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Subtractive Phage Display Selection from Canine Visceral Leishmaniasis Identifies Novel Epitopes That Mimic *Leishmania infantum* Antigens with Potential Serodiagnosis Applications

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Visceral leishmaniasis (VL) is a zoonotic disease that is endemic to Brazil, where dogs are the main domestic parasite reservoirs, and the percentages of infected dogs living in regions where canine VL (CVL) is endemic have ranged from 10% to 62%. Despite technological advances, some problems have been reported with CVL serodiagnosis. The present study describes a sequential subtractive selection through phage display technology from polyclonal antibodies of negative and positive sera that resulted in the identification of potential bacteriophage-fused peptides that were highly sensitive and specific to antibodies of CVL. A negative selection was performed in which phage clones were adhered to purified IgGs from healthy and *Trypanosoma cruzi*-infected dogs to eliminate cross-reactive phages. The remaining supernatant nonadhered phages were submitted to positive selection against IgG from the blood serum of dogs that were infected with *Leishmania infantum*. Phage clones that adhered to purified IgGs from the CVL-infected serum samples were selected. Eighteen clones were identified and their reactivities tested by a phage enzyme-linked immunosorbent assay (phage-ELISA) against the serum samples from infected dogs ($n = 31$) compared to those from vaccinated dogs ($n = 21$), experimentally infected dogs with cross-reactive parasites ($n = 23$), and healthy controls ($n = 17$). Eight clones presented sensitivity, specificity, and positive and negative predictive values of 100%, and they showed no cross-reactivity with *T. cruzi*- or *Ehrlichia canis*-infected dogs or with dogs vaccinated with two different commercial CVL vaccines in Brazil. Our study identified eight mimotopes of *L. infantum* antigens with 100% accuracy for CVL serodiagnosis. The use of these mimotopes by phage-ELISA proved to be an excellent assay that was reproducible, simple, fast, and inexpensive, and it can be applied in CVL-monitoring programs.

Leishmaniasis consists of a wide range of diseases found in 98 countries and three territories, with approximately 1.6 million cases occurring each year and with an incidence of 40,000 deaths registered per year (1). Canine visceral leishmaniasis (CVL) caused by *Leishmania infantum* is a major global zoonosis that is potentially fatal to humans and dogs; it can be found in the regions of southern Europe, Africa, Asia, and Central and South America, and it is considered endemic to approximately 70 countries worldwide (2, 3). However, CVL is expanding in its geographic distribution throughout the Western Hemisphere, where it now is found from northern Argentina to the United States (4), even reaching as far as the provinces of southern Canada (5). CVL is also an important concern in countries that are not endemic for the disease, as imported sick or infected dogs constitute a veterinary and public health problem (6). Brazilian public health authorities currently use the enzyme-linked immunosorbent assay (ELISA) as a screening test and the indirect immunofluorescence antibody test (IFAT), with a cutoff titer of 1:40, as a confirmatory analysis to determine the clinical status of dogs (7). Moreover, controlling the spread of CVL in Brazil is mainly based on the elimination of seropositive dogs (8).

The serodiagnosis of CVL, however, is hampered by various factors, mainly due to the variable sensitivity and/or specificity values obtained with the different antigens employed, leading to the occurrence of a large number of false-positive results caused by cross-reactivity with other organisms, such as *Trypanosoma cruzi* and *Trypanosoma caninum* (9–12), *Leishmania braziliensis* (9, 13), and *Ehrlichia canis* (14, 15). Additionally, there are two commercially available Brazilian vaccines used to prevent CVL that can induce the production of high levels of *Leishmania*-specific antibodies in vaccinated animals, causing them to be diagnosed as infected animals in distinct serological trials (16).

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Phage display is a technology based on DNA recombination, resulting in the expression of foreign peptide variants on the outer surface of phage clones. Using a refined *in vitro* selection process based on binding affinity and so-called biopanning cycles, peptides exposed in the selected phage clones are analyzed by DNA sequencing and then identified (17–19). One of the earliest applications of phage display was the study of antigen-antibody binding aimed at identifying mimotopes (peptides that mimic linear, discontinuous, and even nonpeptide epitopes [20]), which were recognized by specific monoclonal or polyclonal antibodies applied in the diagnoses and/or vaccines against some diseases, such as malaria (21–23), toxoplasmosis (24, 25), and Chagas' disease (26).

In the present study, a subtractive phage display selection was employed to identify mimotopes that may be promptly used in CVL serodiagnosis. These selected phage clones were serologically relevant in a panel of several controls using a direct phage-ELISA. Therefore, this work analyzes this significant improvement in CVL serodiagnosis with high sensitivity and specificity, and it has implications in animal control programs in regions that are endemic for the disease.

MATERIALS AND METHODS

Ethics statement. The experiments in this study were performed in compliance with the Committee on the Ethical Handling of Research Animals of the Federal University of Minas Gerais (UFMG), which approved this study under protocol no. 043/2011.

