) or 1:200 (for SLA ELISA) in PBST containing 0.5% casein. Plates were washed seven times using PBST and were incubated with an anti-dog IgG horseradish peroxidase-conjugated antibody (1:10,000; Sigma, St. Louis, MO) for 1 h at 37°C. After seven washes with PBST, the reaction was developed through incubation with H_2O_2, *ortho*-phenylenediamine, and citrate phosphate buffer (pH 5.0) for 30 min in the dark. The reaction was stopped by adding 25 μl of 2 N H_2SO_4, and the optical density was read in an ELISA microplate spectrophotometer (SpectraMax Plus; Molecular Devices, Canada), at 492 nm.

Data analysis. All of the statistical analyses were performed using GraphPad Prism (version 5.0 for Windows). The cutoff values for assessing the sensitivity and specificity of synthetic antigens were determined using receiver operating characteristic (ROC) analysis, and the area under the curve (AUC) was calculated to assess the accuracy of the tests. First, ROC curves were plotted with values for healthy control dogs versus values for symptomatic CVL dogs (all peptides). Cutoff values were chosen to obtain a minimum of 95% specificity. Second, ROC curves were plotted with values for *T. cruzi*-infected dogs versus values for symptomatic CVL and values for healthy control dogs versus values for asymptomatic CVL (selected peptides; see Table 3). The cutoff values for each peptide were those obtained in the previous analysis (see Table 2). Then, sensitivity, specificity, and AUC values were assessed. The same procedure was adopted to assess the sensitivity, specificity, and AUC values for synthetic antigen mixes.

RESULTS

Peptide identification. In the present work, 8 of 26 previously identified hypothetical proteins were recognized as *Leishmania*-

TABLE 1 Number of trypanosomatide sequences exhibiting similarity to hypothetical proteins selected from *Leishmania infantum*

GenBank accession no.	No. of sequences with similarity to:				
	L. braziliensis	*L. major*	*L. mexicana*	*T. cruzi*	*T. brucei*
XP_888524.1	4	5	1	ND[a]	ND
XP_001463134.1	1	1	1	ND	ND
XP_001466114.1	1	1	1	ND	ND
XP_001467567.1	1	1	1	ND	ND
XP_001468309.1	1	1	1	ND	ND
XP_001564657.1	1	1	1	ND	ND
XP_001568117.1	1	1	1	ND	ND
XP_001686061.1	1	1	1	ND	ND
XP_001568364.1	1	1	1	1	1
XP_001568689.1	1	1	1	1	1
XP_001567688.1	1	1	1	ND	2
XP_001466647.1	1	1	1	ND	2
XP_001467784.1	1	1	1	ND	2
XP_001564693.1	1	1	1	2	ND
XP_001465588	1	1	1	2	ND
XP_001565846.1	1	1	1	2	2
XP_001467126.1	1	1	1	2	2
XP_001564596.1	1	1	1	2	2
XP_843545.1	1	1	1	2	2
XP_001469969.1	1	1	1	2	2
XP_001468941.1	1	1	1	2	2
XP_001682223.1	1	1	1	2	2
XP_001463668.1	1	1	1	2	2
XP_001684096.1	1	1	1	2	2
XP_001684884.1	1	1	1	2	2
CAJ09012.1	1	1	1	2	2

[a] ND, not detected.

specific antigens by database searches (Table 1). The other 18 proteins presented primary sequences similar to those of proteins of *T. cruzi* and/or *T. brucei* species and were not evaluated in this study. The 8 selected proteins were mapped *in silico* to predict the B-cell-specific epitopes, and a total of 17 peptides were identified and synthesized. The sequences of the peptides were AAVC VAAALYAL (PepLi1), AGQSVPNSL (PepLi2), CTECDTGYSLTS DYQCKAITT (PepLi3), FTVTRDVTMSSTSFDDYTMVLDLS (PepLi4), SGALFSFPAGLEDASE (PepLi5), TMMPDTPSADASP SPRITRI (PepLi6), GTSAVYERYLLLTP (PepLi7), KLLFPLPPPP LRLPEALQELSPECH (PepLi8), VLVAAAALVIAAEQLRMPLPA (PepLi9), SGPGAGAAL (PepLi10), QGPPPLASV (PepLi11), SVL KGYQALKQSTAGSD (PepLi12), QEEAEEEEAAAVAGSAQPHP (PepLi13), DMVALQEEAKSVRDRRLALEEIMR (PepLi14), DK KQKAREERFAASLQRRLERRKA (PepLi15), PVEAVEEAVAT (PepLi16), and QPQQPVTQQPVYQPPPPMEPV (PepLi17).

