ESCOLA BAHIANA DE MEDICINA E SAÚDE PÚBLICA

CURSO BIOMEDICINA

FERNANDA LOPES HABIB

CHRONIC CHAGAS DISEASE IN DOGS: A PHASE II STUDY TO ASSESS THE PERFORMANCE OF FOUR CHIMERIC *Trypanosoma cruzi* RECOMBINANT ANTIGENS IN THE SEROLOGICAL DIAGNOSIS OF DOGS

SALVADOR – BA

2021

FERNANDA LOPES HABIB

CHRONIC CHAGAS DISEASE IN DOGS: A PHASE II STUDY TO ASSESS THE PERFORMANCE OF FOUR CHIMERIC *Trypanosoma cruzi* RECOMBINANT ANTIGENS IN THE SEROLOGICAL DIAGNOSIS OF DOGS.

Trabalho de Conclusão de Curso apresentado à Escola Bahiana de Medicina e Saúde Pública, como parte dos requisitos para obtenção do título de Bacharel em Biomedicina.

Orientador: Prof. Dr. Fred Luciano Neves Santos

SALVADOR – BA

2021

FERNANDA LOPES HABIB

CHRONIC CHAGAS DISEASE IN DOGS: A PHASE II STUDY TO ASSESS THE PERFORMANCE OF FOUR CHIMERIC *Trypanosoma cruzi* RECOMBINANT ANTIGENS IN THE SEROLOGICAL DIAGNOSIS OF DOGS

Este Trabalho de Conclusão de Curso foi julgado adequado à obtenção do grau de Bacharel em Biomedicina e aprovada em sua forma final pelo Curso de Biomedicina da Escola Bahiana de Medicina e Saúde Pública.

Salvador – BA, 31 de outubro de 2022.

Prof. Dr. Fred Luciano Santos Neves

Instituto Gonçalo Muniz, Fundação Oswaldo Cruz

Prof. Dra. Maria Fernanda Rios Grassi

Instituto Gonçalo Muniz, Fundação Oswaldo Cruz

Prof. Dr. Gabriel Nonato Queiroz

Escola Bahiana de Medicina e Saúde Pública

RESUMO

INTRODUÇÃO: Devido ao convívio próximo com humanos e papel na manutenção do ciclo de transmissão doméstica e peridoméstica, os cães são considerados os animais reservatórios/sentinela de maior importância epidemiológica para doença de Chagas (DC). Há uma correlação entre a infecção em cães e a prevalência da doença em humanos. Apesar da sua importância epidemiológica, inexiste no mercado mundial testes sorológicos comerciais para a identificação da infecção nestes animais. Assim, é evidente a necessidade do desenvolvimento de testes comerciais para esta finalidade, os quais poderão ser constituídos por preparações antigênicas contendo antígenos recombinantes quiméricos do *Trypanosoma cruzi*, proporcionando alto desempenho diagnóstico e elevada reprodutibilidade. Em 2019, nosso grupo investigou o potencial diagnóstico de quatro antígenos quiméricos em um estudo de fase I, encontrando elevados valores de sensibilidade e especificidade, o que nos motivou a aprofundar as avaliações e realizar o presente estudo de fase II, o qual empregou maior quantitativo amostral para avaliar o potencial diagnóstico dos quatro antígenos na detecção do *T. cruzi* por meio do ELISA indireto**. OBJETIVO:** O objetivo do estudo foi avaliar o potencial diagnóstico de quatro antígenos recombinantes quiméricos do *Trypanosoma cruzi* (IBMP-8.1, IBMP-8.2, IBMP-8.3 e IBMP-8.4) em imunoensaios para detecção de anticorpos IgG anti-*T. cruzi* em cães na forma crônica da doença de Chagas. **MATERIAL E MÉTODOS:** Os imunoensaios foram otimizados por ***c**heckerboard titration*. No estudo de fase II, o desempenho diagnóstico dos antígenos IBMP foi avaliado utilizando 1.260 amostras caninas. Para análise de reatividade cruzada, foram utilizadas 752 amostras referentes a outras doenças infecto-parasitárias. O desempenho das moléculas quiméricas foi comparado com o desempenho de testes comerciais para humanos adaptado para espécie canina (Gold Elisa Chagas). **RESULTADOS:** Os antígenos IBMP atingiram valores de AUC entre 89,0-97,4%. A acurácia foi entre 87,4-96%. O maior valor de sensibilidade foi atribuído ao IBMP-8.2 (90,3%), enquanto o IBMP-8.1, IBMP-8.3 e IBMP-8.4 atingiram valores de 74,8%, 72,6% e 79,6%, respectivamente. Quanto à especificidade, o maior valor foi observado para o IBMP-8.4 (99,6%), seguido pelo IBMP-8.1, IBMP-8.2 e IBMP-8.3 com 90,6%, 96,5% e 99%, respectivamente. O kit Gold Elisa Chagas apresentou uma sensibilidade de 62,3%, especificidade de 98,6% e acurácia de 89,9%. O menor índice de reatividade cruzada foi observado na IBMP-8.2 (0.9%), sendo esta molécula que mais se aproximou de um teste ideal**.** **CONCLUSÃO:** Diante do alto desempenho das moléculas IBMP, conclui-se que são moléculas promissoras para uso em imunoensaios para diagnosticar a infecção causada pelo *T. cruzi* em cães. O uso combinado dos antígenos é uma alternativa para aumento dos valores de sensibilidade e especificidade em estudos futuros.

Palavras-chave: Doença de Chagas, *Trypanosoma cruzi*, imunodiagnóstico antígenos recombinantes quiméricos.

ABSTRACT

INTRODUCTION: Dogs are considered a reservoir of epidemiological importance for Chagas disease because of their sentinel function, their proximity to humans, and their role in maintaining the transmission cycle in the domestic and peridomestic environment. There is also a correlation with the prevalence of Chagas disease in humans. Despite the epidemiological importance of Chagas disease, the lack of commercial and high-performance diagnostic tests to detect infected dogs is a major constraint that poses a public health risk. Therefore, a strategy to address this limitation is the use of chimeric antigens to achieve better diagnostic performance and good applicability. Our group has previously investigated the diagnostic potential of four chimeric antigens in a phase I study with results similar to those obtained in humans. This study follows the phase I study with an expanded sample size to evaluate the four chimeric antigens in detecting *T. cruzi* infection by indirect ELISA.**OBJECTIVE:** The objective of this study was to evaluate the potential diagnostic performance of chimeric recombinant *T. cruzi* antigens (IBMP-8.1, IBMP-8.2, IBMP-8.3, and IBMP-8.4) in immunoassays for the detection of IgG anti-*T. cruzi* in dogs with the chronic form of Chagas disease. **MATERIALS AND METHODS:** Assays were optimized by checkerboard titration. In the II phase, the diagnostic performance of the IBMPs was evaluated using 1,260 canine serum samples. Cross-reactivity to other infectious diseases was also evaluated in 752 samples. The performance of the chimeric molecules was compared to a commercial human assay adapted to canine species (Gold Elisa Chagas). **RESULTS:** IBMP antigens reached AUC values between 89.0-97.4%. The accuracy was 87.4-96%. The highest sensitivity was attributed to IBMP-8.2 (90.3%), while IBMP-8.1, IBMP-8.3, and IBMP-8.4 achieved 74.8%, 72.6%, and 79.6%, respectively. The highest specificity was observed for IBMP-8.4 (99.6%), followed by IBMP-8.1, IBMP-8.2, and IBMP-8.3 with 90.6%, 96.5%, and 99%, respectively. The Gold Elisa Chagas had a sensitivity of 62.3%, specificity of 98.6%, and accuracy of 89.9%. The

lowest cross-reactivity index was obtained with IBMP-8.2 at 0.9%, and this molecule was the surrogate for an ideal assay. **CONCLUSION:** Because of the good performance of IBMP molecules in the diagnosis of *T. cruzi* infections in dogs, this is a promising tool that, when used in an indirect ELISA, increases immunoassay performance and reduces diagnostic failures mainly due to cross-reactivity. The combined use of these antigens is an alternative to increase sensitivity and specificity values in future studies.

Keywords: Chagas disease, *Trypanosoma cruzi*, immunodiagnostics, recombinant chimeric antigens, dogs.

SUMÁRIO

1 ARTIGO CIENTÍFICO 10

BACKGROUND 12

MATERIAL AND METHODS 14

RESULTS 22

DISCUSSION 35

REFERENCES 40

2 PROPOSTA DE SUBMISSÃO 49

REVISTA 49

REGRAS PARA SUBMISSÃO 49

2 **ARTIGO CIENTÍFICO**

3

4 **Chronic Chagas disease in dogs: a phase II study to assess the performance of**
5 **four chimeric *Trypanosoma cruzi* recombinant antigens in the serological diagnosis of**
6 **dogs**

7

8 Natália Dantas Fontes[1#], Fernanda Lopes Habib[1#], Leonardo Maia Leony[1], Natalia Erdens
9 Maron Freitas[1], Ângelo Antônio Oliveira Silva[1], Filipe Dantas-Torres[2], Kamila Gaudêncio da
10 Silva Sales[2], Andréa Pereira da Costa[3], Thaliane França Costa[3], Lileia Gonçalves Diotaiuti[4,5],
11 Carlota Josefovcz Belisario[4], Cláudia Moura de Melo[6,7], Antônia Cláudia Jácome da Câmara[8],
12 Vicente Toscano de Araújo Neto[8], Leila Denise Alves Ferreira Amorim[9], Déborah Bittencourt
13 Mothé Fraga[10], Paola Alejandra Fiorani Celedon[11], Nilson Ivo Tonin Zanchin[12], Fred Luciano
14 Neves Santos[1,5]

15

16 [1]Advanced Public Health Laboratory, Gonçalo Moniz Institute, Oswaldo Cruz Foundation,
17 Salvador, Bahia, Brazil
18 [2]Laboratory of Immunoparasitology, Department of Immunology, Aggeu Magalhães Institute,
19 Oswaldo Cruz Foundation, Recife, Pernambuco, Brazil
20 [3]Veterinary Medicine Studies Unit, State University of Maranhão, São Luís, Maranhão, Brazil
21 [4]Triatomines and Epidemiology of Chagas Disease, René Rachou Institute, Oswaldo Cruz
22 Foundation, Belo Horizonte, Minas Gerais, Brazil
23 [5]Integrated Translational Program in Chagas disease from Fiocruz – Fio-Chagas, Rio de
24 Janeiro, Rio de Janeiro, Brazil
25 [6]Postgraduate Program in Health and Environment, Tiradentes University, Aracaju, Sergipe,
26 Brazil
27 [7]Institute of Technology and Research, Aracaju, Sergipe, Brazil
28 [8]Department of Clinical and Toxicological Analysis, Health Sciences Center, Federal
29 University of Rio Grande do Norte, Natal, Rio Grande do Norte, Brazil
30 [9]Department of Statistics, Institute of Mathematics and Statistics, Federal University of Bahia,
31 Salvador, Brazil
32 [10]Laboratory of Parasite-Host Interaction and Epidemiology, Gonçalo Moniz Institute, Oswaldo
33 Cruz Foundation, Salvador, Bahia, Brazil
34 [11]Laboratory for Applied Science and Technology in Health, Carlos Institute, Oswaldo Cruz
35 Foundation, Curitiba, Paraná, Brazil

36 [12]Structural Biology Protein Engineering, Carlos Institute, Oswaldo Cruz Foundation, Curitiba,
37 Paraná Brazil
38
39 [#]Natália D. Fontes and Fernanda L. Habib contributed equally to this work.
40
41 *Corresonding atuhor: Fred Luciano Neves Santos, Advanced Public Health Laboratory,
42 Gonçalo Moniz Institute, Waldemar Falcão Street, 121, Candeal, 40296-710, Salvador, Bahia,
43 Brazil; e-mail: fred.santos@fiocruz.br.
44
45
46
47
48
49
50
51
52
53
54
55
56
57

58 **BACKGROUND**

59 Chagas disease (CD) is an infectoparasitic neglected disease caused by the pathogen
60 *Trypanosoma cruzi*, a hemoflagellate protozoan that can infect multiple species. The disease is
61 endemic in 21 Latin American countries, where an estimated 5.7-6 million people are infected
62 and 10,000 deaths occur annually [1]. In endemic countries, the parasite is transmitted primarily
63 through the feces/urine of infected bloodsucking triatomines (family Reduviidae). Due to the
64 persistent presence of the vector, 70 million people in this region are at risk of contracting the
65 disease [1]. Transmission can also occur through blood transfusion, from mother-to-child, and
66 orally through ingestion of contaminated food and beverages [2]. In recent decades, CD is no
67 longer confined to the Americas. Increasing migration flows and shifts of infected individuals
68 to nonendemic countries have contributed to the worldwide spread of the disease, especially in
69 some European, North American, Asian, and Ocenian countries, making it a global public
70 health problem [3,4].

