Immuno-enzymatic evaluation of the recombinant TSSA-II protein of *Trypanosoma cruzi* in dogs and human sera: a tool for epidemiological studies

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SUMMARY

The rTSSA-II (recombinant Trypomastigote Small Surface II) antigen was evaluated by ELISA to detect anti-*Trypanosoma cruzi* antibodies in sera from naturally infected dogs and humans. For this evaluation ELISA-rTSSA-II was standardized and groups were classified according to the results obtained through xenodiagnosis, ELISA and PCR. Sensitivity (Se), Specificity (Sp), Kappa index (KI) and area under curve (AUC) were determined. The Se was determined by using 14 sera from dogs infected with *T. cruzi* VI (TcVI) whereas Sp was determined by using 95 non-chagasic sera by xenodiagnosis, ELISA-Homogenate and PCR. The performance of ELISA-rTSSA-II in dog sera was high (AUC = 0·93 and KI = 0·91). The Se was 92·85% (1 false negative) and Sp was 100%. Two sera from dogs infected with TcI and 1 with TcIII were negative. For patients infected with *T. cruzi*, reactivity was 87·8% (36/41), there was only 1 indeterminate, and Sp was 100%. Fifty-four sera from non-chagasic and 68 sera from patients with cutaneous leishmaniasis did not react with rTSS-II. ELISA-rTSSA-II showed a high performance when studying sera from naturally infected dogs and it also presented 100% Sp. This assay could be an important tool to carry out sero-epidemiological surveys on the prevalence of *T. cruzi* circulating lineages in the region.

Key words: *Trypanosoma cruzi*, Chagas' disease, lineage, ELISA, TSSA-II, diagnosis, Argentina.

INTRODUCTION

Chagas' disease or American Trypanosomiasis is a parasitosis that affects approximately 15 million people in Latin America (Coura and Dias, 2009). *Trypanosoma cruzi*, the causal agent of the disease, infects several mammals, including humans, dogs, cats, opossums and other domestic and sylvatic hosts (Minter, 1978).

Serological diagnosis of Chagas' disease assays includes indirect haemagglutination (IHAs), indirect immunofluorescence (IFI) and enzyme-linked immunosorbent assay (ELISA). These serological methods are used in the diagnosis of chagasic infection in humans and dogs due to their simplicity, low cost and good performance in terms of specificity and sensitivity (Lauricella *et al*. 1993, 1998; da Silveira *et al*. 2001). Crude antigens or homogenates of *T. cruzi* often present cross-reaction with other parasites, mainly *Leishmania* spp. The use of recombinant antigens, mixed antigens, synthetic peptides and quimeric proteins (multi-epitope fused protein) has been proposed to improve specificity and sensitivity.

Recombinant antigens such as SAPA and *trans*-sialidase are used for the diagnosis of acute and congenital infections, whereas CRA, B13 and FRA are specific for chronic infection. The diagnostic test of these antigens through multi-centre studies has shown sensitivity and specificity between 0·86–1·00 and 0·95–1·00, respectively (Affranchino *et al*. 1989;

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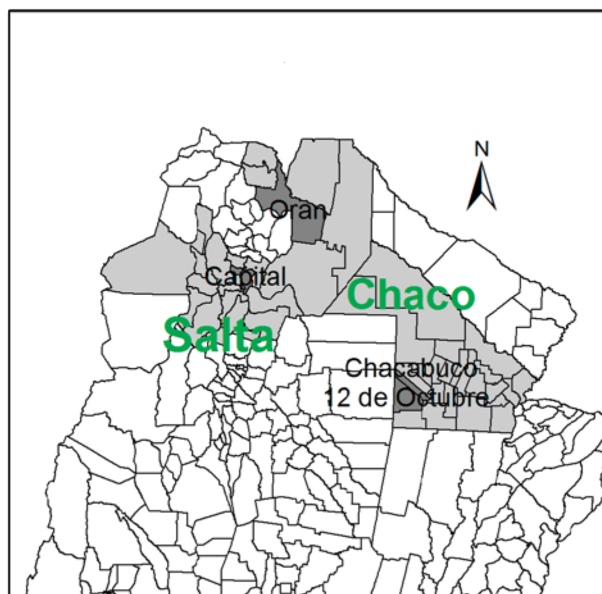

Fig. 1. Map of the northern part of Argentina showing the areas of study and sources for the samples. (1) Endemic area for ATL, (2) endemic area for Chagas's disease and for vector transmission active, (3) non-endemic area (Salta, Capital City).