Study animals. The sample size of this study consisted of 77 domestic dogs (*Canis lupus familiaris*) made up of males and females of different breeds and ages, collected from an area of Belo Horizonte, Minas Gerais, Brazil, that is endemic for CVL. The blood serum samples from animals with CVL were selected on the basis of two serological tests (IFAT [IFAT-LVC Bio-Manguinhos kit] and ELISA [EIE-LVC Bio-Manguinhos kit], both from Bio-Manguinhos, Fiocruz, Brazil) for *Leishmania* spp. Dogs with an IFAT titer of <1:40 or ELISA reactivity below the cutoff value indicated by the manufacturer were considered to be seronegative. Animals with an IFAT titer of >1:40 and an ELISA value over the cutoff were considered to be seropositive. Thus, symptomatic dogs ($n = 16$) were those that were positive by IFAT and ELISA but that also had positive parasitological results by restriction fragment length polymorphism PCR (PCR-RFLP) in blood samples, and which presented more than three clinical symptoms (weight loss, alopecia, adenopathy, onychogryphosis, hepatomegaly, conjunctivitis, and exfoliative dermatitis on the nose, tail, and ear tips). Asymptomatic dogs ($n = 15$) also presented positive serological (IFAT and ELISA) and parasitological (PCR-RFLP in blood samples) results, but they did not present any clinical signals or symptoms of leishmaniasis. Healthy dogs ($n = 17$) were selected from an area of Belo Horizonte that is endemic for CVL, and they presented negative serological (IFAT and ELISA) and parasitological (PCR) results and they were free of any clinical signs or symptoms of leishmaniasis. Healthy animals vaccinated with Leish-Tec ($n = 15$) or Leishmune ($n = 6$) were isolated in kennels to prevent their contact with transmitting vectors of leishmaniasis and with dogs that had been experimentally infected with *T. cruzi* ($n = 15$) or *E. canis* ($n = 8$), which also were used in this study. These blood serum samples were obtained from previous projects that evaluated the immune responses in these infected animals (27, 28). The serum samples were provided by Alexandre Barbosa Reis (Departamento de Análises Clínicas, Federal University of Ouro Preto, Ouro Preto, Brazil), Maria Norma Mello (Departamento de Parasitologia, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil), and Ana Paula Fernandes (Departamento de Análises Clínicas e Toxicológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil).

Purification of antibodies by coupling in protein G microspheres. The purification of IgG antibodies from the serum samples of the different groups was performed by coupling to magnetic microspheres (magnetic beads) conjugated to protein G (Dynabeads; Invitrogen). To this end, 2×10^9 particles of microspheres were washed 3 times in 1 ml of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.0), and the following were added: 375 μl of pooled blood serum samples from healthy dogs, 150 μl of pooled serum samples from *T. cruzi*-infected dogs, 300 μl of pooled serum samples from dogs with symptomatic VL or 195 μl of pooled serum samples from dogs with asymptomatic VL. An incubation of 40 min was performed with constant stirring at room temperature. The microspheres coupled with the antibodies were washed 3 times using 1 ml of 0.1 M MES buffer in order to remove the nonadhered antibodies. Next, the bead-antibody system was washed twice with 1 ml of 0.2 M triethanolamine buffer (pH 8.2) and resuspended in 1 ml of covalent coupling buffer (containing 20 mM dimethyl pimelimidate-HCl diluted in triethanolamine buffer) for 30 min, with constant stirring and at room temperature. The neutralization of unbound reactive sites was made by incubating 1 ml of 50 mM Tris-base (pH 7.5) for 15 min at room temperature. The microspheres were washed 3 times with 1 ml of TBS-T (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% of Tween 20), blocked by the addition of 2 ml of blocking solution (5% bovine serum albumin [BSA] diluted in TBS-T) for 1 h at 37°C and then resuspended in 200 μl of TBS buffer. To verify the coupling, 5 μl of beads coupled using IgGs was incubated for 1 h at 37°C with an anti-dog IgG peroxidase antibody (1:5,000 dilution), and then they were washed 3 times with 1 ml of TBS-T, and the reaction was developed by adding 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. The reaction was stopped by adding 25 μl H_2SO_4 (2 N), and the optical density at 450 nm was read in an ELISA microplate spectrophotometer (SpectraMax Plus; Molecular Devices, Canada).

Biopanning cycles: negative selection. To carry out negative selection in the biopanning cycles, 1×10^{11} viral particles from a phage library containing random seven peptides fused to a minor coat protein of M13 filamentous phages (Ph.D.-C7C library; New England BioLabs, USA) were diluted in 190 μl of TBS-T buffer. The mixture was incubated for 30 min at room temperature with the microspheres coupled to IgGs that had been purified from healthy dogs, and then it was precipitated by magnetic attraction to produce a Dynal Biotech support. The supernatant containing the clones that were not adhered to the IgGs was recovered and transferred to a new tube, and this procedure using IgGs from healthy dogs was repeated three times. After this, the supernatant was recovered and transferred to a new tube containing microspheres coupled to IgGs from *T. cruzi*-infected dogs, and this procedure of negative selection was also repeated three times; the supernatant containing the phage clones that did not adhere to the IgGs from *T. cruzi*-infected dogs was recovered, titrated, and used in the positive selection.

Positive selection. For positive selection, the previously recovered phage clones were transferred to a tube containing microspheres coupled to IgGs purified from serum samples of dogs with asymptomatic VL and incubated for 30 min at room temperature. After, the supernatant was removed and the remaining phages that were bound to the IgGs were washed 10 times with 1 ml of TBS-T and eluted in 500 μl of 0.2 M glycine buffer (pH 2.0). Next, 75 μl of 1 M Tris-base (pH 9.0) was added to neutralize the acidic pH. Subsequently, the recovered phage clones were transferred to a new tube containing IgGs that had been purified from serum samples of dogs with symptomatic VL. The process was repeated three times with serum samples of dogs with asymptomatic and symptomatic VL, and the selected phage clones were recovered and titrated.

