Immunoagglutination test to diagnose Chagas disease: comparison of different latex–antigen complexes

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Abstract

OBJECTIVE To evaluate the diagnostic performance of novel latex–protein complexes obtained from different antigens of *Trypanosoma cruzi* through immunoagglutination test using a panel of *T. cruzi*-positive sera, leishmaniasis-positive sera and negative sera for both parasites.

METHODS Complexes' behaviour using total parasite homogenate (TPH), two simple recombinant proteins (RP1 and RP5) and two chimeric recombinant proteins (CP1 and CP2) was comparatively evaluated. The area under ROC curves was used as an index of accuracy. Sensitivity, specificity and discrimination efficiency were assessed.

RESULTS All recombinant antigens showed higher specificity than TPH. The lower specificity of TPH was mainly due to cross-reacting peptides between *T. cruzi* and *Leishmania* spp. In turn, all performance indicators were higher for CP1 and CP2 than for RP1 and RP5. The carboxylated latex–CP2 (C2–CP2) complex was able to detect antibodies against *T. cruzi*. The values of area under ROC curve (0.96), sensitivity (92.3%, 95% CI: 79.4–100.0%) and specificity (84.0%, 95% CI: 67.6–100.0%) indicate that the assay could be used as a screening test.

CONCLUSION The C2–CP2 complex could be an important tool to carry out sero-epidemiological studies.

keywords immunodiagnosis, Chagas disease, latex–protein complexes

Introduction

Chagas disease is caused by the infection with the protozoan *Trypanosoma cruzi* and affects million people in Latin American. Lately, it has also become a growing problem in the United States and Europe due to migration of people (WHO 2002). As the infection by *T. cruzi* highly stimulates host antibodies (Ab) production during the chronic phase, serology may be accurately used to diagnose *T. cruzi* infection in chronical-infected patients. The assays currently used in clinical practice to detect antibodies to *T. cruzi* are indirect hemagglutination (HAI), indirect immunofluorescence (IFI) and enzyme-linked immunosorbent assay (ELISA) (WHO 2002), even though there are also immunochromatographic tests on the market. For the same purpose, our group has recently proposed an immunoagglutination assay (IA), which is faster than the above-mentioned assays (Gonzalez *et al.* 2010; Garcia *et al.* 2012, 2013). The IA for the detection of Ab comprises mixing serum or plasma with a suspension containing antigens (Ag) bound to latex particles. This method uses small-volume samples, allows results in 5 min, is easy to implement and does not require sophisticated equipment. Furthermore, it is a technique that may be used in the field when immunoagglutination is visually detected.

Traditionally, total parasite homogenate (TPH) extracted from laboratory strains of *T. cruzi* epimastigotes cultures has been the source of Ag used for serological diagnosis. TPH has diagnostic sensitivity to detect even low Ab levels (Guhl *et al.* 2002). However, when using the complex mixture of Ag present in the TPH, it appears that not only specificity problems to cross-reactivity with phylogenetically related microorganisms such as *T. rangeli* (believed to be non-pathogenic to humans) and *Leishmania* spp. (responsible of cutaneous, mucocutaneous and visceral leishmaniasis) occur, but also difficulties in standardising methods (Saez-Alquezar *et al.* 2000; Da Silveira *et al.* 2001).

To avoid such problems, recombinant proteins have been expressed by recombinant DNA methods. In this way, the specific regions responsible for cross-reactivity are excised. Recombinant proteins have been used as Ag for serological diagnosis mainly in ELISAs (Saez-Alquezar

et al. 2000; Umezawa *et al.* 2003, 2004; Aguirre *et al.* 2006). However, none of them have been recognised by all of the chagasic human sera.

To address this last drawback, the use of multipeptide or chimeric proteins expressing several unrelated antigenic determinants has been proposed (Umezawa *et al.* 2004; Aguirre *et al.* 2006; Camussone *et al.* 2009). Although the specificity of the test does not change using chimeric or simple recombinant proteins, the use of multiepitope proteins enhances sensitivity because a greater number of epitopes would be available to capture the Ab present in samples; it also facilitates the standardisation procedure by lowering purification and immobilisation steps and by balancing the number of epitopes on the particle surface.

