The TcTASV proteins are novel promising antigens to detect active *Trypanosoma cruzi* infection in dogs

N. FLORIDIA-YAPUR[1,2], M. MONJE RUMI[3], P. RAGONE[3], J. J. LAUTHIER[3], N. TOMASINI[3], A. ALBERTI D'AMATO[3], P. DIOSQUE[3], R. CIMINO[1,2], J. D. MARCO[3], P. BARROSO[3], D. O. SANCHEZ[4], J. R. NASSER[1,2] *and* V. TEKIEL[4]*

[1] *Cátedra de Química Biológica, Facultad de Ciencias Naturales, Universidad Nacional de Salta, Av. Bolivia 5150, Salta (4400), Argentina*
[2] *Instituto de Investigaciones de Enfermedades Tropicales (IIET), Sede Regional Orán, Universidad Nacional de Salta, Alvarado 751, Orán-Salta (4530), Argentina*
[3] *Instituto de Patología Experimental (IPE-CONICET), Facultad de Ciencias de la Salud, Universidad Nacional de Salta, Av. Bolivia 5150, Salta (4400), Argentina*
[4] *Instituto de Investigaciones Biotecnológicas "Dr. Rodolfo A. Ugalde" (IIB-INTECH), Universidad Nacional de San Martín, UNSAM-CONICET, Av. 25 de Mayo, Partido de San Martín, Buenos Aires (1650), Argentina*

(*Received 15 February 2016; revised 5 April 2016; accepted 12 April 2016*)

SUMMARY

In regions where Chagas disease is endemic, canine *Trypanosoma cruzi* infection is highly correlated with the risk of transmission of the parasite to humans. Herein we evaluated the novel TcTASV protein family (subfamilies A, B, C), differentially expressed in bloodstream trypomastigotes, for the detection of naturally infected dogs. A gene of each TcTASV subfamily was cloned and expressed. Indirect enzyme-linked immunosorbent assays (ELISA) were developed using recombinant antigens individually or mixed together. Our results showed that dogs with active *T. cruzi* infection differentially reacted against the TcTASV-C subfamily. The use of both TcTASV-C plus TcTASV-A proteins (Mix A+C-ELISA) enhanced the reactivity of sera from dogs with active infection, detecting 94% of the evaluated samples. These findings agree with our previous observations, where the infected animals exhibited a quick anti-TcTASV-C antibody response, coincident with the beginning of parasitaemia, in a murine model of the disease. Results obtained in the present work prove that the Mix A+C-ELISA is a specific, simple and cheap technique to be applied in endemic areas in screening studies. The Mix A+C-ELISA could help to differentially detect canine hosts with active infection and therefore with high impact in the risk of transmission to humans.

Key words: *Trypanosoma cruzi*, dogs, TcTASV antigens, immunodiagnosis.

INTRODUCTION

Chagas disease, caused by the kinetoplastid parasite *Trypanosoma cruzi*, is a neglected tropical disease endemic in 21 Latin American countries that affects more than 7 millions of people (World Health Organization, 2006). This complex zoonosis involves several species of triatomine bugs as vectors, and several domestic and sylvatic mammals as vertebrate hosts. In spite of the efforts to eliminate vectors, triatomine-mediated transmission still originates more than 40 000 annual new cases of infection in endemic regions (World Health Organization, 2006). The Gran Chaco is an ecoregion that includes part of the northern of Argentina and certain regions of Bolivia and Paraguay, where still high prevalence levels of human infection with *T. cruzi* are detected, mostly because of household infestation by *Triatoma infestans*, the main vector in this area (Gürtler, 2009; Moretti *et al.* 2010; Samuels *et al.* 2013). In these and other rural areas, dogs are the most important domestic reservoirs of *T. cruzi* (Cardinal *et al.* 2006a; Gürtler *et al.* 2007). Dogs are not only the preferred *T. infestans* source of blood meals (over humans, cats and chickens) but are also highly infectious to bugs (Gürtler *et al.* 2005, 2007, 2009; Enriquez *et al.* 2014). Habits of domestic dogs like permanence inside dwellings, especially in sleeping rooms, increase the risk of transmission to humans. In this sense, Gürtler *et al.* (2005) have shown that the presence and number of infected dogs in households are associated to the incidence of infection in children (Gürtler *et al.* 2005); and incident cases in dog population occur even at very low infestation levels of triatomines (Gürtler *et al.* 2007). All the mentioned constitutes strong evidences that dogs can act as sentinels of the human infection in endemic regions (Castañera *et al.* 1998; Cardinal *et al.* 2006a). Moreover, the detection of active canine *T. cruzi* infection is an accurate tool to evaluate the effectiveness of vector control campaigns and to estimate the risk of transmission towards humans.

* Corresponding author: Instituto de Investigaciones Biotecnológicas "Dr. Rodolfo A. Ugalde" (IIB-INTECH), Universidad Nacional de San Martín, UNSAM-CONICET, Av. 25 de Mayo y Francia, Partido de San Martín, Buenos Aires (1650), Argentina. E-mail: valet@iib.unsam.edu.ar or vtekiel@gmail.com