71 In addition to humans, *T. cruzi* infects over 100 species of domestic and wild mammals.
72 The presence of domestic animals in the household is a risk factor for human infection because
73 they attract triatomines as a blood meal. Indeed, triatomines typically feed on chickens, pigs,
74 dogs, and cats. Similar to humans, animals can also become infected via transfusion, vertical,
75 and oral routes transmamária [5,6]. Among domestic animals, dogs play an important role in
76 maintaining the domestic and peridomestic cycle of CD, as these animals are susceptible to
77 various forms of infection [7] and are considered the main host, sentinel, and reservoir of *T.*
78 *cruzi* in endemic countries [5,8]. From an epidemiological perspective, infected animals may
79 pose a risk to human health, as confirmed by the correlation of seropositivity between species
80 and close contact. It is estimated that adult humans have up to a 6-fold increased risk of
81 infection, while children living in a household with seropositive dogs have a 17-fold increased
82 risk of becoming infected [9]. In addition, dogs have higher parasitemia, can attract vectors,
83 and are highly susceptible to infection [10–12]. Dogs are also considered a biological barrier

84 that prevents human infection. For this reason, these animals are considered sentinels because
85 their infection precedes that of humans and they can indicate an active parasite transmission
86 cycle and thus human susceptibility to infection [13]. Apart from their epidemiological
87 importance, dogs may develop similar clinical signs and physiopathogenesis as humans. They
88 are used as an experimental model for CD [14–16] and may also exhibit morphofunctional
89 changes in the cardiac and digestive systems. In severe cases, sudden death may occur [17].
90 Despite its veterinary and epidemiological importance, there are no commercially available
91 tests to detect Chagas disease in dogs.

92 Laboratory diagnosis of CD infection depends on the phase of the disease. In the acute
93 phase, which is short and usually asymptomatic, the parasites are easily detected
94 microscopically in the blood of infected animals. In the chronic phase, due to low and
95 intermittent parasitemia and high levels of specific anti-*T. cruzi* antibodies, the diagnosis
96 requires the use of antibody-antigen methods, including indirect immunofluorescence (IIF),
97 rapid diagnostic tests (RTDs), and enzymatic immunoassays (ELISA). Despite several
98 available methods, operational and technical issues result irregular performance of serological
99 tests. Reasons for this include the high genetic and phenotypic intraspecific diversity of *T. cruzi*
100 [18], the choice of antigens used to sensitize the solid phase of immunoassays [19], the variable
101 prevalence of the disease [20,21], the variable immune responses in *T. cruzi* infected individuals
102 [22], and the occurrence of cross-reactivity, particularly with *Leishmania* spp. [23,24].
103 Accordingly, the World Health Organization (WHO) recommends the simultaneous use of two
104 different serological tests for diagnosis in humans CD. Because no commercial tests are
105 available and there is no recommendation for diagnosis canine CD, studies of CD in dogs have
106 used in-house or modified commercial tests for humans [25–29], resulting in underreporting
107 and delays in confirming clinical suspicion.

108 In order to improve the diagnosis of chronic infections in humans CD, chimeric
109 recombinant proteins consisting of repeating and conserved epitopes of several proteins of the

110 prasite have been used in several diagnostic platforms as antigens for the detection of anti-*T.*
111 *cruzi* antibodies [30–33]. Among them, the chimeric IBMP proteins (IBMP-8.1, IBMP-8.2,
112 IBMP-8.3, and IBMP-8.4) have been extensively studied for human diagnosis [24,34–40], both
113 in endemic and non-endemic areas in South America [41], as well as in Barcelona (Spain) [42],
114 where high performance was observed, and for human vaccines [43]. Moreover, these antigens
115 performed remarkably well when used in a phase I study for serodiagnosis of chronic CD in
116 naturally and experimentally infected dog populations from different Brazilian states [40,44–
117 46]. Considering the high diagnostic performance of chimeric IBMP antigens in the diagnosis
118 of CD in dogs and the possibility of obtaining an accurate test that supports an assertive
119 therapeutic protocol, better monitoring of clinical cases, and improvement of the quality of life
120 of these affected patients, the present study, a phase II study, sought to evaluate the performance
121 of these antigens for the detection of anti-*T. cruzi* in serum samples of dogs from different
122 Brazilian endemic areas.

123

124 **MATERIAL AND METHODS**

125

126 **Ethical considerations.** The present study was approved by the Ethics Committee for
127 the Use of Animals at the Instituto Gonçalo Moniz - Fiocruz/ BA, Salvador, Bahia-Brazil
128 (protocol number 002/2017).

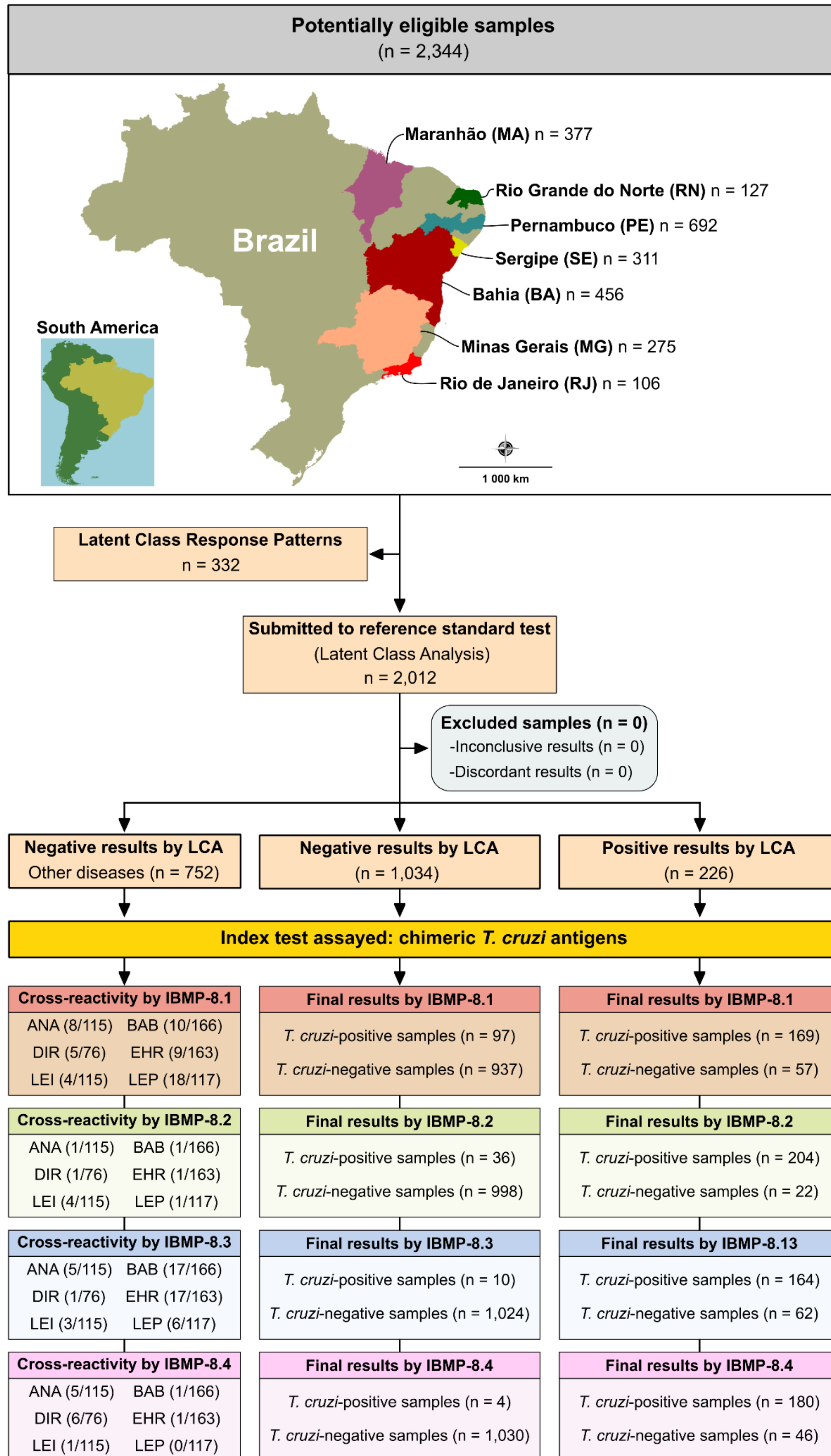
129

130 **Synthesis of chimeric antigens**. The chimeric antigens used in this study were prepared
131 as described by Santos et al. [34]. Briefly, nucleotide sequences were obtained by cloning into
132 the pET28a vector and subsequent expression in *Escherichia coli* BL21-Star DE3 cultured in
133 Luria-Bertani medium (LB) supplemented with 0.5 M isopropyl-β-D-1-thiogalactopyranoside
134 (IPTG). Bacteria were then lysed by microfluidification and purified by affinity and ion

135 exchange chromatography. Finally, chimeric antigens were quantified by fluorimetry (Qubit
136 2.0; Invitrogen Technologies, Carlsbad-CA, USA).

137

138 **Sample collection.** The sample size required to conduct this study was statistically
139 determined using the statistical program OpenEpi [47], considering a sensitivity of 99%, a
140 specificity of 99%, an absolute error of 1.5%, and a confidence level of 95%. Based on these
141 parameters, the minimum quantity required to perform this study was 169 sera from *T. cruzi*-
142 positive dogs and 169 sera from *T. cruzi*-negative dogs. A total of 2,344 serum samples from
143 dogs were included in the sample panel to evaluate the diagnostic performance of all four IBMP
144 molecules by ELISA (Figure 1). Sera were obtained from different Brazilian states: Bahia (n =
145 322), Maranhão (n = 377), Minas Gerais (n = 267), Rio de Janeiro (n = 106), Sergipe (n = 311),
146 Rio Grande do Norte (n = 127), and Pernambuco (n = 82). In addition, 752 samples from dogs
147 with other infectious and parasitic diseases of veterinary interest, previously defined by their
148 serological or parasitological diagnoses, were included in the present panel sample to evaluate
149 cross-reactivity. Unrelated diseases studied included: anaplasmosis (n = 115), babesiosis (n =
150 166), dirofilariasis (n = 76), ehrlichiosis (n = 163), visceral leishmaniasis (n = 115), and
151 leptospirosis (n = 117). All serum samples were tested by indirect ELISA using the four
152 chimeric molecules as antigens (IBMP-ELISA), and each result obtained was analyzed by latent
153 class (LCA). Samples were previously labeled with unique codes to ensure blinded analysis by
154 the operator.



155

156 **Figure 1.** Flowchart depicting study design in accordance with Standards for Reporting of
157 Diagnostic Accuracy Studies (STARD) guidelines. The digital map was freely obtained from
158 the Brazilian Institute of Geography and Statistics (IBGE) cartographic database in shapefile

159 (.shp) format and then reformatted and analyzed using QGIS version 3.10 (Geographic
160 Information System, Open Source Geospatial Foundation Project. http://qgis.osgeo.org).

161 **IBMP-ELISA**. Immunoassays were performed as described by Leony et al [44]. Flat-
162 bottom 96-well polystyrene microtiter plates (Nunc Maxisorp®, USA) were coated with one of
163 the chimeric IBMP antigens at concentrations of 25 ng per well in 100 µl coating buffer (0.05
164 M carbonate/bicarbonate buffer solution, pH 9.6). Sensitization, stabilization, and blocking
165 were performed simultaneously with a synthetic buffer (batch 130703; WellChampion; Kem-
166 En-Tec Diagnostics A/S, Taastrup, Denmark) according to the manufacturer's instructions.
167 Serum samples were added to the coated wells diluted 1:100 in 0.05 M phosphate-buffered
168 saline (PBS; pH 7.4), and the microtiter plates were incubated at 37°C for 60 minutes.
169 Subsequently, the wells were washed five times with 250 µl of wash solution (PBS-Tween; 10
170 mM sodium phosphate, 150 mM sodium chloride, and 0.5% Tween-20, pH 7.4) to remove non-
171 adsorbed material and incubated again at 37°C for 30 minutes with 100 µl of HRP-conjugated
172 anti-dog globulin IgG (Bio-Manguinhos, Fiocruz, Rio de Janeiro-RJ, Brazil) diluted 1:20,000
173 (IBMP-8.3) and 1:40,000 (IBMP-8.1, IBMP-8.2, and IBMP-8.4) in PBS. After another wash
174 cycle, 100 µl of chromogenic TBM substrate (Kem-En-Tec Diagnostics A/S, Taastrup,
175 Denmark) was added to each well, and microtiter plates were incubated for 10 minutes at room
176 temperature in the dark. The colorimetric reactions were interrupted by adding 50 µl of 0.3 M
177 H_2SO_4 to each well. Optical density was determined in a microplate reader with a 450 nm filter
178 (SPECTRAmax 340PC®; Molecular Devices, San Jose- CA, USA) and background values were
179 subtracted from the measurement experiments.

180

181 **Latent Class Analysis (LCA).** Latent class analysis was performed using a statistical
182 model to define a latent variable and then use it as a gold standard. To define the latent variable
183 that can accurately identify *T. cruzi* infection, four indicators were defined representing the

184 chimeric antigens IBMP-8.1, IBMP-8.2, IBMP-8.3, and IBMP-8.4. Sera were divided into two
185 categories: "negative" and "positive". Latent class analysis is a multivariate statistical approach
186 based on categorical indicators expressing a categorical construct/latent variable. Latent classes
187 were characterized based on the response patterns of negative/positive outcomes of the four
188 chimeric antigens (Figure 2) and the conditional probabilities, i.e., the probability of having a
189 given outcome (negative/positive) for a chimeric antigen relative to an individual diagnosis
190 (negative/positive). The present LCA uses maximum likelihood estimation. The following
191 criteria were used to evaluate the LCA model: AIC (Akaike information criteria), BIC
192 (Bayesian information criteria), and entropy. For AIC and BIC, a lower value is better, while
193 for entropy, a value close to one indicates good classification quality. Conditional independence
194 was tested using the bivariate residuals. All analyzes were performed using Mplus v5.2 software
195 (Muthe' n & Muthe' n, Los Angeles-CA, USA). Considering the entire sample panel,
196 approximately 16% (332/2,344; 47 samples for each of the states: BA, MA, MG, RJ, RN, PE;
197 and 50 samples from SE) were randomly selected to define the gold standard for determining
198 *T. cruzi* infection at LCA. The other part of the sample was used to obtain estimates of
199 diagnostic performance (n = 1,260) for each chimeric antigen using the previously defined
200 latent class response patterns, with a corresponding CI of 95%, and cross-reaction analysis (n
201 = 752). The area under the receiver operating characteristic curves was used to estimate
202 diagnostic accuracy, i.e., to describe the ability of the chimeric protein assay to discriminate
203 between healthy and infected populations. These analyzes were performed using the diagt
204 function of STATA v12 software (StataCorp., College Station-TX, USA).