Titration of selected phage clones. Phage clones were diluted 10^{-1} to 10^{-11} in 500 μl of sterile phosphate-buffered saline (PBS), mixed with an *Escherichia coli* culture (optical density at 600 nm [OD_{600}], ~0.5), and plated on LB agar plates containing 1 ml of isopropyl-β-D-thiogalactopyranoside (IPTG)–X-Gal (1.25 g IPTG, 1 g 5-bromo-4-chloro-3-indolyl-β-D-galactoside, and 25 ml of dimethyl sulfoxide [DMSO]). The colonies were individually quantified, and the titration was performed for each

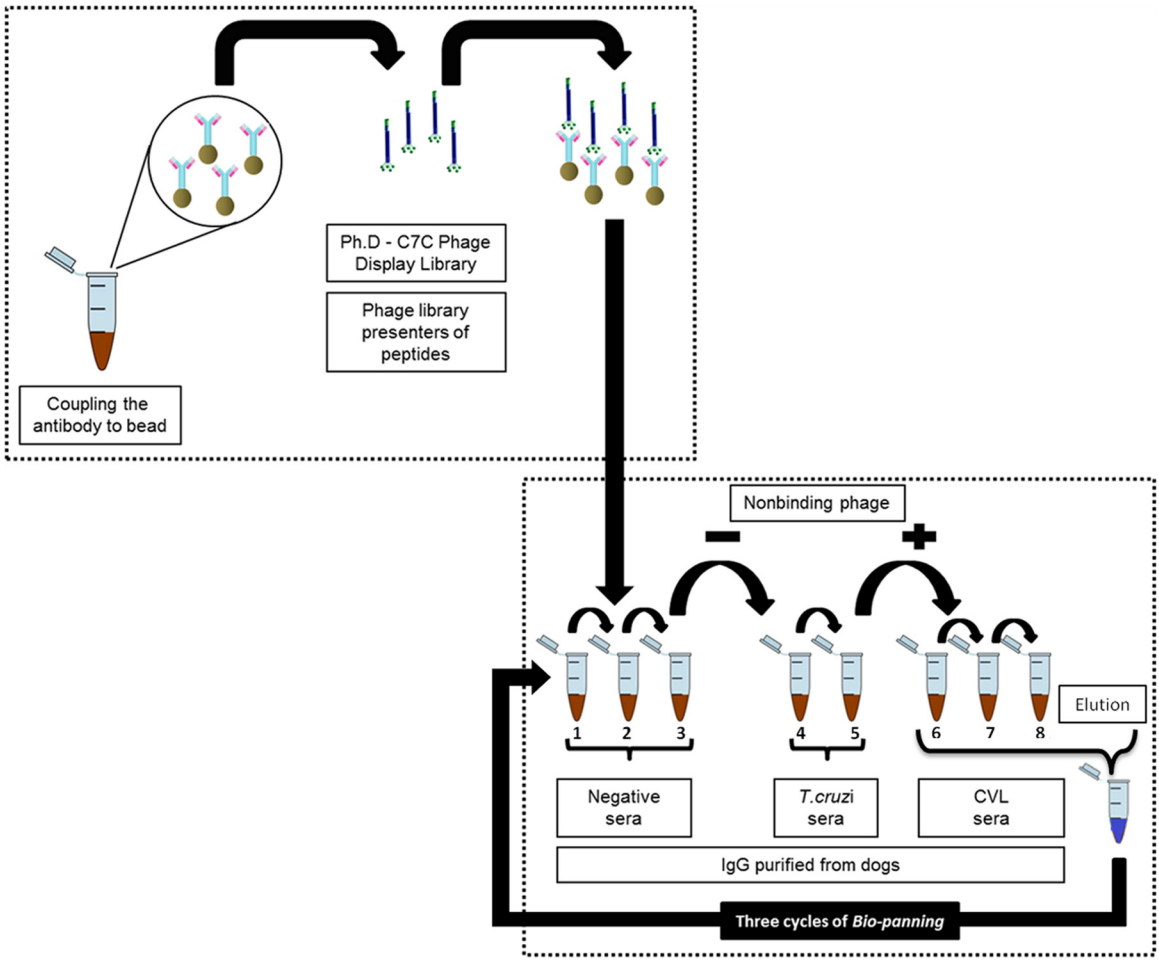

FIG 1 Schedule of the technical protocol used in subtractive selection by phage display technology.

biopanning cycle. After the 3rd cycle of positive selection, 96 colonies were selected from the plate and added to 200 μl of LB in a sterile culture plate (BD Falcon clear 96-well Microtest plate), and then the plate was sealed and incubated for 5 h with constant stirring at 37°C. After incubation, the plate was centrifuged for 20 min at 2,250 × *g* and the supernatant was transferred to a new plate, in which a polyethylene glycol (PEG)-NaCl (20% PEG 8000 and 2.5 M NaCl) solution was added (1/6 of the total volume of the supernatant), and the plate was incubated for 16 h at 4°C. Afterward, the plate was centrifuged for 1 h, the supernatant was removed, and the pellet was resuspended in 500 μl of a solution composed of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 4 M NaI. The plate was shaken vigorously for 5 min, and 250 μl of a 70% ethanol solution was added. After incubation for 10 min, the plate was centrifuged (2,250 × *g* at 4°C for 10 min) and the supernatant was discarded. The pellet containing the DNA of interest of each phage clone was washed with 500 μl of 70% ethanol and centrifuged again. Finally, the DNA was diluted in 20 μl of ultrapure water and its quality was evaluated in a 1% agarose gel, which was stained with an ethidium bromide solution (10 μg/ml). The DNA from individual clones was used for the sequencing and identification of target peptides.