Reyes *et al*. 1990; da Silveira *et al*. 2001, Umezawa *et al*. 2003; Pereira-Chioccola *et al*. 2003).

Evaluation of a mixture of recombinant antigens or synthetic peptides showed high performance (sensitivity and specificity 100%). The construction of quimeric proteins, (multi-epitope fused antigens), has shown high performance by ELISA (Gadelha *et al*. 2003; Aguirre *et al*. 2006; Camussone *et al*. 2009).

The TSSA protein (Trypomastigote Small Surface Antigen) of *T. cruzi* is located on the parasite surface and it is highly immunogenic. The TSSA antigen is encoded by the dimorphic tssa gene, showing a sound polymorphism between *T. cruzi* I (TSSA I) and the formerly named *T. cruzi* II group lineage (TSSA II). Until 2009. *cruzi* was identified by 2 main lineages: TcI and TcII (this one including 5 sublineages TcIIa-e), at present known as 6 discrete typing units (DTUs): TcI to TcVI (Zingales *et al*. 2009). TSSA is the first serological marker to identify *T. cruzi* lineages in human infection (Di Noia *et al*. 2002; Buscaglia and Di Noia, 2003).

Molecular analysis of the TcII-TCVI TSSA has demonstrated great diversity in the nucleotide sequence and amino acids (Bhattacharyya *et al*. 2010).

The aim of this study was to evaluate the diagnostic performance of recombinant TSSA II antigen of *T. cruzi* (CL Brener reference strain) through the ELISA technique using serum samples from naturally infected dogs and humans. The specificity of this antigen was evaluated using serum samples from patients with American Tegumentary Leishmaniasis (ATL) from the province of Salta.

MATERIALS AND METHODS

Origin of samples

We analysed sera of dogs and humans from an endemic area for Chagas' disease in the province of Chaco, Argentina (Diosque *et al*. 2004). The sera of patients with ATL were obtained from patients evaluated at the Instituto de Investigaciones de Enfermedades Tropicales at the Universidad Nacional Salta- Sede Regional Orán, a regional reference centre for diagnosis of leishmaniasis (Fig. 1). The *T. cruzi* infection of the patients was established by conventional serological (CS), Chagatest ELISA recombinant v.3.0 and haemagglutination (Chagatest IHA) from Wiener Laboratories, Rosario, Argentina. All tests were performed according to the manufacturer's instructions. All patients with ATL presented classic cutaneous leishmaniasis lesions. All were positive by smear and/or Montenegro reaction, and positive serology. The serum samples of dogs and humans that were used as normal controls were collected at Salta-city, an area non-endemic for Chagas' disease and ATL (Fig. 1).

The protocol used was reviewed and approved by the Bioethic Commission of the Facultad de Ciencias de la Salud (Universidad Nacional de Salta, Argentina).

Xenodiagnosis (Xe)

We applied 3 boxes, each of them containing 10 nymphs of uninfected *T. infestans* (third-fifth instar) in all the studied dogs, for a period of at least 15 min. Insect feces were examined (400 ×) for the presence of *T. cruzi* infection on days 30 and 60 post-feeding.

Polymerase Chain Reaction (PCR)

PCR to detect *T. cruzi* DNA was performed in duplicate according to the method previously described (Wincker *et al*. 1994); primers used were 121 and 122, to amplify the hypervariable region of minicircles (HVRm). The PCR amplification protocol was performed according to Britto *et al*. (1995).

ELISA-Homogenate (ELISA-H) dogs

The parasites were cultivated in liver infusion tryptose (LIT) medium from 8 to 12 days and concentrated by centrifugation at 966 ***g*** for 20 min. The extracts of the soluble fraction were obtained by lysis of the pellets using a buffer lysis (50 mM Tris base, pH 8, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 0·1% Triton x100) followed by sucrose (final concentration 10%). The lysed parasites were centrifuged at 20 000 ***g*** for 60 min at 4 °C and the supernatant was obtained (Nasser, 1997). Aliquots were taken for protein quantification by the Bradford method (Bradford, 1976). Extracts were stored at −20 °C for a posterior use.