205

ELISA		P1	P2				P3						P4				P5
IBMP-8.1		-	+	-	-	-	+	+	+	-	-	-	+	+	+	-	+
IBMP-8.2		-	-	+	-	-	+	-	-	+	+	-	+	+	-	+	+
IBMP-8.3		-	-	-	+	-	-	+	-	+	-	+	+	-	+	+	+
IBMP-8.4		-	-	-	-	+	-	-	+	-	+	+	-	+	+	+	+
LCS		Neg	Neg	Neg	Neg	Neg	Pos	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
PP %		0.0	1.6	30.9	1.9	5.0	94.9	44.8	68.7	95.8	98.4	73.0	99.9	100	99.1	100	100
Phase II study																	
Chagas disease	n	888	96	36	9	4	29	1	1	8	20	9	9	12	12	20	106
Cross reactivity																	
Anaplasmosis	n	97	7	1	4	5	0	1	0	0	0	0	0	0	0	0	0
Babesiosis	n	140	7	1	14	1	0	3	0	0	0	0	0	0	0	0	0
Dirofilariosis	n	63	5	1	1	6	0	0	0	0	0	0	0	0	0	0	0
Ehrlichiosis	n	138	6	1	14	1	0	3	0	0	0	0	0	0	0	0	0
Leishmaniosis	n	104	3	4	2	1	0	1	0	0	0	0	0	0	0	0	0
Leptospirosis	n	92	18	1	6	0	0	0	0	0	0	0	0	0	0	0	0
Total	n	634	46	9	41	14	0	8	0	0	0	0	0	0	0	0	0
TOTAL																	
Total panel	N	**1,522**	**142**	**45**	**50**	**18**	**29**	**9**	**1**	**8**	**20**	**9**	**9**	**12**	**12**	**20**	**106**

Figure 2. Latent class response patterns, posteriori probability and classification of canine serum samples assayed with four chimeric *Trypanosoma cruzi* antigens for accurate diagnosis of Chagas disease. Samples are categorized P1-P5 according to the response pattern of the chimeric assay. Blue and red squares represent negative and positive results, respectively, for a single IBMP chimeric antigen assay. LCS = latent class status; N = number of samples; NEG = negative; POS = positive; PP = posteriori probability.

Comparison of IBMP performance with commercial ELISA tests adapted for dogs. A total of 154 *T. cruzi*-positive and 490 *T. cruzi*-negative samples were randomly selected to compare the performance and strength of agreement between IBMP chimeric antigen assays and commercial human *T. cruzi* ELISA kits adapted for diagnosis in dogs. The selection of

219 these kits was based on the active registration of the kits for commercial use in Brazil and their
220 previous use for the diagnosis of DC in dogs, as described in the scientific literature.
221 Accordingly, only the following commercial Chagas disease-specific enzyme immunoassay
222 was selected: Gold ELISA Chagas (REM Indústria e Comércio Ltda, São Paulo- SP, Brazil),
223 which uses both recombinant antigens and lysates of epimastigotes of *T. cruzi* strains circulating
224 in Brazil. This kit was adapted in 2019 by Leony et al [44]. for the diagnosis of CD in dogs.
225 Immunoassays were performed according to the manufacturer's recommendations, with some
226 modifications. In brief, serum samples were diluted 1:800 in Sample Dilution Buffer and 100
227 µl were added to each well. After incubation at 37 °C for 30 minutes, the microtiter plates were
228 washed with Wahsing Buffer to remove unbound antibodies. HRP-conjugated goat anti-dog
229 IgG (Bio-Manguinhos, FIOCRUZ, Rio de Janeiro-RJ, Brazil) was diluted 1:40,000 in PBS-T
230 and 100 µl was added to each well. Then, the microtiter plates were incubated at 37 °C for 30
231 minutes. The wells were washed again and the immune complexes were detected by adding 100
232 µl of TMB Solution. After another incubation cycle of 10 minutes at room temperature in the
233 dark, the reaction was stopped by adding 50 µl Stop Solution and the absorbance was measured
234 at 450 nm using a spectrophotometer (SPECTRAmax 340PC®, Molecular Devices, San Jose-
235 CA, USA).

236

237 **Statistical analysis.** Data were coded and entered using computer graphics software
238 (GraphPad Prism v8 Software Inc, San Diego- CA, USA). Descriptive data were presented as
239 arithmetic and geometric means ± standard deviation and coefficient of variation. The Shapiro-
240 Wilk test followed by Student's t- test was used to test the normality of the data. If the assumed
241 homogeneity could not be confirmed, Wilcoxon's signed rank test was used. All analyzes were
242 two-tailed, and p values of less than 5% were considered significant (p value < 0.05). Cut-off
243 values were established by determining the largest area under the Receiver Operating
244 Characteristic (ROC) curve (AUC). The AUC values were also calculated to assess the global

accuracy of the IBMP-ELISA, which was classified as low (0.51-0.61), moderate (0.62-0.81), elevated (0.82-0.99), or outstanding (1.0) [48]. All results were expressed by plotting the values in an index format, which represents the ratio between the OD of a given sample and the cut-off OD for each microplate. This index is called the reactivity index (RI) and all results < 1.00 were considered negative. If the RI value of a sample was $1.0 \pm 10\%$, it was classified as indeterminate (or in the gray zone); these samples were considered inconclusive. ELISA performance was evaluated using a dichotomous approach and compared in terms of sensitivity (Sen), specificity (Spe), accuracy (Acc), likelihood ratios (LR), diagnostic odds ratio (DOR), and pretest and posttest probability. To better assess the diagnostic performance of the four IBMP chimeras, multiple testing (serial and parallel approaches) was applied to individual test characteristics. Multiple tests can be ordered simultaneously (parallel tests), in which case a positive result in any of the tests is evidence of disease, or they can be ordered sequentially (serial tests), as new tests are requested depending on the result of the previous test. In this case, all results must be positive to establish a diagnosis of disease. [49]. A 95% confidence interval (95% CI) was calculated to account for the precision of the proportion estimates. The strength of agreement between the latent class analysis and the ELISA tests was determined by Cohen's kappa (κ) analysis, which was interpreted as follows: poor ($\kappa \leq 0$), slight ($0 < \kappa \leq 0.20$), fair ($0.21 < \kappa \leq 0.40$), moderate ($0.41 < \kappa \leq 0.60$), substantial ($0.61 < \kappa \leq 0.80$) and near perfect agreement ($0.81 < \kappa \leq 1.0$) [50]. A flowchart (Figure 1) and checklist (Table S1) were prepared according to STARD (Standards for Reporting of Diagnostic Accuracy Studies) guidelines [51].

269 **RESULTS**

270 **Latent class analysis.** A total of 2,344 samples were included in the present study. Of
271 these, approximately 16% (332/2,344) were randomly selected to estimate response patterns
272 and accuracy at LCA using the four chimeric *T. cruzi* antigens. The probability that each
273 chimeric antigen accurately predicted positivity in *T. cruzi*-positive samples was 79.1% for
274 IBMP-8.1, 88.1% for IBMP-8.2, 73.4% for IBMP-8.3, and 73.2% for IBMP-8.4. Conversely,
275 the probability of a given *T. cruzi*-negative sample being classified as *T. cruzi*-positive was
276 estimated to be 8.3% for IBMP-8.1, 0.6% for IBMP-8.2, 5.1 for IBMP-8.3, and 1.9 for IBMP-
277 8.4. Accordingly, an entropy value of 0.949 was calculated, indicating a clear delineation
278 among the latent class response patterns. Figure 2 shows the latent class response patterns
279 ordered by diagnostic results for *T. cruzi*-positive and *T. cruzi*-negative samples tested with the
280 four chimeric IBMP antigens. Latent class response patterns were classified according to the
281 number of positive assays: P1 (100% negative results), P2 (75% negative results), P3 (50%
282 negative results), P4 (25% negative results), and P5 (no negative results). Despite variations in
283 the number of samples classified in each pattern, the highest frequencies were observed in the
284 P1 (n = 888), P2 (n = 145), and P5 (n = 106) categories. Samples were classified as *T. cruzi*-
285 positive if at least two chimeric antigens were positive (P3-P5), with posteriori probability (PP)
286 > 68%. However, if a given sample was positive for IBMP-8.1 + IBMP-8.3, it was classified as
287 *T. cruzi*-negative because PP less than 50% (PP = 44.8%). Similarly, samples were classified
288 as negative when no or only one IBMP tested positive (P1 and P2), with PP < 31%.

289

290 **Individual IBMP-ELISA performance.** Sera from 1,260 dogs were tested for *T. cruzi*
291 infection using LCA to evaluate the individual diagnostic performance of four chimeric
292 recombinant IBMP antigens. LCA classified 1,034 (82.1%) samples as negative, while 226
293 (17.9%) samples were predicted to be positive for *T. cruzi* antibody. Most samples classified as
294 negative by LCA were also negative for all four chimeric antigens by ELISA (85.9%;

295 888/1,034) (Figure 2). Negative samples by LCA with positive results for only one antigen were
296 observed in 145 (14.0%; 145/1,034) samples: 96 IBMP-8.1, 36 for IBMP-8.2, nine for IBMP-
297 8.3, and four for IBMP-8.4. The a posteriori probability that these samples were positive is less
298 than 31%, indicating that they were correctly classified as negative (Figure 2). Only one
299 negative sample was positive for both IBMP-8.1 and IBMP-8.3, and the a posteriori probability
300 that this sample was positive is less than 45%. Regarding samples predicted to be positive by
301 LCA, 106 of 226 (46.9%) samples were positive for all IBMP antigens, whereas 67 of 226
302 samples showed positivity for two sets of antigens: 29 for IBMP-8.1 + IBMP-8.2, one for
303 IBMP-8.1 + IBMP-8.4, eight for IBMP-8.2 + IBMP-8.3, 20 for IBMP-8.2 + IBMP-8.4, and
304 nine for IBMP-8.3 + IBMP-8.4. The probability of these samples being positive is greater than
305 68.7%, indicating a high probability of being correctly classified as positive. Fifty-three
306 samples were found to be positive for three antigens: nine for IBMP-8.1 + IBMP-8.2 + IBMP-
307 8.3, twelve for each set of IBMP-8.1 + IBMP-8.2 + IBMP-8.4 and IBMP-8.1 + IBMP-8.3 +
308 IBMP-8.4, and 20 for IBMP-8.2 + IBMP-8.3 + IBMP-8.4, with a posteriori probability of these
309 samples being positive greater than 99.0% (Figure 2).

310 The results of the latent class status determined by the response patterns were used as a
311 gold standard to obtain a reliable estimate of the performance of each chimeric assay. AUC
312 analysis yielded values ranging from 0.89 for IBMP-8.1 to 0.97 for IBMP-8.2, IBMP-8.3, and
313 IBMP-8.4, indicating elevated overall capacity of all four IBMP chimeric antigens to correctly
314 detect positivity and negativity in serum samples. Because of the lack of overlap of the 95%
315 CIs, the AUC value determined for IBMP-8.1 was significantly lower than for the other
316 molecules. With respect to *T. cruzi*-positive sera, IBMP-8.2 produced the highest IgG levels
317 (RI = 1.51) and the lowest level was observed for IBMP-8.1 (RI = 1.27), with a significant
318 difference between them. No significant differences were observed between the RIs of IBMP-
319 8.3 (RI = 1.37) and IBMP-8.4 (RI = 1.35). Of 226 *T. cruzi*-positive samples, IBMP-8.2 showed
320 a sensitivity of 90.3%, with 22 cases classified as false-negative; of these samples, 9 were also

321 classified as false-negative for IBMP-8.1 and one for IBMP-8.3. A higher number of false-
322 negative results were observed for IBMP-8.1 (57 cases), IBMP-8.3 (62 cases), and IBMP-8.4
323 (46 cases), with corresponding sensitivity values of 74.8%, 72.6%, and 79.6%, respectively. No
324 statistically significant differences were observed in the sensitivity of IBMP-8.1, IBMP-8.3,
325 and IBMP-8.4 proteins. However, IBMP-8.2 was more sensitive compared with the other
326 antigens, as there was no 95% CI overlap. In the *T. cruzi*-negative samples, IBMP-8.3 and
327 IBMP-8.4 chimeras showed specificity values ≥ 99.0%, whereas IBMP-8.1 and IBMP-8.2
328 produced a higher number of false positives with corresponding specificity values of 90.6% (97
329 false positives) and 96.5% (36 false positives), respectively. IBMP-8.2 (RI = 0.37), IBMP-8.3
330 (RI = 0.34), and IBMP-8.4 (RI = 0.33) produced the lowest IgG levels without differing among
331 themselves. Conversely, IBMP-8.1 produced the highest level (RI = 0.44) with a significant
332 difference compared to the other antigens (Figure 3).