DNA sequencing and bioinformatics. The sequencing reaction was performed with 500 ng of DNA for each selected phage clone, 5 pmol primer 96 gIII (5′-OH CC TCA TAG TTA GCG TAA CG-3′; New England BioLabs), plus a premix (Dye Terminator cycle ET journal kit; Amersham Biosciences). Thirty-five cycles were performed in a thermocycler under the following conditions: denaturation at 95°C for 20 s, ringing at 58°C for 15 s, and extension at 60°C for 60 s. The generated amplicons were precipitated with 1 μl of ammonium acetate and 27.5 μl of ethanol. The plate was centrifuged for 45 min at 2,432 × *g*, the supernatant was discarded, and 150 μl of 70% ethanol was added to the pellet. The resuspended DNA was centrifuged for 10 min at 2,432 × *g*, and the supernatant was discarded. The plate was inverted on a paper towel and was centrifuged in that position at 486 × *g* for 1 min. Next, the plate was covered for 5 min until complete evaporation of the remaining ethanol had been achieved. The pellet was resuspended in dilution buffer, and sequencing was performed in a MegaBACE 1000 automatic sequencer (Amersham Biosciences). The peptide sequences were deduced using the ExPASy server (see www.expasy.org), and they were analyzed by BLAST (see http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the protein and motifs of the GenBank database. Further analyses of multiple sequence alignments were performed using the Clustal W2 program (see www.ebi.ac.uk). In addition, calculations of the frequency of each amino acid and the amino acid diversity within the population of peptides in the selected phage clones were performed using the AADIV program (see http://www.northeastern.edu/xray/downloads/aadiv-program/). The frequencies of the amino acid residues of the original library were obtained according to the manufacturer's instructions (E8102L, Ph.D.-C7C library; New England BioLabs, USA).

Phage-ELISA. Previous titration curves were performed to determine the most appropriate phage concentration and antibody dilution to be used. Falcon flexible microtiter plates (Becton, Dickinson) were coated, each containing 18 individual clones (1×10^9 phages per well) diluted in

TABLE 1 Amino acid sequences from target peptides present in the selected phage clones

Clone	Amino acid sequence
A1	TSPPLHA
A8	DPRAADP
A9	QSTSGSS
C7	NPFLADP
C9	SPDSLFP
D9	PQDPKQW
E5	LYHDTHY
E7	NPFCGSR
E8	PRSSLPS
F1	TFSPLSV
F10	VHSPFWP
F12	PSPSRFP
G7	PRSTSHL
G8	FGAPVSL
H1	FPHLSRY
H5	SPWPLSY
H7	YPGMLWT
H9	IWSPFFD
RP[a]	NMSDFLRIQLRS

[a] RP, random phage.

a 100-μl coating buffer (50 mM carbonate buffer) (pH 9.6) for 16 h at 4°C. A random nonspecific phage clone (NMSDFLRIQLRS) was used as a control. After sensitization, free binding sites were blocked using 200 μl of a blocking solution (consisting of TBS, 0.05% Tween 20, and 5% nonfat dry milk solution) for 1 h at 37°C. After plates were washed 5 times with TBS-T, they were incubated with 100 μl of individual canine blood serum (diluted 1:100) for 1 h at 37°C. The plates were washed 7 times using TBS-T and incubated with a 1:8,000 anti-dog IgG horseradish peroxidase-conjugated antibody (Sigma, St. Louis, MO, USA) for 1 h at 37°C. After the plates were washed 7 times with TBS-T, the reaction was developed through incubation with H_2O_2, *o*-phenylenediamine, and citrate-phosphate buffer (pH 5.0) for 30 min in the dark. The reaction was stopped by adding 25 μl H_2SO_4 (2 N), and the optical density at 492 nm was read in an ELISA microplate spectrophotometer.

Data analysis. Statistical analyses were performed using GraphPad Prism (version 5.0 for Windows), Win Episcope (version 2.0 for Windows), and Microsoft Excel (version 10.0). Two different approaches were used to measure the reactivity of each serum sample for each selected phage clone. First, the mean optical density (OD) value was calculated by subtracting the mean blank OD from mean OD for each sample by using specific values for the phage-ELISA. In a second approach, the normalized absorbance ratio (NAR) was computed by further correcting the mean OD value for each sample from each phage-ELISA by dividing it by the equivalent mean result from the wild-type phage. As a consequence, the NARs represent multiples of reactivity with respect to that of the wild-type phage (29). In addition, for each plate, outliers were evaluated using Grubb's test (30) and excluded from statistical analyses. The cutoff values for each phage were determined by using a receiver operating characteristic (ROC) analysis and by obtaining 100% specificity. The ROC curves were plotted with the values from serum samples from dogs with symptomatic VL versus those from the control groups (*T. cruzi*-infected, *E. canis*-infected, Leishmune- or Leish-Tec-immunized, and healthy dogs), according to a sick/nonsick rating method, where the criteria of inclusion in each group was the positivity or negativity of PCR-RFLP in the blood samples (31). Tables of contingency and Fisher's exact test ($P < 0.05$) were used to compare the diagnostic performances. This was estimated by assessing sensitivity (95% confidence interval [CI]), specificity (95% CI), positive predictive value (PPV), negative predictive value (NPV), and diagnostic odds ratios (DORs). The accuracy was evaluated according to the area under the curve (AUC) relative to the ROC curve (95% CI) and by the Youden index (J) (95% CI) (32). Agreement beyond chance was assessed using the kappa (κ) coefficient (95% CI) (33) and interpreted according to the following scale: 0.00 to 0.20 (negligible), 0.21 to 0.40 (weak), 0.41 to 0.60 (moderate), 0.61 to 0.80 (good), and 0.81 to 1.00 (excellent) (34). The Kruskal-Wallis test, followed by the Dunn posttest for multiple comparisons, was used to compare differences between the CVL and control groups ($P < 0.05$).