doi:10.1017/S0031182016000822

The several phases of the *T. cruzi* life cycle in the mammal host influence the ability to detect the parasite by direct or indirect diagnostic methods. Direct methods, such as microscopic observation of trypomastigotes or PCR, are appropriate to detect the parasite in the early acute phase, when parasitaemia is patent, and treatment is more effective. However, this phase is usually short (4–8 weeks) and causes no specific clinical signs, with the consequence that the infection go through unnoticed during this first stage, losing the possibility to eliminate parasites. Then, the host's immune response partially controls the infection, diminishing the amount of circulating parasites to loads that are hardly detectable by the mentioned methods. The infected patients evolve to a chronic phase, where around 30% of the infected adults develop cardiac or digestive manifestations and where treatment is much less effective (Morillo *et al.* 2015). During this phase, the detection of the infection is mainly based on the detection of anti-*T. cruzi* antibodies. Furthermore, in contrast to direct methods, serodiagnosis is independent of the level of parasitaemia of the infected host. PCR, although being highly specific, should be used in combination with serology to achieve a reliable diagnosis, as has been previously demonstrated in naturally and experimentally infected dogs (Araújo *et al.* 2002; Enriquez *et al.* 2013). Conventional serological methods, such as enzyme-linked immunosorbent assay (ELISA) and indirect haemagglutination assay, employed for the detection of chronic human *T. cruzi* infection have been standardized or adapted for its use in dogs (Lauricella *et al.* 1993, 1998). However, a limitation of the serological diagnosis is that the parasite's crude extracts, which are routinely used as antigens in conventional serology, share epitopes with proteins of other pathogens, such as *Leishmania* spp. (Umezawa *et al.* 2009). This may cause cross-reactivity when samples from areas of co-endemicity, like some regions of the Gran Chaco (i.e. humid Chaco), are evaluated. The use of defined specie-specific antigens produced by recombinant technologies, is an effective substitute of protein homogenates of the parasite, and an adequate alternative to overcome the problem of cross-reactivity (Umezawa *et al.* 1999; Da Silveira *et al.* 2001). Nevertheless, up to now, few antigens have been assayed for the immunodiagnosis of canine infection and further studies to find optimal diagnostic antigens are needed (Cimino *et al.* 2011, 2012; Floridia-Yapur *et al.* 2014).

TcTASV is a *T. cruzi*-specific protein family identified few years ago (García *et al.* 2010). Several features make TcTASV proteins promising candidates for the diagnosis of the infection in dogs. First, TcTASVs members are expressed mainly in trypomastigotes, the mammalian infective and blood-circulating stage. Second, the family is conserved at least in the TcI and TcVI lineages, which predominate in canine infections from the Gran Chaco region (Monje-Rumi *et al.* 2015). Third, the TcTASV family has no orthologues in *Leishmania* spp. or *Trypanosoma rangeli*, anticipating their specificity as a diagnostic tool. On the other hand, the TcTASV family is splitted into three subfamilies according to their molecular weight and sequence identity: TcTASV-A, TcTASV-B and TcTASV-C. The TcTASV-C subfamily is expressed at the surface of trypomastigotes and secreted (Bernabó *et al.* 2013), which ensures its contact with the host's immune system during the natural infection. Furthermore, anti-TcTASV-C antibodies have been detected both in humans infected with *T. cruzi* as well as in experimentally infected animals (Bernabó *et al.* 2013). In the present study, we evaluate the performance of an ELISA assay for the detection of anti-TcTASV-A, TcTASV-B and/or TcTASV-C antibodies in sera of naturally infected dogs from an endemic region, the Gran Chaco in Argentina.

MATERIALS AND METHODS

Ethics statement

All serum samples from animal origin that were employed in this work had been obtained according to the ethical guidelines protocols of our Institutions that adhere the international regulations of animal care. The protocols for sample extraction were reviewed and approved by the Bioethics Committee of the Facultad de Ciencias de la Salud, Universidad Nacional de Salta, Argentina (N° 052–10) and Bioethics Committee of the Facultad de Ciencias Agrarias y Veterinarias, Universidad Católica de Salta (23 April 2014), Argentina.

Serum samples

Sera from infected dogs ($N = 70$) that were collected during a cross-sectional study carried out in 12 de Octubre Department, located at the southwest of Chaco Province, Argentina, were used as positive controls of *T. cruzi* infection (Tc+) (Monje-Rumi *et al.* 2015). All dogs were diagnosed as *T. cruzi* infected, by the detection of antibodies by ELISA. Although an homogenate of epimastigote proteins was employed as antigen (H-ELISA), the possibility of these sera being false-positives by cross-reaction with *Leishmania* spp. antigens is unlikely because this pathogen was never reported in the studied area; additionally, some of these sera were reactive with *T. cruzi*-specific recombinant antigens (Cimino *et al.* 2011; Floridia-Yapur *et al.* 2014). Tc+ sera were classified as group I (GI) or II (GII) according to previous results of xenodiagnosis and/or PCR diagnostic tests (Fig. 1). Dogs that had a positive PCR

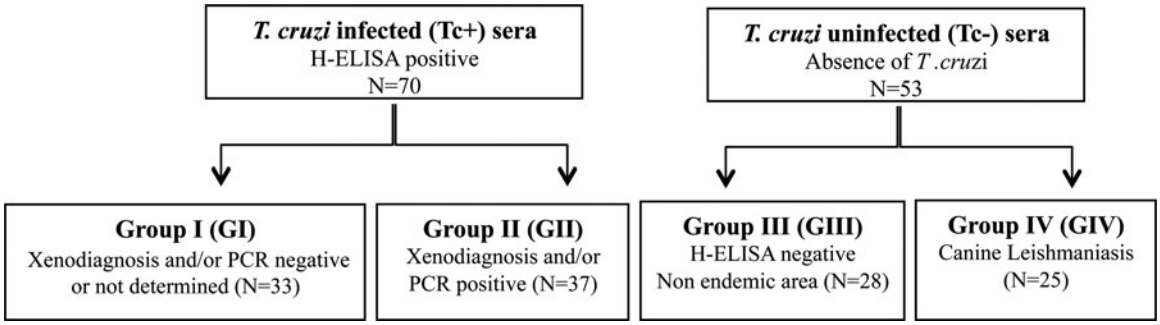

Figure 1. Flowchart and classification of serum samples employed in this work (H-ELISA: ELISA with epimastigotes protein homogenate antigen).

reaction and/or a positive xenodiagnosis were included in GII, while dogs in GI dogs were only positive by H-ELISA.

To evaluate specificity, samples from dogs without *T. cruzi* infection were used: 28 healthy dogs H-ELISA negative, from a non-endemic area (Buenos Aires city, Argentina) (sera group GIII); and 25 dogs with canine leishmaniasis (CanL) (sera group IV) (Fig. 1). The CanL cases were diagnosed by a combination of parasitological, molecular and serological methods. *Leishmania* (*Leishmania*) *infantum* was the causal agent, as was described previously (Barroso *et al*. 2015a, b).

