The recombinant cysteine proteinase B (CPB) from *Leishmania braziliensis* and its domains: promising antigens for serodiagnosis of cutaneous and visceral leishmaniasis in dogs.

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Abstract

Leishmaniasis represents a group of parasitic diseases caused by protozoan of the genus *Leishmania* and is widely distributed in tropical and subtropical regions. Leishmaniasis is one of the major tropical neglected diseases, with 1.5-2 million new cases occurring annually. Diagnosis remains a challenge despite advances in parasitological, serological and molecular methods.

Dogs are important host for the parasite and develop both visceral and cutaneous lesions. Our goal was to contribute to the diagnosis of canine cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) using the recombinant cysteine proteinase B (F-CPB) from *Leishmania braziliensis* and its N- and C-terminal domains (N-CPB and C-CPB) as antigens in an ELISA assay. Sera of dogs from the Northwest Argentina diagnosed with CL were tested by ELISA against a supernatant of *L. braziliensis* lysate, the F-CPB protein and its domains. We found values of sensitivity (Se) of 90.7, 94.4 and 94.3 % and specificity (Sp) of 95.5, 90.9 and 91.3% for F-CPB

and its N- and C-terminal domains, respectively. In dog sera from Northeast Argentina diagnosed with VL we found Se of 93.3, 73.3 and 66.7 and Sp of 92.3, 76.9 and 88.5 for F-CPB and its N- and C-terminal domains. These results support CPB as a relevant antigen for canine leishmaniasis diagnosis in its different clinical presentations. More interestingly, the amino acid sequence of CPB showed high percentages of identity in several *Leishmania* species, suggesting that the CPB from *L. braziliensis* qualifies as a good antigen for the diagnosis of leishmaniasis caused by different species.

Introduction

Leishmaniasis is endemic in 88 countries, with an estimated 350 million people at risk of becoming infected. Leishmaniasis is transmitted by the bite of infected female phlebotomine sandflies and is caused by different flagellate protozoans of the family Trypanosomatidae belonging to the genus *Leishmania* (1). These intracellular protozoa have a complex digenetic life cycle, requiring a susceptible vertebrate host and a permissive insect vector, which allow their transmission. The main epidemiological reservoirs of *Leishmania infantum* are dogs, which can remain asymptomatic for long periods of time, to finally develop cutaneous or systemic symptoms (2, 3). In Latin America, canine leishmaniasis is widespread, being one of the most important canine zoonotic vector-borne diseases (4).

More than 20 species and subspecies of *Leishmania* infect humans and dogs causing a wide spectrum of diseases, ranging from: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL) and visceral leishmaniasis (VL), depending on the parasite virulence factors and the immune response established by the host (5). In America, CL, MCL, and DCL taken together are also known as American tegumentary leishmaniasis (TL), with

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69 a wide geographical distribution from Southern United States to Northern Argentina. In
70 Northwestern Argentina (NWA) there have been several CL outbreaks, mainly in the forest of
71 Salta (6,7).

72 In 2006 the first autochthonous human VL case was reported in Posadas, province of Misiones
73 (Northeastern Argentina, NEA) (8, 9). Since then, climate change has contributed to the spread of
74 VL in Argentina. Dogs have been found to be naturally infected with species such as *Leishmania*
75 (*Viannia*) *peruviana*, *L.* (*Leishmania*) *major*, and *L.* (*L.*) *tropica*, among others, in several countries
76 (10). In Argentina, *L.* (*V.*) *braziliensis* and *L.* (*L.*) *infantum* have been incriminated as the causal
77 agents of canine leishmaniasis in the cities of Orán and Posadas, NWA and NEA, respectively (11,
78 12, 13).

79 Traditionally, the diagnosis of leishmaniasis is based on the microscopic detection of
80 amastigotes in tissue macrophages obtained by aspiration, scraping, or skin biopsy for CL, and in
81 bone marrow, nodes and spleen for VL. However, the presence of amastigotes depends on several
82 factors, and they can be morphologically misidentified as fungi, *Toxoplasma*, *Histoplasma* or even
83 artifacts (14). In order to increase diagnostic sensitivity and specificity, cultured lesion material
84 and molecular biology techniques such as PCR and real-time PCR (qPCR) have been proposed
85 (15, 16). However, not all *Leishmania* strains grow at the same rate and not all tissues have a
86 similar parasite load. Moreover, these techniques are expensive and require sophisticated
87 laboratories.

88 As VL infection develops, large amounts of polyclonal antibodies are produced in the host
89 (hypergammaglobulinemia). Therefore, various methods of detection of non-specific antibodies
90 have been used, which have subsequently been discarded for lack of sensitivity and specificity.
91 Other methods such as electrophoresis, hemagglutination, complement fixation test and gel

diffusion test have been performed in different endemic areas. Currently, only the direct agglutination test, the immunofluorescent antibody test (IFAT), ELISA and immunochromatography are being used (17-19). Improving serological tests for the diagnosis of leishmaniasis is important because they are rapid, easy to perform and can be easily implemented under the conditions commonly encountered in developing countries.

Antibodies against a wide range of parasitic antigens such as rK39 (a kinesin-related antigen), rK9 and rK26, heat shock proteins (HSP-70), histones (H-2A, 2B-H, H-3 and H-4), cysteine proteinases (CPA and CPB), gp63 and gp70 proteins, ribosomal proteins P (P0, P2a, P2b), iron-superoxide dismutases (Fe-SODe) and the cathepsin L-like protein, among others, have been detected in *Leishmania* spp. infection (20-23). The rK39 antigen is one of the most used antigens for the diagnosis of canine and human VL, showing excellent results mainly in India, where sensitivity and specificity are almost 100% (24-28). Although antigen rK39 has been important for VL serodiagnosis, it does not allow the diagnosis of CL or MCL (29-30).

The identification of new antigens to be employed in sensitive and specific serological assays is highly desirable. Extensive studies on the parasitic protozoan *Leishmania* have shown that cysteine proteinases (CPs) are involved in parasite survival, replication and the onset of disease (31). The cysteine proteinase B (CPB) from *Leishmania* spp. is present in all strains and stages of the parasite and plays a crucial role in host-parasite interaction. The genes that code for the CPBs in trypanosomatids are organized as follows: a pre-region, a propeptide, the catalytic domain, and a C-terminal extension (32, 33). The latter, as those of other CP orthologues, presents different immunogenic properties. We have demonstrated that the immune response in *T. cruzi* infection is directed mostly against the C-terminal domain (34). This part of the antigen may operate as a diversion of the immune system, concentrating the antibody response against the C-terminal

115 domain, and preserving the enzymatic activity of the N-terminal domain. Accordingly, our overall
116 objective was to contribute to the diagnosis of cutaneous and visceral leishmaniasis in dogs using
117 the recombinant CPB from *L. braziliensis* and its domains for the detection of specific antibodies
118 against *Leishmania* spp.
119