TABLE 2 Analysis of the expected and present amino acid frequencies in the peptides expressed on selected phage clones display after selection procedures

	Frequency (%)	
Amino acid	Expected	Present
Alanine	6.20	3.97
Cysteine	3.10	0.79
Aspartic acid	3.10	5.56
Glutamic acid	3.10	0.00
Phenylalanine	3.10	7.94
Glycine	6.20	3.17
Histidine	3.10	4.76
Isoleucine	3.10	0.79
Lysine	3.10	0.79
Leucine	9.40	8.73
Methionine	3.10	0.79
Asparagine	3.10	1.59
Proline	6.20	20.63
Glutamine	6.20	2.38
Arginine	9.40	4.76
Serine	9.40	18.25
Threonine	6.20	4.76
Valine	6.20	2.38
Tryptophan	3.10	3.97
Tyrosine	3.10	3.97

RESULTS

Affinity selection of phage clones selected by CVL sera. Negative selection was performed by excluding phage clones that were recognized by antibodies present in serum samples from healthy and *T. cruzi*-infected dogs. Afterward, the positive selection process was performed by recovering the phage clones from the previous step, and those that were recognized by antibodies present in the serum samples from dogs with asymptomatic and symptomatic CVL were selected. The technical protocol representing each of three biopanning cycles is summarized in Fig. 1. Approximately 96 clones were randomly selected from individual colonies, and their DNA sequences were PCR amplified and sequenced. Eighteen clones had their sequences clearly identified (Table 1), and an alignment showed that no identical consensus motif could be detected between them (data not shown). Table 2 shows a comparison between the expected and found frequencies of 20 amino acids in the ligand peptides present in the selected phage clones obtained from the original library.

Assessment of the diagnostic performance of selected phage clones. To investigate the diagnostic performance of 18 selected phage clones, the individual phages were analyzed by phage-ELISA regarding their reactivities using a diverse serological panel (Table 3). From the 18 selected phage clones, nine (A1, A8, C7, C9, E7, E8, F12, G8, and H7) clones presented a perfect agreement beyond chance, with kappa values ranging from 0.917 to 1.0. The cutoff values were chosen to obtain 100% specificity. Considering

TABLE 3 Diagnostic performance of clones by phage-ELISA using ROC curves, data validation, and agreement to identify *L. infantum*-infected dogs

Clone	AUC[b]	95% CI[c]	No. of serum samples used	No. of samples with indicated result[d]				P[e]	κ	95% CI
				TP	TN	FP	FN			
A1	1.000	1.000–1.000	77	16	61	0	0	<0.0001	1.000	1.000–1.000
A8	1.000	1.000–1.000	78	16	62	0	0	<0.0001	1.000	1.000–1.000
A9	0.989	0.974–1.000	78	10	62	6	0	<0.0001	0.726	0.521- 0.931
C7	1.000	1.000–1.000	78	16	62	0	0	<0.0001	1.000	1.000–1.000
C9	0.873	0.711–1.000	74	13	60	1	0	<0.0001	0.955	0.867–1.000
D9	0.993	0.981–1.000	76	11	62	3	0	<0.0001	0.857	0.699.1.000
E5	0.998	0.992–1.000	76	13	61	2	0	<0.0001	0.913	0.793–1.000
E7	1.000	1.000–1.000	78	16	62	0	0	<0.0001	1.000	1.000–1.000
E8	1.000	1.000–1.000	78	16	62	0	0	<0.0001	1.000	1.000–1.000
F1	0.989	0.974–1.000	78	11	62	5	0	<0.0001	0.778	0.593- 0.963
F10	0.949	0.889–1.000	77	12	61	4	0	<0.0001	0.826	0.662- 0.990
F12	1.000	1.000–1.000	77	16	61	0	0	<0.0001	1.000	1.000–1.000
G7	0.949	1.000–1.000	77	6	61	10	0	<0.0001	0.487	0.207–0.768
G8	1.000	1.000–1.000	76	16	60	0	0	<0.0001	1.000	1.000–1.000
H1	0.571	1.000–1.000	78	2	62	14	0	0.0400	0.185	−0.185 to 0.555
H5	0.962	0.922–1.000	78	8	62	8	0	<0.0001	0.614	0.371–0.857
H7	0.998	0.923–1.000	77	14	61	2	0	<0.0001	0.917	0.805–1.000
H9	0.795	0.964–0.897	78	1	62	15	0	NS	0.096	−0.307 to 0.499
RP[a]	0.579	0.419–0.739	78	0	62	16	0	NS		

[a] RP, random phage.
[b] AUC, area under the curve.
[c] CI, confidence interval.
[d] TP, true positive; TN, true negative; FP, false positive; FN, false negative.
[e] Statistical significance determined by Fisher's exact test. NS, no significance.

this requirement, eight clones (A1, A8, C7, C9, E7, E8, F12, and G8) accurately differentiated *L. infantum*-infected dogs from the control groups, thus avoiding false-negative results. Additionally, these selected phage clones showed highly accurate ROC curves (AUC, 1.0) and statistically significant values ($P < 0.0001$) when analyzed by Fisher's exact test (Table 3). The measures of individual performance for each evaluated phage are shown in Table 4. The higher sensitivity (100%) and Youden index (1.00) were obtained by eight selected phage clones. These clones showed specificities and PPVs of 100% and NPVs ranging from 90% to 100%. The highest DORs were achieved by A8, C7, E7, and E8, presenting a value of 4,125, while the A1 and F12 clones achieved a DOR of 4,059 and the G8 clone achieved a DOR of 3,993. Figure 2 shows the results from the NAR of the serum samples, and Fig. 3 shows the ROC curves in relation to the selected phage clones that presented the best results. Additionally, all phage clones differentiated *L. infantum*-infected dogs from the control groups, with statistical significance evaluated by the Kruskal-Wallis test followed by the Dunn posttest ($P < 0.0001$).