Cloning, expression and purification of recombinant proteins

TcTASVs genes were cloned into pGEX vectors and expressed as glutathione-S-tranferase (GST)-fusion proteins.

A TcTASV-A gene (nucleotides 132–463 of GenBank AM492202·1, corresponding to amino acids 37–173) was amplified from the TcTASV-A-7 clone (García *et al*. 2010) by PCR using Pfu DNA polymerase and primers with restriction sites for BamHI and HindIII to allow further subcloning. The PCR amplicons were A-tailed, gel-purifed and cloned first into pGEM-T Easy vector (Promega, USA), to check sequence identity by sequencing. The TcTASV-A insert was then restriction enzyme-digested and subcloned into pGEX-1lambdaT to produce the TcTASV-A protein tagged with GST (TcTASV-A_{GST}). TcTASV-A_{GST} was expressed in *Escherichia coli* DH10B cells co-transformed with pGTf2 plasmid (Takara Bio Inc., Japan) carrying GroEL–GroES chaperone genes, to enhance the solubility of the protein of interest. The chaperones and the fusion protein TcTASV-A_{GST} were induced with 5 ng ml^{-1} tetracycline and 0·1 mM isopropyl-β-D-thiogalactopiranoside (IPTG), respectively, and this last protein was purified using columns packed with glutathione-sepharose (GE Healthcare Life Sciences, UK). Both Coomassie Brilliant Blue staining and Western-blot assays (with mouse anti-GST serum) were performed to check the presence of TcTASV-A_{GST} protein in the supernatant of induced bacteria lysates (solubility test) and in the eluted fractions after purification. Briefly, an aliquot of each fraction was electrophoresed on a 10% denaturing polyacrylamide gel and transferred to nitrocellulose membranes by standard methodologies. The membranes were blocked with phosphate-buffered saline (PBS)-3% non-fat milk, and incubated with anti-GST serum (O.N., 4 °C). Peroxidase-labelled goat anti-mouse (1/10000, Thermo Scientific, USA) was used as secondary antibody. SuperSignal West Pico (Thermo Scientific, USA) was used as chemiluminescent substrates to develop TcTASV-A_{GST}.

Similarly, a TcTASV-B gene (TcCLB.511877·10, TritrypDB.org) was amplified from TASV-B2 gene with Pfu DNA polymerase using primers with restriction sites BamHI and EcoRI. After A-tailing, amplicons were cloned into pGEM-T Easy vector (Promega, USA) and sequenced. The TcTASV-B insert was subcloned into pGEX-1lambdaT and the vector construction was used to transform BL21 *E. coli*. Expression of TcTASV-B_{GST} was induced by 0·1 mM IPTG and purification of the fusion protein was also carried out using columns packed with glutathione-sepharose.

TcTASV-C_{GST} was already cloned in our laboratory, and was expressed and purified as previously described (Bernabó *et al*. 2013). GST was used as a control.

Purity of proteins was analysed by sodium dodecyl sulphate–polyacrylamide electrophoresis (SDS–PAGE), followed by staining with Commasie Brilliant Blue. All proteins were quantified by Bradford assay and Picodrop and dyalized against PBS. Recombinant proteins were aliquoted and stored at −80 °C until use.

ELISA assays

To standardize concentration of antigens (individually or combined in a mixture) and serum dilutions a checkerboard titration was carried out, as described previously (Crowther, 2009). Two dilutions of secondary antibody anti-dog IgG whole molecule,

Table 1. Antigens and sera dilutions used in ELISA assays

Antigen*	Coating conditions	Sera dilution
TcTASV-A	0·5 μg well^{-1} in PBS	1/200
TcTASV-B	0·5 μg well^{-1} in PBS	1/200
TcTASV-C	0·125 μg well^{-1} in PBS	1/200
Mix A + C	0·25 μg well^{-1} TcTASV-A + 0·25 μg well^{-1} TcTASV-C in PBS	1/400

*For each ELISA with TcTASV antigens, GST was assayed as background control using the same coating conditions and sera dilution as for the evaluated antigen.

biotin conjugate (SIGMA) (1/1250;1/2500), and two dilutions of avidin-peroxidase conjugate (SIGMA) (1/8000; 1/16000) were also assessed. Optimum conditions were those that allowed the best discrimination of positive and negative sera. Table 1 shows the standardized conditions of antigen concentration and serum dilution for each assay.

ELISA assays were performed to assess each antigen separately or as a mixture. Polyestyrene microplates were sensitized with TcTASV antigens in PBS buffer (pH = 7·4); GST was used throughout as a background control. To eliminate unbound molecules washings between incubations were carried out using PBS–Tween 20 0·1% buffer. Nonspecific binding was prevented by adding PBS–skimmed milk 5%–Tween 20 0·05% blocking solution for 1 h. Sera from dogs, in the optimum dilution, were incubated for 1 h, whereas secondary antibody and avidin–peroxidase conjugate were left for 30 m. All incubations were performed at 37 °C. The reaction was developed by adding tetrametilbenzidine (SIGMA) and H_2O_2 30% in the citrate buffer, stopped with 0·5 N sulphuric acid and the absorbance was measured at 450 nm (OD_{450}) using a BIOTEK ELX800 spectrophotometer.

Data analysis

Each serum sample was tested by duplicate against TcTASV antigens, plated individually or combined in a mixture and against GST. Reactivity was first normalized as the ratio between the mean OD_{450} for TcTASV and GST (ratio = OD_{450} TcTASV/OD_{450} GST) for each serum, to rule out a possible effect of GST in the absorbance (Bernabó *et al.* 2013). Then, each ratio was normalized to a cut-off to obtain the plotted values 'ratios/cut-off'. The aim of the last procedure was to render comparable results between different ELISA-datasets (i.e. ELISA results using different TcTASVs), since ratios were transformed to meet the same scale (Caballero *et al.* 2007; Floridia-Yapur *et al.* 2014). The cut-off was selected for each antigen as the value that provided the maximum sensitivity for 100% specificity, through the analysis of the multiple pairs of sensitivity-specificity using a receiver-operating characteristic (ROC) curve (Linnet *et al.* 2012; Menezes-Souza *et al.* 2015). Samples were considered positive when the ratio/cut-off was equal or greater than 1·0, and negative when the ratio/cut-off was smaller than 1·0. Normal distribution of data was assessed by Shapiro–Wilk normality test. Since data were not distributed normally, the Mann–Whitney *U*-test ($P < 0·05$) was applied to assess differences. All data analysis and statistical tests were carried out with the GraphPad Prism v.5 software.