333 Considering RI values of 1.0 ± 0.10 as the gray zone interval for inconclusive results,
334 we observed that only eight *T. cruzi*-negative samples (0.8%; 8/1,034) fell into the inconclusive
335 zone when tested with the IBMP-8.3 chimeric priotein, whereas 16 samples (1.6%; 16/1,034),
336 53 (5.1%; 53/1,034), and 59 (5.7%; 59/1,034) fell into the inconclusive zone when tested with
337 IBMP-8.4, IBMP-8.1, and IBMP-8.2, respectively. Among the *T. cruzi*-positive samples, we
338 observed the following number of samples in the gray zone: 19 (8.4%; 19/226) tested with
339 IBMP-8.2, 29 (12.8%; 29/226) tested with IBMP-8.1, 42 (18.6%) tested with IBMP-8.4, and
340 43 (19%; 43/226) tested with IBMP-8.3. Overall analysis showed that 4.05% (51/1,260) of
341 samples tested with IBMP-8.3, 4.60% (58/1,060) of samples tested with IBMP-8.4, 6.19%
342 (78/1,260) of samples tested with IBMP-8.2, and 6.518% (82/1,26) of samples tested with
343 IBMP-8.1 had RI values that were within the gray zone.

344 Assays with IBMP antigens exhibited an accuracy of 96.0% for IBMP-8.4, 96.5% for
345 IBMP-8.2, and 94.3% for IBMP-8.3. Due to the high number of both false negative and false
346 positive results in samples assayed with IBMP-8.1, the accuracy of this molecule was

347 significantly lower compared to the others (87.8%). As shown in Figure 3, IBMP-8.4 presented
348 the best DOR (DOR ~ 1,008), which as calculated based on the likelihood ratios. This analysis
349 revealed values of 270.9 for IBMP-8.3, 257.1 for IBMP-8.2 and 28.6 for IBMP-8.1. Cohen's
350 Kappa index revealed substantial agreement (κ = 0.61 for IBMP-8.1, and κ = 0.79 for IBMP-
351 8.3) and almost perfect (κ = 0.85 for IBMP-8.2, and κ = 0.85 for IBMP-8.4) with LCA. Among
352 the chimeric proteins tested, IBMP-8.4 presented the best performance, as noted by the
353 parameters obtained upon ROC analysis, especially regarding the extremely high diagnostic
354 odds ratio shown by this chimera. Conversely, IBMP-8.1 presented the lowest performance. It
355 is important to mention that IBMP-8.2 presented the highest sensibility, while IBMP-8.3 and
356 IBMP-8.4 presented the best specificity.

357

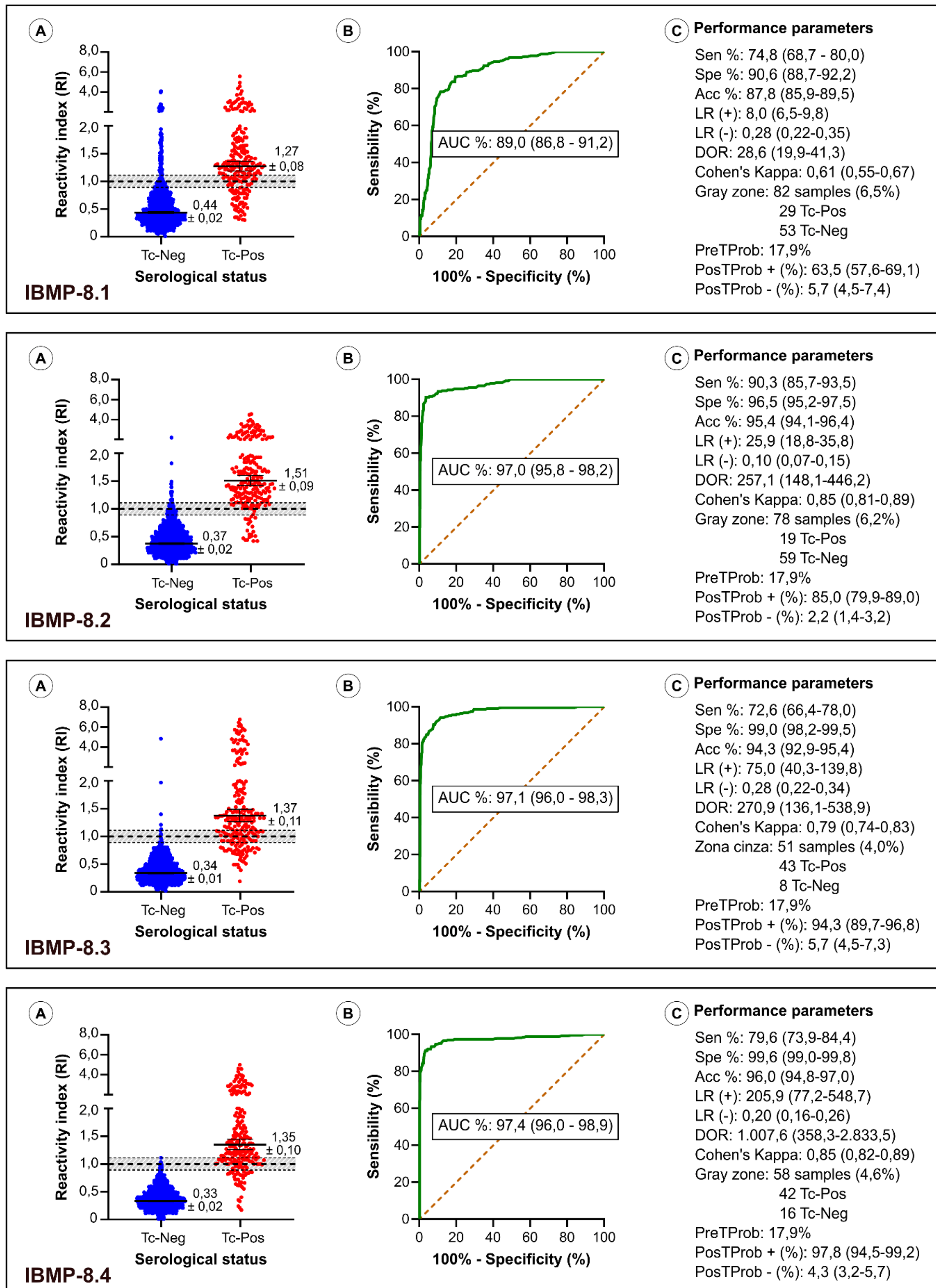
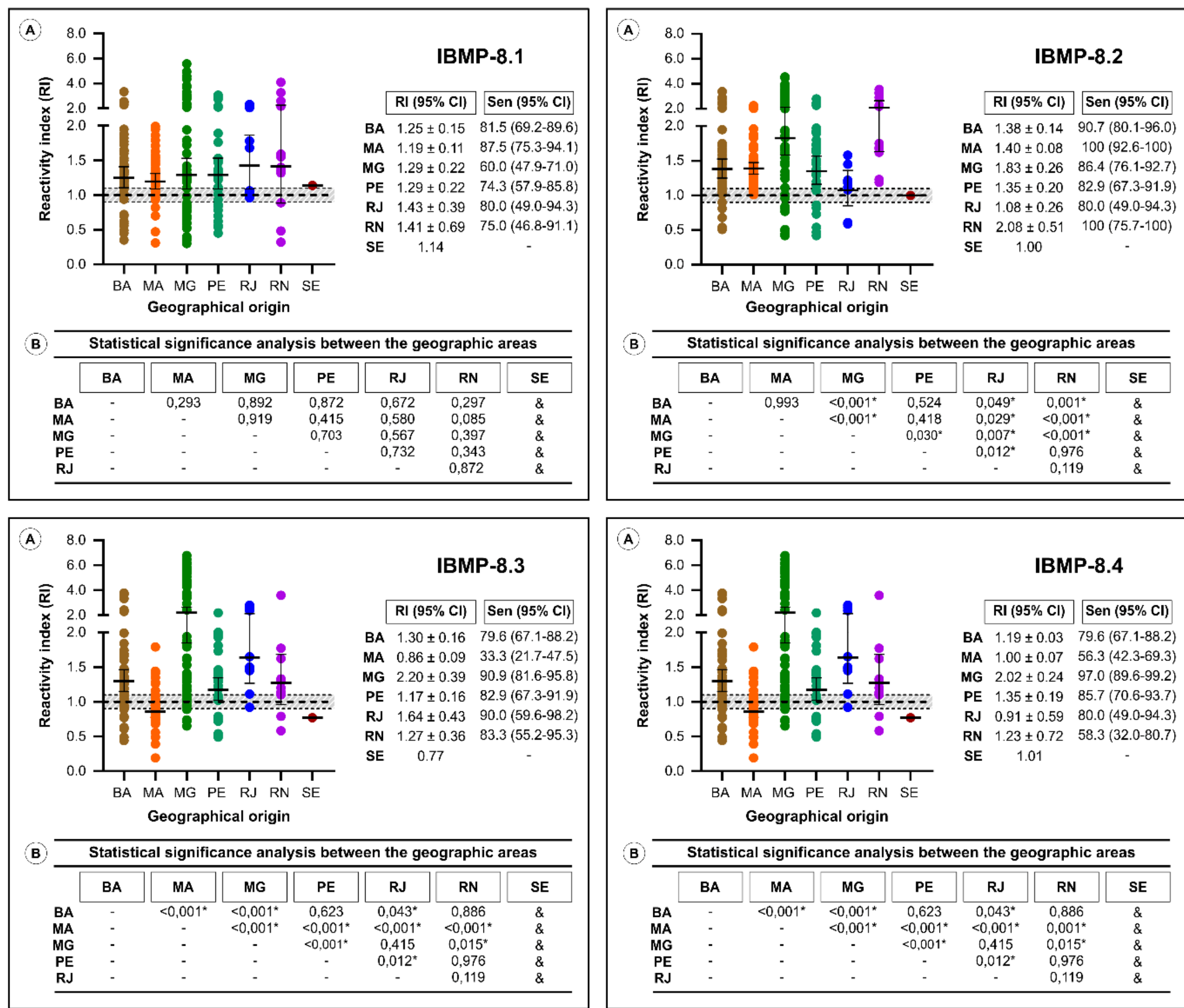


Figure 3. Evaluation of chimeric recombinant *Trypanosoma cruzi* antigens performance using ELISA. (A) Graphs showing the RI for each antigen tested against a panel of 226 *T. cruzi*-positive samples and 1,034 *T. cruzi*-negative samples. The cutoff value is 1.0, and the shaded

362 area represents the gray zone. The horizontal lines and numbers for each group of results
363 represent the geometric means (95% CIs). (B) Receiver Operating Characteristic Curce (ROC)
364 and AUC determined for each IBMP antigen. (C) Antigen performance parameters determined
365 using the assays shown in panel A. Acc (accuracy); AUC (area under the ROC curve); DOR
366 (diagnostic odds ratio); LR (likelihood ratio); PosTProb (posttest probability); PreTProb
367 (pretest probability); Sen (sensitivity); Spe (specificity); Tc-Neg (*T. cruzi*-negative samples);
368 Tc-Pos (*T. cruzi*-positive samples).

369 **Analysis of antigen sensitivity by geographical origin.** Considering the genetic
370 variability of *T. cruzi*, the positive samples were stratified by geographic area of origin to
371 analyze the extent of humoral response to IBMP antigens in infected dogs from different
372 geographic areas of Brazil (Figure 4). Graphical analysis shows that the IBMP-8.1 molecule
373 was recognized by samples from all geographic regions, with no differences in signal among
374 them. In contrast, the signal for the IBMP-8.2, IBMP-8.3, and IBMP-8.4 molecules varied by
375 geographic region, with significant differences, as shown in Figure 4. Sensitivity was also
376 determined in order to assess the extent of response to IBMP antigens in *T. cruzi*-positive dogs
377 from different geographic regions of Brazil.



378

379 **Figure 4.** Evaluation of chimeric recombinant *Trypanosoma cruzi* antigens performance using

380 ELISA in different Brazilian geographical areas (A) Reactivity index and sensitivity for *T.*

381 *cruzi*-positive samples from different geographical areas. The graphs show the RI and

382 sensitivity for each antigen tested against a panel of 226 *T. cruzi*-positive samples from different

383 geographical areas. The cutoff value is 1.0 and the shaded area represents the gray zone (RI =

384 1.0 ± 0.10). The horizontal lines represent the geometric means (± 95% CI). (B)Statistical

385 significance analysis of RI signal between geographic areas. BA (Bahia state); MA (Maranhão

386 state); MG (Minas Gerais state); PE (Pernambuco state); RJ (Rio de Janeiro state); RI (reactivity

387 index); RN (Rio Grande do Norte state); SE (Sergipe state); Sen (sensitivity); CI (confidence

388 interval). *Significance stastical; &Analysis was not performed due to the small number of

389 positive samples (n = 1) classified by LCA.