In this work, the diagnostic potential of latex IA obtained using the TPH and four recombinant Ag of *T. cruzi* (RP1, RP5, CP1 and CP2) was determined. To this effect, a panel of 25 *T. cruzi*-positive sera, 12 leishmaniasis-positive sera and 25 negative sera for both parasites was used. The objective was to determine the latex–protein complex which performs best under the optimal reaction conditions previously obtained (Garcia *et al.* 2014). The diagnostic test evaluation was realised on the basis of ROC curves (receiver operating characteristic).

Materials and methods

All chemicals used in this study were of analytical grade and were used without further purification. Molecular biology reagents were purchased from Promega (Madison, WI, USA), unless otherwise stated. The latex–protein complexes were synthesised in our laboratory and resuspended in borate buffer 2 mM, pH 8 (Anedra) with bovine serum albumin, 0.5 mg/ml (BSA – Sigma), sodium azide, 0.1% w/w (Sigma), glycine 0.1 M (Sigma) and PEG 8000, 3% w/v (Sigma) before their use in immunoassays.

Carboxylated latex (indicated by C2) with a mean diameter of 418 nm was synthesised through a semibatch copolymerisation of styrene and methacrylic acid onto a uniform polystyrene (PS) latex seed. The polymerisation reaction conditions and the characterisation of PS and carboxylated latexes were previously reported (Gonzalez *et al.* 2008a,b; Garcia *et al.* 2012). The unreacted comonomers and initiator were eliminated from the final latex by serum replacement.

Total parasite homogenate

Trypanosoma cruzi epimastigotes culture (Tulahuen strain) was kindly provided by Centro de Investigaciones sobre Endemias Nacionales, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral (Argentina). Cultured epimastigote forms were kept at 28 °C in liver infusion tryptose medium (Camargo 1964) supplemented with 10% foetal bovine serum and 100 mg/ml penicillin G. Then, parasites were washed three times by centrifugation in phosphate-buffered saline (PBS) pH 7.2 0.1 M. The total volume of the suspension, 10 ml, was placed in a sonicator (Sonics) and subjected to three cycles of 2 min at an intensity of 20 kHz, 30 W. The lysate containing the parasite Ag was cleared by centrifugation at 10 000 ***g*** for 15 min. The supernatant was aliquoted and kept at −20 °C until use. Main characteristics of the Total parasite homogenate (TPH) are presented in Table 1.

Recombinant Ags of *T. cruzi* used were the single proteins RP1 and RP5, and the multipeptide proteins CP1 and CP2 (Camussone *et al.* 2009). The chimeric protein CP1 is a unique macromolecule built as the tandem expression of two highly antigenic peptides, RP1 and RP2, whereas CP2 included these two peptides plus RP5. *E. coli* BL21 (DE3) cells bearing the different plasmidic constructions pET-32a/RP1, pET-32a/CP1 and pET-32a/

Table 1 Characteristics of the employed proteins

		Recombinant proteins			
	TPH	RP1	RP5	CP1	CP2
Molar mass (kDa)	20–90	27.9*	26.0*	34.9*	40.8*
Isoelectric point: Ip	5.9–6.3†	5.4*/6.2†	6.1*/6.3†	5.3*/6.2†	5.4*/5.8†
Negatively charged residues*	–	45	33	52	57
Positively charged residues*	–	31	26	31	40
Concentration (mg/ml)‡	–	3.6	2.9	4.1	4.4

*Calculated through ExPasy Program (ExPasy Proteomic Service, http://www.expasy.org/tools/protparam.html).
†Measured by isoelectric focusing.
‡Calculated through Gel-Pro Program.