RESULTS

Expression and purification of TcTASV recombinant antigens

To evaluate the performance of TcTASV antigens as serological markers of *T. cruzi* infection in dogs, we produced a member of each TcTASV-A and -B subfamilies (when this work was initiated a TcTASV-C gene was already cloned and expressed). Purification steps of TcTASV-A_{GST} antigen are shown in Fig. 2A. Although multiple bands between 47·8 and 34 kDa weight markers were observed in TcTASV-A_{GST}, we confirmed that these bands corresponded to the protein of interest (Fig. 2B). Previous results in our laboratory showed that the TcTASV-C_{GST} protein undergo proteolysis at specific sites producing polypetides of lower molecular weight that can be observed in SDS–PAGE gels. We hypothesize something similar could happen with TcTASV-A_{GST} and TcTASV-B_{GST}. Figure 3 shows the TcTASV-A_{GST}, TcTASV-B_{GST} and TcTASV-C_{GST} purified proteins (predicted molecular weights: 45, 54 and 64 kDa, respectively) that were used in the ELISAs.

Reactivity of infected (Tc+) and uninfected (Tc−) dog sera against TcTASVs proteins

Sera from dogs infected with *T. cruzi* (Tc+) and uninfected controls (Tc−) were first evaluated against TcTASVs antigens plated separately. Antibody levels against TcTASV-A and TcTASV-C were significantly higher in Tc+ dogs than in Tc−; moreover, all control sera from Tc− were classified as negative by TcTASV-A and TcTASV-C (0/53) (Table 2). The positivity of *T. cruzi* infected sera was 57·1% (40/70) for TcTASV-A, and 52·9% (37/70) for TcTASV-C (Table 2). TcTASV-B_{GST}-ELISA had poor specificity (50%) and no further analyses were carried out with this antigen.

Although TcTASV-A- and TcTASV-C-ELISAs had similar percentages of positive results, the assays agreed only in 21 Tc+ sera. The combination of the results of both assays allowed us to classify 56 Tc+ sera as TcTASVs positive, which suggested that

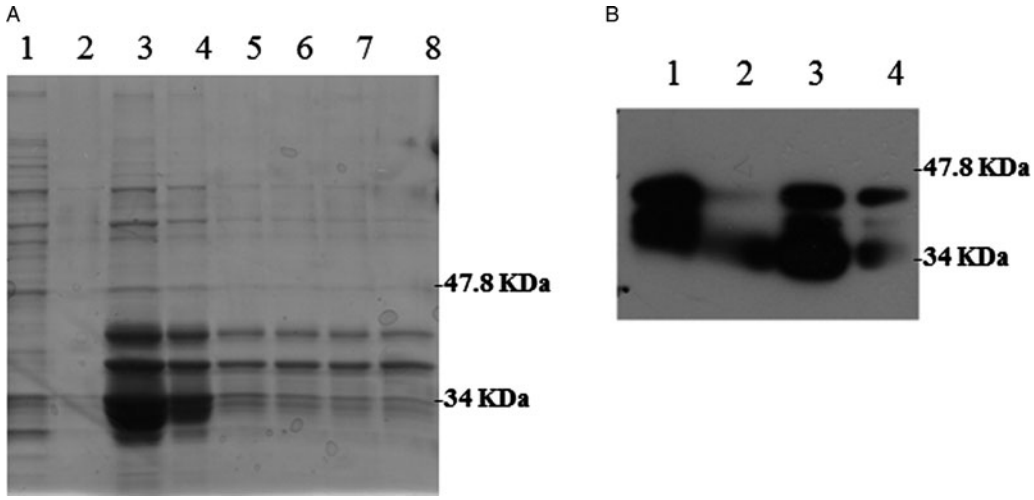

Figure 2. Purification of TcTASV-A_{GST} antigen. (A) TcTASV-A_{GST} purification (SDS–PAGE). Lanes 1 and 2: washes; lanes 3–8: eluted fractions. (B) Induced culture (lane 1), flow-through (lane 2), and eluates (lanes 3–4) of TcTASV-A_{GST} expression probed with anti-GST antisera (Western Blot).

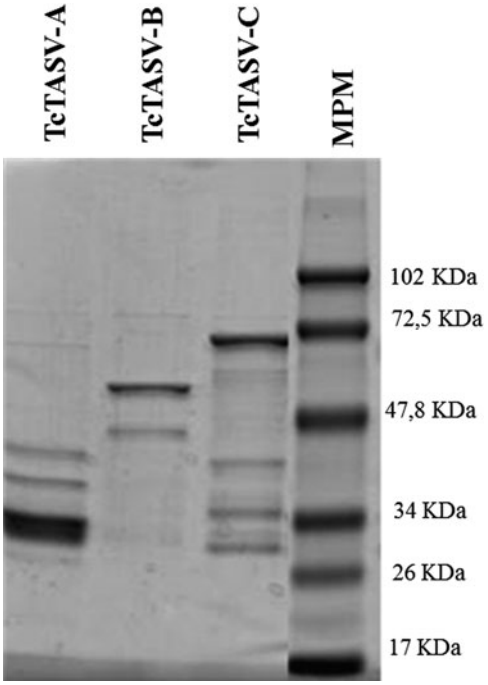

Figure 3. Purified $TcTASV_{GST}$ antigens used in ELISAs.

carrying out an ELISA with a TcTASV-A+C mixture could improve the sensitivity of the test. As expected, 70% (49/70) of Tc+ group of sera were reactive with the TcTASV-A+TcTASV-C mixture (Mix A+C-ELISA), proving that the reactivity of sera against each antigen can be summed in one assay (Table 2).

