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Evaluation of recombinant antigens of *Trypanosoma cruzi* to diagnose infection in naturally infected dogs from Chaco region, Argentina

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SUMMARY

Dogs are considered the main mammal reservoir of Trypanosoma cruzi *in domiciliary environments. Consequently, accurate detection of* T. cruzi *infection in canine populations is epidemiologically relevant. Here, we analysed the utility of the* T. cruzi *recombinant antigens FRA, SAPA, CP1, Ag1 and a SAPA/TSSA VI mixture, in an ELISA format. We used a positive control group of sera obtained from 38 dogs from the Chaco region in Argentina with positive homogenate-ELISA reaction, all of them also positive by xenodiagnosis and/or PCR. The negative group included 19 dogs from a nonendemic area. Sensitivity, specificity, area under the curve (AUC) of the receiver operating characteristic (ROC) curve and Kappa index were obtained to compare the diagnostic efficiency of the tests. The SAPA/TSSA VI had the highest performance, with a sensitivity of 94·7% and an AUC ROC of 0·99 that indicates high accuracy. Among individual antigens, SAPA-ELISA yielded the highest sensitivity (86·8%) and AUC ROC (0·96), whereas FRA-ELISA was the least efficient test (sensitivity = 36·8%; AUC ROC = 0·53). Our results showed that the use of SAPA/TSSA VI in ELISAs could be a useful tool to study dogs naturally infected with* T. cruzi *in endemic areas.*

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INTRODUCTION

Chagas disease, caused by *Trypanosoma cruzi,* affects an estimated number of 7 million people throughout Mexico, central and southern America (1). Although it is autochthonous in Latin America, it has become a health problem worldwide due to transfusional and vertical transmission associated with migratory processes (2). Particularly, the Chaco region in north-west Argentina is an endemic area for *T. cruzi* infection, where estimations of canine prevalence between 15% and 60·3% were recorded (3, 4). Dogs are considered epidemiologically relevant as reservoirs of the parasite in the domestic cycle of transmission, mainly due to their permanence inside the dwellings, the high level of infectiousness to bugs, and the feeding preference of *Triatoma infestans* for these mammals (4–6). Detecting *T. cruzi* infection in dogs, from areas with active vectorial transmission, is important to estimate the risk of transmission to humans and to evaluate the effectiveness of insecticides (7, 8).

The diagnosis of Chagas disease in human patients has been largely studied. Many recombinant antigens of *T. cruzi* have been described and used for serodiagnosis of the infection in the last 30 years due to their specificity (9). Moreover, the use of mixtures of peptides or synthetic constructions bearing epitopes of different antigens has proved to enhance the detection of antibodies against *T. cruzi* in immunoassays (10, 11). In this study, we examined three recombinant antigens individually: FRA (flagellar repetitive antigen), SAPA (shed acute phase antigen),

CP1, Ag1 and a combination of SAPA with TSSA VI (trypomastigote small surface antigen). These peptides have demonstrated to be useful in terms of sensitivity/reactivity and specificity in humans (9, 11–15). FRA and SAPA are both repetitive antigens proposed as markers of chronic and acute phase of *T. cruzi* infection in humans, respectively (12–14). Camussone *et al.* (11) produced CP1, which is an assemblage of SAPA and FRA antigens. The authors demonstrated that CP1 showed a greater discrimination efficiency than the independent antigens or their mixture (11). Ag1 is another recombinant antigen that was included as it is homologous to FRA (9). In addition, we included the TSSA VI antigen, that is the only protein that proved to be useful for detecting infections with the *T. cruzi* discrete typing units (DTUs) TcII, TcV and/or TcVI (15).

There are few studies focused on the diagnosis of *T. cruzi* infection in dogs and even fewer in naturally infected dogs using recombinant antigens. We reported the effectiveness of SAPA for diagnosing *T. cruzi* infection in dogs in a preliminary study including a small group of animals from an endemic area in Chaco province (16). TSSA VI was also evaluated in another study in dog serum samples with the same origin and demonstrated to be a useful tool for epidemiological studies (17). On the other hand, to our knowledge, FRA and CP1 have never been used for antibodies detection in dog sera.