120 ## Materials and methods

121 **Cloning, expression and purification of CPB and its domains in prokaryotic cells.**
122 The cloning of the recombinant proteins will be described elsewhere (Bivona AE, unpublished
123 results). Briefly, the CPB gene of *L. braziliensis* (LbrM08_V2.0820, accession XM_001562090)
124 was synthesized by GenScript, optimizing the sequence between nucleotides 373 to 954 for
125 expression in prokaryotic cells. From this gene, using specific primers containing cleavage sites
126 for restriction enzymes and a tail of six histidines, we synthesized by PCR sequences of 954, 657
127 and 297 bp corresponding to the full length CPB and its N-and C- terminal domains, respectively.
128 The purified PCR products were digested with restriction enzymes and ligated to plasmid pET23a.
129 Bacterium *Escherichia coli* DH5 was transformed with the constructs and after selecting positive
130 clones for their resistance to ampicillin, the presence of the inserts was confirmed by digestion
131 with restriction enzymes. Constructs showed at least 97% identity with the previously reported
132 sequence (LbrM08_V2.0820) for the entire CPB and N- and C-terminal domains.
133 The resulting vectors were then transformed into *E. coli* BL21 (DE3) cells for expression.
134 Recombinant proteins were obtained by inducing bacterial cultures with 1mM isopropyl-L-thio-β-
135 D-galactoside (IPTG) for 4 h. Cells were harvested, centrifuged, and resuspended in lysis buffer
136 pH 8.0 containing 100 mM NaH_2PO4, 10 mM Tris-HCl, 8 M urea, 1 mM PMSF, 1 µM E-64. The
137 cells were stirred at room temperature for 60 min and then centrifuged at 10,000 ×g for 20 min to

pellet the cell debris. Proteins were purified under denaturing conditions from the supernatant using a Ni^{2+}-nitrilotriacetic acid-Sepharose matrix. Properly folded proteins were obtained by extensive dialysis against buffer 2 M urea, 50 mM Tris, 5% sucrose, 10% glycerol, 0.3 M NaCl, 0.5 mM EDTA followed by dialysis in phosphate-buffered saline (PBS)-20% glycerol and stored at -70°C until use. Protein concentration was determined by the Bradford protein assay (Bio-Rad, Hercules, CA), using bovine serum albumin (Sigma) as a standard.

Dog serum samples

Study 1: Samples were taken in the localities of Colonia Santa Rosa, Pichanal and Orán, Province of Salta, NWA. The study area is included within the biogeographic "Yungas" rainforest (6). The Province of Salta has been the area of Argentina with high incidence of CL, with most cases originating in the Orán Department (7, 35, 36). Moreover, *L. braziliensis* has been acknowledged as the main causative agent for CL in this area of Argentina (7, 35).

Samples stored at -20°C, were collected from 76 dogs previously diagnosed with leishmaniasis by the identification of amastigotes in Giemsa-stained material obtained by touch print, scraping, exudate, or aspirate obtained by injecting 0.1–0.4 ml of buffered saline solution plus penicillin-streptomycin followed by an aspirate of the fluid (6). Clinical signs support the diagnosis of CL (6, 37). The entire skin surface of the dogs was carefully inspected in the search for lesions or scars. Particular attention was paid to the limbs, ears, nose and scrotum, since ulcerous lesions were most often found in these areas. The clinical criteria used to define "suspected leishmaniasis lesions" were: ulcerative character, long duration, and rounded, raised and indurated edges, coupled with swollen lymph node. Lesions probably induced by trauma were not considered to be *Leishmania* spp. infection.

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161 Canines classified as no leishmaniasis (noL) were dogs without any sign of leishmaniasis and
162 negative for the ELISA serological test (6).

163 ***Study 2:***

164 Thirty-three dogs were submitted to a careful clinical evaluation by veterinarians from
165 "Veterinaria del Oeste" in the city of Posadas, Province of Misiones, NE Argentina and diagnosed
166 with VL or no leishmaniasis (NoL) based on parasitological and serological tests and supported
167 by clinical signs (38). We have recently found *Leishmania (Leishmania) infantum* as the causative
168 agents of canine VL cases in the city of Posadas (39).

169 Amastigotes observed on smears from aspirates were analyzed for the parasitological diagnosis
170 of canine VL. A puncture aspiration was aseptically performed on the dogs using 2.5 ml syringes
171 and 21-gauge needles. The aspirates were taken from enlarged lymph nodes, especially the
172 popliteal ones. When lymph nodes could not be found, the samples were taken from the bone
173 marrow or the spleen. A fraction from each sample obtained by aspiration or the scrapings were
174 stained with Giemsa and observed under an optical microscope.

175 Clinical suspicion of VL was defined by the presence of three or more of the following signs:
176 weight loss, alopecia, lymphadenopathy, renal azotemia, onychogryphosis, hepatomegaly, and
177 splenomegaly. Signs such as exfoliative dermatitis on the nose, tail, and ear tips were also
178 recorded. Skin features such as periocular and generalized alopecia, hair loss, seborrhea, and
179 depigmentation in the muzzle were recorded to note the presence of skin disease without
180 ulceration. Asymptomatic dogs appeared completely healthy at the clinical examination (no blood
181 counts performed).

182 Blood samples were collected from the jugular vein and sera were kept frozen until tested. The
183 diagnosis of canine VL was confirmed in the laboratory based on the positive results of IFAT,

antigen rk39 (Kalazar Detect™ Rapid Test, Canine, InBios International, Inc) and SNAP Leishmania (IDEXX) (40).

Additionally, sera from dogs living in a VL non-endemic area (Buenos Aires Province) not presenting any clinical signs of leishmaniasis and negative by the serological evaluation were also included.

Ethics statement

The dog owners voluntarily requested the medical attention of their animals. Under clinical suspicion of the disease, they gave their informed consent to include the dogs in this study. The procedures were approved by the Bioethics Committee of the Faculty of Agricultural and Veterinary Sciences, the Catholic University of Salta, Argentina (No. 442837/0052. October 14, 2014).

Parasites

Leishmania braziliensis promastigotes (MHOM/BR/75/M2903 strain) were grown in liver infusion tryptose (LIT) medium, which was prepared as follows: 5 g/l liver infusion (Sigma 2023-072K1066), 5 g/l tryptose (Britania), 2 g/l glucose (Sigma), 68 mM NaCl, 5.4 mM KCl, 22 mM HPO_4Na_2, supplemented with 20 mg/l hemin (Sigma) and 10% (vol/vol) fetal calf serum (FCS) (Internegocios). Culture maintenance was performed by weekly passages at 26 ºC.

***Leishmania braziliensis* supernatant lysate**

Promastigotes of *L. braziliensis* were centrifuged for 15 min at 5000 *g*, re-suspended in 0.25 M sucrose, 5 mM KCl containing protease inhibitors (2 µM PMSF, 5µM leupeptin, 5 µM pepstatin

and 5 µM E-64; Sigma, St. Louis, MO) and broken by three cycles of freeze-thawing and sonication by 4 cycles of 30 sec on ice. The homogenate obtained was centrifuged at 45,000 *g*, obtaining a supernatant fraction called F45 that was conserved at −20 °C until use. Protein concentration determination was performed by the Bradford method (BIO-RAD, Protein Assay Cat. 500-0006), using bovine serum albumin as a standard.

IFAT

Leishmania braziliensis promastigotes harvested during the exponential growth phase by centrifugation at 5000 *g* for 15 min were washed three times with 0.1 M phosphate buffered saline (PBS) pH 7.2 and re-suspended with 2% formalin solution in PBS. Formalin-treated promastigotes ($1x10^5$ parasites/field) placed in immunofluorescence glasses were fixed by heat, washed twice with PBS and finally with H_2O. Sera were assayed at 1/60 dilution and added to the coverslips and incubated for 16 h at 4 ºC. As secondary antibody, anti-dog IgG (whole molecule)-FITC antibody produced in rabbit (Sigma-F4012) in 0.001% Evans blue was used and observed under a fluorescence microscope.