Differential reactivity of TcTASV-A+C ELISA in dogs with positive results for PCR and/or xenodiagnosis

Despite of the difference in the media of reactivity between Tc+ and Tc– sera, some Tc+ sera did not react with TcTASV-A, TcTASV-C or with the mixture of both antigens. Consequently, we analysed if these differences in positivity could be associated with the status of the infection of the sampled dogs. Sera from dogs that were diagnosed as Tc+ both by H-ELISA and xenodiagnosis and/or PCR (GII sera), reacted preferentially against TcTASVs antigens (Fig. 4). Both the number of reactive sera and the mean of ratios were higher in GII than in the GI group. Those differences were significant for TcTASV-C-ELISA and Mix A+C-ELISA (Fig 4, Table 2).

Table 2 resumes the parameters of ELISA performance calculated for the studied antigens. Sensitivity and area under the curve (AUC) ROC parameters varied according to the group of sera considered as the positive controls. When the whole Tc+ group (GI + GII) was considered, the sensitivity was always an intermediate value between the percentages obtained in GI and GII groups (Table 2). The highest sensitivity of the evaluated TcTASV-ELISAs was obtained when sera of group GII were considered as the positive control group, while the lowest values were obtained with group GI as positive group. The described pattern was consistent for all the assays evaluated. The AUC ROC values varied in a similar pattern except for TcTASV-A-ELISA when a value of 0·8 was obtained for all of the groups of sera considered. Specificity was maximum for all assays (100%). Altogether, these results show a differential reactivity against TcTASVs antigens, especially against TcTASV-C or the Mix A + C, of sera from dogs going through an active infection, i.e. those with parasites detected in peripheral blood by xenodiagnosis, or *T. cruzi* DNA detected by PCR.

DISCUSSION

Dogs are considered to be the most important domestic reservoir of *T. cruzi* in endemic regions, like

Table 2. Performance of TcTASV-ELISAs.

ELISA	Group of sera	Sensitivity [CI 95%] % (posit/total) [CI 95%]	Specificity [CI 95%]	AUC ROC [CI 95%]
TcTASV-A	Tc+ (GI + GII)	57·1% (40/70) [44·8–68·9]	100% (0/53) [93·3–100]	0·8 [0·72–0·88]
	GI	51·5% (17/33) [33·5–69·2]		0·8 [0·7–0·9]
	GII	62·2% (23/37) [44·8–77·5]		0·8 [0·7–0·91]
TcTASV-C	Tc+ (GI + GII)	52·9% (37/70) [40·6–64·9]	100% (0/53) [93·3–100]	0·76 [0·68–0·85]
	GI	27·3% (9/33) [13·3–45·5]		0·59 [0·46–0·72]
	GII	75·7% (28/37) [58·8–88·2]		0·91 [0·85–0·98]
Mix A + C	Tc+ (GI + GII)	70% (49/70) [57·9–80·4)]	100% (0/53) [93·3–100]	0·89 [0·84–0·95]
	GI	42·4% (14/33) [25·5–60·8]		0·79 [0·68–0·89]
	GII	94·6% (35/37) [81·8–99·3]		0·99 [0·97–1]

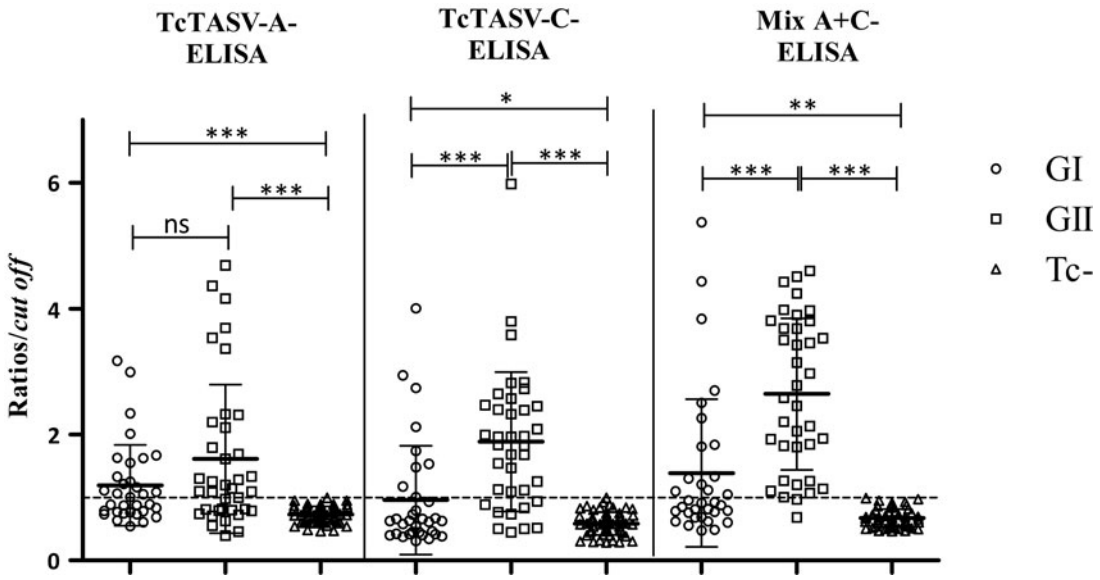

Figure 4. Reactivity against TcTASVs of sera from GI (Tc+ and PCR/Xd negative or unknown), GII (Tc+ and PCR/Xd positive) and Tc− groups evaluated by ELISA. Normalized ratios (ratios/cut off) are shown. ***: $P < 0{\cdot}05$, Mann–Whitney *U*-test; ns: non-significant.

the Gran Chaco, and not only constitute a risk factor for transmission to humans, but also can act as sentinels of human infection (Castañera *et al.* 1998; Cardinal *et al.* 2006a; Gürtler *et al.* 2007). Therefore, studies on the detection of the parasite in this reservoir are relevant. Since the sensitivity of PCR and parasitological methods extremely depends on parasitaemia, serological methods using defined recombinant antigens could represent a sensitive, specific and affordable alternative to achieve a reliable diagnosis. In this sense, the members of the TcTASV protein family could be promising diagnostic candidates since they are differentially expressed in trypomastigotes and have no orthologues in related trypanosomatids (García *et al.* 2010; Bernabó *et al.* 2013). Moreover, these proteins are conserved in TcI and TcVI lineages that predominate in canine infections in the Gran Chaco region (Monje-Rumi *et al.* 2015). All the mentioned made us to initially propose the assessment of the diagnostic performance of TcTASVs antigens for the detection of *T. cruzi* infection in naturally infected dogs from an endemic area.