CP2 were grown overnight in Luria–Bertani (LB) medium, supplemented with 0.1 mg/ml ampicillin at 37 °C, with agitation (180 rpm). In the next morning, a 1:50 culture dilution in LB/ampicillin was incubated at 37 °C under shaking at 200 rpm. When the optical density at 600 nm (OD_{600}) reached 0.5–0.6, isopropyl-β-D-thiogalactopyranoside was added at a final concentration of 0.5 mM to induce gene expression for 3 h at 37 °C. Then, cells were collected by centrifuging at 2286 ***g*** for 15 min and resuspended in column buffer. The suspension was sonicated to achieve complete cell lysis and centrifuged at 4500 rpm for 10 min. The soluble proteins, present in the supernatant, were purified by nickel affinity chromatography as previously described (Camussone *et al.* 2009). The purity of the recombinant proteins was analysed by 15% polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue, according to the method described by Laemmli (1970). Proteins were quantified by electrophoresis by measuring the band intensity and interpolating these values with those obtained using different concentrations of bovine serum albumin. Gel-Pro analysis program was used to determine bands intensity (Figure 1). The main characteristics of the employed recombinant Ag of *T. cruzi* are presented in Table 1.

Latex–protein complexes were obtained by covalent coupling of the antigenic proteins onto the surface carboxyl groups of the latex particles. Assays were performed in duplicate (coefficient of variation 5%). In all cases, high fractions of antigens were chemically bound to the carboxyl groups. The employed recipes and reaction conditions were previously reported (Gonzalez *et al.* 2008a,b, 2010; Garcia *et al.* 2012, 2013). In all cases, antigenic proteins were added to the C2 latex in the presence of N-N-(3-dimethylamine propyl) N'-ethyl carbodiimide activator, and they were shaken during 5 h at room temperature. The latex–protein complexes were isolated by ultracentrifugation, and the non-covalently coupled proteins were desorbed with Triton X-100 during 24 h. The surface densities of covalently bound proteins were calculated from the total linked protein and the amount that remains in solution after a desorption process with Triton X-100. The concentrations of dissolved protein were determined through the copper reduction/bicinchoninic acid (BCA) method (Ortega-Vinuesa *et al.* 1995). Finally, the complexes were redispersed in borate buffer 2 mM, pH 8.0 until use.

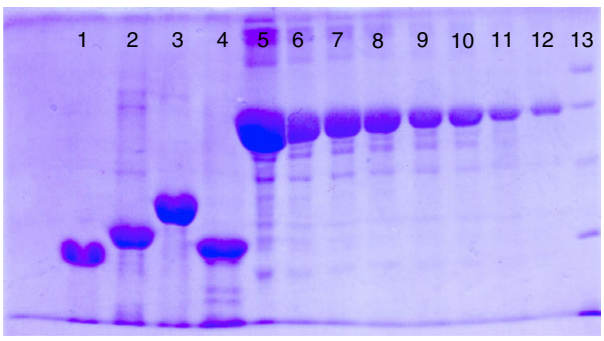

Figure 1 Picture of the gel showing the purity and concentration of the produced recombinants. From left to right: lane 1: RP1; lane 2: CP1; lane 3: CP2; lane 4: RP5, lanes 5–12: pattern of bovine serum albumin (5, 4, 3, 2.5, 2, 1.5, 0.75, 0.325 mg/ml, respectively); and lane 13: molecular weight marker.

Sera used in this study were obtained from samples discarded after routine diagnosis in patients. No personal identifiers were used nor any clinical investigation carried out as part of the present work. Information about the patient's clinical diagnosis is not available. Serum samples from *T. cruzi*-infected patients ($n = 25$) were obtained from a region of endemicity located in northeast Argentina. *T. cruzi* infection status of the patients was established using two different conventional tests, commercial Chagatest ELISA and Chagatest HAI, both from Wiener Lab (Argentina). Sera from individuals infected with American cutaneous leishmaniasis ($n = 12$) were obtained from the Instituto de Investigaciones en Enfermedades Tropicales, Sede Regional Orán, Universidad Nacional de Salta (Argentina). Infection status of the patients was established by the intradermal test (or Montenegro test). Negative sera ($n = 25$) were obtained from healthy blood donors from northeast Argentina. Donors of these negative samples were clinically healthy individuals whose serum samples rendered negative results when tested for both Chagas disease (Chagatest ELISA and Chagatest HAI, both from Wiener Lab) and Leishmaniasis (Montenegro test).