ELISA

An indirect ELISA test for antibody detection was used as described elsewhere (41). Briefly, flat polystyrene bottom plates (Nunc, Roskilde, Denmark) were sensitized with 1 µg per well of the soluble fraction of *L. braziliensis* (F45) promastigote lysate or with 0.2 µg per well of the full length CPB (F-CPB) and its N and C-terminal domains (N-CPB and C-CPB). Blocking was done with 3% bovine serum albumin (BSA) and 0.1% gelatin in PBS during 1 h at 37 ºC. Plates were then washed three times with 0.05% Tween in PBS. Sera were assayed at a serial dilution of 1/100

and incubated for 18 h at 4ºC. Peroxidase-conjugated immunoglobulins to dog IgG (Sigma) diluted 1/25000 were used as a secondary antibody. Plates were developed by adding OPD/H_2O_2, incubated for 10 min in the dark and the reaction was stopped using 4N H_2SO_4. Optical density was read by an ELISA reader (Bio-Rad Laboratories, Hercules, CA) at 490 nm. Cutoff values were calculated using receiver operating characteristic (ROC) curves. Titers were calculated as the dilution in which the optical density (OD) obtained was equal to the mean of controls ± 2.23 SD (equivalent to 99% confidence in the one-tailed test hypothesis), where applicable.

Multiple sequence alignment

The amino acid sequence of the *L. braziliensis* CPB (XP_001562140.1), without the pro-domain region (aa 1 to 124), was aligned with the sequences registered in the NCBI database as 'cpb' or 'cysteine proteinase b' from other *Leishmania* species. Namely, *L. guyanensis* (ACS66748.1), *L. panamensis* (ABX74953.1), *L. major* (XP_001681135.1), *L. infantum* (SUZ39418.1), *L. donovani* (AGI92544.1), *L. mexicana* (CAA90236.1), *L. tropica* (AFN27127.1), *L. aethiopica* (AAZ23596.1). The multiple sequence alignment was performed and the phylogenetic tree was constructed using the ClustalW2 software tool (42).

Statistics

The cutoff point for optimal sensitivity and specificity, as well as the other statistical parameters, were determined using the receiver operating characteristic (ROC) curve analysis to assess ELISA F-CPB, ELISA N-CPB and ELISA C-CPB using the XL-STAT statistical software/program (Excel).

Graphs were performed using the GraphPad Prism program (version 5.0). Statistical comparisons between groups were performed using the Mann-Whitney U test. P-values of < 0.05 were considered statistically significant.

Results

CPB and its domains in the diagnosis of canine CL

Dogs from NWA had been previously checked for lesions compatible with CL and for parasite microscopic observation in stained material from lesions (6). Accordingly, sera were classified into cutaneous leishmaniasis (CL) and no leishmaniasis (NoL) sera. We analyzed 76 stored sera by the immunofluorescence antibody test (IFAT). In slides containing fixed promastigotes of *Leishmania braziliensis*, a cutoff value of 1/60 was established for the in-house IFAT test. Later, we analyzed the samples, finding reactivity in 98.15 % of the dogs diagnosed as CL. By contrast, 18.18% of dogs without leishmaniasis was positive against promastigotes of *L. braziliensis* by IFAT **(Fig 1).** These results indicate values of Se: 98.1 and Sp: 81.8% for the IFAT test in the diagnosis of canine CL.

Titration curves were performed to determine the most appropriate concentration of the *L. braziliensis* antigens to be used in the ELISA experiments (data not shown). Then, an ELISA assay was performed to determine specific IgG antibodies against *L. braziliensis* promastigote lysate (F45), the recombinant full-length CPB (F-CPB) and its domains (N-CPB and C-CPB), respectively. **Fig 2** shows that IgG specific antibodies against F45, F-CPB and its domains were significantly higher in CL than in non-leishmaniasis dogs ($p< 0.0001$).

We analyzed the accuracy of the ELISA tests to correctly classify the samples as CL. As shown in **Fig 3** the AUC 0.9722 (95% confidence interval 0.9372 to 1.0070), 0.9722 (CI: 0.9347 to 1.010),

0.9562 (CI: 0.9055 to 1.007), and 0.9423 (CI: 0.8831 to 1.002) were determined for F45, F-CPB, N-CPB and C-CPB, respectively. According to the traditional academic point system, all the antigens showed an AUC between 0.90-1.0, which means they were excellent ligands to correctly discriminate between the two groups (43).

Interestingly, the detection of antibodies against the recombinant antigens in the ELISA matrix, showed sensitivities of 0.907, 0.944 and 0.943 for F-CPB, N-CPB and C-CPB, respectively, which were equal or close to those observed when a mixture of *Leishmania* antigen (F45) was used (0.944). Moreover, F-CPB presented higher specificity and predictive positive value (0.955 and 0.980) than its domains (0.909 and 0.962; and 0.913 and 0.962 for the N-CPB and C-CPB, respectively) **(Table 1).** Overall**,** these results endorse F-CPB and its domains as effective tools in the diagnosis of CL in dogs, with high sensitivity and specificity.

Based on a thorough analysis of clinical and epidemiological data, CL dogs were then subdivided as follows: **A**: dogs bearing ulcerative lesions typical of CL; **B:** dogs without ulcers, living in the houses of humans or other dogs with leishmaniasis; **C:** dogs with atypical ulcers, living in the houses of humans with leishmaniasis; **D**: asymptomatic dogs living in houses with human or other dogs without leishmaniasis. Interestingly, specific antibodies against all the antigens tested were significantly higher in groups A, B and C, which corresponded to dogs that had or could have been exposed to *Leishmania* parasites, with respect to those observed in asymptomatic dogs (group D). Titers of specific antibodies against the recombinant proteins agreed with those observed against the parasite lysate **(Fig 4)**.

CPB and its domains in the diagnosis of VL

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297 We then analyzed the efficiency of the different antigens in the diagnosis of VL in dogs. As
298 shown in **Fig 5,** significant differences in reactivity against F-CPB and its domains were observed
299 among dogs suffering from VL or not.

300 The ELISA containing F-CPB exhibited the best performance compared to the other antigens
301 tested (AUC: 0.879, 0.789 and 0.723, for F-CPB, N-CPB and C-CPB respectively). These results
302 mean that F-CPB as a coating antigen in an ELISA assay is a good candidate for the diagnosis of
303 VL in dogs (**Fig 6**). In addition, we observed higher sensitivity (Se) (93.3%) and specificity (Sp)
304 (92.30 %) for F-CPB compared to the N- (Se: 73.3 % and Sp 76.9%) and C-terminal domains (Se:
305 66.7 and Sp: 88.5 %) **(Table 2)**.

306

307 **The CPB amino acid sequence is highly conserved among *Leishmania* species**

308 In order to further analyze whether CPB could be a promising antigen for the diagnosis of
309 leishmaniasis caused by the infection of several species, the amino acid sequence of the CPB from
310 *L. braziliensis* was aligned with its orthologous sequence in different *Leishmania* species. As
311 shown in **Fig 7**, high percentages of identity sere found: 91.5% for *L. guyanensis* (ACS66748.1),
312 76.1% for *L. panamensis* (ABX74953.1), 68.1% for *L. major* (XP_001681135.1), 62.8% for *L.*
313 *infantum* (SUZ39418.1), 62.5% for *L. donovani* (AGI92544.1), 62.2% for *L. mexicana*
314 (CAA90236.1), 61.5% for *L. tropica* (AFN27127.1), 61.5% for *L. aethiopica* (AAZ23596.1) and
315 the CPB of *L. braziliensis*. These results suggest that the CPB from *L. braziliensis* qualifies as a
316 good target for the diagnosis of *Leishmania* spp. infection caused by different species of the
317 parasite However, an exhaustive study of the ELISA performance of the CPB of *L. braziliensis* in
318 *Leishmania* infection caused by all the mentioned strains should be carried out in the near future.

