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Research paper

Is the infectiousness of dogs naturally infected with *Trypanosoma cruzi* associated with poly-parasitism?

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ABSTRACT

Interactions among different species of parasites co-infecting the same host could be synergistic or antagonistic. These interactions may modify both the frequency of infected hosts and their infectiousness, and therefore impact on transmission dynamics. This study determined the infectiousness of *Trypanosoma cruzi*-seropositive dogs (using xenodiagnosis) and their parasite load (quantified by qPCR), and tested the association between both variables and the presence of concomitant endoparasites. A cross-sectional serosurvey conducted in eight rural villages from Pampa del Indio and neighboring municipalities (north-eastern Argentina) detected 32 *T. cruzi*-seropositive dogs out of 217 individuals examined for infection. Both the infectiousness to the vector *Triatoma infestans* and parasite load of *T. cruzi*-seropositive dogs examined were heterogeneous. A statistically significant, nine-fold higher mean infectiousness was registered in *T. cruzi*-seropositive dogs co-infected with *Ancylostoma caninum* and a trematode than in *T. cruzi*-seropositive dogs without these infections. The median parasite load of *T. cruzi* was also significantly higher in dogs co-infected with these helminths. An opposite trend was observed in *T. cruzi*-seropositive dogs that were serologically positive to *Toxoplasma gondii* or *Neospora caninum* relative to dogs seronegative for these parasites. Using multiple logistic regression analysis with random effects, we found a positive and significant association between the infectiousness of *T. cruzi*-seropositive dogs and co-infections with *A. caninum* and a trematode. Our results suggest that co-infections may be a modifier of host infectiousness in dogs naturally infected with *T. cruzi*.

1. Introduction

When a parasite trespasses the physical barriers of a host (i.e., the resistance exerted by the skin to parasite internalization), it finds an "immunoenvironment" that is determined by previous and current parasitic infections and by intrinsic factors such as host age, sex, nutritional status and genotype (Telfer et al., 2008). Therefore, the susceptibility to and duration of a parasitic infection, its intensity, transmission dynamics and pathology may be altered by infections with other parasites (Telfer et al., 2008; Graham, 2002). These alterations may change the number of infected individuals or their infectiousness thus impacting on transmission dynamics (Graham et al., 2007). A mechanism suggested for this interaction among parasite species, or genotypes, is by down-regulation of the immune system (Lafferty, 2010; Telfer et al., 2010). Other factors probably involved could be competition for nutrients or place of accommodation in the host, and cross-reactivity (i.e., components of the immune response developed by a host against a parasite species or genotype recognize other species or genotypes), among others.

In general, intracellular parasites (e.g., *Trypanosoma cruzi*, *Toxoplasma gondii*) stimulate a Th1 type immune response producing IFN-γ, IL-2 and macrophage activation (Hoft et al., 2000), in opposition to extracellular parasites (e.g., helminths) which stimulate a Th2 immune response producing IL-4, IL-5, IL-6 and IL-10

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(Mosmann and Coffman, 1989). The polarization towards a certain type of immune response reduces the degree of the opposite immune response that a host can exert (Rodríguez et al., 1999), in a mechanism called "immunomodulation" (Graham, 2008; Telfer et al., 2010).

Trypanosoma cruzi, the etiological agent of Chagas disease, is characterized by a large genetic diversity and has been classified into six Discrete Typing Units (DTUs), TcI-VI (Zingales et al., 2009). DTUs are distributed differentially among vectors, mammalian hosts and geographical regions (Miles et al., 2009). In Argentina, TcVI is the most frequent DTU found in peripheral blood of dogs, whereas TcV and mixed infections have frequently been reported in humans (Cardinal et al., 2014; Enriquez et al., 2013a; Monje-Rumi et al., 2015).

Interspecies interactions between *T. cruzi* and other pathogens in humans and mammalian hosts suggest that infectious agents and parasites may affect the transmission and the pathology of *T. cruzi*. de Freitas et al. (2011) found a higher *T. cruzi* load and infectiousness to the vector *Triatoma infestans* in HIV-infected humans. In a murine model of *T. cruzi* with a helminth co-infection, the intensity of *T. cruzi* parasitemia was associated with both the time since the primary infection and the sequence of infection (Galán-Puchades and Osuna, 2012). The intensity of parasitemia by *T. cruzi* was higher in *Leontopithecus rosalia* and *L. chrysomelas* monkeys naturally co-infected with nematode worms (Trichostrongylidae) than in monkeys not co-infected with nematodes (Monteiro et al., 2007). *Trypanosoma cruzi*-seropositive dogs, naturally co-infected with *Dirofilaria immitis*, showed less intense heart inflammatory response than those free of *D. immitis* suggesting immunomodulation by the worm (Cruz-Chan et al., 2010).