Immunoagglutination assay was performed as recently reported (Garcia *et al.* 2014). In all cases, the agglutination reaction was carried out with 3.11×10^9 particles/ml (with a density of covalently coupled protein about 3.0 mg/m^2) at low ionic strength (20 mM), BSA 0.5 mg/ml and Glycine 0.1 M as blocking agents and PEG 8000 (3% w/v) as a potentiator of the Ab–Ag reaction. The agglutination reaction was detected by turbidimetry, measuring the optical absorbance (A) at 570 nm in an UV/vis spectrophotometer (Perkin Elmer Lambda 25), and the increment in A (ΔA) was determined by subtracting the absorbance of a blank (the complex without serum) to the absorbance measured for the (complex + serum) sample.

All serum samples were evaluated in triplicate. The ΔA results obtained at 570 nm for each latex–protein

complex were used for the construction of ROC curves using the graphic software MedCald. ROC curves were used for diagnostic test evaluation. The most commonly used global measure for the overall accuracy of a diagnostic marker is the area under the ROC curve (AUC) (DeLong *et al.* 1988). In general, AUC values from 0.5 to 0.7 represent low accuracy and do not allow disease health discrimination; values from 0.7 to 0.8 represent acceptable ability of the test to discriminate positive from negative sera; and for AUC values ≥ 0.9, a test can discriminate between positive and negative sera (DeLong *et al.* 1988).

From the *cut-off* values obtained in ROC curves, the relative ΔA (ΔA/*cut-off*) were distributed using the scatter computer graphic software (GraphPad Prism, version 2.0).

Results and discussion

The antigenic performance of the different proteins of *T. cruzi* was evaluated when the Ag was as mixtures (TPH), as a single recombinant protein (RP1 or RP5) or as a unique chimeric construction with two (CP1) or three (CP2) antigenic determinants (RP1 + RP2 and RP1 + RP2 + RP5, respectively). It is noteworthy that, although RP2 is included in both CP1 and CP2 antigens, it was not employed in the present study for producing single Ag–latex complexes due to the low response previously described in ELISAs when using RP2 compared with RP1 and RP5 (Camussone *et al.* 2009). Also, the same panel of *T. cruzi*-positive sera, leishmaniasis-positive sera and negative sera for both parasites was used to compare the reactivities of TPH, RP1, RP5, CP1 and CP2.

Accuracy of diagnostic – area under curve ROC

Figure 2 shows the ROC curves obtained for each complex. Considering the AUC values, TPH (AUC = 0.54 ± 0.11) does not provide an adequate discrimination power. However, the single recombinant proteins RP1 (AUC = 0.85 ± 0.05) and RP5 (AUC = 0.85 ± 0.04) have a moderate discrimination power; the chimeric recombinant proteins CP1 (AUC = 0.92 ± 0.03) and CP2 (AUC = 0.96 ± 0.02) exhibit a good performance and a high diagnostic value.

When evaluating the statistical significance between the different AUCs, the recombinant Ags exhibit a significantly greater AUC than the TPH ($P < 0.05$). The AUCs obtained with recombinant Ag showed no significant difference between chimerics (CP1 *vs.* CP2; $P = 0.23$) nor singles (RP1 *vs.* RP5; $P = 0.50$). However, there is a difference between chimerics and single recombinant Ag (chimerics recombinant *vs.* single recombinant $P = 0.06$). The obtained results showed that by increasing the number of antigenic determinants, the immunoassay discrimination efficiency is enhanced.

Efficiency of diagnosis – sensitivity and specificity

Results of relative ΔA distributions (ΔA/*cut-off*) obtained for each complex are shown in Figure 3. When evaluating the serum samples against TPH, the relative ΔA distributions were similar for the panel of positive sera (mean value: mv = 1.62 ± standard deviation: SD = 0.83) and negative sera (mv = 1.49 ± SD = 0.73), not allowing the discrimination between both types of sera. The TPH produced a sensitivity of 86.7% (95% CI: 63.6–100.0%) and an extremely low specificity (33.3%; 95% CI: 11.7–52.3%).