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ELISA was carried out as described by Nasser (1997) and Lauricella *et al*. (1998) with modifications. Polyestyrene microplates were coated with 2 μg per well of the homogenate dissolved in carbonate buffer (pH 9·6). The plates were incubated at 4 °C, washed 5 times with 0·1% Tween in PBS, and blocked for 1 at room temperature with 5% skimmed milk. After 5 washes with Tween-PBS, microplates were incubated with serum samples 1/100 diluted in 1% skimmed milk in PBS. Then anti-dog immunoglobulin G (IgG) biotin (Sigma) and peroxidase-avidin were used, these were diluted 1:2500 and 1:16 000, respectively. The reaction was developed using *o*-phenylenediamine in H_2O_2 (Sigma) diluted in citrate buffer (pH 5·3) and incubated for 30 min at room temperature. In order to stop the reaction 2 M H_2SO_4 was used, and absorbance was read at 490 nm using a Bioteck ELx800 apparatus. All samples were tested in duplicate.

ELISA-rTSSA-II dogs

The plasmid (pGEX2) was cloned with the sequence expressing GST-TSSA-II (CL-Brener reference strain). *Escherichia coli* (BL21) cells were transformed; the protein expression was carried out with isopropyl-*β*-D-thiogalactopyranoside (0·1 mM final concentration) at 30 °C for 3 h. The cellular lysis was carried out by using mixed lysozyme-DNAse, and centrifuging at 20 000 ***g***. The supernatants were used to purify the protein with a glutation-agarose column (GE), then they were washed with Tris-buffered saline and eluted with buffer containing 5 mM glutation (Sigma). The concentration of the purified protein was measured using the Bradford assay. The purity of the recombinant protein was analysed by 10% SDS-PAGE and staining with Coomasie Blue (Laemmli, 1970; Sambrook *et al*. 1989)

ELISA was previously standardized evaluating the following: (a) Ag optimal concentration (0·1 and 0·5 μg per well/100 μl); (b) 2 buffers as diluents of Ag, (PBS, pH 7. 2 and carbonate, pH 9·6); (c) absorption of non-specific antibodies (anti-GST), sera incubated for 1 h at room temperature in microplates coated with GST (0·5 μg per well/100 μl); (d) different dilutions of serum, 1:100, 1:200, 1:400, 1:500 and 1:1000; and (e) 2 conjugate (peroxidase-biotin) dilutions, 1:8000 and 1:16 000. Optimal conditions were those that best discriminated between positive and negative sera. Polystyrene plates were then coated with 0·1 μg per well/100 μl in PBS. The plates were incubated at 4 °C overnight, washed 5 times with 0·1% Tween in PBS during 2 min and blocked for 1 h at room temperature with 5% skimmed milk in PBS. Microplates were incubated with a 1:500 of dog serum in 1% skimmed milk in PBS, after absorption of non-specific antibodies. After 5 washes, microplates were incubated with anti-dog IgG biotin (Sigma) diluted 1:2500 and then with 1:16 000 peroxidase-avidin (conjugated) for 30 min at room temperature. The reaction was developed using tetramethyl benzidine-TMB-(Zymed) in H_2O_2. The reaction was stopped with 0·5N H_2SO_4 and the absorbance was measured with an ELISA reader (Bioteck ELx800) with 450 nm filter. All samples were measured in duplicate.

ELISA-rTSSA-II humans

Polystyrene plates were then coated with 0·1 μg per well/100 μl in PBS and serum dilutions of 1:200 and 1:10 000 peroxidase-conjugated anti-human IgG (Sigma) were used. All samples were measured in duplicate. The reaction was developed using TMB in H_2O_2, stopped with 0·5 N H_2SO_4 and the absorbance read at 450 nm.