Host nutritional status can affect the susceptibility and severity of the infection in a great variety of pathogens (Louria, 2007). A cross-sectional study in Panama found that the body condition of rural dogs was negatively associated with the number of infecting parasite species (Fung et al., 2014). A vicious cycle was suggested (Beldomenico et al., 2008), in which poor nutritional conditions predispose to parasite infections and these infections undermine the host's nutritional status. A general sign of malnutrition is the presence of anemia, which could also be caused by gastrointestinal helminths (Crompton and Neishem, 2002; Dias et al., 2013).

Dogs and cats are major reservoir hosts of *T. cruzi* in domestic transmission cycles throughout the Americas (Crisante et al., 2006; Gürtler and Cardinal, 2015). Therefore, they could be the target of innovative strategies developed for the interruption of domestic transmission. In this study we evaluate the association between infectiousness (as determined by xenodiagnosis), bloodstream parasite load (as assessed by qPCR) and co-infections with helminths or intestinal protozoa and intracellular protozoa in dogs naturally infected with *T. cruzi* from a highly endemic district in the Gran Chaco region. As body condition (a surrogate of nutritional state) was negatively associated with the infectiousness of *T. cruzi*-seropositive dogs (Petersen et al., 2001; Enriquez et al., 2014), we also assessed whether the body condition of dogs and/or the presence of anemia were associated with infectiousness to *T. infestans* and bloodstream parasite load.

2. Materials and methods

2.1. Study area

This study is part of a broader research project on the transmission and control of Chagas disease in the municipality of Pampa del Indio (26° 2′ 0″ S, 59° 55′ 0″ W), Chaco Province, Argentina. Field work was conducted in five villages (El Gramillar, Tacuruzal, Campo Nuevo, Lote Cuatro and Ex Parque) in the municipality of Pampa del Indio in April and October-November 2013. Three villages neighboring to Pampa del Indio municipality were included and visited: Santa Carmen (General Güemes Department, 25° 55′ 0″ S 60° 37′ 0″ W), Pampa Bandera (25 de Mayo Department, 26° 55′ 0″ S 60° 02′ 0″ W) and El Palmar (Quitilipi Department, 52° 0′ 0″ S 60° 13′ 0″ W). The rural area of Pampa del Indio (1721 km^2) has been under sustained vector surveillance including a community participation component since 2007. As a consequence of these interventions, local vector-borne transmission of *T. cruzi* was significantly reduced or interrupted; therefore, the search for infected dogs was extended to neighboring rural villages from other municipalities, where vector control actions had been sporadic or absent, to increase the likelihood of finding *T. cruzi*-infected dogs.

2.2. Study design

To maximize the probability of finding *T. cruzi*-seropositive dogs, sampling was targeted to dogs residing in households infested with *T. cruzi*-infected *T. infestans*. Therefore, some households from Pampa del Indio were selected because of prior collection of infected bugs in 2012 or because their owners reported the presence of *T. infestans* to the surveillance system. Given that few houses fulfilled the first two criteria, a third criterion for selection of dwellings was based on having mud walls which had a higher probability of harboring triatomines (Gurevitz et al., 2011). For this study we selected 7 of 208 inhabited dwellings of Tacuruzal, 4 of 9 from El Gramillar, 3 of 32 from Campo Nuevo, 2 of 122 from Lote Cuatro, 1 of 27 from Ex Parque, 24 of 70 from Santa Carmen, 17 of 91 from Pampa Bandera, and 11 of 21 from El Palmar. All dogs were examined at their household. Of 226 dogs registered 217 were bled for serological tests.

Processing of blood samples for serum separation and DNA extraction was described elsewhere (Enriquez et al., 2014). The host's body condition was used as an index of the nutritional status of dogs (Petersen et al., 2001). The body condition of each dog (good, regular or poor) was established by a single member of our team (GFE) based on the degree of development of muscles; external evidence of bone structure, state of fur coat, the existence of fat deposits, and facial expression (Petersen et al., 2001; Enriquez et al., 2014). Only dogs aged 1 year or more were classified to avoid potential confounders due to growth effects and acute infections. Handling and examination of dogs were conducted according to the protocol approved by the 'Dr. Carlos Barclay' Independent Ethical Committee for Clinical Research (Protocol No. TW-01-004).