390

391 **IBMP-ELISA performance in serial and parallel approaches.** In an attempt to
392 reduce diagnostic uncertainty, analyzes were performed with serial and parallel approaches
393 using the results of individual *T. cruzi* IBMP chimera ELISA assays (Table 1). These
394 approaches are strategies for combining two diagnostic test results. Sensitivity was found to
395 increase consistently when ELISA test results were analyzed in parallel compared to the results
396 of the individual chimera tests or when a serial approach was used. We found that each
397 combination of IBMP proteins in parallel offered higher sensitivity than when the chimeric
398 antigens were tested individually, with the pair IBMP-8.1 + IBMP-8.3 having the lowest
399 combined sensitivity (> 93%). In terms of specificity, samples analyzed with either IBMP-8.1,
400 IBMP-8.2, or IBMP-8.4 individually or with any combination of these three proteins in series
401 or in parallel achieved values of 90%, with the exception of IBMP-8.1 + IBMP-8.2 and IBMP-
402 8.1 + IBMP-8.3, which had specificity values of 87.4% and 89.7%, respectively. Parallel
403 analysis showed that each combination of chimeras containing IBMP-8.3 protein provided a
404 specificity of 90%. When diagnostic accuracy was analyzed by the serial approach, most
405 combinations showed no differences in accuracy values compared with individual test results,
406 with the exception of IBMP-8.1 alone. However, in the parallel analysis, the IBMP-8.2 + IBMP-
407 8.4 and IBMP-8.3 + IBMP-8.4 test combinations provided the higher accuracy values, which
408 were superior to all individual analyzes except IBMP-8.4.

409

410

411

412

413

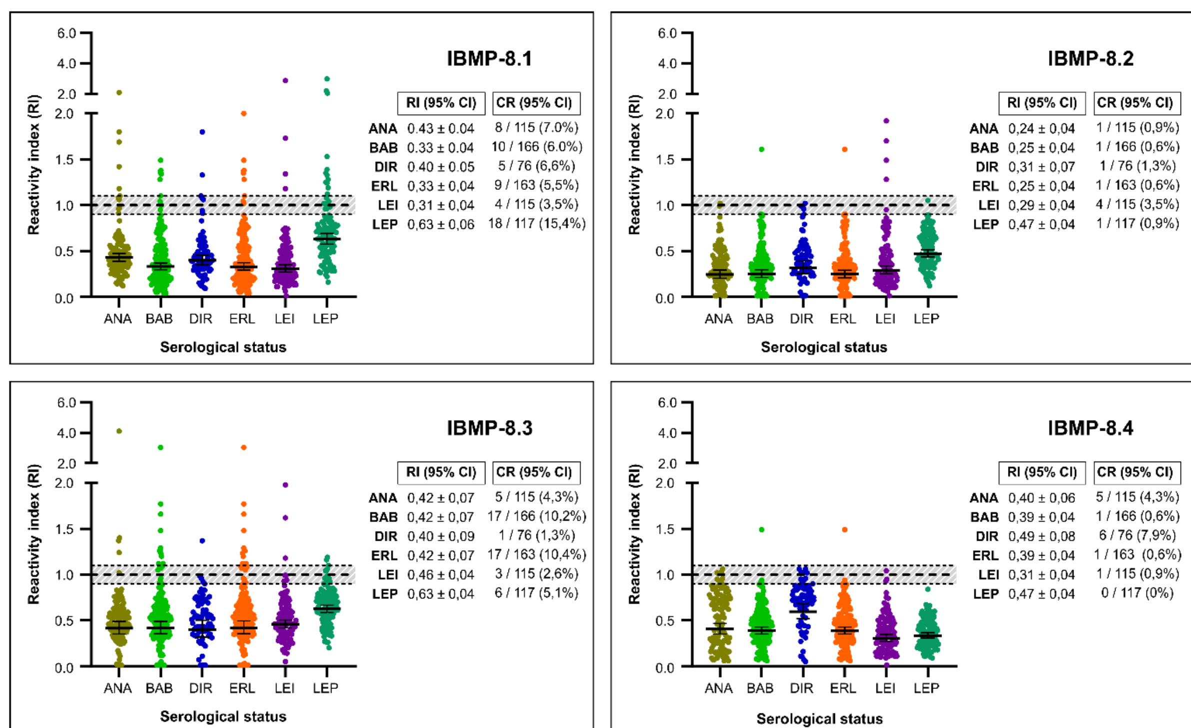
414 **Table 1.** IBMP-ELISA diagnostic test results compared both individually and among various
415 combinations of IBMP chimeras; additional analyzes were performed using serial and parallel
416 approaches.

IBMP-ELISA	Approach	Sen (95% CI)	Spe (95% CI)	Acc (95% CI)
IBMP-8.1	Individual	74.8 (68.7-80.0)	90.6 (88.7-92.2)	87.8 (85.9-89.5)
IBMP-8.2	Individual	90.3 (85.7-93.5)	96.5 (95.2-97.5)	95.4 (94.1-96.4)
IBMP-8.3	Individual	72.6 (66.4-78.0)	99.0 (98.2-99.5)	94.3 (92.9-95.4)
IBMP-8.4	Individual	79.9 (73.9-84.4)	99.6 (99.0-99.8)	96.0 (94.8-97.0)
IBMP-8.1+ IBMP-8.2	Serial	67.5 (58.9-74.8)	99.7 (99.5-99.8)	93.9 (92.2-95.3)
	Parallel	97.6 (95.5-98.7)	87.4 (84.4-89.9)	89.2 (86.4-91.5)
IBMP-8.1+IBMP-8.3	Serial	54.3 (45.6-62.4)	99.9 (99.8-100)	91.7 (90.1-93.2)
	Parallel	93.1 (89.5-95.6)	89.7 (87.1-91.7)	90.3 (87.5-92.4)
IBMP-8.1+ IBMP-8.4	Serial	59.5 (50.8-67.5)	100 (99.9-100)	92,7 (91.1-94.2)
	Parallel	94.9 (91.8-96.9)	90.2 (87.8-92.0)	91.1 (88.5-92.9)
IBMP-8.2+ IBMP-8.3	Serial	65.6 (56.9-72.9)	100 (99.9-100)	93.8 (92.2-95.1)
	Parallel	97.3 (95.2-98.6)	95.5 (93.5-97.0)	95.9 (93.8-97.3)
IBMP-8.2+ IBMP-8.4	Serial	71.9 (63.3-78.9)	100 (99.9-100)	94.9 (93.3-96.2)
	Parallel	98.0 (96.3-99.0)	96.1 (94.2-97.3)	96.4 (94.6-97.6)
IBMP-8.3+ IBMP-8.4	Serial	57.8 (49.1-65.8)	100 (99.9-100)	94.9 (93.3-96.2)
	Parallel	94.4 (91.2-96.6)	98.6 (97.2-99.3)	97.9 (96.1-98.8)

417 Sen (Sensitivity); Spe (Specificity); Acc (Accuracy); CI (Confidence interval)

418

419 **Analysis of cross-reactivity with other infections.** The potential cross-reactivity (RI ≥
420 1.0) of IBMP chimeric antigens was evaluated using serum samples from 752 samples from
421 dogs with unrelated diseases. As shown in Figure 5, the frequency of cross-reactivity was 7.2%
422 (54/752) for IBMP-8.1, 0.9% (7/752) for IBMP-8.2, 6.5% (49/752) for IBMP-8.3, and 1.9%
423 (14/752) for IBMP-8.4. Notably, the index of inconclusive results for the protein IBMP-8.2
424 (1.73%; 13/752) was low compared with other proteins: 3.83% (29/752) for IBMP-8.1, 4.26%
425 (32/752) for IBMP-8.4, and 5.72% (43/752) for IBMP-8.3. Among the samples of *Leishmania*
426 spp. only one sample showed cross-reactivity with IBMP-8.4 antigen, while three samples
427 cross-reacted for IBMP-8.3 and four samples each for IBMP-8.1 and IBMP-8.2 antigens.



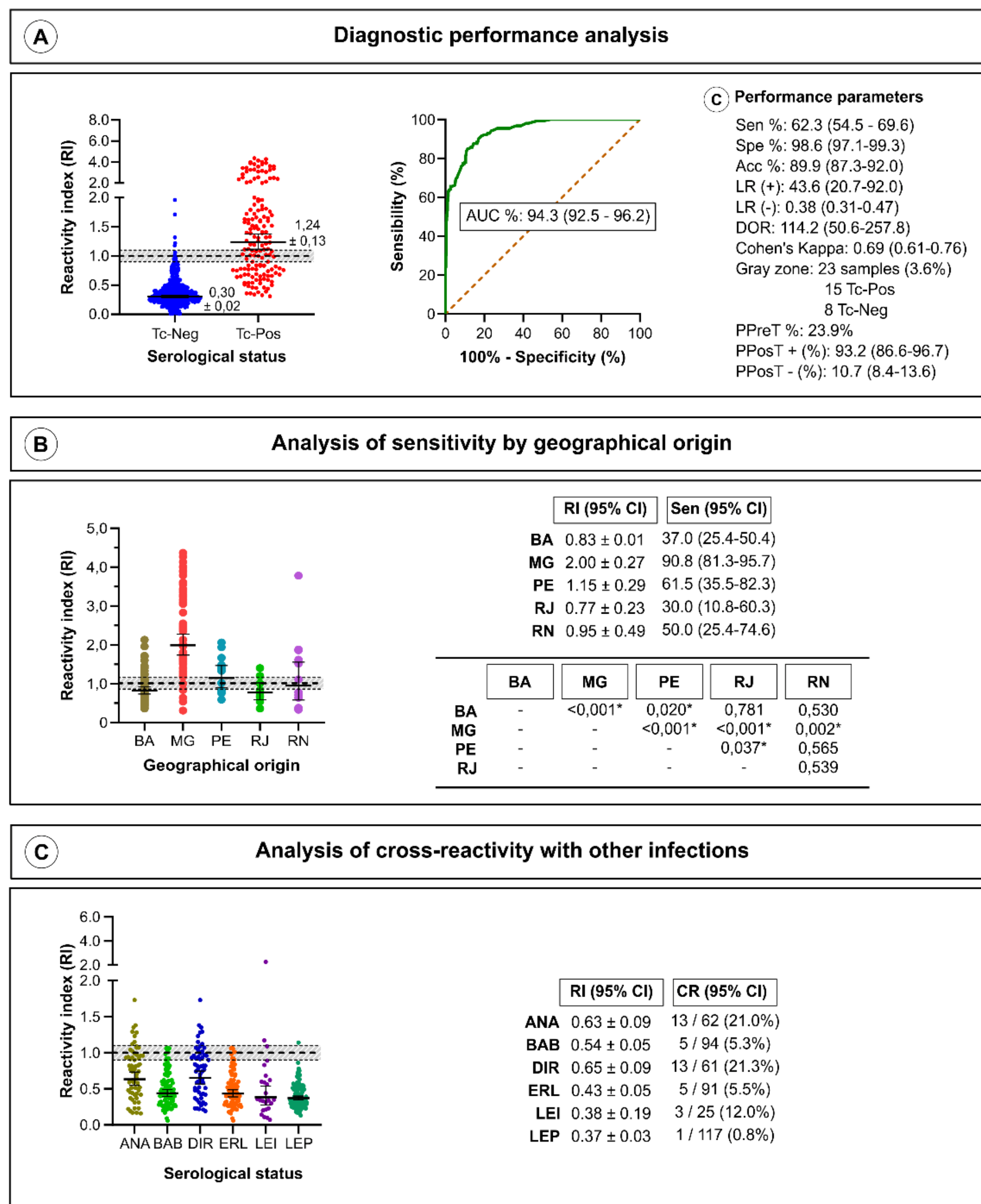
428

429 **Figure 5.** Cross-reactivity analysis of chimeric IBMP *Trypanosoma cruzi* proteins with sera
430 from dogs with unrelated diseases. The cutoff value is 1.0 and the shaded area represents the
431 gray zone (RI = 1.0 ± 0.10). The horizontal lines represent the geometric mean values. ANA
432 (anaplasmosis); BAB (babesiosis); CI (confidence interval); DIR (dirophilariasis); ERL
433 (erlichiosis); LEI (leishmaniasis); LEP (leptospirosis); RI (reactivity index).

434

435 **Comparison with commercial human-adapted *T. cruzi* ELISA.** To compare the
436 accuracy of IBMP ELISAs with a commercial human-adapted *T. cruzi* ELISA, we used 154 *T.*
437 *cruzi*-positive and 490 *T. cruzi*-negative dog samples. The Gold ELISA Chagas test showed a
438 sensitivity of 62.3% (95% CI: 54.5-69.6%) (Figure 6; see Table S8 for individual RI values).
439 This value was not different from the IBMP-8.1 and IBMP-8.3 values because the 95% CIs
440 overlapped. However, the IBMP-8.2 and IBMP-8.4 antigens were more sensitive compared
441 with the commercial kit. In terms of specificity, the Gold ELISA Chagas showed a value of
442 98.6% (95% CI: 97.1-99.3%), which was not different from the values for the IBMP-8.2, IBMP-
443 8.3 and IBMP-8.4 antigens. However, the specificity value of IBMP-8.1 antigen was
444 statistically lower than that of the Gold ELISA Chagas kit. Analysis of the DOR values indicates
445 that the Gold ELISA Chagas test and the IBMP-8.3 and IBMP-8.2 chimeric antigens have
446 similar performance in diagnosing *T. cruzi*-positive and *T. cruzi*-negative dogs. However, the
447 Gold ELISA Chagas test had a lower DOR value compared with IBMP-8.4 and a higher DOR
448 value compared with IBMP-8.1. Figure 6 also shows that the signal generated by the Gold
449 ELISA Chagas test varied by geographic region, with significant differences between regions.
450 The frequency of cross-reactivity was 8.9% (40/450): 7.2% (54/752) for IBMP-8.1, 0.9%
451 (7/752) for IBMP-8.2, 6.5% (49/752) for IBMP-8.3, and 1.9% (14/752) for IBMP-8.4. Of note,
452 the index of inconclusive results for the protein IBMP-8.2 (1.73%; 13/752) was low compared
453 with other proteins: 3.83% (29/752) for IBMP-8.1, 4.26% (32/752) for IBMP-8.4, and 5.72%
454 (43/752) for IBMP-8.3. Among *Leishmania* spp. samples, three samples showed cross-
455 reactivity with the commenrcial kit (Figure 6).