To characterize phage ligands, specificity is defined as the ability of a phage probe to associate with its target due to the presence of a specific peptide sequence displayed on the surface of the phage, whereas selectivity is the ability of a phage probe to identify its cognate target from a mixture of targets (35). In this context, a random phage selected by biopanning cycles using serum samples from dogs without CVL was used as a control in the phage-ELISA, in order to demonstrate that the specificity and selectivity of the selected clones were in fact due to the exogenous peptide of interest and not to any other endogenous phage component. As shown in Tables 3 and 4, this random phage clone failed to differentiate between the sera from *L. infantum*-infected dogs and that from the control groups, and it provided poor diagnostic performance for CVL.

DISCUSSION

The present study describes a subtractive phage display strategy that led to the identification of novel antigens to aid in CVL serodiagnosis. The selected phage clones were able to discriminate the serum samples from *L. infantum*-infected dogs from the cross-reactive serum samples infected by other parasites, as well as from the serum samples from vaccinated dogs using a phage-ELISA technique. Since its first description (36) and introduction into laboratory practice (37), phage display technology has proven to be useful in selecting specific peptides that are highly reactive against many pathogen targets. Its biological applications have ranged from use in studies of vaccine candidates for rabies (38), hepatitis C (39), *E. coli* (40), *Plasmodium vivax* (23), and *Schistosoma japonicum* (41) to use with diagnostic antigens for the detection of antibodies to pneumonia (42), neurocysticercosis (43–45), equine viral arteritis (46), rabies (47), and anaplasmosis (48). These phage-displayed peptides, also called mimotopes, have shown either linear or conformational recognition with high affinity to paratope sequences of antibodies. In this context, the present study used a subtractive approach through phage display in order to identify new antigens for the serodiagnosis of CVL so as to identify phage clones that express target peptides capable of being recognized by antibodies present in the serum samples from *L. infantum*-infected dogs. Aimed at identifying more refined antigens for CVL serodiagnosis, the selected phage clones were tested in a serological panel in phage-ELISAs.

TABLE 4 Diagnostic performance of the phage-ELISA using different blood serum samples

Clone	Sensitivity		Specificity					
	%	95% CI[b]	%	95% CI	J[c]	PPV[d]	NPV[e]	DOR[f]
A1	100.00	79.41–100.00	100.00	94.13–100.00	1.000	100.00	100.00	4,059
A8	100.00	79.41–100.00	100.00	94.13–100.00	1.000	100.00	100.00	4,125
A9	62.50	35.43–84.80	100.00	94.22–100.00	0.625	100.00	91.18	202
C7	100.00	79.41–100.00	100.00	94.13–100.00	1.000	100.00	100.00	4,125
C9	92.86	66.13–99.82	100.00	94.13–100.00	0.929	100.00	98.36	1,089
D9	78.57	49.20–95.34	100.00	94.22–100.00	0.786	100.00	95.38	411
E5	86.67	59.54–98.34	100.00	94.13–100.00	0.867	100.00	96.83	664
E7	100.00	79.41–100.00	100.00	94.13–100.00	1.000	100.00	100.00	4,125
E8	100.00	79.41–100.00	100.00	94.13–100.00	1.000	100.00	100.00	4125
F1	68.75	41.34- 88.98	100.00	94.22–100.00	0.687	100.00	92.54	261
F10	75.00	47.62–92.73	100.00	94.13–100.00	0.750	100.00	93.85	342
F12	100.00	79.41–100.00	100.00	94.13–100.00	1.000	100.00	100.00	4059
G7	37.50	15.20- 64.57	100.00	94.13–100.00	0.375	100.00	85.92	76
G8	100.00	79.41–100.00	100.00	94.13–100.00	1.000	100.00	100.00	3993
H1	12.50	01.55–38.35	100.00	94.22–100.00	0.012	100.00	100.00	21
H5	50.00	24.65–75.35	100.00	94.22–100.00	0.500	100.00	88.57	125
H7	87.50	61.65–98.45	100.00	94.13–100.00	0.875	100.00	96.83	713
H9	6.25	00.16–30.23	100.00	94.22–100.00	0.062	100.00	80.52	12
RP[a]	0	0.00–00.20	100.00	91.34–99.96	0	100.00	79.22	1

[a] RP, random phage.
[b] CI, confidence interval.
[c] J, Youden index.
[d] PPV, positive predictive value.
[e] NPV, negative predictive value.
[f] DOR, diagnostic odds ratio.

Serological tests are currently recommended for the laboratorial diagnosis of CVL. IFAT and ELISA are the most widely used diagnostic assays that are applicable to leishmaniasis; however, their low sensitivities for detecting cases with low levels of *Leishmania*-specific antibodies, as well as their cross-reactivity with other diseases, including Chagas' disease represent important limitations for their use in the CVL serodiagnosis (49, 50). In addition, crude antigens are also limited by the difficulty in producing large quantities in a standardized manner. Moreover, a high percentage of dogs, though infected by *Leishmania*, can remain seronegative for various periods of time, which can present as false-negative results in the serological trials (7, 51, 52).

In evaluating the amino acid residues present in the peptide sequence exposed in the selected phage clones, it is possible to conclude that the negative and positive selection processes we employed in the phage display strategy are effective once cysteine, lysine, and glycine amino acids were selected as presenting a frequency lower than the one expected, while the proline amino acid presented a frequency three times higher than expected, suggesting that this amino acid might be involved in antigen-antibody interactions. In fact, it is known that proline limits the flexibility of peptides and may therefore favor the formation of the monoclonal antibody (MAb)-peptide complex (53). In addition, this amino acid presents a chemically cohesive and rigid structure, resulting in a cyclic format that strongly influences the architecture of proteins (54, 55). Thus, the presence of proline in the majority of the selected peptides might suggest that this amino acid confers a more stable spatial conformation that interacts better with the IgG antibodies used in the positive and negative selection processes.