ELISA. The synthetic peptides were then employed as antigens in ELISA to compare their diagnostic performance, using sera from *L. infantum*-infected dogs and healthy animals. Of the 17 peptides, 6 (namely, PepLi1, PepLi2, PepLi3, PepLi6, PepLi7, and PepLi15) presented sensitivity and specificity values of ≥90% and ≥95%, respectively, in identifying *L. infantum*-infected dogs (Table 2). In this context, these 6 peptides were selected for the next phase of experiments in which their sensitivity and specificity were investigated using 20 serum samples from asymptomatic CVL dogs and 19 serum samples from *T. cruzi*-infected animals (Table 3). Of these evaluated antigens, PepLi1, PepLi2, and PepLi7 presented sensitivity values of 100%, 100%, and 93% and specificity values of 95%, 93%, and 95%, respectively. In a comparison of serum samples from asymptomatic and healthy dogs, the sensitivity values were 75%, 100%, and 75% and the specificity values were 72%, 96%, and 96% for PepLi1, PepLi2, and PepLi7, respectively.

Peptide mix for the CVL diagnosis. Finally, the 3 selected peptides were combined in different mixtures to evaluate their diag-

TABLE 2 Diagnostic performance of selected peptides using sera from symptomatic CVL dogs and serologically negative dogs[a]

GenBank accession no.	Peptide	Sensitivity (% [95% CI[b]])	Specificity (% [95% CI])	AUC
XP_888524.1	PepLi1	100 (69.00–100)	95.00 (75.10–99.90)	1
	PepLi2	100 (69.00–100)	95.00 (74.00–99.90)	1
	PepLi3	93.00 (68.00–99.80)	96.00 (79.70–99.90)	0.9307
	PepLi4	67.00 (38.40–88.20)	96.00 (79.70–99.90)	0.8373
	PepLi5	53.00 (26.60–78.70)	96.00 (79.70–99.90)	0.8653
XP_001463134.1	PepLi6	100 (78.20–100)	96.00 (79.70–99.90)	0.9840
	PepLi7	90.00 (55.50–99.80)	95.00 (75.10–99.90)	1
XP_001466114.1	PepLi8	87.00 (59.50–98.30)	96.00 (79.70- 99.90)	0.9387
	PepLi9	73.00 (44.90–92.20)	96.00 (79.70–99.90)	0.9520
	PepLi10	53.00 (26.60–78.70)	96.00 (79.70–99.90)	0.9067
	PepLi11	47.00 (21.30–73.40)	96.00 (79.70- 99.90)	0.9147
XP_001467567.1	PepLi12	47.00 (21.30–73.40)	96.00 (79.70–99.90)	0.6053
XP_001468309.1	PepLi13	67.00 (38.40–88.20)	96.00 (79.70–99.90)	0.9027
XP_001564657.1	PepLi14	73.00 (44.90–92.20)	96.00 (79.70–99.90)	0.9173
XP_001568117.1	PepLi15	93.00 (68.10–99.80)	96.00 (79.70–99.90)	1
	PepLi16	67.00 (38.40–88.20)	96.00 (79.70–99.90)	0.8533
XP_001686061.1	PepLi17	27.00 (7.80–55.10)	96.00 (79.70–99.90)	0.7467
SLA[c]		100 (78.20–100)	96.00 (79.70–99.90)	1

[a] Samples from symptomatic *L. infantum*-infected dogs ($n = 25$) and animals with no clinical signs of CVL and negative parasitological and serological results for *Leishmania* antigens ($n = 40$) were tested in ELISA. Receiver operating characteristic (ROC) curves were used to determine sensitivity, specificity, and AUC values.
[b] CI, confidence interval.
[c] SLA, soluble *Leishmania infantum* antigen extract.