2.3. Serodiagnosis

At the end of each workday, dog sera was separated and tested at the field laboratory for antibodies to *T. cruzi* by indirect hemagglutination assay (IHA) following the manufacturer's instructions (Wiener Laboratories S.A.I.C., Buenos Aires, Argentina) and an in-house enzyme-linked immunosorbent assay (ELISA). Serodiagnostic methods were described elsewhere (Enriquez et al., 2013b). An individual was considered seropositive when it was reactive to at least two assays. All *T. cruzi*-seropositive dogs were selected for stool collection.

Serological examination of *T. cruzi*-seropositive dogs were conducted to detect the presence of antibodies against *T. gondii* and *Neospora caninum* by indirect immunofluorescence assay test (IFAT) by the Laboratory of Immunology, Department of Epizootiology and Public Health, Faculty of Veterinary Science, National University of La Plata, Argentina. The cut-off titer employed was 100. One dog was not tested because of insufficient serum.

2.4. Xenodiagnosis

Trypanosoma cruzi-seropositive dogs were examined by xenodiagnosis with 20 uninfected, laboratory-reared fourth-instar nymphs of *T. infestans* per dog. Bugs were exposed to the animal's belly during 20 min, followed by a 10 min re-exposure period if most of the bugs had not blood-fed to repletion (Enriquez et al., 2014). Each bug was individually examined by optical microscopy (400×) for *T. cruzi* infection 30 and 60 days after exposure. The infectiousness to the vector of dogs seropositive for *T. cruzi* was computed as the total number of insects infected with *T. cruzi* divided by the total number of insects exposed and examined for infection at least once (Gürtler et al., 2007).

2.5. Molecular analysis

2.5.1. DNA extraction

Guanidine-EDTA blood samples (GEB) were heated in boiling water for 15 min. Prior to DNA extraction, 200 pg of an internal amplification control DNA (IAC) was added to 400 μL GEB aliquot (Bua et al., 2012). Total DNA was purified using a commercial kit (DNeasy Blood & Tissue Kit, QIAGEN Sciences, Maryland, USA) following manufacturer's instructions. However, the use of proteinase K and the addition of buffer AL were avoided as reported (Duffy et al., 2009). Purified DNA was eluted in 200 μL of distilled water and used as template for PCR and qPCR amplification.

2.5.2. Qualitative polymerase chain reaction

DNA samples from *T. cruzi*-seropositive dogs that had been also examined by xenodiagnosis were tested by means of a qualitative PCR assay targeted to the minicircles of the kinetoplast (kDNA-PCR) as reported elsewhere (Enriquez et al., 2013b). kDNA-PCR was also employed to the detect subpatent infections in xenodiagnosis bugs as described (Enriquez et al., 2014).

2.5.3. Quantitative polymerase chain reaction

Bloodstream parasite load was quantified by amplifying a *T. cruzi* satellite DNA flanked by the Sat Fw and Sat Rv oligonucleotides (Duffy et al., 2009). The standard curve for DNA quantification (generated with *T. cruzi* CL Brener clone), reagents, control samples and cycling profile for *T. cruzi* DNA amplification were performed as described (Bua et al., 2012; Enriquez et al., 2014). Given that the number of satellite DNA repeats differs among different *T. cruzi* DTUs, DNA quantification was normalized according to the identified DTU (Duffy et al., 2009). Recovery of IAC DNA was 70–95% and the parasite load was normalized accordingly. Parasite DNA concentration was expressed as equivalent amounts of parasite DNA per ml (Pe/mL).

2.5.4. Identification of Trypanosoma cruzi discrete typing units

We identified DTUs in each *T. cruzi*-infected dog employing a PCR strategy based on three molecular markers as described in Enriquez et al. (2013a). In order to normalize parasite quantifications, identified DTUs were classified into three groups: TcI, TcIII, and the remainder (TcII/TcV/TcVI) in which we could not confirm or exclude the presence of any of them (Duffy et al., 2009; Enriquez et al., 2014).

2.6. Stool sampling

Stool collection was organized as follows: we visited the dwellings where the selected dogs lived and asked the owners to restrain the dogs all night until our arrival on the next morning. Animals remained tied overnight in a sheltered place. On the next morning dogs were released one at a time and followed to the place where they defecated. An aliquot of stool was deposited in collecting vials containing 5% formalin (1:3) which were immediately homogenized to ensure proper fixation and preservation.