In our study, we expressed a representative member of each TcTASV subfamily and analysed the reactivity of sera from naturally infected dogs against these proteins using an ELISA format. For this purpose, positive controls of infection were grouped depending on whether they presented or not direct evidence of the presence of the parasite (Group II and Group I of Tc+ sera, respectively). It is common to find studies where only parasitological positive samples are taken as positive controls, since the presence of the parasite is an undeniable proof of infection (Umezawa *et al.* 2009; Floridia-Yapur *et al.* 2014). However, in endemic areas, reservoirs can be coursing either an acute phase (with detectable levels of parasitaemia, although sometimes only after amplification) or an indeterminate/chronic phase (with unapparent parasitaemia) at a given time. Therefore, we consider that the approach applied here is proper for evaluation

of a novel diagnostic test in infected reservoirs from endemic areas. It is also important to mention that samples used in this work were collected from an area where neither vectors nor infected hosts with *Leishmania* were ever reported. Besides, considering that a great number of samples of GI were reactive to recombinant antigens (Cimino *et al*. 2011; Floridia-Yapur *et al*. 2014), we are confident to think that Tc+ sera positive by H-ELISA are true positive samples.

We observed that sera from dogs with detectable levels of parasitaemia by PCR and/or xenodiagnosis (named GII in this work) had a differential reactivity against TcTASV-C antigen. This reactivity was enhanced by using a mixture of this antigen plus TcTASV-A, which allowed us to detect almost all (94·6%) of the GII sera studied with maximum accuracy (AUC ROC = 0·99).

Previous findings showed that in mice infected with the highly virulent RA strain of *T. cruzi* (lineage TcVI), the peak of parasitaemia is coincident with the occurrence of anti-TcTASV-C IgG antibodies, which suggests that TcTASV-C is in close contact with the host's immune system during the early infection (Bernabó *et al*. 2013). Despite of the fact that similar studies with TcTASV-A are yet to be performed, the previous evidence on TcTASV-C, together with the results of this work, suggest that at least TcTASV-C preferably reacts with sera from mammals with active infection. Similarly, other results of our group are in line with this hypothesis (Floridia-Yapur *et al*. 2014). Most sera of GII (32/37) had been previously evaluated by ELISA against the shed acute phase antigen (SAPA), and all the sera that were SAPA-positive were also positive by the Mix A + C; moreover, almost 80% of these sera were also reactive with TcTASV-C.

It is noteworthy that the low reactivity of TcTASV-C antigen in GI group (27.3%) is coincident with a previous study where a ~30% reactivity was obtained for TcTASV-C-ELISA in a group of chronic humans (Bernabó *et al*. 2013). Since TcTASV-C is expressed at the surface of trypomastigotes that mostly circulate in the bloodstream during the acute phase (Bernabó *et al*. 2013), anti-TcTASVC antibodies probably persist shortly a while after the end of this phase, explaining the low reactivity of the hosts against this antigen in the indeterminate or chronic phase. However, in the present study, we cannot assure that all of these dogs were coursing a chronic phase, since most of these dogs lacked PCR or xenodiagnosis tests. Further studies are needed in order to confirm this observation. On the other hand, the differences in reactivity between TcTASV-A and TcTASV-C observed mainly in group I, could be due to the fact that these proteins present different expression patterns: while TcTASV-C is expressed at the surface of blood trypomastigotes and secreted (Bernabó *et al*. 2013), TcTASV-A has an intracellular location (unpublished data). Regarding the anti-TcTASV-A reactivity, the lower reactivity of GI sera against the mixture – compared with the TcTASV-A antigen alone – could probably be explained by the different amounts of protein used in the assays (Table 1).

Only 70% of the dogs that were positive by conventional serology – which employs total homogenates of parasites (H-ELISA) – reacted with a TcTASV protein by ELISAs in this work. However, we must consider that the dogs that reacted with TcTASV A + C antigens could have higher level of parasitaemia, as mentioned above. There is strong evidence that the amount blood circulating parasites in *T. cruzi* infected dogs is positively correlated with infectiousness to *T. infestans* (proportion of uninfected insects that become infected after blood-feeding once on an infected host) (Enriquez *et al*. 2014). Furthermore, the dog populations in endemic areas can be clustered according to their parasitaemia level, which is highly correlated with nutritional status, immunocompetence and/or general health condition, and determine the capacity to spread parasites to uninfected vectors feeding from them (Gürtler *et al*. 2007; Enriquez *et al*. 2014). We therefore postulate that dogs reactive to TcTASV-A and -C antigens could represent a greater source of parasites to an uninfected vector feeding from it and, therefore, have a major epidemiologic impact. On the other hand, there are cases where the standard serology using parasite extracts failed to detect infection in dogs with positive parasitology (Cardinal *et al*. 2006b). The TcTASVs antigens mixture evaluated here could be useful in combination with antigens that are reactive in the later phases of the disease to enhance a maximum sensitivity in the immunodiagnosis. Lastly, TcTASV-A, -C and the antigenic mixture ELISAs presented no cross-reactivity (100% specificity), neither in CanL cases nor in healthy individuals, which represents a relevant advantage compared with conventional serology, when diagnosing the infection in areas of probable co-endemicity with *Leishmania* spp.