319

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Discussion

A rapid and accurate diagnosis of *Leishmania* spp. infection followed by the early implementation of an effective treatment in infected individuals is essential for the control of a disease that has spread for several reasons. Domestic dogs are considered the main reservoirs of *L. infantum*, playing an important role in the epidemiology of VL (44, 45). The number of infected dogs in South America is estimated in millions, and there are high infection rates associated with a high risk of human disease (44-46). Although the development of sensitive molecular diagnostic techniques has improved the detection of clinically healthy infected dogs, those methods are not always available to researchers in Latin America.

Immunoserological tests have evolved as useful tools in the diagnosis of leishmaniasis in dogs since the humoral response in general is intense, with high levels of specific immunoglobulins (47-49). We showed in an ELISA assay that the CPB from *L. braziliensis* and its domains, mainly F-CPB, is a promising antigen for the diagnosis of both cutaneous and visceral clinical presentations of leishmaniasis in dogs with high sensitivity and specificity (Se: 90.7, Sp: 95.5, AUC: 0.97 and Se: 93.3, Sp 92.3, AUC: 0.88, respectively **(Fig 2, 5 and Tables 1, 2).** Moreover, the high sensitivity of the CPB from *L. braziliensis* in the diagnosis of VL (93.3%) could be explained considering the higher stimulation of the immune system in the visceral form compared to a localized cutaneous presentation (Se: 90.7). In that regard, several reports (50-52) have shown the importance of the CPB from *L. infantum* and *L. (L.) chagasi* as targets of the humoral and cellular immune response and their potential use for the diagnosis of VL in humans and dogs.

Bearing in mind that the species that cause CL and VL disease are generally different, the ability of the CPB from *L. braziliensis* to detect the infection caused by different *Leishmania* strains highlights its value as a candidate for the universal diagnosis of leishmaniasis. This is also

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supported by the conserved amino acid sequence of this antigen among several *Leishmania* species **(Fig 7)**.

One limitation of most serological tests is their inefficiency to detect VL in dogs during the early stages of infection. Early detection of canine VL is highly desirable in order to shorten the contact time between the infected reservoirs and the vectors. In that regard, Faria (53) has reported an ELISA for two multiepitope proteins, PQ10 and PQ20, which was able to detect *Leishmania* infection at earlier time points as compared with kDNA PCR-RFLP in anti-IgG and anti-IgM assays. In **Fig 4,** we observed that dogs without ulcers living in contact with humans with leishmaniasis (Group B) displayed a significant increase in IgG titers against F-CPB and its domains, in comparison with asymptomatic dogs (Group D). These results indicate that the CPB of *L. braziliensis* can be a good predictor of *Leishmania* spp. infection yielding significant serum IgG antibodies in the host before the onset of leishmaniasis symptoms. This hypothesis needs to be further explored in future studies.

Recently, Lima (54) showed high sensitivity and specificity of an ELISA assay from a *L. braziliensis* kinesin-like hypothetical protein (LbHyM) for the serodiagnosis of human cutaneous and mucosal leishmaniasis. Nearly 78% similarities were found in the amino acid sequence comparison between LbHyM and the *T. cruzi* hypothetical protein. The strong cross-reactivity between *Leishmania* and *T. cruzi* makes their differential serodiagnosis difficult. Since the drugs used for the treatment of both parasitoses are different, an accurate diagnosis is necessary. In a preliminary study we have recently observed no cross-reactivity between *T. cruzi*-infected patients and the CPB of *L. braziliensis*, by ELISA. Additionally, sera from patients that were positive for the F-CPB from *L. braziliensis* and its domains did not recognize in an immunoblotting assay, *T. cruzi* specific antigens like cruzipain, thiol-transferase (Tc52) and the flagellar calcium-binding

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protein (Tc24). By contrast, samples from patients with Chagas disease recognized all these *T. cruzi* antigens (data not shown).

We conclude that the performance of the CPB from *L. braziliensis* and its domains turns them into promising antigens for the diagnosis of leishmaniasis in dogs caused by different *Leishmania* species. Furthermore, it must be considered that the ELISA assay, with potential application in endemic areas, could be further improved by the addition of other antigens, using different blocking reagents or different detection systems, such as streptavidin-peroxidase. The analysis of potential cross-reactivity with other co-endemic diseases and pathogens must be further investigated as the next step to validate CPB in the diagnosis of *Leishmania* spp. infection.

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Figure Legends

Figure 1. Immunofluorescence antibody test (IFAT) of dog sera from Northwestern Argentina (NWA). Dogs previously diagnosed with (A) cutaneous leishmaniasis (CL), or (B) no leishmaniasis (noL), by direct methods, epidemiological, and clinical examination were tested for their reactivity against promastigotes of *L. braziliensis* by an IFAT test. Fixed *Leishmania braziliensis* promastigotes were incubated overnight with dog sera with CL (C) and noL (D and

564 E); and then stained with an anti-dog IgG FITC-labeled antibody. The figures show representative
565 images of epifluorescence (C and D) and brightfield (E) microscopy. Magnifications 40X.
566
567 **Figure 2**. **ELISA of sera from dogs living in the Northwest of Argentina.** Canines classified
568 as diagnosed with cutaneous leishmaniasis (CL) or no leishmaniasis (NoL) were assayed for the
569 presence of IgG antibodies against *L. braziliensis*: **(A)** promastigote lysate (F45), **(B)** full-length
570 CPB, **(C)** N-terminal domain of the CPB and **(D)** C-terminal domain of the CPB. The results were
571 expressed as the DO_{490} nm, and the cutoff (CO) was calculated using the ROC curve. Lines
572 represent the mean ± S.E.M. ****<p 0.0001.
573
574 **Figure 3. ROC curves for ELISAs coated with the recombinant antigens**. Sera from dogs from
575 the Northwest of Argentina were analyzed by an ELISA assay against *L. braziliensis*: **(A)**
576 promastigote lysate (F45), **(B)** full-length CPB, **(C)** N-terminal domain of the CPB and **(D)** C-
577 terminal domain of the CPB. True positive rate (Sensitivity) was plotted as a function of the false
578 positive rate (100-Specificity) for the different *Leishmania* antigens at different cutoff points. An
579 area of 1 represents a perfect test while an area of 0.5 represents a worthless test. The accuracy of
580 a diagnostic test is: 0.90-1 = excellent, 0.80-0.90 = good, 0.70-0.80 = fair, 0.60-0.70 = poor, 0.50-
581 0.60 = fail.
582
583 **Figure 4**. **ELISA test of sera from dogs living in the Northwest of Argentina.** Sera were assayed
584 for the presence of IgG antibodies against *L. braziliensis* promastigote lysate (F45), full -length
585 CPB, N and C-terminal domains. Groups: **A**: dogs bearing ulcerative lesions typical of CL; **B**:
586 dogs without ulcers, but living in the houses of humans or other dogs with leishmaniasis; **C**: dogs

with atypical ulcers, living in the houses of humans with leishmaniasis; **D**: asymptomatic dogs from endemic areas living in houses with human or other dogs without leishmaniasis. Results are expressed as the titers of specific antibodies. Titers were calculated as the dilution in which the optical density (OD) obtained was equal to the mean of controls ± 2.23 SD for each antigen. **p < 0.01 and ***p < 0 0.005 and ****p< 0.0001.

Figure 5. CPB and its domains in the diagnosis of canine visceral leishmaniasis. Sera from dogs from the Northeast and center of Argentina were assayed for the presence of IgG antibodies against *L. braziliensis* promastigote lysate (F45); full -length CPB; N- and C-Terminal domains. Results are expressed as OD490 nm. Lines represent the mean ± S.E.M. The cutoff (CO) for the different antigens was determined using the ROC curve. *p < 0.05, **p < 0.01 and ***p< 0.0005 and ****p< 0.0001.