The use of recombinant Ag instead of TPH allowed overcoming the low-specificity problem. Thus, the results obtained with recombinant Ag showed specificities of 88.0% (95% CI: 71.3–95.0%), 87.5% (95% CI: 70.3–98.0%), 88.0% (95% CI: 73.3–100.0%) and 84.0% (95% CI: 67.6–100.0%), for RP1, RP5, CP1 and CP2, respectively (Figure 2). Most of the negative sera assayed with complexes obtained from recombinant Ag exhibited ΔA values below the *cut-off* line (Figure 3).

Although similar values of specificity were obtained with all the employed recombinant Ag, sensitivity increased when chimeric proteins were used. Thus, assays with RP1, RP5, CP1 and CP2 exhibited sensitivities of 70.4% (95% CI: 47.7–88.3%), 74.3% (95% CI: 57.2–94.7%), 77.8% (95% CI: 57.3–94.7%) and 92.3% (95% CI: 79.4–100.0%), respectively (Figure 2). When using the multipeptide protein CP2, many of the results that appeared as false negatives with the other complexes disappeared, and the number of undetermined results was reduced.

The sensitivity obtained with the C2–CP2 complex (92.3%) is in the order of other screening tests. Sosa-Estani *et al.* (2008) conducted a cross-sectional study of Chagas disease in five endemic areas in Argentina, Bolivia, Honduras and México to assess the use of a lateral flow rapid test (Chagas Stat-Pak; Chembio Diagnostic System Inc) to screen for *T. cruzi* infection at the time of delivery. The Chagas Stat-Pak sensitivity and specificity were 94.6% and 99.0%, respectively. Remesar *et al.* (2009) estimated the sensitivity of two enzyme immunoassays, an HAI test (HAI Chagas; PolyChaco), and a particle agglutination assay (PA) (Serodia-Chagas; Fujirebio Inc.). They observed that assay sensitivities varied from 84% for PA, and 66 to 74% for HAI tests. Specificities were higher than 96% for both evaluated tests. Reithin-