Although the standardized immunodiagnosis technique using a mixture of TcTASV-A and -C antigens did not reach the sensitivity of conventional crude extract-ELISA, we found that relevant facts can be highlighted. First of all, the use of Mix A + C-ELISA in screening studies could help to detect canine hosts with active infection and therefore with high impact in the risk of transmission to humans, being a specific, simple and cheap technique to be applied in endemic areas. Secondly, by means of this research we were able to acquire evidence on the expression of these recently discovered proteins during the course of the natural infection with *T. cruzi*. Further studies are needed to elucidate the role of the TcTASV protein family in the biology of *T. cruzi* and its interaction with the mammalian hosts.

FINANCIAL SUPPORT

This work was supported by grants from CONICET (PIP-329 to V. T.), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT-2014-1151 to V. T.) and Consejo de Investigación de la Universidad Nacional de Salta (CIUNSA N° 2056), Argentina. V. T., D. O. S., P. D., D. M., P. B. and N. T. are members of the Research Career of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). N. F. Y. has a Ph.D. scholarship funded by CONICET.

REFERENCES

Araújo, F. M. G., Bahia, M. T., Magalhaes, N. M., Martins-Filho, O. A., Veloso, V. M., Carneiro, C. M., Tafuri, W. L. and Lana, M. (2002). Follow-up of experimental chronic Chagas' disease in dogs: use of polymerase chain reaction (PCR) compared with parasitological and serological methods. *Acta Tropica* **81**, 21–31.

Barroso, P. A., Nevot, M. C., Hoyos, C. L., Locatelli, F. M., Lauthier, J. J., Ruybal, P., Cardozo, R. M., Russo, P. D., Vassiliades, C. N., Mora, M. C., Estévez, J. O., Hashiguchi, Y., Korenaga, M., Basombrío, M. A. and Marco, J. D. (2015a). Genetic and clinical characterization of canine leishmaniasis caused by *Leishmania* (*Leishmania*) *infantum* in northeastern Argentina. *Acta Tropica* **150**, 218–223.

Barroso, P. A., Marco, J. D., Locatelli, F. M., Cardozo, R. M., Hoyos, C. L., Mora, M. C., García Bustos, M. F., López-Quiroga, I., Mimori, T., Gentile, A. G., Barrio, A. B., Korenaga, M., Hashiguchi, Y. and Basombrío, M. A. (2015b). Visceral Leishmaniasis caused by *Leishmania infantum* in Salta, Argentina: possible reservoirs and vectors. *American Journal of Tropical Medicine and Hygiene* **93**, 334–339.

Bernabó, G., Levy, G., Ziliani, M., Caeiro, L. D., Sánchez, D. O. and Tekiel, V. (2013). TcTASV-C, a protein family in *Trypanosoma cruzi* that is predominantly trypomastigote-stage specific and secreted to the medium. *Plos One* **8**, 1–10.

Caballero, Z. C.; Sousa, O. E.; Marques, W. P.; Saez-Alquezar, A.; Umezawa, E. S. (2007). Evaluation of serological tests to identify *Trypanosoma cruzi* infection in humans and determine cross-reactivity with *Trypanosoma rangeli* and *Leishmania* spp. *Clinical and Vaccine Immunology*. **14** (8), 1045–1049.

Cardinal, M. V., Castañera, M., Lauricella, M., Cecere, M., Ceballos, L., Vazquez-Prokopec, G., Kitron, U. and Gürtler, R. (2006a). A prospective study of the effects of sustained vector surveillance following community-wide insecticide application on *Trypanosoma cruzi* infection of dogs and cats in rural northwestern Argentina. *The American Society of Tropical Medicine and Hygiene* **75**, 753–761.

Cardinal, M. V., Reithinger, R. and Gürtler, R. E. (2006b). Use of an immunochromatographic dipstick test for rapid detection of *Trypanosoma cruzi* in sera from animal reservoir hosts. *Journal of Clinical Microbiology* **44**, 3005–3007.

Castañera, M. B., Lauricella, M. A., Chuit, R. and Gürtler, R. E. (1998). Evaluation of dogs as sentinels of the transmission of *Trypanosoma cruzi* in a rural area of north-western Argentina. *Annals of Tropical Medicine and Parasitology* **92**, 671–683.

Cimino, R. O., Monje Rumi, M., Ragone, P., Lauthier, J. J., Alberti D ´ Amato, A., Lopez Quiroga, I. R., Gil, J. F., Cajal, S. P., Acosta, N., Juarez, M., Krolewiecki, A., Orellana, V., Zacca, R., Marcipar, I., Diosque, P. and Nasser, J. R. (2011). Immuno-enzimatic evaluation of the recombinant TSSA-II protein of *Trypanosoma cruzi* in dogs and human sera: a tool for epidemiological studies. *Parasitology* **138**, 1–8.

Cimino, R. O., Diosque, P., Lopez Quiroga, I. R., Gil, J. F. and Nasser, J. R. (2012). Evaluación inmunoenzimática del antígeno recombinante SAPA en perros infectados naturalmente por *Trypanosoma cruzi*. *Revista Argentina de Microbiologia* **44**, 1–5.

Crowther, J. R. (2009). *The ELISA Guidebook*. Second. Humana Press, Hatfield, Hertfordshire, UK.

Da Silveira, J. F., Umezawa, E. S. and Luquetti, A. O. (2001). Chagas disease: recombinant *Trypanosoma cruzi* antigens for serological diagnosis. *Trends in Parasitology* **17**, 286–291.

Enriquez, G. F., Cardinal, M. V., Orozco, M. M., Schijman, A. G. and Gürtler, R. E. (2013). Detection of *Trypanosoma cruzi* infection in naturally infected dogs and cats using serological, parasitological and molecular methods. *Acta Tropica* **126**, 211–217.

Enriquez, G. F., Bua, J., Orozco, M. M., Wirth, S., Schijman, A. G., Gürtler, R. E. and Cardinal, M. V. (2014). High levels of *Trypanosoma cruzi* DNA determined by qPCR and infectiousness to *Triatoma infestans* support dogs and cats are major sources of parasites for domestic transmission. *Infection, Genetics and Evolution* **25**, 36–43.