Figure 6. Diagnostic efficacy of the recombinant antigens in canine VL using ROC curves. Sera from dogs from the Northeast and center of Argentina were analyzed in an ELISA matrix against *L. braziliensis*: **(A)** promastigote lysate (F45), **(B)** full-length CPB, **(C)** N-terminal and **(D)** C-terminal domains of the CPB. True positive rate (Sensitivity) was plotted as a function of the false positive rate (100-Specificity) for the different *Leishmania* antigens at different cutoff points. An area of 1 represents a perfect test while an area of 0.5 represents a worthless test. The accuracy of a diagnostic test is: 0.90-1 = excellent, 0.80-0.90 = good, 0.70-0.80 = fair, 0.60-0.70 = poor, 0.50-0.60 = fail.

Figure 7. Conservation of the amino acid sequence of cysteine proteinase B (CPB) in different *Leishmania* species. **(A)** Alignment of the CPB from *L. braziliensis* with its orthologous sequences from *L. guyanensis* (ACS66748.1), *L. panamensis* (ABX74953.1), *L. major* (XP_001681135.1), *L. infantum* (SUZ39418.1), *L. donovani* (AGI92544.1), *L. mexicana* (CAA90236.1), *L. tropica* (AFN27127.1) and *L. aethiopica* (AAZ23596.1). **(B)** Phylogenetic tree based on the amino acid sequence of the CPB in *Leishmania*.

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630 **Table 1.** Statistic parameters of the ELISA test against the cysteine proteinase B from *L.*
631 *braziliensis* (CPB) and its domains for the diagnosis of CL in dogs.

STATISTICS	ANTIGEN			
	F45	**F-CPB**	**N-CPB**	**C-CPB**
Se	0.944	0.907	0.944	0.943
Sp	0.909	0.955	0.909	0.913
TP	51	49	51	50
FP	2	1	2	2
TN	20	21	20	21
FN	3	5	3	3
PPV	0.870	0.980	0.962	0.962
NPV	0.962	0.808	0.870	0.875
AUC	0.972	0.972	0.956	0.948

632

633 Abbreviations: **Se**: sensitivity; **Sp**: specificity; **TP**: true positive; **FP**: false positive; **TN**: true
634 negative; **FN**: false negative; **PPV**: positive predictive values; **NPV**: negative predictive values;
635 **AUC**: area under the curve.

636

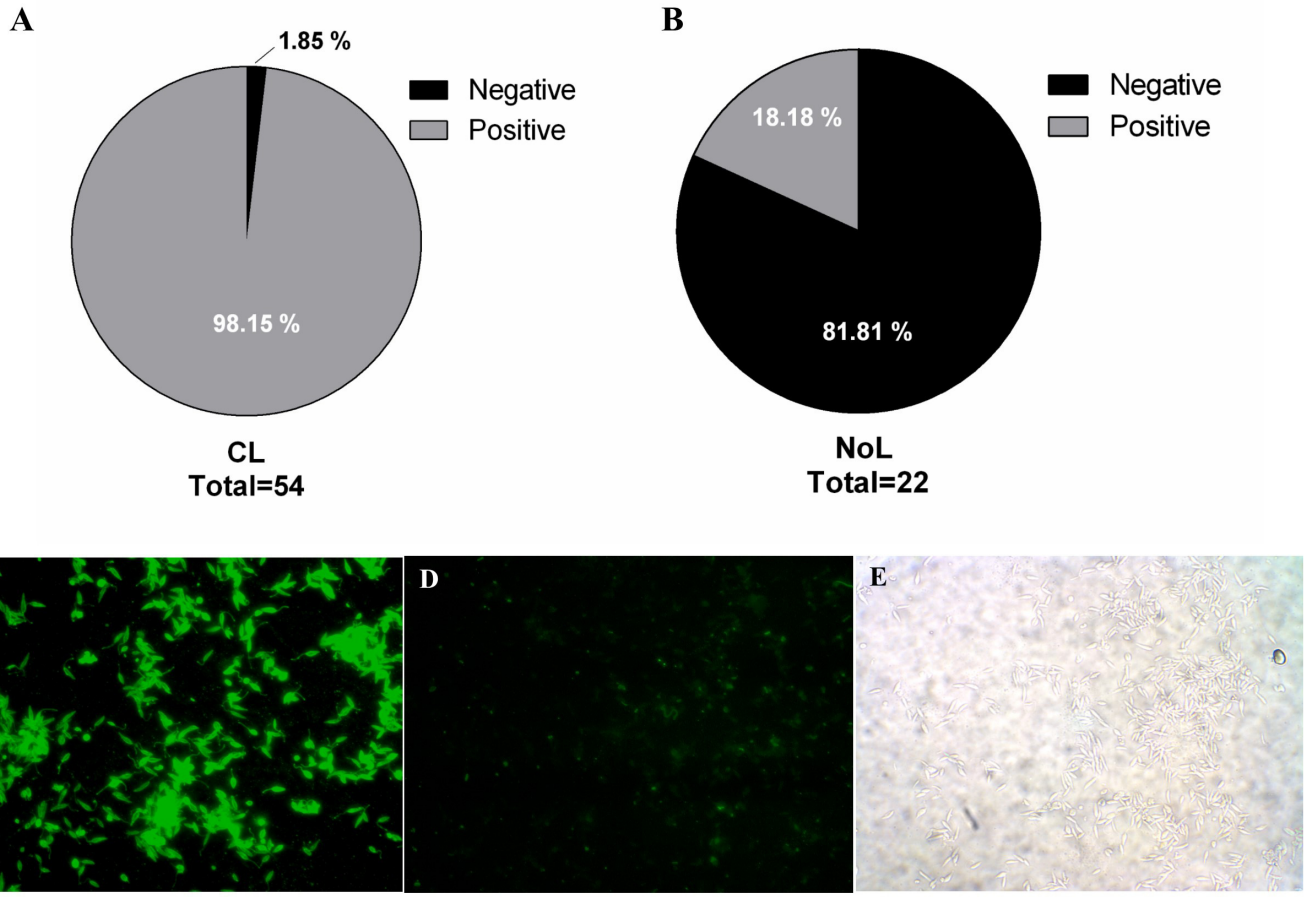
637 **Table 2.** Statistic parameters of the ELISA test against cysteine proteinase B from *L. braziliensis*
638 (CPB) and its domains for the diagnosis of VL in dogs.