456



457

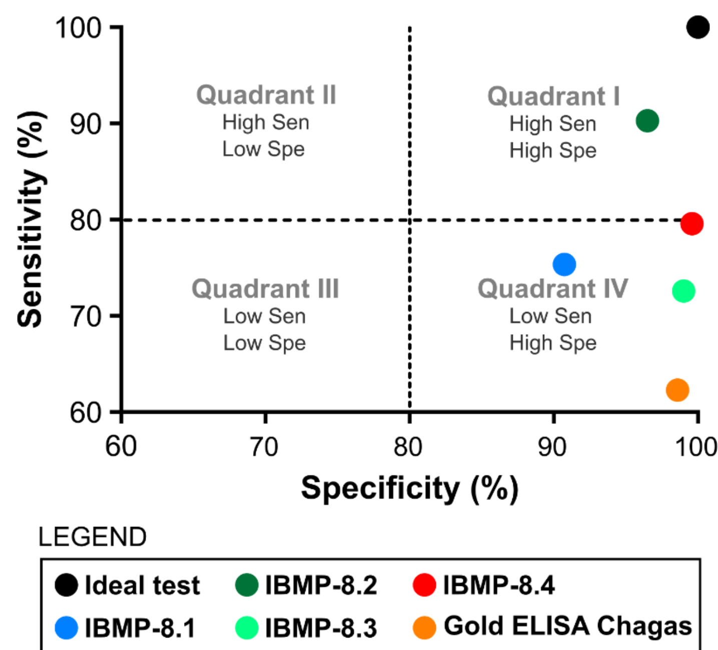
458 **Figure 6.** Evaluation of chimeric recombinant *Trypanosoma cruzi* antigens performance using
459 ELISA. (A) Graphs showing the RI for each antigen tested against a panel of 226 *T. cruzi*-
460 positive samples and 1,034 *T. cruzi*-negative samples. The cutoff value is 1.0, and the shaded
461 area represents the gray zone. The horizontal lines and numbers for each group of results
462 represent the geometric means (95% CIs). (B) Receiver Operating Characteristic Curce (ROC)
463 and AUC determined for each IBMP antigen. (C) Antigen performance parameters determined
464 using the assays shown in panel A. Acc (accuracy); AUC (area under the ROC curve); DOR
465 (diagnostic odds ratio); LR (likelihood ratio); PosTProb (posttest probability); PreTProb

466 (pretest probability); Sen (sensitivity); Spe (specificity); Tc-Neg (*T. cruzi*-negative samples);
467 Tc-Pos (*T. cruzi*-positive samples).

468

469 Figure 7 shows the relationship between the specificity and sensitivity of the IBMP antigens
470 and the commercial ELISA *T. cruzi* assay. For comparison purposes, the figure also shows the
471 maximum performance of an ideal assay (black dot, 100% accurate). The IBMP-8.2 chimera
472 produced the best results compared to the other chimeras and the commercial assay. The
473 accuracy of the IBMP-8.2 chimera was followed by IBMP-8.4, IBMP-8.3, IBMP-8.1, and Gold
474 ELISA Chagas assay. The IBMP-8.2 and IBMP-8.4 antigens had high sensitivity and high
475 specificity (Quadrant I in Figure 7), whereas IBMP-8.1, IBMP-8.3, and the Gold ELISA Chagas
476 assay had low sensitivity and high specificity (Quadrant IV in Figure 7). No test was classified
477 in any of the quadrants II (high sensitivity and low specificity) and III (low sensitivity and low
478 specificity).

479



480

481 **Figure 7.** Sensitivity vs. specificity data from IBMP chimeras and a commercial ELISA *T. cruzi*
482 assay. The black dot represents an ideal diagnostic assay that is 100% accurate. Sen
483 (sensitivity); Spe (specificity).

484 **DISCUSSION**

485 Although dogs are considered important for maintaining the domestic CD cycle and *T.*
486 *cruzi* infections can cause symptoms similar to those in humans, there are no commercially
487 available tests to detect anti-*T. cruzi* antibodies in these animals. For this reason, in-house or
488 modified commercial tests for humans have been used in studies of CD in dogs worldwide [25–
489 29]. In the present study, we evaluated the diagnostic performance of four chimeric recombinant
490 *T. cruzi* proteins for the detection of anti-*T. cruzi* antibodies in sera of dogs from different
491 Brazilian endemic and non-endemic areas. All IBMP proteins showed high discriminatory
492 power between *T. cruzi*-positive and negative samples. Indeed, AUC values ranged from 89.0
493 to 97.4%, with IBMP-8.4 antigen showing the highest value. These results are consistent with
494 the phase I study, in which IBMP-8.4 achieved the highest AUC value (AUC = 100%) [44].
495 AUC values for all four molecules were also above 99% in studies with human samples [35,39–
496 42,52]. Compared with a commercial human test adapted for the diagnosis of CD in dogs (Gold
497 ELISA Chagas), AUC values were higher for IBMP-8.2, IBMP-8.3, and IBMP-8.4 and lower
498 for IBMP-8.1; however, no statistical differences were observed.

499 Diagnostic sensitivity was higher for IBMP-8.2 than for the other molecules and Gold
500 ELISA Chaga. Although there was no significant difference between the IBMP-8.1 and IBMP-
501 8.3 proteins and the commercial kit, the IBMP-8.2 assay was the only assay with a sensitivity
502 greater than 90%. The results described here contrast with those previously reported in the
503 phase I study, in which IBMP-8.3 was the molecule with the highest sensitivity. In the present
504 study, IBMP-8.3 (and IBMP-8.1) had the lowest sensitivity values [44]. This discrepancy may
505 be due to the inclusion of a larger number of samples from different Brazilian endemic areas
506 and, thus, samples from dogs infected with different *T. cruzi* strains. It is known that *T. cruzi*

507 has a high genetic diversity in humans, which leads to different results in serological tests when
508 only a few antigens are used. In fact, the parasite is classified into seven genotypes with
509 subclassifications for regional strains called clonets [53–55]. We believe that the genetic
510 diversity of *T. cruzi* may also contribute to the discrepant results in the diagnosis of CD in dogs.
511 In addition, the discrepancy in sensitivity may also be explained by the different amino acid
512 composition of IBMP antigens, which may not be detected by the completeness of strains
513 circulating in animals from different study areas. This discrepancy was confirmed when
514 positive samples were stratified by geographic area of origin and the extent of humoral response
515 to IBMP antigens was considered. To increase the number of *T. cruzi*-positive samples
516 recognized by IBMP proteins, we analyzed pairs of antigens using serial and parallel
517 approaches. It was found that the parallel approach was able to increase sensitivity values,
518 especially when IBMP-8.2 was combined with IBMP-8.4 or IBMP-8.1, with sensitivity values
519 higher than 97%, making the use of pairs an interesting alternative for a diagnostic tool designed
520 for screening.

521 With respect to *T. cruzi*-negative samples, specificity values ranged from 90.6% to
522 99.6%. IBMP-8.3 and IBMP-8.4 provided the highest values (Spe ≥ 99.0%). This result is
523 consistent with the phase I study, in which IBMP-8.3 was the molecule with the highest
524 specificity value [44]. Following the parallel approach, the pair IBMP-8.3 + IBMP-8.4 reached
525 the highest specificity value (> 98%), while the values for the serial approach ranged from 99.7
526 to 100% regardless of the pairs considered. Thus, the use of the serial or parallel approach
527 (IBMP-8.3+IBMP-8.4) could be used for diagnostic purposes, especially for confirmatory tests.
528 The accuracy values were similar for IBMP-8.2, IBMP-8.3, and IBMP-8.4 proteins. Due to the
529 high proportion of misdiagnosed samples detected with the IBMP-8.1 protein, its accuracy was
530 significantly lower compared with the other proteins. For human diagnosis, on the other hand,
531 IBMP-8.1 and IBMP-8.4 proteins showed the highest performance parameters [39–42,52];
532 therefore, the new commercial lateral flow immunromatographic assay TR Chagas (Bio-

533 Manguinhos, Fiocruz-RJ, Brazil) uses these two antigens to detect human anti-*T. cruzi*
534 antibodies with an accuracy of 100% [39]. The lower performance of IBMP-8.1 protein for the
535 diagnosis of CD in dogs compared to humans might be due to the characteristics of the
536 expressed immunoglobulin VH and VL repertoires in different breeds of dogs compared to
537 those in humans [56]. Unless the IBMP-8.1 molecule is used in the latent class model for the
538 diagnosis of CD in dogs, its use alone is not recommended for reliable diagnosis in dogs.

539 Evaluating diagnostic tests using sensitivity, specificity, and accuracy is insufficient to
540 measure their impact on clinical decisions. A diagnostic test is useful only if its results alter the
541 probability of disease occurrence. Determination of the likelihood ratio (LR) is useful to
542 describe the discriminatory power of a test and defines the probability of a given result in
543 infected individuals versus the probability of the same result in healthy individuals [33]. In this
544 study, IBMP-8.4 showed a positive LR of 250.9, which means that a dog infected with *T. cruzi*
545 is approximately 251 times more likely to be diagnosed with chronic CD when tested with
546 IBMP-8.4 protein. The estimated DOR for IBMP-8.4 (1,007.6) was higher than the values for
547 IBMP-8.3 (270.9), IBMP-8.2 (257.1), and IBMP-8.1 (28.6). Although DOR values varied
548 according to the protein, IBMP-8.2, IBMP-8.3, and IBMP-8.4 were all above 250 and
549 performed better than the commercial Gold ELISA Chagas (114.2). However, the Gold ELISA
550 Chagas performed better than the IBMP-8.1 protein. LR and DOR are relevant and stable tools
551 in phase II studies since these parameters do not depend on disease prevalence.

552 Serological cross-reactivity for IBMP proteins was not surprising given the weak
553 seropositivity for unrelated diseases. Indeed, these molecules consist of specific *T. cruzi*
554 fragments, and review of amino acid sequences in the NCBI database using the Protein BLAST
555 software revealed modest similarity to nonpathogenic canine and human microorganisms [52].
556 Among the total unrelated diseases sera, IBMP-8.1 and IBMP-8.3 proteins provided the highest
557 cross-reactivity values (7.2% and 6.5%, respectively). In contrast to these data, IBMP-8.2 and
558 IBMP-8.4 showed cross-reactivity of 0.9% and 1.9%, respectively, indicating that they can be

559 used to diagnose *T. cruzi* infection in dogs in co-endemic areas. It is already well documented
560 that anti-*Leishmania* spp. antibodies are a significant cause of cross-reactivity in serological
561 tests for chronic CD, especially in conventional tests [23,34,57–59]. In the present study, cross-
562 reactivity to *Leihmania* ranged from 0.9% to 3.5%. These values are higher than in the phase I
563 study, in which samples showed no cross-reactivity when tested with IBMP-8.1, IBMP-8.2, and
564 IBMP-8.4. Although *Leishmania* spp. is important for accurate chronic CD diagnosis, other
565 unrelated pathogens can also cause cross-reactivity. In contrast to IBMP-8.2 and IBMP-8.4,
566 cross-reactivity with babesiosis, ehrlichiosis, and leptospirosis was observed when samples
567 were tested with IBMP-8.1 and IBMP-8.3. Cross-reactivity was insignificant with the other
568 pathogens tested. The number of samples that cross-reacted with IBMP chimeras was very low,
569 especially for the IBMP-8.2 protein. Overall, our data suggest that IBMP chimeras can be used
570 in areas of co-endemism between *T. cruzi* and other diseases.

571 The main limitation of the study was the lack of a validated standard test to preclassify
572 the sera that would be used to evaluate the efficacy of the antigens. To address this limitation,
573 we used a reference array of chimeric *T. cruzi* antigens at LCA as a surrogate in the absence of
574 a gold standard. LCA provided more precise diagnostic precision for the evaluation of
575 diagnostic tests in the absence of a gold standard. The present study was also limited by testing
576 samples from restricted geographic areas of Brazil, representing a limited number of circulating
577 discrete typing units, and by the absence of *Trypanosoma rangeli* samples. It is important that
578 this last limitation be addressed in future studies that consider comprehensive samples from
579 other Brazilian states, especially from the northern region, and from different Latin American
580 endemic countries. Nevertheless, our analysis confirmed the remarkable performance of these
581 chimeric IBMP antigens in the context of chronic CD diagnosis in dogs, with IBMP-8.2 and
582 IBMP-8.4 proteins showing higher accuracy.

583 In summary, the results described here indicate that these four chimeric recombinant
584 *T. cruzi* IBMP antigens reliably distinguish *T. cruzi*-positive from negative samples. In addition,

585 the accuracy of IBMP-8.2, IBMP-8.3, and IBMP-8.2 has not been shown to vary regardless of
586 the geographic origin of the samples, suggesting that these proteins could be useful in
587 commercial test kits. Accordingly, a lateral flow immunocromatographic assay using two
588 different IBMP antigens could potentially be employed to reliably monitor the parasite
589 transmission cycle of *T. cruzi* in endemic areas and for veterinary purposes.