Groups of sera from dogs

In total, 121 dogs were tested using xenodiagnosis, ELISA and PCR. According to the results the following groups were formed. Group A, 18 negative control (NC) sera, corresponding to dogs from Salta City, a non-endemic area for *T. cruzi*, all of them negative for serology. Group B, 75 sera corresponding to dogs from an area with active vector transmission, all of them negative for Xe, serology and PCR. Group C, 15 positive control (PC) sera, corresponding to naturally infected dogs in which *T. cruzi* isolates were obtained and studied by multilocus enzyme electrophoresis (MLEE) (Diosque *et al*. 2003). Fourteen of them were identified as belonging to TcVI and 1 of them as belonging to TcIII. Group D, sera from 2 dogs infected by TcI, according to the MLEE typing of the obtained isolates. Group E, 12 sera positive by serology and negative for Xe; 6 of these dogs were found to be positive by PCR.

Groups of human sera

A total of 211 serum samples was used in this study. The samples were classified in 6 different groups. *Group I (n=54)* sera from non-chagasic patients, which were used as NC in all trials; *Group II (n=41)* samples from chagasic patients that were positive by IHA and ELISA; *Group III (n=31)* samples from patients with cutaneous leishmaniasis and positive for CS (ELISA) Chagas' disease; *Group IV (n=11)* samples from patients with cutaneous leishmaniasis and negative for CS Chagas' disease and PCR; *Group V (n=6) Leishmania spp- T. cruzi* infection (mixed infection), samples from patients with cutaneous leishmaniasis positive for CS Chagas' disease and PCR; *Group VI (n=68)* samples from patients with cutaneous leishmaniasis and negative for CS Chagas' disease.

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Table 1. Results obtained using ELISA-rTSSA-II in sera from dogs

(Number of reactive sera/total sera given in parentheses. ND, not determined.)

Groups	n	Xenodiagnosis	ELISA- H	PCR	ELISA-rTSSA-II
A	18	ND	0% (0/18)	ND	0% (0/18)
B	75	0% (0/75)	0% (0/75)	0% (0/75)	0% (0/75)
C	15	100% (15/15)	100% (15/15)	33,33%[a] (5/15)	86,67%[b] (13/15)
D	2	100% (2/2)	100% (2/2)	100% (2/2)	0% (0/2)
E	11	0% (0/12)	100% (12/12)	50,0% (6/12)	50,0% (6/12)

[a]Performed only PCR on 5 dogs.
[b]One false negative from a dog infected with TcIII and another one with TcVI.

Statistical analysis

ELISA cut-off was calculated as the mean optical density (OD) of the true negative serum samples plus 3 standard deviations. Relative optical density (ROD) was calculated and defined as OD/ cut-off, where OD is the value of serum samples and cut-off is the OD value. Values greater than 1·0 were considered reactive. Sensitivity (Se) was calculated by dividing the number of seropositive samples by the number of PC. Specificity (Sp) was calculated by dividing the number of seronegative samples by the number of NC. Kappa index (KI) and ROC (receiver operating characteristic) curves were calculated and the 95% confidence intervals (CI) were determined (Robertson and Zwieg, 1981; Sim and Wright, 2005; Gardner and Greiner, 2006). EPIDAT (v.3·1) software was used to determine the performance of the test (http://dxsp.sergas.es).

RESULTS

Standardization of ELISA-rTSSA-II

Prior to diagnostic evaluation, standardization of ELISA was performed to obtain a better discrimination between positive and negative controls. The pH diluents of rTSSA-II significantly affected the test performance; the use of PBS (pH7·2) was better than the use of the carbonate buffer. The absorption of non-specific antibodies using coated plates with GST allowed improvement of Sp without affecting S. The immunogenicity of the Ag was important, serum dilutions to 1/1000 gave positive reactions (data not shown).

Evaluation of ELISA-H in dog sera

In Table 1, different serum groups and status are presented for each diagnostic test. The ROC area (AUC) was calculated using 26 sera from chagasic dogs with positive parasitology and/or PCR, and 26 sera from dogs with negative parasitology and PCR. The AUC was 0·97 (IC 95%: 0·955–1·00). The ELISA-H performance was optimal at a cut-off optical density value of 0·31. At this cut-off, the Se of ELISA was 96·15% (25/26) and the Sp was 100% (0/26). The OD mean and standard deviations (mean ± S.D.) of positive and negative serum controls were the following: 1·094 ± 0·51 and 0·15 ± 0·09, respectively. The agreement was excellent (KI = 0·96) according to Landis and Koch (see Sim and Wright, 2005).