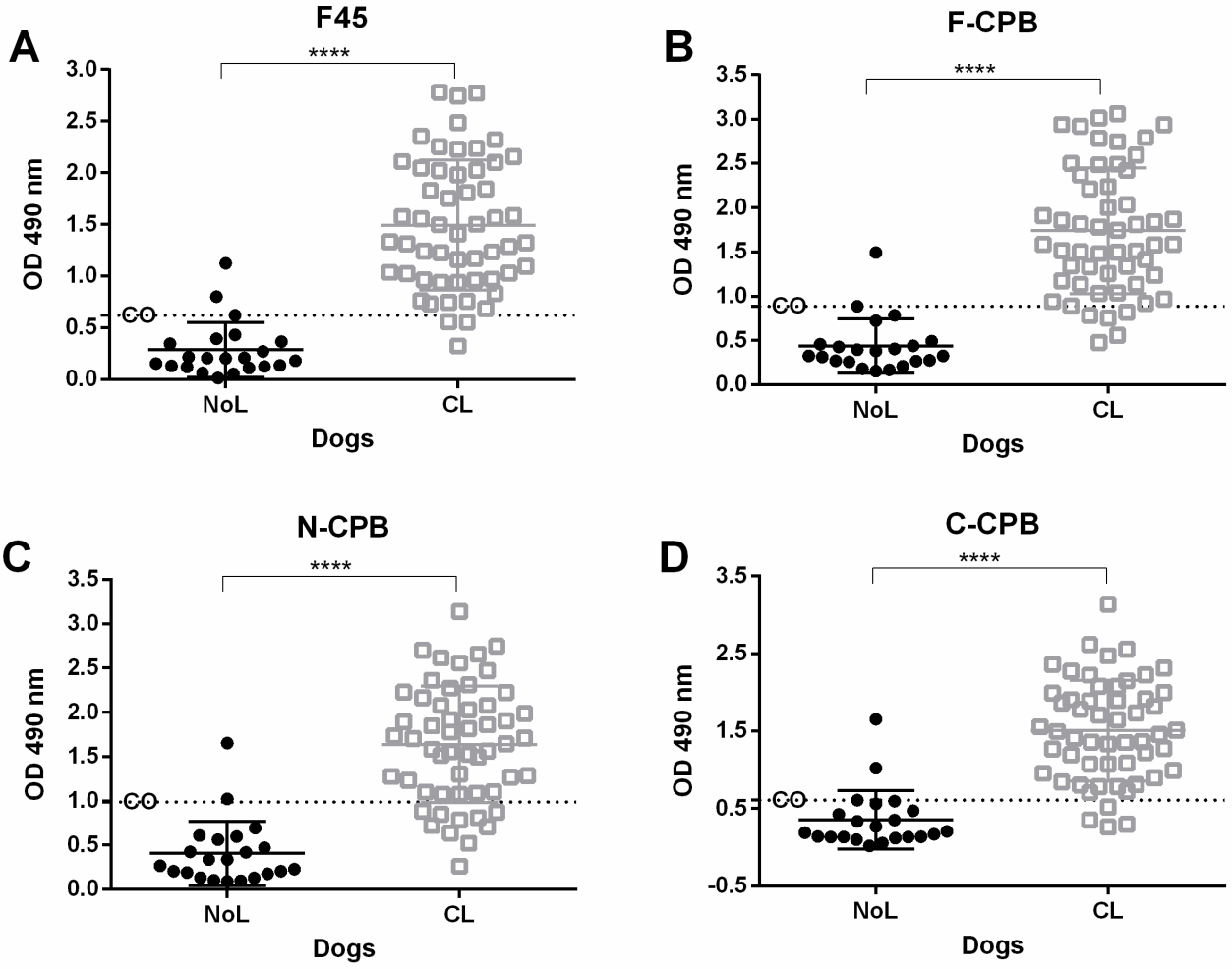
STATISTICS	ANTIGEN			
	F45	**F-CPB**	**N-CPB**	**C-CPB**
Se	0.867	0.933	0.733	0.667
Sp	1.000	0.923	0.769	0.885
TP	13	14	11	10
FP	0	2	6	3
TN	26	24	20	23
FN	2	1	4	5
PPV	1.0	0.875	0.647	0.769
NPV	0.929	0.960	0.833	0.821
AUC	0.941	0.879	0.7897	0.723