Due to the epidemiological importance of dogs as potential reservoirs of parasite and the lack of background in assessing recombinants antigens for diagnosis of *T. cruzi* infection in dogs, there is special interest in validating new recombinants antigens with diagnostic purposes. The aim of this work was to assess the diagnostic potential of the recombinant antigens FRA, SAPA, CP1, Ag1 and a SAPA/TSSA VI mixture for *T. cruzi* infection in naturally infected dogs from an endemic area in Chaco province, Argentina.

MATERIALS AND METHODS

Serum samples

Sera from dogs (n = 320) were obtained from a rural area in 12 de Octubre county, Chaco province (Argentina), from 2008 to 2010. Sera from dogs residing in Salta city, an area where there is no vectorial transmission of *T. cruzi* at present, were also included in this study.

The protocol for sample extraction used was reviewed and approved by the Bioethics Commission of the Facultad de Ciencias de la Salud, Universidad Nacional de Salta, Argentina (N° 052-10).

Control groups

The positive control group of samples was defined according to the results of the combination of reference tests: xenodiagnosis (XD), polymerase chain reaction (PCR) and homogenate-ELISA (H-ELISA) (see below). Of the total of samples collected in Chaco, we selected 38 sera from dogs with XD and/or PCR positive that were used as positive controls (PC), all of them were reactive by H-ELISA. Nineteen sera from dogs from Salta city, nonreactive by H-ELISA, were used as negative controls (NC). Figure 1 shows the flowchart describing inclusion of sera in control groups.

Xenodiagnosis

Dogs were examined by this method using 10 uninfected third and fourth instar nymphs of *T. infestans*. Three XD boxes were exposed during 10 min on the belly of each individual animal. Bugs faeces were examined with microscope (400×) for *T. cruzi* infection at 30 and 60 days post-feeding (17).

Polymerase chain reaction

This technique was performed to detect *T. cruzi* DNA in blood samples using primers 121 and 122 targeting the kinetoplast DNA minicircle, as described by Monje Rumi *et al.* (18).

Homogenate-ELISA

The preparation of protein homogenate of *T. cruzi* and the ELISAs were carried out as described previously (17).

Trypanosoma cruzi recombinant antigens (Ags)

We worked with the Ags FRA, SAPA, CP1, Ag1 and TSSA VI produced according to the specifications described previously (11, 14, 17, 19).

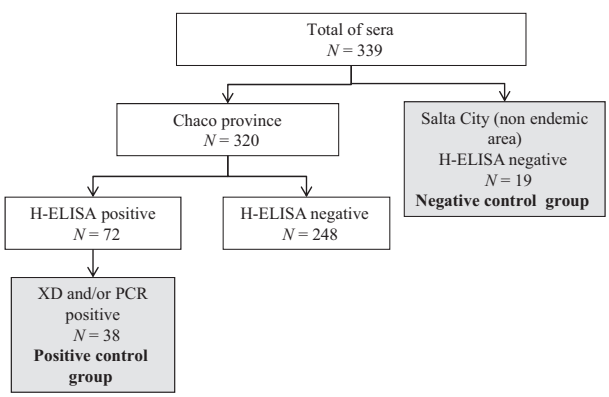

Figure 1 Flowchart describing the inclusion of dog sera and their classification in positive and negative control groups.

ELISA with individual recombinant antigens

The following conditions were evaluated to standardize the ELISAs: (I) two buffers as diluents of Ags (PBS, pH = 7·4 and carbonate, pH = 9·6); (2) eleven dilutions of antigen beginning with 1 μg in 100 μL of buffer per well to $9{\cdot}8{\cdot}10^{-4}$ μg in 100 μL per well; (3) four dilutions of sera (1 : 50; 1 : 100; 1 : 200 and 1 : 400); (4) two biotin-conjugated anti-dog immunoglobulin G (anti-dog IgG) (Sigma, St. Louis, MO, USA) dilutions (1 : 1250; 1 : 2500); and e) two conjugate (peroxidase–avidin) (Sigma, St. Louis, MO, USA) dilutions (1 : 8000; 1 : 16 000). The optimal conditions chosen were those that allowed the best discrimination between positive and negative control sera.