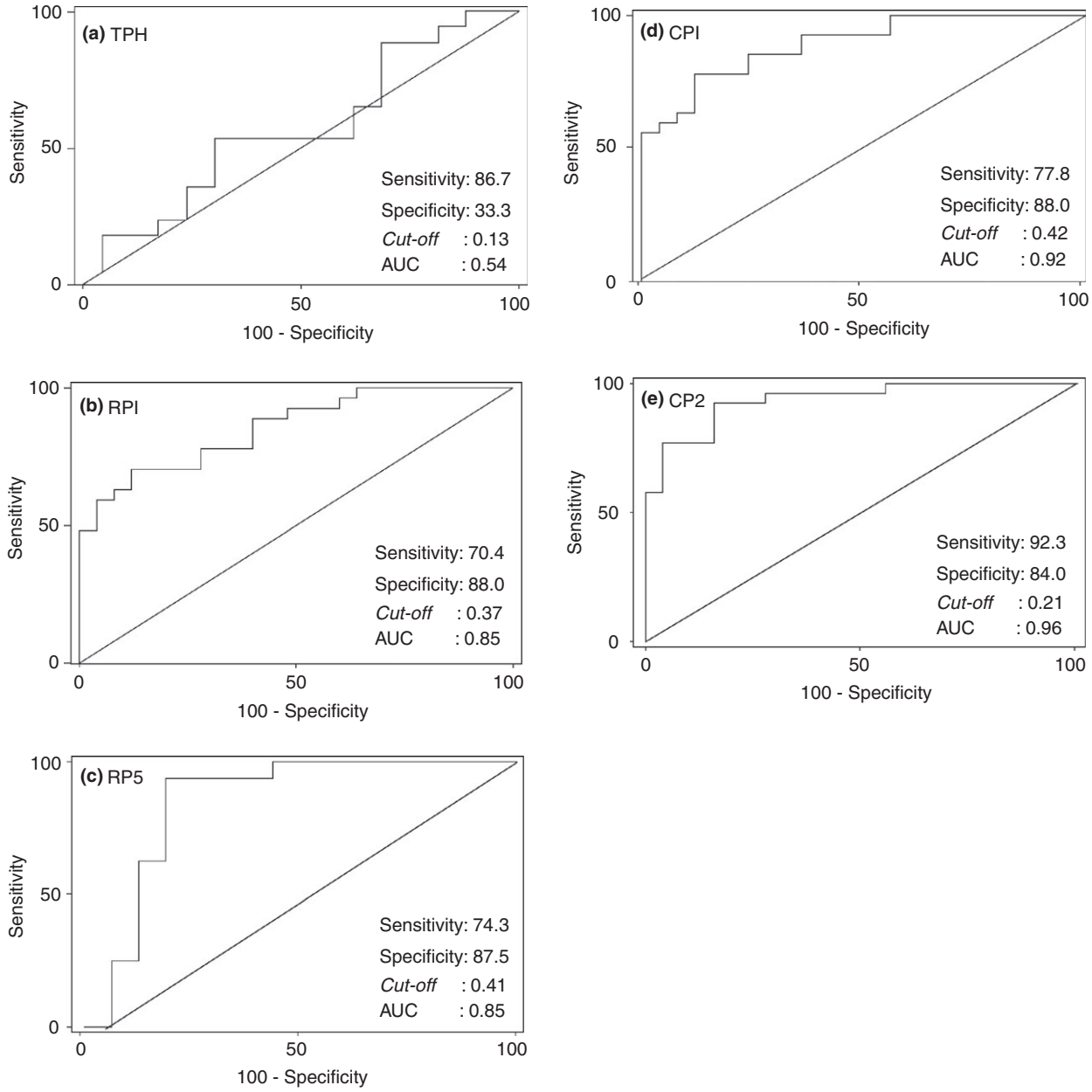

Figure 2 ROC curves obtained from a panel of 25 Chagas disease-positive and 25 Chagas disease-negative serum samples for the different latex–protein complexes analysed: (a) C2–TPH; (b) C2–RP1; (c) C2–RP5; (d) C2–CP1 and (e) C2–CP2.

ger *et al.* (2010) evaluated a commercially available immunochromatographic dipstick test to detect *Trypanosoma cruzi* infection (Chagas Detect Plus; Inbios). The Chagas Detect Plus sensitivity was calculated to be 84.8% and specificity was 97.9%.

In general terms, the use of multipeptide Ag enhanced the immunoagglutination diagnostic potential compared with the single Ag. This result can be due to the fact that when sensitising particles with chimeric constructions (instead of single proteins), sensitivity of the assay is increased because a higher number of freely accessible peptides are available to capture the Ab present in samples. Thus, even though epitope masking may occur, other peptides could still be exposed appropriately to