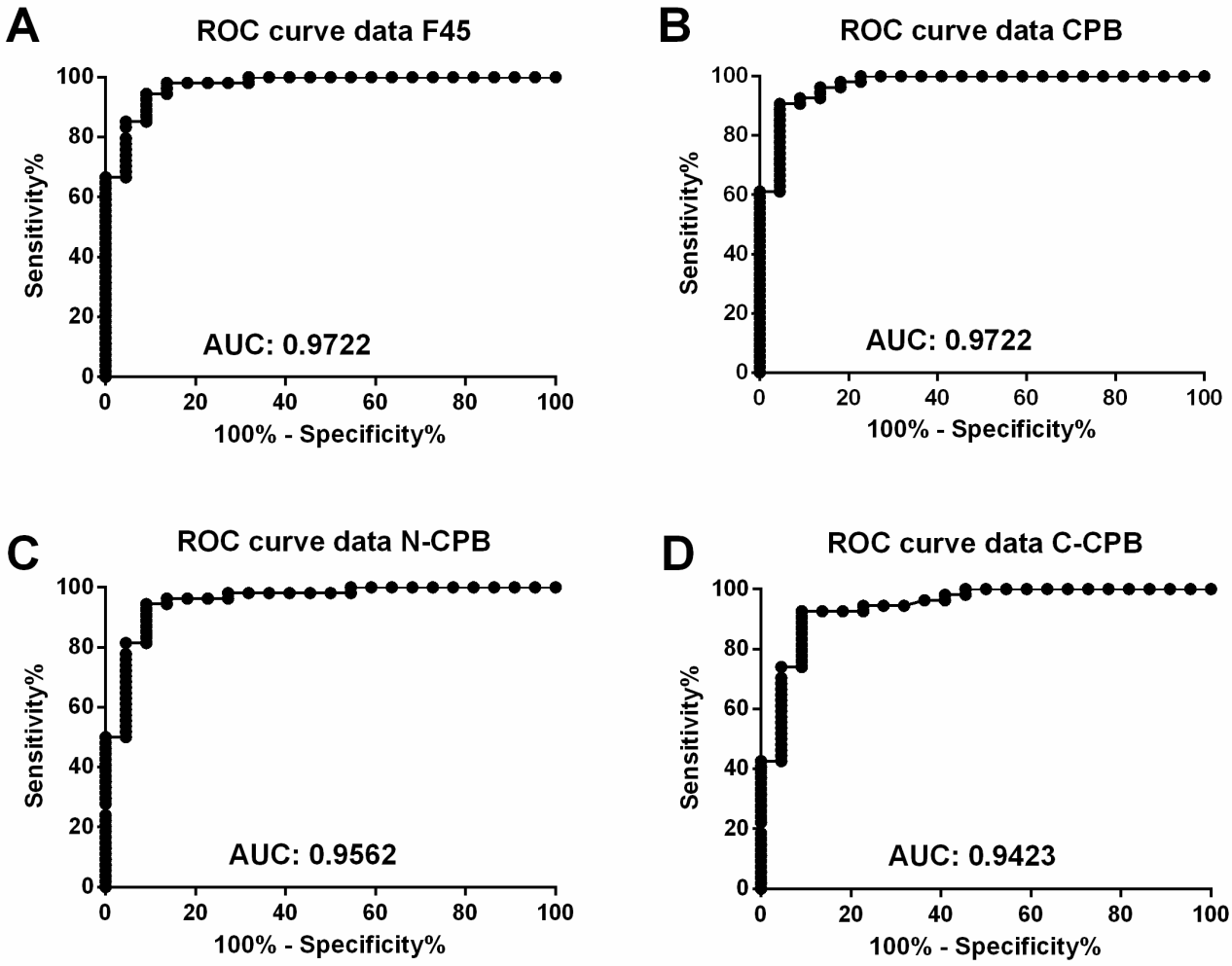
639

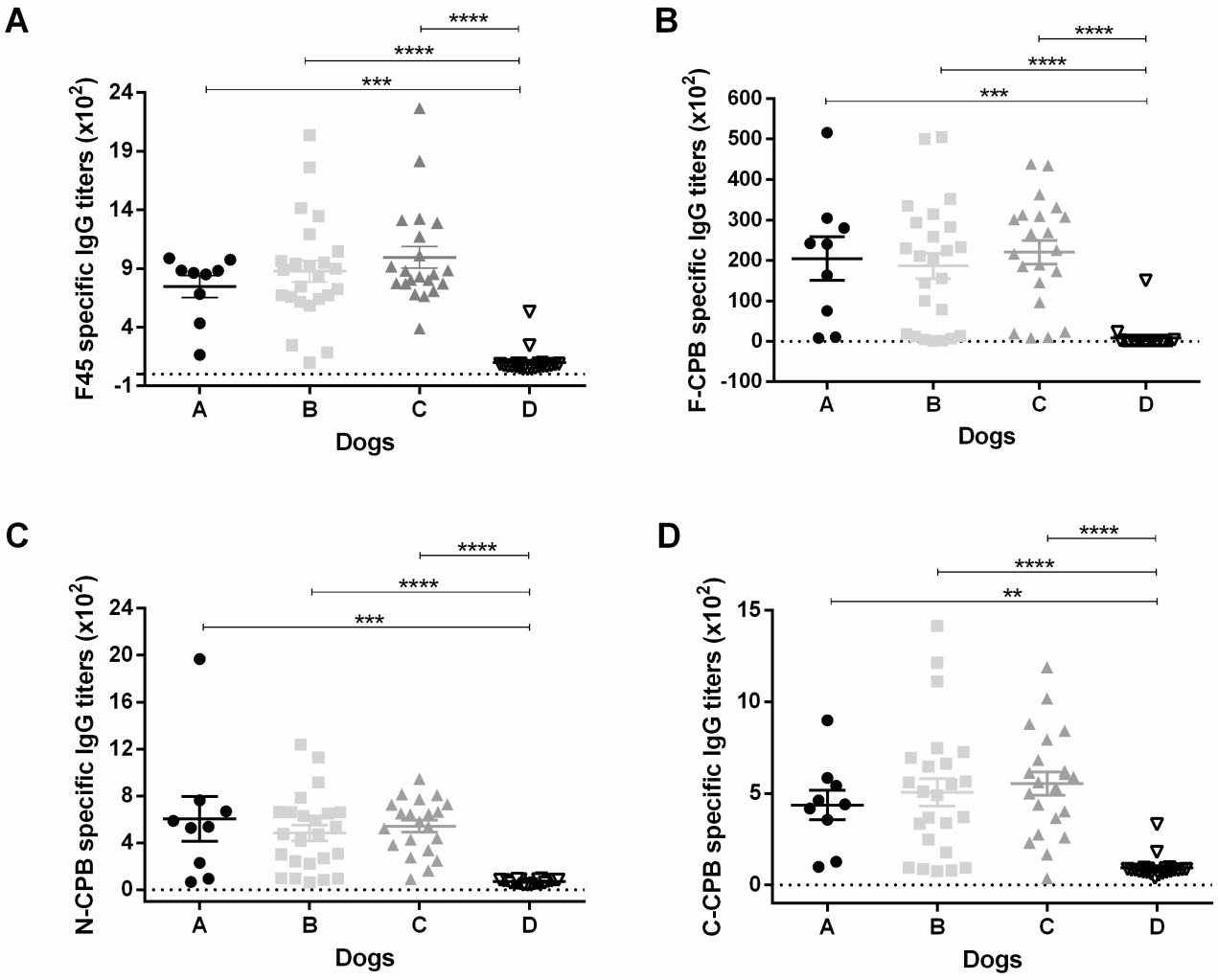
640 Abbreviations: **Se**: sensitivity; **Sp**: specificity; **TP**: true positive; **FP**: false positive; **TN**: true
641 negative; **FN**: false negative; **PPV**: positive predictive values; **NPV**: negative predictive values;
642 **AUC**: area under the curve.