Regarding the sensitivity and specificity found in this study for selected phage clones, the high performance observed for phage-ELISA using the A1, A8, C7, E7, E8, F12, and G8 clones is compatible with the diagnostic performance observed in recent studies using recombinant proteins (56, 57), multiple-epitope chimeric antigens (58, 59), and synthetic peptides (60, 61). Nevertheless, the results of this study show improvements in sensitivity (100%) and specificity (100%), given that the cutoff values were selected in an attempt to obtain 100% specificity. Moreover, no false-positive result was observed when ROC curves were plotted, despite regular cross-reactive conditions, such as dogs that were infected with *T. cruzi* or *E. canis* and animals immunized with Leishmune or Leish-Tec Brazilian commercial vaccine. Recently, Faria et al. (62) performed an ELISA using a mixture of synthetic antigens and obtained high sensitivity and specificity values (78.5% and 80%, respectively); however, all of the synthetic antigens and the mixture presented high cross-reactivity with serum samples from *T. cruzi*-infected dogs. In addition, Marcondes et al. (63), who performed a rapid ELISA, showed a high rate of false-positive results in which high percentages of cross-reactivity occurred between *Leishmania* spp. and *T. cruzi* (57%), as well as between *Leishmania* spp. and *E. canis* (57%).

In this study, the PPV, NPV, and DOR parameters were used to compare the performances of the selected clones in the phage-ELISAs, indicating those that were true positives or true negatives to infection. All phage clones showed high PPV (100%); therefore, a positive result using any one of these indicates almost certain ocurrence of infection. Regarding the NPV, only the G7, H5, and H9 clones obtained lower values, ranging from 80.5% to 88.6%. The highest DORs were achieved by the A8, C7, E7, and E8 phage clones, which reached a value of 4,125. This parameter is the measure of the ratio between the DOR of the positivity found in those with the disease and the DOR of the positivity found in those with

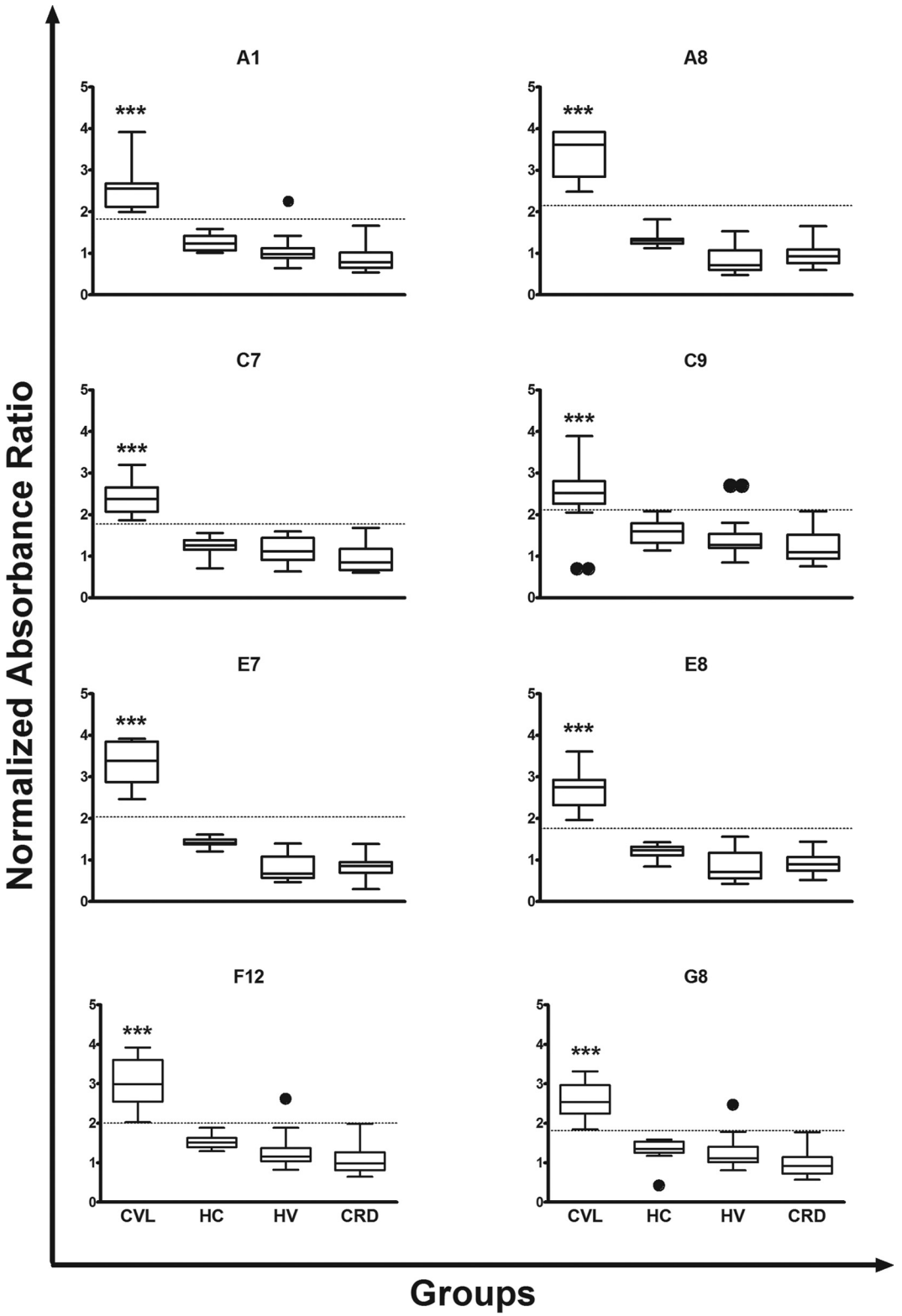

FIG 2 Phage-ELISA reactivity using the selected phage clones against different blood serum samples. Shown are box plots with minimum and maximum values of normalized absorbance ratios (NARs) between dogs with CVL ($n = 16$) and healthy ($n = 17$) or vaccinated ($n = 21$) dogs or dogs with cross-reacting diseases (*T. cruzi* and *E. canis*; $n = 23$). ●, outliers; ***, P values of <0.0001 represent statistically significant differences between CVL and control groups, as determined by the Kruskal-Wallis test, followed by Dunn's posttest for multiple comparisons.