TABLE 3 Diagnostic performance of selected peptides using other serum samples[a]

Peptide	*T. cruzi*-infected vs symptomatic dogs			Control vs asymptomatic dogs		
	Sensitivity (%)	Specificity (%)	AUC	Sensitivity (%)	Specificity (%)	AUC
PepLi1	100	95.00	1	75.00	72.00	0.51
PepLi2	100	93.00	1	100	96.00	1
PepLi3	60.00	95.00	0.88	30.00	92.00	0.56
PepLi6	53.00	95.00	0.79	70.00	96.00	0.86
PepLi7	93.00	95.00	0.97	75.00	96.00	0.75
PepLi15	40.00	95.00	0.63	45.00	96.00	0.89
SLA[b]	100	36.00	0.95	4.00	100	0.56

[a] Samples from *T. cruzi*-infected dogs ($n = 19$), asymptomatic ($n = 20$) and symptomatic ($n = 25$) *L. infantum*-infected dogs, and animals with no clinical signs of CVL and negative parasitological and serological results for *Leishmania* antigens ($n = 40$) were used. Receiver operating characteristic (ROC) curves were used to determine ELISA sensitivity, specificity, and AUC values.
[b] SLA, soluble *Leishmania infantum* antigen extract.

nostic performance in serological tests by ELISA. In this context, the different mixtures were composed as follows: mix 1, PepLi plus PepLi2; mix 2, PepLi1 plus PepLi7; mix 3, PepLi2 plus PepLi7; mix 4, PepLi1 plus PepLi2 plus PepLi7. Figure 1 shows the results of the individual distribution of the serum samples in relation to the different peptide mixtures. When the sensitivity of the different mixtures to differentiate among the serum samples of symptomatic *L. infantum*-infected and healthy dogs was evaluated, mix 1, mix 2, mix 3, and mix 4 presented sensitivities of 100%, 100%, 80%, and 95%, respectively (Fig. 2). Regarding the specificity to differentiate the serum samples of symptomatic *L. infantum*-infected dogs from those of *T. cruzi*-infected animals, the mixtures presented values of 100%, 100%, 76%, and 95% for mix 1, mix 2, mix 3, and mix 4, respectively (Table 4).

DISCUSSION

Serological tests are currently recommended for the laboratory diagnosis of CVL. IFAT and ELISA are the most widespread diagnostic assays applicable to leishmaniasis; however, their low sensitivity to detect cases with low or absent levels of *Leishmania*-