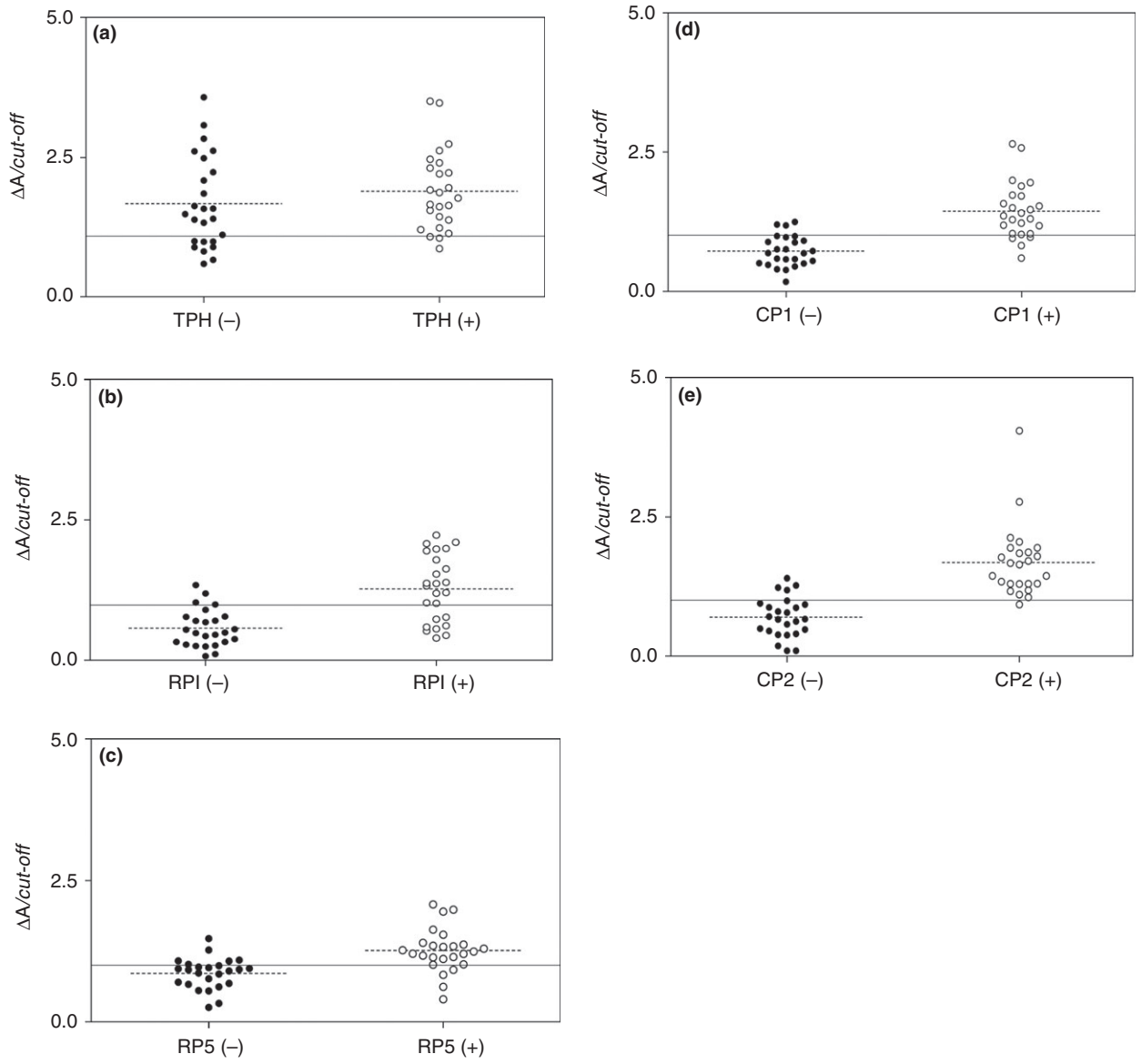

Figure 3 Relative optical distributions (ΔA/*cut-off*) obtained from a panel of 25 Chagas disease-positive and 25 Chagas disease-negative serum samples for the complexes: (a) C2–TPH; (b) C2–RP1; (c) C2–RP5; (d) C2–CP1 and (e) C2–CP2. The dashed lines show the relative mean values for each assay, and the continuous line indicates the relative *cut-off* value (ΔA/*cut-off* = 1). Values greater than 1.0 were considered reactive.

further interact with their specific Ab (Camussone *et al.* 2009).

Cross-reactivity test

Because *T. cruzi* shares antigenic determinants with other pathogens, particularly with members of *Leishmania* spp., we used 12 serum samples from parasitologically diagnosed patients with American cutaneous leishmaniasis, to determine the cross-reactivity. Note that it is important to differentiate these infections because treatment and clinical follow-up are largely different, and seroepidemiological studies could underestimate or overestimate the prevalence of these parasitic diseases.