Polystyrene microplates (Kima®, Arzergrande (PD) Italy) were coated with each antigen in diluent buffer and incubated overnight at 4°C. After incubation, the plates were washed with 0·1% Tween in PBS and unspecific sites were blocked with 5% skimmed milk PBS for 1 h. The sera, anti-IgG and peroxidase–avidin were added to the concentrations chosen in the optimization process. Incubation with sera was performed for 1 h while anti-IgG and peroxidase–avidin were incubated for 30 min each. The colorimetric reaction was developed using ortho-phenylenediamine (OPD) diluted in citrate buffer and stopped with 2 M sulphuric acid. The absorbance was measured using an ELISA reader ELx800 (BIO-TEK INSTRUMENTS, INC. Vermont, USA) with 490 nm-filter. All samples were analysed in duplicate.

ELISA with antigenic mixture SAPA/TSSA VI

To standardize the assay conditions with the antigen mixture, the previously determined optimal concentrations for both antigens were considered. Three serial dilutions of a mixture of SAPA and TSSA VI were evaluated: 0·25 μg of SAPA and 0·1 μg of TSSA VI per 100 μL of coating buffer; 0·125 μg of SAPA and 0·05 μg of TSSA VI per 100 μL of coating buffer; and 0·0625 μg of SAPA and 0·025 μg of TSSA VI per 100 μL of coating buffer. Also, four dilutions of serum (1 : 50; 1 : 100; 1 : 200; 1 : 400) were evaluated. The dilutions of biotin conjugated anti-dog IgG and peroxidase–avidin used were 1 : 2500 and 1 : 16 000, respectively.

Data analysis

The mean of the optical density (OD) values was calculated for each sample. These data were used to perform a receiver operating characteristic (ROC) curve analysis using the GraphPad Prism v.5 software (GraphPad Software, San Diego, SA, USA). The cut-off point for each assay was selected considering the parameters of sensitivity and specificity that resulted from the mentioned analysis. The parameter area under the curve (AUC) ROC obtained for each assay was interpreted according to Swets (1988) as follows: values between 0·5 and 0·7 indicate low accuracy; between 0·7 and 0·9, indicate moderate accuracy and can be useful for some purposes; a value bigger than 0·9 indicates high accuracy (20).

Relative optical density (ROD) values were calculated as the mean OD/cut-off for each serum. Samples with a ROD of 1 or higher were considered positive. Agreement between assays was analysed by estimating Kappa index (KI) using epidat 3.1 software (http://dxsp.sergas.es). We used Landis and Kochs scale to interpret this index (KI: ≤0 = poor agreement; 0·01–0·2 = weak; 0·21–0·4 = regular; 0·41–0·6 = moderate; 0·61–0·8 = substantial; 0·81–1 = almost perfect; 1 = perfect).

RESULTS

The optimal conditions selected for each ELISA during the standardization process are detailed in Table 1. The parameters of sensitivity, specificity, concordance and AUC ROC ($P < 0{\cdot}05$) were estimated for each ELISA (Table 2). Among individual antigens, FRA and SAPA antigens exhibited the lowest and highest sensitivity values (36·8% and 86·8%, respectively). Moreover, SAPA-ELISA showed the highest accuracy according to its AUC ROC value (0·96), followed by CP1 (0·93), Ag1 (0·78) and FRA (0·53) ELISAs. All the tests evaluated showed maximum specificity (100%).

Table 1 Optimal conditions selected in the standardization process for each ELISA

Test	Antigen dilution (in 100 μL of buffer per well)	Sera dilution	Anti-dog IgG-biotin dilution	Peroxidase-avidin dilution
FRA-ELISA	0·5 μg of FRA antigen in buffer carbonate	1/100	1/1250	1/8000
SAPA-ELISA	0·25 μg of SAPA antigen in PBS buffer	1/50	1/2500	1/16 000
CP1-ELISA	0·5 μg of CP1 antigen in buffer carbonate	1/50	1/2500	1/16 000
Ag1-ELISA	0·5 μg of Ag1 antigen in buffer carbonate	1/50	1/1250	1/8000
SAPA/TSSA VI-ELISA	0·25 μg of SAPA and 0·1 μg of TSSA VI antigens in buffer carbonate	1/100	1/2500	1/16 000

Table 2 Diagnostic efficiency parameters and the number of positive and negative samples for each ELISA after evaluating control groups employing recombinant antigens