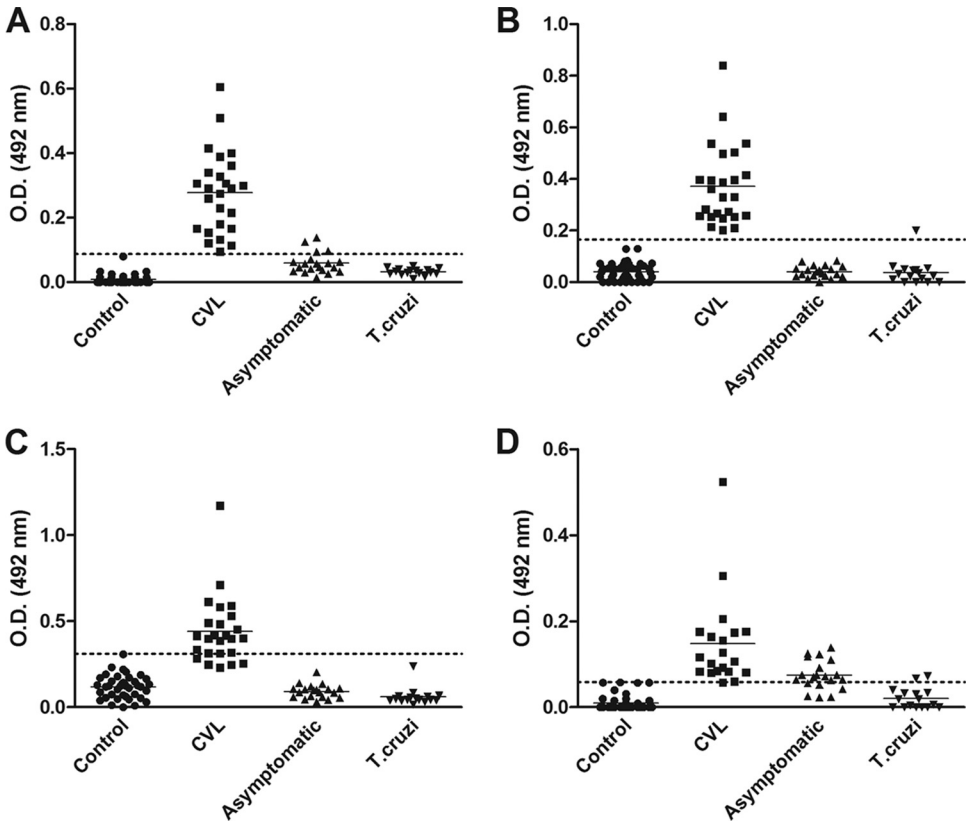

FIG 1 Evaluation of ELISA reactivity using peptide mixtures with different serum samples. ELISAs were performed using serum samples from *T. cruzi*-infected ($n = 19$), asymptomatic ($n = 20$) and symptomatic ($n = 25$) *L. infantum*-infected, and healthy ($n = 40$) dogs. Reactions against mix 1 (A), mix 2 (B), mix 3 (C), and mix 4 (D) are shown. Mixes were as follows: mix 1, PepLi1 plus PepLi2; mix 2, PepLi1 plus PepLi7; mix 3, PepLi2 plus PepLi7; and mix 4, PepLi1 plus PepLi2 plus PepLi7. O.D., optical density.