Evaluation of ELISA rTSSA-II in dog sera

AUC was determined using 15 sera from chagasic dogs (Group C) and 45 sera from dogs with negative parasitology, serology (ELISA-H) and PCR. The AUC was 0·93 (IC 95%: 0·831–0·98). Comparison between ELISA-H AUC and ELISA-rTSSA-II AUC did not show statistically significant differences ($P > 0{\cdot}05$) (Fig. 2). The Se was 92·86% (13/14) and the Sp was 100% (0/95), all NC sera were non-reactive. One serum sample from an infected dog with TcIII was negative and 2 serum samples from infected dogs with TcI were negative for rTSSA-II. The agreement according to KI (0·91) was very good (Table 2). From group E dogs (seropositive by ELISA-H), 50·0% (6/12) were reactive for rTSSA-II (Fig. 3).

Evaluation for ELISA-rTSSA-II in human sera

The Sp for rTSSA-II was 100%; Groups I, III and IV sera were non-reactive. The reactivity in the chagasic group (Group II) was 90·62% (37/41), whereas KI between CS and ELISA-rTSSA-II was 0·878 (EE: 0·067). From the sera of seropositive leishmaniasic patients, 70·96% (22/31) were positive and 2 cases were indeterminate (8·3%). In Group V (mixed infection), 66·67% (4/6) were positive by rTSSA-II (Fig. 4 and Table 3).

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Table 2. Performance of the ELISA-rTSSA II in dogs' sera; comparison with ELISA-H, (KI) Kappa index and (CI) confidence interval

(S.E., standard error.)

ELISA	Sensitivity (95% CI)	Specificity (95%CI)	KI (S.E.) (95% CI)	AUC (S.E.) (95% CI)
Homogenate	96·15% (86·84–100)	100% (98·08–100)	0·96 (0·03) (0·88–1·00)	0·97 (0·02) (0·88–0·99)
rTSSA II	92·86% (75·80–100)	100% (99·33–100)	0·91 (0·05) (0·80–1·00)	0·93 (0·046) (0·831–0·98)

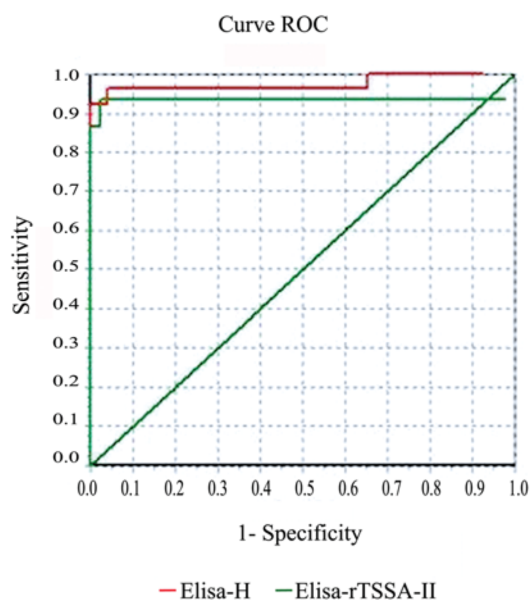

Fig. 2. Comparison of the ROC curves between ELISA-H and ELISA-rTSSA-II. Red line: ELISA-H. Green line: ELISA-rTSSA-II.

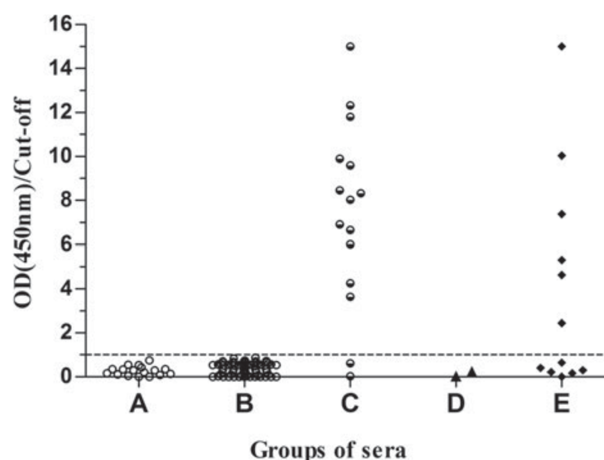

Fig. 3. Reactivities of sera from dog against recombinant TSSA-II by the ELISA-rTSSA-II. The results were calculated as the ratio of the absorbance of each serum sample at an optical density of 450 nm to the cut-off value. Values greater than 1.0 were considered reactive. (A) Negative control non-endemic area sera, (B) negative control endemic area sera, (C) positive control sera, 14 of them infected with TcVI and 1 with TcIII, (D) sera from dogs infected with *Trypanosoma cruzi* I, (E) sera from dogs seroreactive by serology.