590 **ANCKNOLEDGEMENTS**

591 We are grateful to Adriana and her team for providing us with a room in the electron microscopy
592 platform to perform the immunoassays when our laboratory was renovated. Sintam-se a vontade
593 de explorar este tópico.

594

595 **Data Availability Statement**

596 The raw data supporting the conclusions of this article will be made available by the authors,
597 without undue reservation.

598

599 **Conflict of Interest**

600 The authors declare that the research was conducted in the absence of any commercial or
601 financial relationships that could be construed as a potential conflict of interest.

602

603 **Autor Contributions**

604 All authors contributed substantially to the work described in this article. FLNS, PAFC, and
605 NITZ designed the experimental procedure. NITZ selected T. cruzi epitopes and designed the
606 chimeric recombinant IBMP proteins. PAFC expressed and purified the chimeric recombinant
607 IBMP proteins. FDT and KGSS collected and characterized all positive samples for

608 anaplasmosis, babesiosis, dirofilaosis, erliquiosis, and leishmaniasis. APC and TFC collected
609 and characterized all samples from the state of Maranhão. LGD and CJB collected and
610 characterized all samples from the state of Minas Gerais. CMM collected and characterized all
611 samples from the state of Sergipe. ACJC and VTAN collected and characterized all samples
612 from the state of Rio Grande do Norte. DBMF KGSS collected and characterized all positive
613 samples for leptospirosis. NDF and FLH performed the ELISA experiments. NDF, FLH, LML,
614 NEMF and AAOS performed the data collection, analysis and interpretation. LDAFA
615 performed the latent class analysis. NDF, FLH, and FLNS wrote the article. NDF and FLNS
616 produced the figures. FLNS provided the laboratory space and obtained funding for this study.
617 FLNS supervised the work. All authors read and agreed to the published version of the
618 manuscript.

619

620 **Funding**

621 This research was supported by the Coordination of Superior Level Staff Improvement-Brazil
622 (CAPES; Finance Code 001), the Research Support Foundation of the State of Bahia
623 (FAPESB); and Inova Fiocruz/VPPCB (grant number VPPCB-008-FIO-18-2-20). Nilson I. T.
624 Zanchin and Fred L. N. Santos are CNPq research fellows (grant numbers 312195/2015-0 and
625 309263/2020-4, respectively). Funders had no influence on the study design, data collection
626 and analysis, decision to publish, or preparation of the manuscript.

627 **REFERENCES**

628 1. World Health Organization. Chagas disease in Latin America: an epidemiological
629 update based on 2010 estimates. Wkly Epidemiol Rec. 2015;90: 33–43. Available:
630 http://www.who.int/wer/2015/wer9006.pdf?ua=1

631 2. Santos EF, Silva ÂAO, Leony LM, Freitas NEM, Daltro RT, Regis-Silva CG, et al.

632 Acute Chagas disease in Brazil from 2001 to 2018: A nationwide spatiotemporal analysis.
633 Almeida IC, editor. PLoS Negl Trop Dis. 2020;14: e0008445.
634 doi:10.1371/journal.pntd.0008445

635 3. Lidani KCF, Andrade FA, Bavia L, Damasceno FS, Beltrame MH, Messias-Reason IJ,
636 et al. Chagas disease: From discovery to a worldwide health problem. Front public Heal.
637 2019;7: 166. doi:10.3389/fpubh.2019.00166

638 4. Manne-Goehler J, Reich MR, Wirtz VJ. Access to care for Chagas disease in the United
639 States: a health systems analysis. Am J Trop Med Hyg. 2015;93: 108–113.
640 doi:10.4269/ajtmh.14-0826

641 5. Barr SC. Canine Chagas' disease (American trypanosomiasis) in North America. Vet
642 Clin North Am Small Anim Pract. 2009;39: 1055–1064. doi:10.1016/j.cvsm.2009.06.004

643 6. Rodríguez-Morales O, Ballinas-Verdugo MA, Alejandre-Aguilar R, Reyes PA, Arce-
644 Fonseca M. Trypanosoma cruzi connatal transmission in dogs with Chagas disease:
645 experimental case report. Vector Borne Zoonotic Dis. 2011;11: 1365–70.
646 doi:10.1089/vbz.2010.0231

647 7. Gürtler RE, Cecere MC, Lauricella MA, Cardinal MV, Kitron U, Cohen JE. Domestic
648 dogs and cats as sources of Trypanosoma cruzi infection in rural northwestern Argentina.
649 Parasitology. 2007;134: 69–82. doi:10.1017/S0031182006001259

650 8. Gürtler RE, Cecere MC, Lauricella MA, Petersen RM, Chuit R, Segura EL, et al.
651 Incidence of Trypanosoma cruzi infection among children following domestic reinfestation
652 after insecticide spraying in rural northwestern Argentina. Am J Trop Med Hyg. 2005;73: 95–
653 103. doi:73/1/95 [pii]

654 9. Gürtler RE, Chuit R, Cécere MC, Castañera MB, Cohen JE, Segura EL. Household
655 prevalence of seropositivity for Trypanosoma cruzi in three rural villages in northwest

656 Argentina: Environmental, demographic, and entomologic associations. Am J Trop Med Hyg.
657 1998;59: 741–749. doi:10.4269/ajtmh.1998.59.741

658 10. Gürtler RE, Kitron U, Cecere MC, Segura EL, Cohen JE. Sustainable vector control and
659 management of Chagas disease in the Gran Chaco, Argentina. Proc Natl Acad Sci U S A.
660 2007;104: 16194–9. doi:10.1073/pnas.0700863104

661 11. Gürtler RE, Cardinal M V. Reservoir host competence and the role of domestic and
662 commensal hosts in the transmission of Trypanosoma cruzi. Acta Trop. 2015;151: 32–50.
663 doi:10.1016/j.actatropica.2015.05.029

664 12. Enriquez GFF, Bua J, Orozco MMM, Wirth S, Schijman AGG, Gürtler REE, et al. High
665 levels of Trypanosoma cruzi DNA determined by qPCR and infectiousness to Triatoma
666 infestans support dogs and cats are major sources of parasites for domestic transmission. Infect
667 Genet Evol. 2014;25: 36–43. doi:10.1016/j.meegid.2014.04.002

668 13. Jansen AM, Luiz A, Roque R, Maria A, Jansen AM. Importância dos animais
669 domésticos sentinelas na identificação de áreas de risco de emergência de doença de Chagas.
670 Rev Soc Bras Med Trop. 2008;41: 191–193.

671 14. Andrade ZA. The canine model of Chagas' disease. Mem Inst Oswaldo Cruz. 1984;79:
672 77–83. doi:10.1590/S0074-02761984000500016

673 15. Andrade ZA, Andrade SG. Pathology of experimental Chagas disease in dogs. Mem
674 Inst Oswaldo Cruz. 1980;75: 77–95. doi:10.1590/s0074-02761980000200008

675 16. Tafuri WL, Lana M de, Chiari E, Caliari MV, Bambirra EA, Rios-Leite VH, et al. O cão
676 como modelo experimental para o estudo da história natural da doença de Chagas. Rev Soc
677 Bras Med Trop. 1988;21: 77–77. doi:10.1590/S0037-86821988000200010

678 17. Lana M de, Chiari E, Tafuri WL. Experimental Chagas' disease in dogs. Mem Inst
679 Oswaldo Cruz. 1992;87: 59–71. doi:10.1590/S0074-02761992000100011

680 18. Zingales B. Trypanosoma cruzi genetic diversity: Something new for something known
681 about Chagas disease manifestations, serodiagnosis and drug sensitivity. Acta Trop. 2018;184:
682 38–52. doi:10.1016/j.actatropica.2017.09.017

683 19. Santos FLN, Souza WV, Barros MS, Nakazawa M, Krieger MA, Gomes YM. Chronic
684 Chagas disease diagnosis: A comparative performance of commercial enzyme immunoassay
685 tests. Am J Trop Med Hyg. 2016;94: 1034–1039. doi:10.4269/ajtmh.15-0820

686 20. Leeflang MMG, Bossuyt PMM, Irwig L. Diagnostic test accuracy may vary with
687 prevalence: implications for evidence-based diagnosis. J Clin Epidemiol. 2009;62: 5–12.
688 doi:10.1016/j.jclinepi.2008.04.007

689 21. Leeflang MMG, Rutjes AWS, Reitsma JB, Hooft L, Bossuyt PMM. Variation of a test's
690 sensitivity and specificity with disease prevalence. CMAJ. 2013;185: E537-44.
691 doi:10.1503/cmaj.121286

692 22. Martin DL, Marks M, Galdos-Cardenas G, Gilman RH, Goodhew B, Ferrufino L, et al.
693 Regional variation in the correlation of antibody and T-cell responses to Trypanosoma cruzi.
694 Am J Trop Med Hyg. 2014;90: 1074–1081. doi:10.4269/ajtmh.13-0391

695 23. Vega Benedetti a. F, Cimino RO, Cajal PS, Juarez MDV, Villalpando C a., Gil JF, et
696 al. Performance of different Trypanosoma cruzi antigens in the diagnosis of Chagas disease in
697 patients with American cutaneous leishmaniasis from a co-endemic region in Argentina. Trop
698 Med Int Heal. 2013;18: 1103–9. doi:10.1111/tmi.12144

699 24. Daltro RT, Leony LM, Freitas NEM, Silva ÂAO, Santos EF, Del-Rei RP, et al. Cross-
700 reactivity using chimeric Trypanosoma cruzi antigens: diagnostic performance in settings co-
701 endemic for Chagas disease and American cutaneous or visceral leishmaniasis. J Clin
702 Microbiol. 2019;57. doi:10.1128/JCM.00762-19

703 25. Longoni SS, López-Cespedes A, Sánchez-Moreno M, Bolio-Gonzalez ME, Sauri-Arceo

704 CH, Rodríguez-Vivas RI, et al. Detection of different Leishmania spp. and Trypanosoma cruzi
705 antibodies in cats from the Yucatan Peninsula (Mexico) using an iron superoxide dismutase
706 excreted as antigen. Comp Immunol Microbiol Infect Dis. 2012;35: 469–476.
707 doi:10.1016/j.cimid.2012.04.003

708 26. Enriquez GF, Cardinal M V, Orozco MM, Schijman AG, Gürtler RE. Detection of
709 Trypanosoma cruzi infection in naturally infected dogs and cats using serological,
710 parasitological and molecular methods. Acta Trop. 2013;126: 211–217.
711 doi:10.1016/j.actatropica.2013.03.001

712 27. González CR, Reyes C, Canals A, Parra A, Muñoz X, Rodríguez K. An entomological
713 and seroepidemiological study of the vectorial-transmission risk of Chagas disease in the coast
714 of northern Chile. Med Vet Entomol. 2015;29: 387–392. doi:10.1111/mve.12131

715 28. Saldaña A, Calzada JE, Pineda V, Perea M, Rigg C, González K, et al. Risk factors
716 associated with Trypanosoma cruzi exposure in domestic dogs from a rural community in
717 Panama. Mem Inst Oswaldo Cruz. 2015;110: 936–944. doi:10.1590/0074-02760150284

718 29. Zecca IB, Hodo CL, Slack S, Auckland L, Rodgers S, Killets KC, et al. Prevalence of
719 Trypanosoma cruzi infection and associated histologic findings in domestic cats (Felis catus).
720 Vet Parasitol. 2020;278: 109014. doi:10.1016/j.vetpar.2019.109014

721 30. Camussone C, Gonzalez VV, Belluzo MSMS, Pujato N, Ribone MEME, Lagier CM, et
722 al. Comparison of recombinant Trypanosoma cruzi peptide mixtures versus multiepitope
723 chimeric proteins as sensitizing antigens for immunodiagnosis. Clin Vaccine Immunol.
724 2009;16: 899–905. doi:10.1128/CVI.00005-09

725 31. Houghton RL, Benson DR, Reynolds LD, McNeill PD, Sleath PR, Lodes MJ, et al. A
726 Multi-Epitope Synthetic Peptide and Recombinant Protein for the Detection of Antibodies to
727 *Trypanosoma cruzi* in Radioimmunoprecipitation-Confirmed and Consensus-Positive Sera. J
728 Infect Dis. 1999;179: 1226–1234. doi:10.1086/314723

729 32. Aguirre S, Silber AM, Brito MEF, Ribone ME, Lagier CM, Marcipar IS. Design,
730 construction, and evaluation of a specific chimeric antigen to diagnose chagasic infection. J
731 Clin Microbiol. 2006;44: 3768–74. doi:10.1128/JCM.01043-06

732 33. Abras A, Ballart C, Fernández-Arévalo A, Llovet T, Gállego M, Muñoz C.
733 ARCHITECT Chagas® as a single test candidate for Chagas disease diagnosis: evaluation of
734 two algorithms implemented in a non-endemic setting (Barcelona, Spain). Clin Microbiol
735 Infect. 2020;27: 782.e1–782.e6. doi:10.1016/j.cmi.2020.07.002

736 34. Santos FLN, Celedon PAF, Zanchin NIT, Brasil TAC, Foti L, Souza WV, et al.
737 Performance assessment of four chimeric Trypanosoma cruzi antigens based on antigen-
738 antibody detection for diagnosis of chronic Chagas disease. Dellagostin OA, editor. PLoS One.
739 2016;11: e0161100. doi:10.1371/journal.pone.0161100