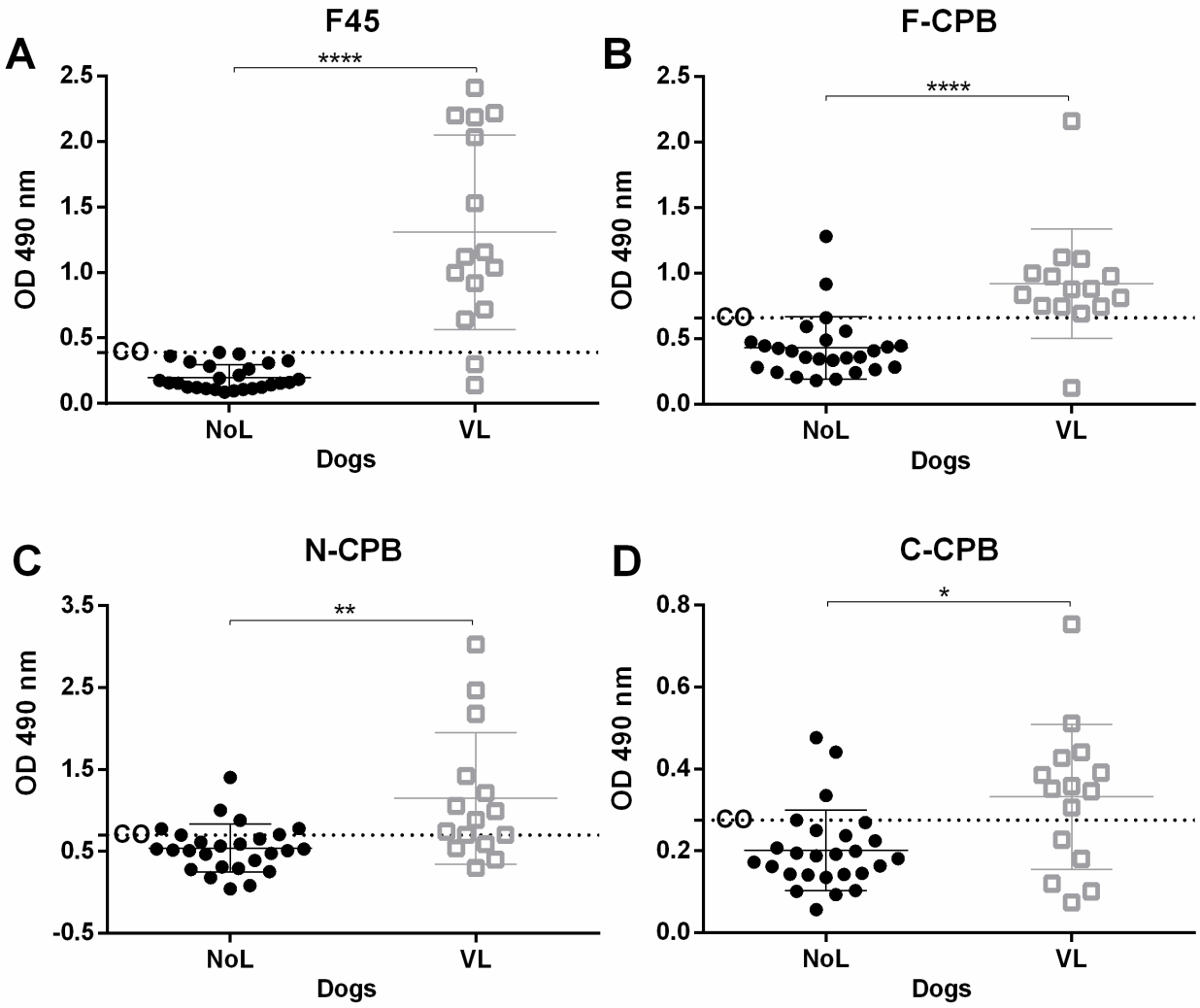
643

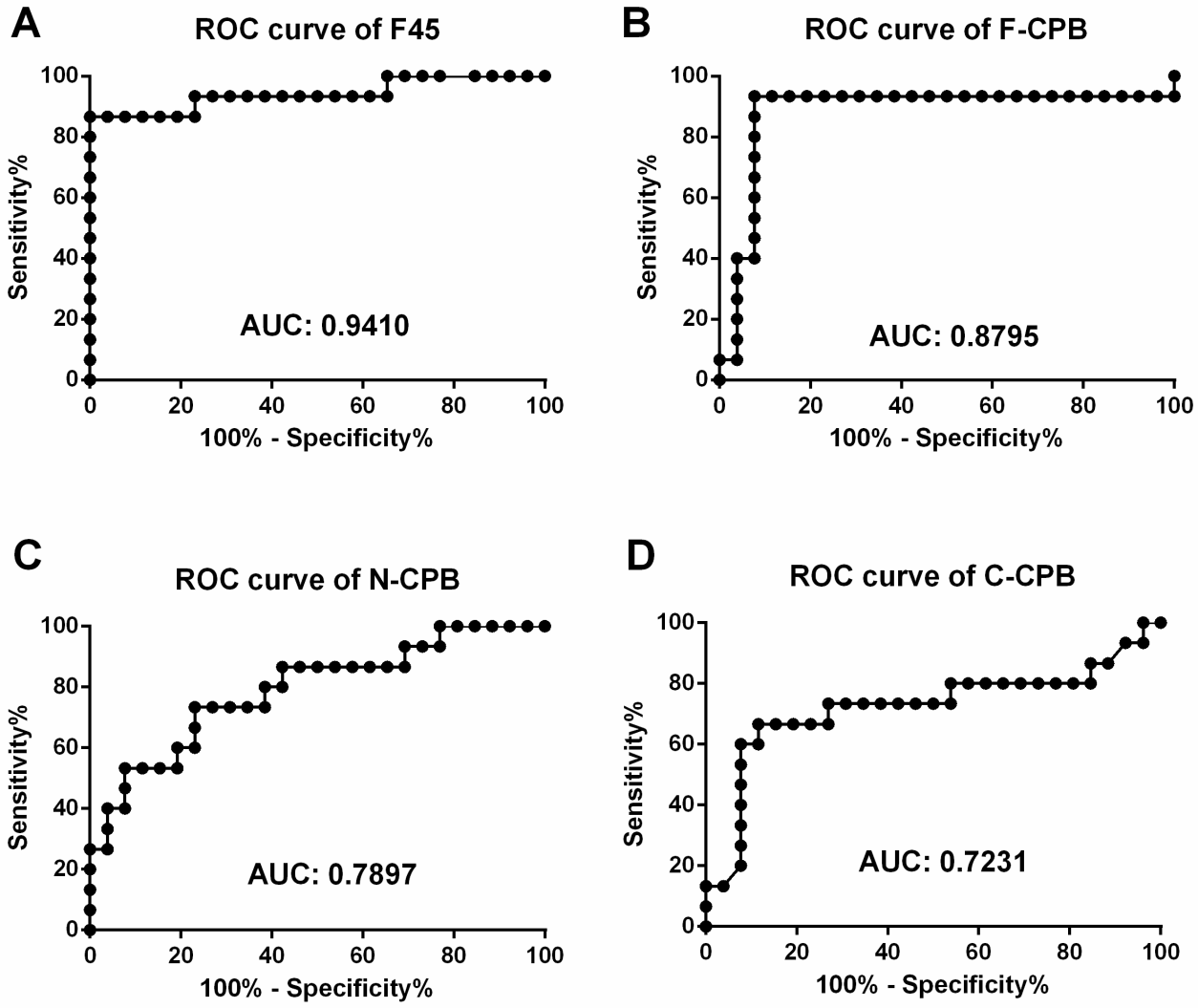












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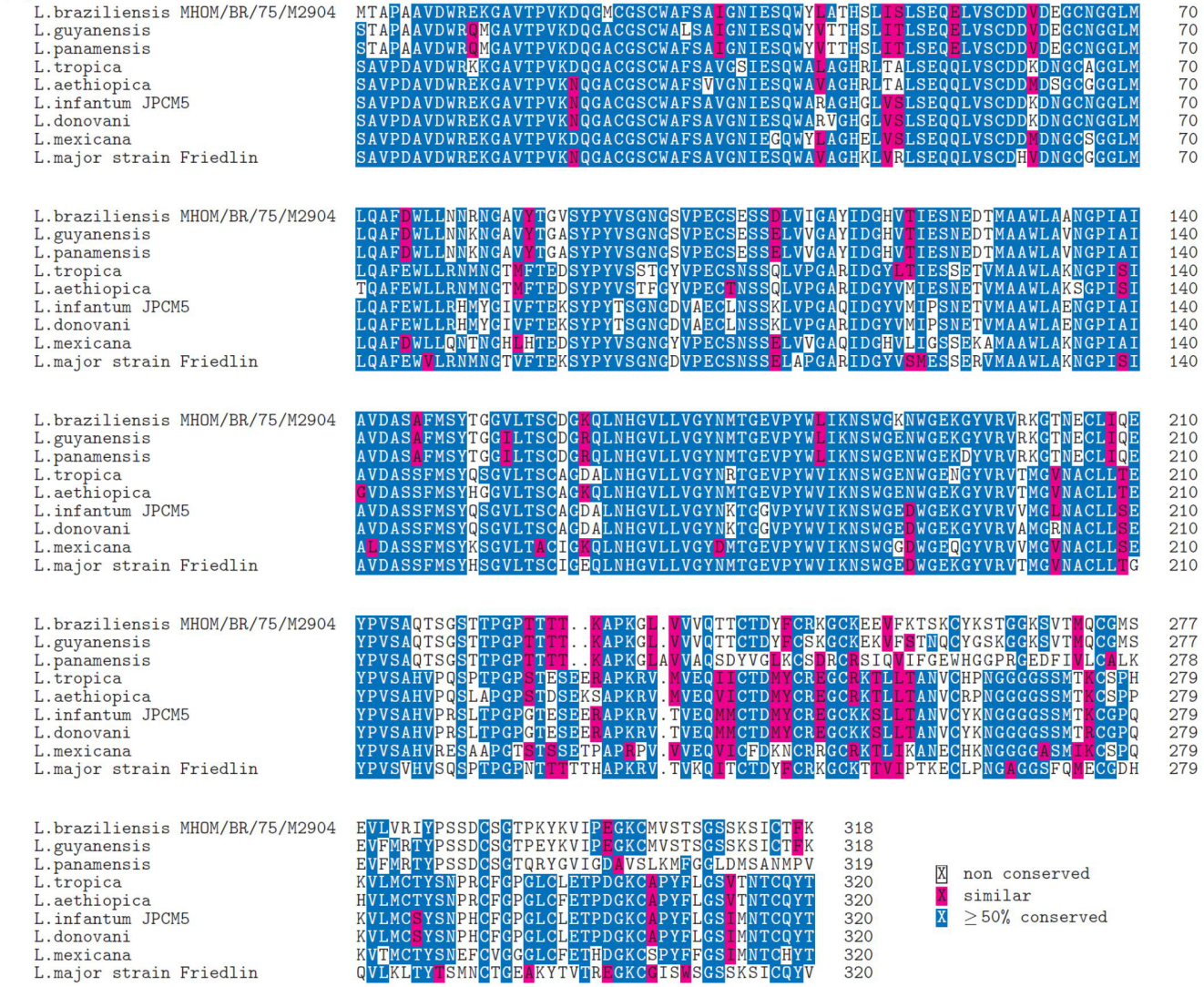

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