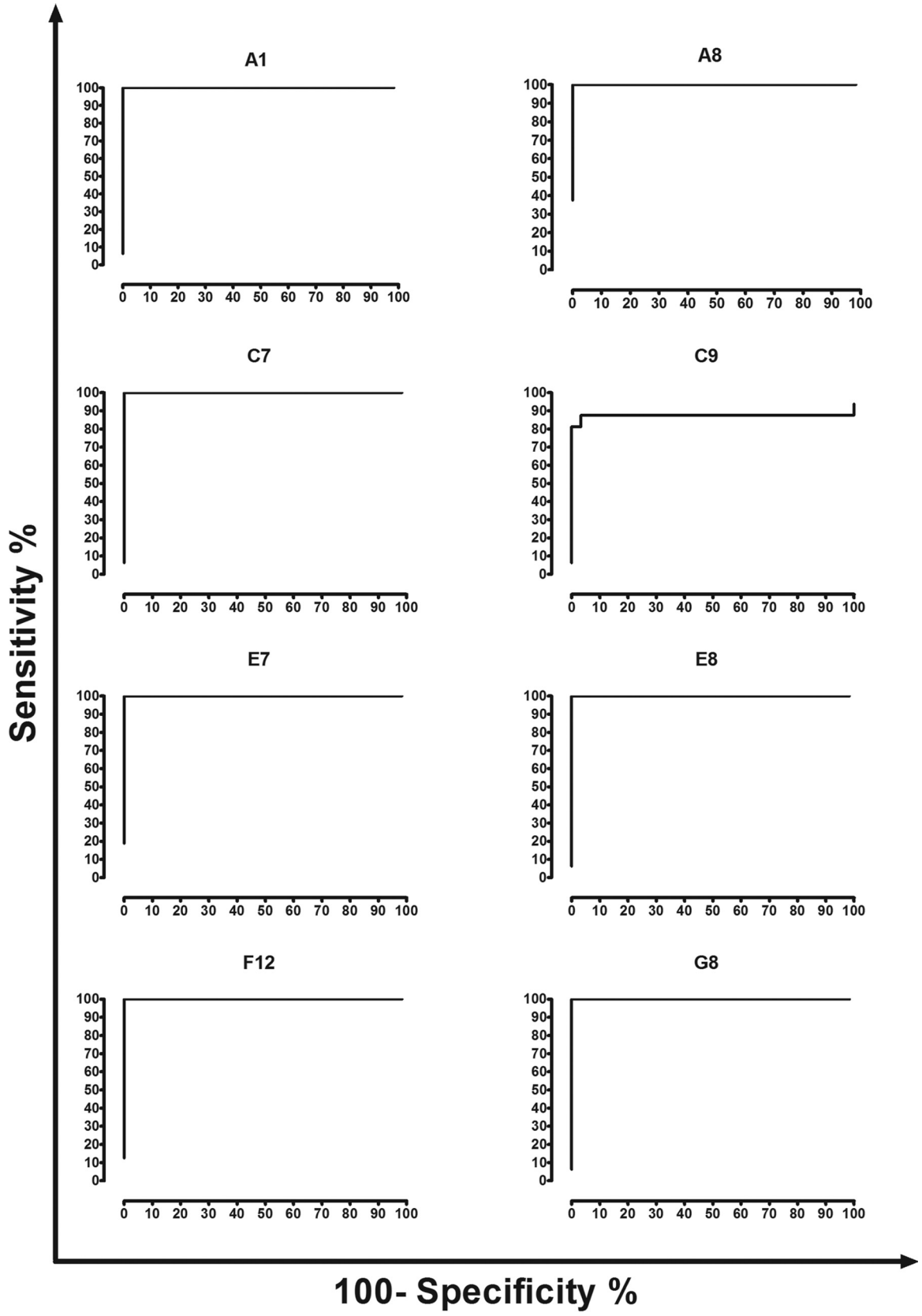

FIG 3 Diagnostic performance (sensitivity and specificity) of selected phage clones using different serum samples. ROC curves were plotted with the values of dogs with VL versus control groups (healthy, immunized with Leishmune or Leish-Tec, *T. cruzi*-infected, or *E. canis*-infected dogs). The ROC curves were calculated to determine the ELISA cutoff values and the AUC.

no disease (64). Recently, Rodríguez-Cortés et al. (65) compared six commercially available tests for the serodiagnosis of CVL and observed that the results presented a PPV of 100%, while the NPV ranged from 50% to 93%. In the same study, the DOR ranged from 47 to 729.

The serological panel employed in the present study did not contain samples from *L. braziliensis*-infected dogs; the collection of the serum samples from dogs was restricted to the urban area of Belo Horizonte, where the low incidence of infection with *L. braziliensis* in dogs was recently described (66). Thus, the data from this study should be taken as a proof-of-concept of the capacity of the proposed synthetic antigens to aid in the serodiagnosis of CVL, and they may well serve as a reference for further assays. However, these novel highly accurate phage-fused peptides and their use in phage-ELISAs may be promptly applied in the serodiagnosis used in CVL-monitoring programs due to their simplicity, ease of use, reproducibility, and low cost.

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