DISCUSSION

A significant number of recombinant antigens have been used for the serological diagnosis of Chagas' disease. The advantage of using recombinant antigens is recognized particularly for the identification different phases of the disease (acute or chronic), and also for the detection of congenital transmission in infants. The evaluation of recombinant antigen-based assays and commercial kits through multicentre studies permitted observation of differences in accuracy at a regional level (Umezawa *et al*. 2003, 2004). The genetic variability of the parasite among lineages and the host-parasite relationship may be responsible for the variability in antibody responses which could also affect the performance of different diagnostic tests. For the diagnostic evaluation of ELISA-rTSSA-II, serum groups were made up *a priori*, characterized by other diagnostic tests like xenodiagnosis, PCR, ELISA-H and commercial kits (ELISA-Recombinant Wiener). ELISA-H showed high quality performance (only 1 false negative was observed). The Se (96·15%) ELISA-H is similar to that identified by Lauricella *et al*. (1998) as being present in infected dogs' sera from the northern region in Argentina. This test, together with the Xe and PCR, were used as tests of reference and to calculate Se, Sp, AUC and KI of ELISA-rTSSA-II.

ELISA-rTSSA-II responses in dogs

The assays to determine the absorption of non-specific anti-GST antibodies enabled reactivity with this fusion protein to be discarded, improving the Sp. A previous report indicates that *Schistosoma japonicum* GST has a low immunogenic capacity (Nasser *et al*. 1997). In studies with human sera, Umezawa *et al*. (1999) concluded that the reactivity of recombinant antigen was specific for the *T. cruzi* component, although chagasic and non-chagasic individuals reacted, at low titres, with GST.

Performance indicators of ELISA-rTSSA-II like Se (92·86%), Sp (100%), AUC (0·93%) and KI (0·91%) confirm the validity of this method as a diagnostic test. Two false negative results were detected, one

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Table 3. Results obtained using ELISA-rTSSA-II in sera from humans

Groups	*n*	Chagatest Rec v3.0	PCR	ELISA-rTSSAII
I	54	0% (0/54)	ND	0% (0/54)
II	41	100% (41/41)	46·34% (19/41)	92·24% (37/41)
III	31	100% (31/31)	ND	70·96% (22/31)
IV	11	0% (0/11)	0% (0/11)	0% (0/11)
V	6	100% (6/6)	100% (6/6)	66·66 (4/6)
VI	68	0% (0/68)	ND	0% (0/68)

ND, not determined.

of them corresponding to a TcIII (before TcIIc) infected dog, which would be in accordance with rTSSA-II specificity for TcII, TcV and TcVI. Indeed, the molecular analysis of the TSSA antigen diversity showed that this has the potential for serologically detecting only infection by TCII, TcV and TCVI, but not infection by TcI or TCVI (Bhattacharyya *et al*. 2010). Co-infection with more than 1 lineage is possible, and it has been demonstrated by Burgos *et al*. (2008). In this study a co-infection case could be detected, TcI-TcVI typified through MLEE, presenting negative serology for rTSSA-II. Although it represents only a single case and therefore no generalization can be made, it should be possible to study more in detail the association between mixed infections and the production of Ac anti-TSSA-II, and whether this affects the Se of the test.