Test	Sensitivity (Number of positive sera/total of positive controls) (95% CI)	Specificity (Number of negative sera/total of negative controls) (95% CI)	AUC ROC (95% CI)	KI (95% CI)
FRA-ELISA	36·8% (14/38) (21·8–54)	100% (19/19) (82·4–100)	0·53 (0·38–0·68)	0·29 (0·13–0·44)
SAPA-ELISA	86·8% (33/38) (71·9–95·6)	100% (19/19) (82·4–100)	0·96 (0·91–1)	0·82 (0·67–0·97)
CP1-ELISA	76·3% (29/38) (59·8–88·6)	100% (19/19) (82·4–100)	0·93 (0·87–1)	0·72 (0·55–0·89)
Ag1-ELISA	55·3% (21/38) (38·3–71·4)	100% (19/19) (82·4–100)	0·78 (0·66–0·9)	0·46 (0·28–0·64)
SAPA/TSSA VI ELISA	94·7% (36/38) (82·2–98·4)	100% (19/19) (82·4–100)	0·99 (0·99–1)	0·89 (0·77–1)

AUC, area under the curve; ROC, receiver operating charactheristic.

Figure 2 shows the ROD values of sera calculated for each assay, clearly indicating that the ELISA with the antigenic mixture had the best reactivity and was the one that best discriminated between control groups. Sensitivity and agreement (KI) with reference tests of SAPA/TSSA VI mixture were 94·7% (36/38) and 0·89, respectively. The AUC ROC of this assay was 0·99, indicating an excellent ability of the test to discriminate between control groups (Table 2).

Table 3 shows the results of the KI comparison between the tests. SAPA and SAPA/TSSA VI ELISAs had the best diagnosis agreement (KI: 0·93), followed by SAPA and CP1 ELISAs (KI: 0·89) and SAPA/TSSA VI and CP1 ELISAs (IK: 0·82) indicating almost perfect agreement for all these groups of tests (Table 3).

DISCUSSION

Dogs are considered important reservoirs of *T. cruzi* in endemic areas (4–6). Taking into account that the infection status of canine populations in endemic areas could represent an indicator of the effectiveness of insecticide-spraying campaigns (7, 8), accurate assays for the diagnostic of *T. cruzi* infection in dogs are desirable. Although the performance of different recombinant antigens used to detect human infections have been widely examined and validated (9), they have been scarcely assessed to diagnose infections in dogs. Furthermore, these few reports are hardly comparable as they have been performed using different techniques, such as: ELISA (17, 21), Western blot (22), immunochromatography (23) and trans-sialidase inhibition assay (24). When serological techniques are applied to large sample sizes, a versatile, simple and economic technique is needed. We think that the ELISA technique, using recombinant antigens, has all these characteristics.

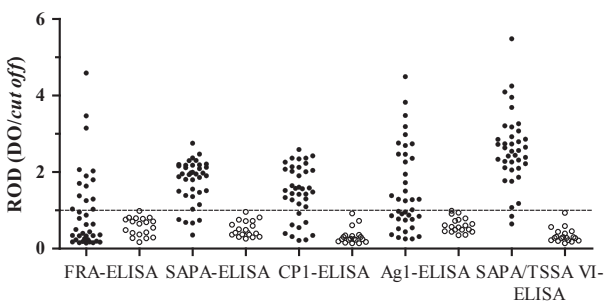

Figure 2 Relative optical density (ROD) values obtained with each ELISA by evaluating control groups. Black circles: positive controls; white circles: negative controls.

All the assays with the recombinant antigens evaluated in this work presented 100% specificity. Although SAPA-ELISA obtained high sensitivity (86·8%), this parameter was lower than the one reported preliminarily in dogs (100%) (16), probably due to the differences in sample sizes between studies. The lower sensitivity of CP1-ELISA (76·3%) when compared to SAPA-ELISA could be explained by CP1 composition, as this chimeric antigen has four repeats of SAPA antigen against 12–14 repeats that compose the natural antigen (25). Nevertheless, the concordance between both tests was almost perfect according to Landis and Kochs interpretation of KI. The AUC ROC calculated showed that these assays had high exactitude to discriminate PC from negative ones.