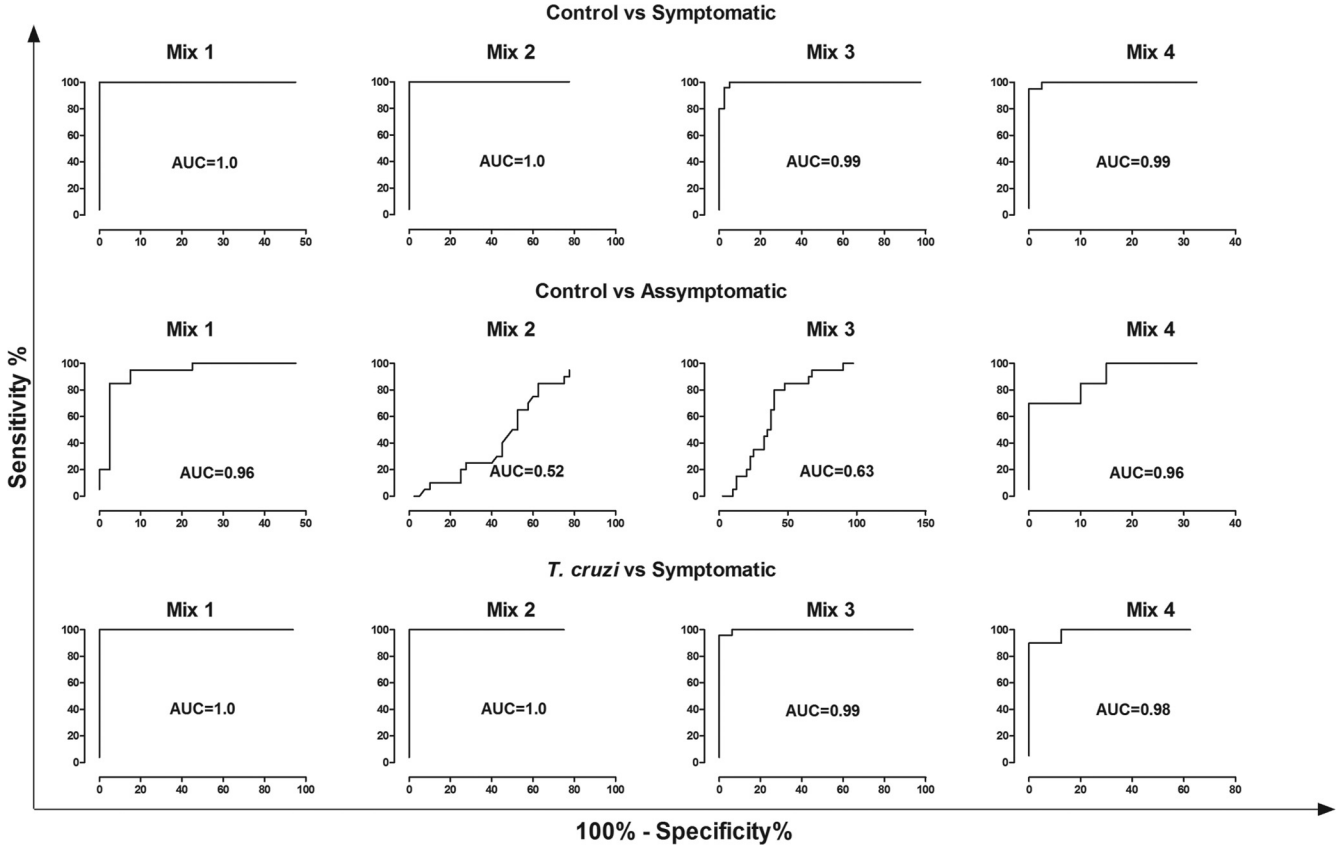

FIG 2 Diagnostic performance of peptide mixtures with different serum samples. Samples from *T. cruzi*-infected ($n = 19$), symptomatic CVL ($n = 25$), and healthy ($n = 40$) dogs were used. Receiver operating characteristic (ROC) curves were used to determine ELISA sensitivity, specificity, and AUC values, and sensitivity and specificity values for the different mixtures with serum samples from *T. cruzi*-infected, symptomatic CVL, and healthy dogs are shown. The mixes were as follows: mix 1, PepLi plus PepLi2; mix 2, PepLi1 plus PepLi7; mix 3, PepLi2 plus PepLi7; and mix 4, PepLi1 plus PepLi2 plus PepLi7.

specific antibodies and their cross-reactivity with other diseases, including Chagas' disease, represent important limitations for their use in laboratory serodiagnosis (37). Moreover, crude antigens also are limited by the difficulty of producing large quantities in a standardized manner (30).

Improvements in sensitivity and specificity might be achieved by using individual *Leishmania* proteins that are recognized by sera from *L. infantum*-infected dogs. After database searches were performed in this study, eight antigens were identified as *Leishmania*-specific proteins. Since they were recognized by sera from *L. infantum*-infected dogs in a previous immunoproteomics study (29), their primary sequences were mapped to obtain B-cell-specific epitopes, which were synthesized and subsequently evaluated as new and more-refined antigens for a more sensitive and specific serodiagnosis of *L. infantum* infections in dogs.

The specificity of ELISA using SLA depends heavily on antigen preparation. Many times, false-positive results are obtained when serum samples are collected from animals with other diseases, such as Chagas' disease (22, 38). Recombinant leishmanial antigens also have been tested with ELISA to develop a more specific test (25); although their specificity is generally greater, sensitivity is hampered, given the much lower availability of B-cell epitopes than for SLA and the high level of heterogeneity of major histocompatibility complex (MHC) molecules of canine populations (39).

Among the tested synthetic antigens, PepL1, PepL2, PepL3,

TABLE 4 Diagnostic performance of peptide mixes using different serum samples[a]

Mix	Control vs symptomatic dogs			Control vs asymptomatic dogs			*T. cruzi*-infected vs symptomatic dogs		
	Sensitivity (%)	Specificity (%)	AUC	Sensitivity (%)	Specificity (%)	AUC	Sensitivity (%)	Specificity (%)	AUC
1	100	100	1	20.00	100	0.96	100	100	1
2	100	100	1	0	95.00	0.52	100	94.00	1
3	80.00	100	1	0	95.00	0.63	76.00	100	1
4	95.00	100	1	70.00	97.50	0.96	95.00	88.00	0.98

[a] Samples from *T. cruzi*-infected dogs ($n = 19$), symptomatic ($n = 25$) and asymptomatic ($n = 20$) visceral leishmaniasis dogs, and animals with no clinical signs of CVL and negative parasitological and serological results for *Leishmania* antigens ($n = 40$) were used. ROC curves were used to determine ELISA sensitivity, specificity, and AUC values.