ELISA-rTSSA-II on human sera

The impossibility of having a significant number of sera from patients with characterized parasite isolates leads to an inability to define Se for the human group. Samples from 2 TcVI-infected patients were used as controls and to confirm rTSSA-II reactivity. Group II (chagasic) samples were studied to calculate the concordance between the commercial kit (which uses a mixture of recombinant antigens) and ELISA-rTSSA-II. The high concordance (KI = 0·878) can be explained by the high prevalence of TcVI in Argentina, more precisely in Chaco province. The 87·8% reactivity of rTSSA-II was less than that reported by Di Noia *et al* (2002); the latter was 95%. Despite this finding, evidence suggests that the prevailing lineage in Argentina is TcVI. The absence of cross-reaction with *Leishmania* spp. was demonstrated by the absence of reactivity to rTSSA-II among Groups I, IV and VI. This is important because it can be used in regions where *T. cruzi* and

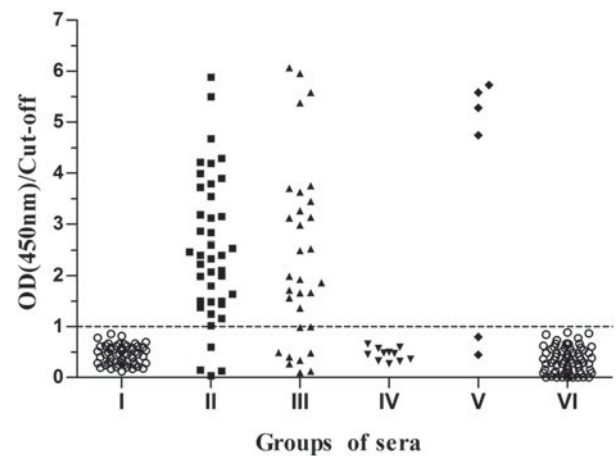

Fig. 4. Reactivities of sera from humans against recombinant TSSA-II by the ELISA-rTSSA-II. The results were calculated as the ratio of the absorbance of each serum sample at an optical density of 450 nm to the cut-off value. Values greater than 1·0 were considered reactive. (I) Non-Chagasic sera, (II) Chagasic sera, (III) ATL patients seropositive by CS, (IV) ATL sera seronegative by CS and negative by PCR, (V) mixed infections of *Trypanosoma cruzi*- *Leishmania* spp., (VI) ATL sera all seronegative by CS. ATL, American Tegumentary Leishmaniasis; CS, Conventional Serology Chagas' disease (Chagatest Rec v3.0)

Leishmania overlap, as is the case in the northern part of Salta. The specificity of the ELISA Chagatest reported by Caballero *et al*. (2007) indicated that the sera corresponding to patients with ACL, found to be seropositive by the Chagatest, could be regarded as potentially chagasic (Group III). On the other hand in those samples from patients with ACL in which infection with *T. cruzi* could be confirmed by PCR (Group V) were classified as mixed infection (*T. cruzi*-*L*.spp.). Cases of mixed infection have been demonstrated by serological tests in patients with ACL (Chiaramonte *et al*. 1996; Frank *et al*. 2003). The reactivity in Groups III and V, which was 70·96% and 66·66% respectively, confirm the infection with *T. cruzi,* and probably with TcVI.

The rTSSA – II was obtained from the CL-Brener reference strain, belonging to TcVI DTU. According to the compelling evidence regarding the large genetic diversity among the different *T. cruzi* DTUs, it would be not surprising to observe diversity in the TSSA – II sequences, even involving epitopes with high antigenic activity. In the present study, we analysed 17 serum samples from dogs (Groups C and D), for which the infecting DTU was identified. It is worth noting that the 14 serum samples from dogs infected by TcVI, were positive by ELISA-rTSSA-II; while the remaining sera from TcIII (1 dog) and TcI (2 dogs) were negative by the same technique. These results suggest that the rTSSA – II antigen could be specific for TcVI infection, according to our results in dogs. However, more studies are needed to test the hypothesis of DTU specificity.

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Evaluation of the test using a panel of sera from leishmaniasis patients showed that there are no cross-reactions with sera from *Leishmania* spp-infected people.

In conclusion, our results show that the rTSSA-II of *T. cruzi* presents high Se and Sp, and high performance according to the results obtained by analysing ROC curves, and it could be a useful tool for sero-epidemiological studies in regions endemic for Chagas' disease and leishmaniasis, such as the northern part of Argentina.

The rTSSA-II could be a useful tool for epidemiological studies in domestic animals and/or humans for investigation of the geographical distribution of *T. cruzi* lineages in Latin America.

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