Floridia-Yapur, N., Vega-Benedetti, F., Monje Rumi, M., Ragone, P., Lauthier, J., Tomasini, N., Alberti D´ Amato, A., Lopez Quiroga, I., Diosque, P., Marcipar, I., Nasser, J. and Cimino, R. (2014). Evaluation of recombinant antigens of *Trypanosoma cruzi* to diagnose infection in naturally infected dogs from Chaco region, Argentina. *Parasite Immunology* **36**, 694–699.

García, E. A., Ziliani, M., Agüero, F., Bernabó, G., Sánchez, D. O. and Tekiel, V. (2010). TcTASV: a novel protein family in *Trypanosoma cruzi* identified from a subtractive trypomastigote cDNA library. *PLoS Neglected Tropical Diseases* **4**, 6.

Gürtler, R. E. (2009). Sustainability of vector control strategies in the Gran Chaco Region: current challenges and possible approaches. *Memórias do Instituto Oswaldo Cruz* **104**, 52–59.

Gürtler, R. E., Cecere, M. C., Lauricella, M. A., Petersen, R., Chuit, R., Segura, E. L. and Cohen, J. E. (2005). Incidence of *Trypanosoma cruzi* infection among children following domestic reinfestation after insecticide spraying in rural northwestern Argentina. *American Journal of Tropical Medicine and Hygiene* **73**, 95–103.

Gürtler, R. E., Cecere, M. C., Lauricella, M. A., Cardinal, M. V., Kitron, U. and Cohen, J. E. (2007). Domestic dogs and cats as sources of *Trypanosoma cruzi* infection in rural northwestern Argentina. *Parasitology* **134**, 69–82.

Gürtler, R. E., Ceballos, L. A., Ordoñez-Krasnowski, P., Lanati, L. A., Stariolo, R. and Kitron, U. (2009). Strong host-feeding preferences of the vector *Triatoma infestans* modified by vector density: implications for the epidemiology of Chagas disease. *PLoS Neglected Tropical Diseases* **3**, 1–12.

Lauricella, M. A., Wisnivesky-Colli, C., Gürtler, R. E., Petersen, R., Bujas, M. and Segura, E. L. (1993). Standarization of serological tests for detecting anti-*Trypanosoma cruzi* antibodies in dogs. *Memórias do Instituto Oswaldo Cruz* **88**, 413–417.

Lauricella, M. A., Castañera, M. B., Gürtler, R. E. and Segura, E. L. (1998). Immunodiagnosis of *Trypanosoma cruzi* (Chagas' disease) infection in naturally infected dogs. *Memórias do Instituto Oswaldo Cruz* **93**, 501–507.

Linnet, K.; Bossuyt, P. M. M.; Moons, K. G. M. and Reitsma, J. B. (2012). Quantifying the accuracy of a diagnostic test or marker. *Clinical Chemistry* **58**, 1292–1301.

Menezes-Souza, D., de Oliveira Mendes, T. A., de Souza Gomes, M. dS., Bartholomeu, D. C., Fujiwara, R. T. (2015). Improving serodiagnosis of human and canine Leishmaniasis with recombinant *Leishmania braziliensis* Cathepsin L-like protein and a synthetic peptide containing its linear B-cell Epitope. *PLoS Negl Trop Dis* **9**(1), e3426.

Monje-Rumi, M., Pérez Brandán, C., Ragone, P., Tomasini, N., Lauthier, J., Alberti D´ Amato, A., Cimino, R., Orellana, V., Basombrío, M. and Diosque, P. (2015). *Trypanosoma cruzi* diversity in the Gran Chaco: mixed infections and differential host distribution of TcV and TcVI. *Infection, Genetics and Evolution* **29**, 53–59.

Moretti, E., Castro, I., Franceschi, C. and Basso, B. (2010). Chagas disease: serological and electrocardiographic studies in Wichi and Creole communities of Misión Nueva Pompeya, Chaco, Argentina. *Memórias do Instituto Oswaldo Cruz* **105**, 621–626.

Morillo, C., Marin-Neto, J., Avezum, A., Sosa-Estani, S., Rassi, A., Rosas, F., Villena, E., Quiroz, R., Bonilla, R., Britto, C., Guhl, F., Velazquez, E., Bonilla, L., Meeks, B., Rao-Melacini, P., Pogue, J., Mattos, A., Lazdins, J., Rassi, A., Connolly, S. J. and Yusuf, S. (2015). Randomized trial of benznidazole for chronic Chagas' cardiomyopathy. *The New England Journal of Medicine* **373**, 1295–1306.

Samuels, A., Clark, E., Galdos-Cardenas, G., Wiegand, R., Ferrufino, L., Menacho, S., Gil, J., Spicer, J., Budde, J., Levy, M., Bozo, R., Gilman, R. and Bern, C. (2013). Epidemiology of and impact of insecticide spraying on Chagas Disease in communities in the Bolivian Chaco. *PLoS Neglected Tropical Diseases* **7**, 1–11.

Umezawa, E. S., Bastos, S. F., Camargo, M. E., Yamauchi, L. M., Santos, M. R., Gonzalez, A., Zingales, B., Levin, M. J., Sousa, O., Rangel-Aldao, R. and da Silveira, J. F. (1999). Evaluation of recombinant antigens for serodiagnosis of Chagas' disease in South and Central America. *Journal of Clinical Microbiology* **37**, 1554–60.

Umezawa, E. S., Souza, A. I., Pinedo-Cancino, V., Marcondes, M., Marcili, A., Camargo, L. M., Camacho, A. A., Stolf, A. M. and Teixeira, M. M. (2009). TESA-blot for the diagnosis of Chagas disease in dogs from co-endemic regions for *Trypanosoma cruzi, Trypanosoma evansi* and *Leishmania chagasi*. *Acta Tropica* **111**, 15–20.

World Health Organization (2006). *Estimación cuantitativa de la Enfermedad de Chagas en las Américas*. Technical Report OPS/HDM/CD/425–06. OPS/HDM/CD, Montevideo, Uruguay.