The results showed that the least efficient assay for diagnosing *T. cruzi* infection in dog sera was

Table 3 Kappa index for all the different pairs of ELISAs

Tests	Ag1-ELISA	FRA-ELISA	CP1-ELISA	SAPA-ELISA	SAPA/TSSA VI-ELISA
Ag1-ELISA	–				
FRA-ELISA	0·64 (0·43–0·84)	–			
CP1-ELISA	0·55 (0·35–0·75)	0·32 (0·11–0·52)	–		
SAPA-ELISA	0·53 (0·33–0·73)	0·38 (0·2–0·56)	0·89 (0·78–1)	–	
SAPA/TSSA VI-ELISA	0·54 (0·35–0·72)	0·34 (0·17–0·51)	0·82 (0·68–0·97)	0·93 (0·83–1)	–

FRA-ELISA, with 36·8% sensitivity and a regular concordance with reference tests. The greater sensitivity of Ag1-ELISA (55·3%) could be explained by the higher number of repetitions that compose this antigen than its homologous FRA, evidenced in Ag1 higher molecular weight in SDS-PAGE polyacrylamide electrophoresis (data not shown). This behaviour is consistent with a previous report in which the influence of the number of repetitions in the antigenicity of FRA antigen has been determined (19). Besides, the agreement between these two tests was not as high as it was expected for being homologous (KI: 0·64).

Interestingly, FRA is one of the most sensitive antigens to diagnose *T. cruzi* infection in humans (9, 26); however, in the present work we found that, the antigen develops a very poor response in sera from *T. cruzi* infected dogs. The comparative study of antigenicity in different species has not been previously described for this antigen. Alvarez *et al.* (27) determined that each repeat of 12 amino acids of SAPA antigen contains multiple epitopes for different species: two for humans and three for rabbits and mice. This kind of information could be useful to explain why recombinant antigens of *T. cruzi* that are highly reactive in human sera have a different sensitivity in other species, confirming that, antigens have to be assessed in different species to be used for diagnostic purposes, as evidenced in this work.

The use of recombinant antigens in the serological diagnosis of *T. cruzi* infections provides advantages against the use of parasite protein extracts, such as specificity, facility of production process and reproducibility (26, 28). The most reactive recombinant antigen studied here could not reach by itself the sensitivity of the protein homogenate in dog sera. However, the antigen mixture SAPA/TSSA VI had a significant reactivity (95%) that could be as useful as the protein homogenate of *T. cruzi* with the advantage of being specific. The specificity of these antigens, compared to other parasite infections, was analysed in humans previously (11, 12, 17, 25, 26, 28, 29, 30).

It is noteworthy that TSSA VI antigen is specific for TcII and/or TcV and/or VI DTUs and so, it is expected that dogs infected with other DTUs will not generate antibodies against this antigen. However, we think that the incorporation of this antigen in a mixture of recombinant proteins is profitable because these lineages are highly prevalent in the countries of the southern cone of America (31). Particularly, the predominant presence of lineage TcVI was evidenced in dogs in the Chaco Region in north-western Argentina (17, 32, 33). The identification of DTU of *T. cruzi* infecting 29 of the PC used in this work resulted in 25 (86%) dogs infected by lineage TcVI (16/25) or mixtures of lineage TcVI and others (TcI and TcIII, mainly) (9/25) (M. Rumi, unpublished data). This confirms the prevalence of lineage TcVI in dogs in the Chaco region (32, 33) and highlights the importance of including TSSA VI antigen in an antigenic mixture for the immunodiagnosis of infection with *T. cruzi* in dogs in this endemic area.

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AUTHORSHIP

N Floridia-Yapur performed the immunoassays, analysed data and wrote the paper; AF Vega-Benedetti, J Nasser and RO Cimino contributed to data analysis and revised the paper critically; M Monje Rumi, P Ragone, JJ Lauthier, N Tomassini, A Alberti dAmato and P Diosque performed sample collection and both parasitologic and PCR analysis; I Lopez-Quiroga contributed with essential technical support; P Diosque revised the paper critically; I Marcipar contributed with recombinant antigens FRA and CP1 and revised the paper critically; N Floridia-Yapur and RO Cimino designed the research study.

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