740 35. Santos FLN, Celedon PAF, Zanchin NIT, Leitolis A, Crestani S, Foti L, et al.
741 Performance assessment of a Trypanosoma cruzi chimeric antigen in multiplex liquid
742 microarray assays. J Clin Microbiol. 2017;55: 2934–2945. doi:10.1128/JCM.00851-17

743 36. Santos FLN, Campos ACP, Amorim LDAF, Silva ED, Zanchin NIT, Celedon PAF, et
744 al. Highly accurate chimeric proteins for the serological diagnosis of chronic Chagas disease:
745 A latent class analysis. Am J Trop Med Hyg. 2018;99: 1174–1179. doi:10.4269/ajtmh.17-0727

746 37. Celedon PAF, Leony LM, Oliveira UD, Freitas NEM, Silva ÂAO, Daltro RT, et al.
747 Stability assessment of four chimeric proteins for human Chagas disease immunodiagnosis.
748 Biosensors. 2021;11: 289. doi:10.3390/bios11080289

749 38. Santos EF, Leony LM, Silva ÂAO, Daltro RT, Freitas NEM, Vasconcelos LCM, et al.
750 Assessment of Liaison <scp>XL</scp> Murex Chagas diagnostic performance in blood
751 screening for Chagas disease using a reference array of chimeric antigens. Transfusion.
752 2021;61: 2701–2709. doi:10.1111/trf.16583

753 39. Silva ED, Silva ÂAO, Santos EF, Leony LM, Freitas NEM, Daltro RT, et al.
754 Development of a new lateral flow assay based on IBMP-8.1 and IBMP-8.4 chimeric antigens
755 to diagnose Chagas disease. Biomed Res Int. 2020;2020: 1803515. doi:10.1155/2020/1803515

756 40. Cordeiro TAR, Martins HR, Franco DL, Santos FLN, Celedon PAF, Cantuária VL, et
757 al. Impedimetric immunosensor for rapid and simultaneous detection of Chagas and visceral
758 leishmaniasis for point of care diagnosis. Biosens Bioelectron. 2020;169: 112573.
759 doi:10.1016/j.bios.2020.112573

760 41. Del-Rei RP, Leony LM, Celedon PAF, Zanchin NIT, Reis MG, Gomes YDM, et al.
761 Detection of anti-Trypanosoma cruzi antibodies by chimeric antigens in chronic Chagas
762 disease-individuals from endemic South American countries. PLoS One. 2019;14: e0215623.
763 doi:10.1371/journal.pone.0215623

764 42. Dopico E, Del-Rei RP, Espinoza B, Ubillos I, Zanchin NIT, Sulleiro E, et al. Immune
765 reactivity to Trypanosoma cruzi chimeric proteins for Chagas disease diagnosis in immigrants
766 living in a non-endemic setting. BMC Infect Dis. 2019;19: 251. doi:10.1186/s12879-019-3872-
767 z

768 43. Freitas GRO, Silva DAO, Yokosawa J, Paula NT, Costa LF, Carneiro BM, et al.
769 Antibody response and avidity of respiratory syncytial virus-specific total IgG, IgG1, and IgG3
770 in young children. J Med Virol. 2011;83: 1826–33. doi:10.1002/jmv.22134

771 44. Leony LM, Freitas NEM, Del-Rei RP, Carneiro CM, Reis AB, Jansen AM, et al.
772 Performance of recombinant chimeric proteins in the serological diagnosis of Trypanosoma
773 cruzi infection in dogs. PLoS Negl Trop Dis. 2019;13: e0007545.
774 doi:10.1371/journal.pntd.0007545

775 45. Santos F, Magalhães-Junior JT, Carneiro I de O, Santos FLN, Silva ÂAO, Novais
776 JMCB, et al. Eco-epidemiology of vectorial Trypanosoma cruzi transmission in a region of
777 northeast Brazil. Acta Trop. 2022;225: 106184. doi:10.1016/j.actatropica.2021.106184

778 46. Costa TF, Rocha AVVO, Miranda LM, Lima LFS, Santos FLN, Silva ÂAO, et al.
779 Seroprevalence and detection of Trypanosoma cruzi in dogs living in a non-endemic area for
780 Chagas disease in the legal Amazon region, Brazil. Vet Parasitol Reg Stud Reports. 2021;26:
781 100648. doi:10.1016/j.vprsr.2021.100648

782 47. Dean AG, Sullivan KM, Soe MM, Mir RA. OpenEpi: Open Source Epidemiologic
783 Statistics for Public Health. Atlanta: Centers for Disease Control and Prevention (CDC); 2013.
784 Available: www.OpenEpi.com

785 48. Swets JA. Measuring the accuracy of diagnostic systems. Science. 1988;240: 1285–93.
786 doi:10.1126/science.3287615

787 49. Cebul RD, Hershey JC, Williams S V. Using multiple tests: Series and parallel
788 approaches. Clin Lab Med. 1982;2: 871–890. doi:10.1016/S0272-2712(18)31018-7

789 50. Landis JR, Koch GG. The measurement of observer agreement for categorical data.
790 Biometrics. 1977;33: 159–74. doi:10.2307/2529310

791 51. Irwig L, Bossuyt P, Glasziou P, Gatsonis C, Lijmer J. Designing studies to ensure that
792 estimates of test accuracy are transferable. BMJ. 2002;324: 669–71. Available:
793 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1122584&tool=pmcentrez&rende
794 rtype=abstract

795 52. Santos FLN, Celedon PA, Zanchin NI, Souza WV, Silva ED, Foti L, et al. Accuracy of
796 chimeric proteins in the serological diagnosis of chronic Chagas disease - a Phase II study.
797 PLoS Negl Trop Dis. 2017;11: e0005433. doi:10.1371/journal.pntd.0005433

798 53. Zingales B, Andrade SG, Briones MR, Campbell DA, Chiari E, Fernandes O, et al. A
799 new consensus for Trypanosoma cruzi intraspecific nomenclature: second revision meeting
800 recommends TcI to TcVI. Mem Inst Oswaldo Cruz. 2009;104: 1051–1054. doi:10.1590/S0074-
801 02762009000700021

802 54. Zingales B, Miles MA, Campbell DA, Tibayrenc M, Macedo AM, Teixeira MM, et al.
803 The revised Trypanosoma cruzi subspecific nomenclature: rationale, epidemiological relevance
804 and research applications. Infect Genet Evol. 2012;12: 240–253.
805 doi:10.1016/j.meegid.2011.12.009

806 55. Tibayrenc M, Ayala FJ. Towards a population genetics of microorganisms: The clonal
807 theory of parasitic protozoa. Parasitol Today. 1991;7: 228–232. doi:10.1016/0169-
808 4758(91)90234-F

809 56. Steiniger SCJ, Dunkle WE, Bammert GF, Wilson TL, Krishnan A, Dunham SA, et al.
810 Fundamental characteristics of the expressed immunoglobulin VH and VL repertoire in
811 different canine breeds in comparison with those of humans and mice. Mol Immunol. 2014;59:
812 71–78. doi:10.1016/j.molimm.2014.01.010

813 57. Malchiodi EL, Chiaramonte MG, Taranto NJ, Zwirner NW, Margni R a. Cross-
814 reactivity studies and differential serodiagnosis of human infections caused by Trypanosoma
815 cruzi and Leishmania spp; use of immunoblotting and ELISA with a purified antigen
816 (Ag163B6). Clin Exp Immunol. 1994;97: 417–423. doi:10.1111/j.1365-2249.1994.tb06104.x

817 58. Caballero ZC, Sousa OE, Marques WP, Saez-Alquezar A, Umezawa ES. Evaluation of
818 serological tests to identify Trypanosoma cruzi infection in humans and determine cross-
819 reactivity with Trypanosoma rangeli and Leishmania spp. Clin Vaccine Immunol. 2007;14:
820 1045–1049. doi:10.1128/CVI.00127-07

821 59. Frank FM, Fernández MM, Taranto NJ, Cajal SP, Margni RA, Castro E, et al.
822 Characterization of human infection by Leishmania spp. in the Northwest of Argentina:
823 immune response, double infection with Trypanosoma cruzi and species of Leishmania
824 involved. Parasitology. 2003;126: 31–9. doi:10.1017/s0031182002002585

825

826 **PROPOSTA DE SUBMISSÃO**

827

828 **REVISTA:** Parasites & Vectors

829

830 **REGRAS PARA SUBMISSÃO:**

831 • Research articles Research articles should not normally exceed 5,000 words in the main
832 body of the text or include more than 100 references. Use of subheadings in the main text
833 is recommended (i.e. Background, Methods, Results and Discussion). Figures and tables
834 are encouraged.

835 • Abstract and keywords Abstracts should not exceed 350 words and should be divided into
836 sections. Provide 3-10 keywords, which should preferentially be available at the Medical
837 Subject Headings (MeSH) database

838 • Abstract and keywords Abstracts should not exceed 350 words and should be divided into
839 sections. Provide 3-10 keywords, which should preferentially be available at the Medical
840 Subject Headings (MeSH) database

841 • Abbreviations All genus names should be fully spelled out at first mention and then
842 abbreviated. When presented in a list, the genus name may be abbreviated (e.g., "Different
843 species of the genus Phlebotomus are implicated in the transmission of Mediterranean
844 Leishmania infantum, including Phlebotomus ariasi, P. balcanicus, P. kandelakii, P.
845 langeroni, P. neglectus, P. perfiliewi, P. perniciosus, P. sergenti and P. tobbi"; from
846 https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-021-04652-2).
847 When presented at the beginning of a sentence, the genus name should be spelled out again
848 (even if already abbreviated). To avoid ambiguity in the text, abbreviations for mosquito
849 genera and 7 subgenera can be made using two and three letters, respectively (see

850 http://www.mosquitocatalog.org/abbreviations.aspx). The most relevant abbreviations used
851 in the manuscript should be listed in the Abbreviation section, as follows:

852 • References All references must be numbered in ascending numerical order; references in
853 figures and tables are numbered last. Reference numbers should be provided in the main
854 manuscript text in square brackets, e.g. Woo et al. [1] or [1–5] or [1, 7, 9, 13–15] or (see [2]
855 for a review). Please follow strictly the format of the examples, including the spacing and
856 punctuation. Note that: (i) abbreviated journal names should be provided; (ii) all organism
857 Latin names should be in italics; (iii) capital letters should be used only for the first word
858 of the article title and proper nouns; (iv) author lists of more than six authors should be
859 presented with ", et al." after the sixth author and (v) non-breaking spaces between words
860 for references copied directly from Internet sources should be removed

861 • Figure formatting and requirements • Do not submit figures in Word or PowerPoint files. •
862 Figure captions (max 300 words) should be provided in the main manuscript (after
863 References), not in the graphic file. For figures with multiple panels (a, b, c, etc), all panels
864 should be mentioned in the figure caption. Only high-resolution figure files will be
865 accepted: - EPS (or PDF): Vector drawings. Embed the font or save the text as ‘graphics‘.
866 9 - TIFF: Colour or grayscale photographs (halftones), use a minimum of 600 dpi. - TIFF:
867 Bitmapped line drawings, use a minimum of 1000 dpi. - TIFF (or JPG): Combinations
868 bitmapped line/halftone (colour or grayscale), use a minimum of 500 dpi. • Each figure of
869 a manuscript should be submitted as a single file that fits on a single page in portrait format
870 (with figure number indicated in the file name). Do not include figures inside the manuscript
871 text or in a separate word document. • Multi-panel figures (those with parts a, b, c, d etc.)
872 should be submitted as a single composite file that contains all parts of the figure. Panels
873 should be labelled with lowercase letters (a, b, c, etc) or capital letters (A, B, C, etc), but
874 not a mixture of them. (e.g.

875 https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-019-3714-
876 2/figures/1 and https://parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-
877 019-3714-2/figures/2). • Figures should be numbered in the order they are first mentioned
878 in the text (e.g. "Fig. 1", “Fig. 2”, and so on) and uploaded in this order. • Each figure should
879 be closely cropped to minimize the white space surrounding the illustration. This improves
880 accuracy when placing the figure in combination with other elements when the manuscript
881 is prepared for publication on our site. • Make sure you use uniform font size and type
882 (preferred font: Arial 12-20-point) in all figures. All figure elements, including letters,
883 numbers, and symbols, must be in highdefinition and legible at their final size. • Make sure
884 all images are free of copyright restrictions. This includes shapefiles use for maps. Open
885 sources are available (e.g. https://qgis.org/en/site/) and should be used. Maps must contain
886 a scale and a north arrow (compass rose). • Abbreviations used in figures should be defined
887 in the figure caption (within parentheses or as a list of abbreviations used; see example
888 below). • Figure captions should follow the journal style, which can be found in previously
889 published papers (e.g., https://parasitesandvector

890 - All references must be numbered in ascending numerical order; references in figures and
891 tables are numbered last. Reference numbers should be provided in the main manuscript
892 text in square brackets, e.g. Woo et al. [1] or [1–5] or [1, 7, 9, 13–15] or (see [2] for a
893 review). Please follow strictly the format of the examples, including the spacing and
894 punctuation. Note that: (i) abbreviated journal names should be provided; (ii) all organism
895 Latin names should be in italics; (iii) capital letters should be used only for the first word
896 of the article title and proper nouns; (iv) author lists of more than six authors should be
897 presented with ", et al." after the sixth author and (v) non-breaking spaces between words
898 for references copied directly from Internet sources should be removed.