PepL6, PepL7, and PepL15 showed the highest sensitivities (100%, 100%, 93%, 100%, 90%, and 93%, respectively) and specificities (95%, 95%, 96%, 96%, 95%, and 96%, respectively). In canine epidemiological screening, a test with high sensitivity and specificity is desirable. The performance observed for the synthetic antigens in this work is consistent with the performance of many antigens (mainly recombinant proteins) that have been developed in recent years for CVL diagnosis by ELISA (23, 24), with the advantages of being cheaper, simpler, reproducible, useful for large-scale testing and, in most cases, more specific and sensitive (26). Our results show an improvement in the sensitivity of the synthetic antigen ELISA, compared to the indirect immunofluorescence assay, in asymptomatic dogs, due to the fact that the IFAT shows a lack of sensitivity for the detection of specific antibodies in asymptomatic dogs (20) and in healthy dogs, due to the large number of false-positive IFAT reactions (18). It should be taken into account that the ELISA technique allows better interpretation of the results than IFAT, because in that technique the interpretation is subjective and depends on the expertise of the operator.

Multiple-epitope chimeric antigens have been evaluated as markers for the serodiagnosis of CVL (25, 40). In an attempt to improve sensitivity and specificity and to avoid cross-reactions, we prepared antigenic mixtures of the synthetic antigens that showed the best results and tested them by ELISA. All of the mixtures containing the synthetic antigens, i.e., mix 1, mix 2, mix 3, and mix 4, showed high sensitivities (100%, 100%, 80%, and 95%, respectively) and specificity (100% in all cases), as well as a perfect accuracy (AUC = 1) when tested in discriminating dogs with symptomatic CVL from healthy animals. When tested in discriminating between dogs with symptomatic visceral leishmaniasis and *T. cruzi*-infected dogs, mix 1, mix 2, and mix 4 showed high sensitivities (100%, 100, and 95%, respectively), while mix 3 showed a sensitivity of 76%. All of the mixes showed high specificities (100%, 94%, 100%, and 88%, respectively).

The detection of asymptomatic *L. infantum*-infected dogs is considered crucial in epidemiological studies, for laboratory diagnosis of the disease. A cohort study showed that the majority of seronegative animals became positive in parasitological tests a few months after presenting serological conversion to *Leishmania* antigens (13). In addition, in experimentally infected dogs, diagnosis was possible as early as 45 days postinfection and before the animals become seropositive. In this context, the diagnostic elucidation of asymptomatic cases would contribute to better characterization of the epidemiology of CVL and evaluation of control actions. Surprisingly, mix 2 and mix 3 failed to discriminate asymptomatic CVL dogs from healthy dogs, while mix 1 and mix 4 showed sensitivities of 20% and 70% and specificities of 100% and 97.5%, respectively. Recently, Costa et al. (27) performed ELISAs using single and mixed synthetic antigens from previously evaluated proteins, which showed high sensitivity in serum samples with low (95%) and intermediate (95%) antibody titers and high specificity (95%); the capacities of these synthetic antigen mixtures were not assayed against asymptomatic dogs, and cross-reactivity assays were not performed. In addition, Faria et al. (28) performed ELISAs using a mixture of synthetic antigens and obtained high sensitivity and specificity values (78.5% and 80%, respectively), but all of the synthetic antigens and the mixture exhibited high cross-reactivity with *T. cruzi* serum samples.

The serum samples used in this work did not contain samples from dogs infected with other *Leishmania* species, such as *L. braziliensis*; the collection of sera from dogs was restricted to the urban area of Belo Horizonte (Minas Gerais, Brazil), where a low incidence of infection with *L. braziliensis* in dogs was noted recently (41). In addition, the sample size used in this work was limited and follow-up evaluation of the asymptomatic dogs was not performed. Thus, our data should be taken as a proof of concept of the capacity of the proposed synthetic antigens for the diagnosis of CVL and might serve as a reference for further assays.

Taken together, the results presented here demonstrate that the 3 synthetic peptides obtained from previously selected hypothetical proteins might be considered an interesting alternative for a more sensitive and specific serodiagnosis of CVL, when used in an isolated or multiple-epitope chimeric mixture format in serological testing by ELISA. This study can be considered relevant mainly in identifying seronegative animals without clinical signs but with positive molecular results for *L. infantum*, represented here as asymptomatic dogs, in epidemiological studies and/or areas in which CVL is endemic.

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