Although the sensitivity of TPH is highly useful to diagnose *T. cruzi* infection, a lack of specificity is

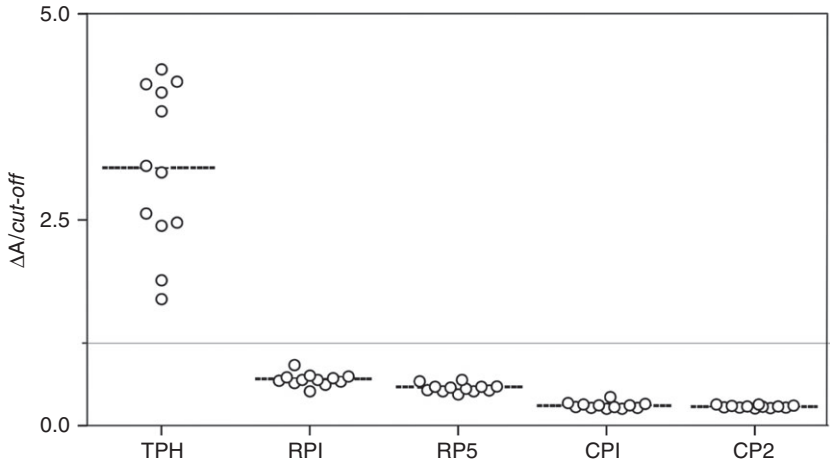

Figure 4 Relative optical distribution (ΔA/*cut-off*) obtained from a panel of 12 Leishmaniasis-positive sera. The dashed lines show the relative mean values for each assay, and the continuous line indicates the relative *cut-off* value (ΔA/*cut-off* = 1). Values greater than 1.0 were considered reactive.

observed in patients with leishmaniasis. As TPH is integrated by a largely undefined complex mixture of Ag, it frequently produces false-positive results and cross-reactivity with *Leishmania* spp. (Chiller *et al.* 1990; Matsumoto *et al.* 1993). The results showed in Figure 3 confirmed the presence of cross-reacting Ag between *T. cruzi* and *Leishmania* spp. when complexes from TPH were used as capture Ag.

Non-cross-reaction was detected with complexes from recombinant Ag, and only sera from chagasic individuals have reacted (Figure 4). From these results, it was concluded that the recombinant Ag is more specific for the detection of anti-*T. cruzi* Ab than TPH. These results were expected as recombinant Ag was synthesised to encode peptide fragments in which the specific regions responsible for cross-reactivity were excised and regions showing high specificity were linked one to another to raise sensitivity.

The absence of cross-reaction with *Leishmania* spp. demonstrated by recombinant Ag is important because this test could be used in regions where *T. cruzi* and *Leishmania* overlap. Interestingly, CP1 and RP1 proteins used in this work were recently assessed with sera from individuals with single *Leishmania* infection or mixed *Leishmania–T. cruzi*-infected patients, and only the last ones gave positive results with these proteins (Vega Benedetti *et al.* 2013).

Conclusion

The antigenic performance of Ag used as mixtures (TPH), as a recombinant individual Ag (RP1 and RP5) or as chimeric constructions (CP1 and CP2) was evaluated with the same panel of *T. cruzi*-positive sera, leishmaniasis-positive sera and negative sera for both parasites. In general terms, the use of recombinant Ag enhanced the performance of IA compared with TPH. The major advantage of the recombinant Ag for the serodiagnosis of Chagas disease was the lack of cross-reaction with leishmaniasis-positive sera. In turn, the use of chimeric Ag displayed better IA accuracy than single recombinant Ag, because chimera rendered a greater available peptide which enhanced the sensitivity of the assay.

The performance comparison of CP1 *vs.* CP2 revealed that the RP5 inclusion into the chimeric construction produced an increase in the discrimination efficiency and a suitable sensitivity (77.8 for CP1 and 92.3 for CP2) to diagnose *T. cruzi* infection. This is an attribute related to the CP2 potential to reduce the number of undetermined results for the tested serum panel.

Our results showed that the immunoagglutination assays performed with the C2–CP2 complex presented high ability to discriminate between positives and negatives sera according to AUC ROC curve (0.96), being the sensitivity (92.3%) and specificity (84.0%) in accord with screening test (Sosa-Estani *et al.* 2008; Remesar *et al.* 2009; Reithinger *et al.* 2010).

The IA could be employed as a method of screening for its use in low-resource settings and then only confirm positive results with reference techniques currently used. The IA is low cost, simple to operate and read, sensitive, specific, and works in a short period of time. Nevertheless, the diagnostic potential of the IA developed in this study needs to be confirmed on the other serum collections.

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