2.7. Coprological examination

A subsample of each fecal sample was processed using two methods of enrichment: a modified sedimentation test to facilitate the recovery of protozoa (Garbossa et al., 2013), and a flotation test (Willis, 1921) followed by optic microscopy examination at 100×.

2.7.1. Helminth identification

We performed four independent observations for the sedimentation test (one of them iodine-stained), and one observation for the flotation technique with the addition of a drop of iodine. In both cases, all helminth eggs detected in each preparation were identified to genus by comparison with pictorial keys (Ash and Orihel, 2010; Bowman et al., 2004). Egg length and width were measured and compared with published records to identify genus and species more accurately (Bowman et al., 2004; Ash and Orihel, 2010).

2.7.2. Intestinal protozoa identification

For detection of intestinal protozoa a preparation was made for each fecal sample obtained by sedimentation and a drop of iodine was added. The preparation was entirely examined by optical microscopy at 400× to detect the presence of protozoa by comparison with pictorial keys (Bowman et al., 2004; Ash and Orihel, 2010) (e.g., *Giardia* sp. and *Entamoeba* sp.). *Cryptosporidium* sp. oocysts were detected in stool smears by staining with a modified Ziehl-Neelsen stain (Dúre et al., 2013). Smears were examined by optical microscopy at 1000× and oocysts recognized by the morphometric characteristics of fuchsia-stained spherical or ovoid elements (length, 4–6 μm).

A dog was considered infected by a helminth species when an egg or larvae was detected and/or infected by a protozoan when cysts or coccidia oocysts were observed in at least one examination.

2.7.3. Trematode infection

Given that trematode eggs were recovered in 15% of the dogs and their presence was associated with increased infectiousness to the vector and *T. cruzi* parasitemia, we collected new samples of feces from four of the 12 trematode-positive dogs in December 2014 (nearly 18 months after the cross-sectional study); the remaining eight dogs had died at that time. In order to rule out an oral ingestion of trematodes, dog owners were provided with dry commercial food to feed them on a controlled diet for 7 days. Three stool samples were collected from each animal in nonconsecutive days. Samples were preserved and analyzed as described above.

All trematode eggs were morphologically indistinguishable from *Fasciola hepatica,* an endemic parasite in the Chaco province according to some authors (Mera y Sierra et al., 2011). Other similar trematodes include *Paragonimus* sp. or *Alaria* sp., although Alaria eggs are grey and half the length of the observed eggs, and *Paragonimus* sp. has not been reported in the region. Samples were also analyzed with a coprological test used for the diagnosis of *F. hepatica* in cows as described by Dennis et al. (1954) excluding the use of detergent in the sedimentation step, and all of them tested positive. Thus molecular identification of *F. hepatica* was attempted. Feces (5 g) were placed in a mortar with liquid nitrogen, crushed with a pestle three times, and DNA was extracted from the whole sample with a commercial kit (QIAmp™ DNA Stool Mini Kit, Qiagen) following manufacturer's instructions. DNA was kept at −20 °C until used as template. Two pairs of primers were employed for PCR amplification reactions targeting a COI region (Ita-8 and Ita-9) and a ribosomal region (ITS1-F and ITS1-R) as described in Itagaki et al. (2005). Cycling conditions were as follows: an initial denaturing

step at 94 °C for 3 min, followed by 40 cycles at 94 °C for 90 s 57 °C for 90 s and 72 °C for 120 s with a final extension step at 72 °C for 10 min. PCR reactions were carried out at a final volume of 30 μL of reaction mix and reagents concentration as described in Itagaki et al. (2005), with 5 μL of DNA sample as template and using Taq Platinum DNA polymerase (Invitrogen, USA). All reactions included negative controls (sterile water) and positive controls (*F. hepatica* DNA extracted from eggs and adult worms).

2.8. Hematological profile

A hematological study was conducted in 25 dogs. Figurative elements of blood were evaluated in terms of quantitative aspects by a hematologic analyzer (Celldyn Emerald) at Hospital Dr. Dante Tardelli, and the white blood cell differential count was performed manually. Values were compared with canine reference values (http://www.labdiagnotest.com/normales.php). A dog was considered anemic when a low value for at least one of these three parameters was detected: hemoglobin, red blood cells and/or hematocrit. The hematological profile of seven *T. cruzi*-seropositive dogs could not be determined because blood samples were inadequately preserved during field work.

2.9. Data analysis

Agresti–Coull binomial 95% confidence intervals (CI) were used for proportions (Brown et al., 2001).

To assess the relationship between infectiousness to the vector of dogs infected with *T. cruzi* and the presence of co-infections with, or exposure to, other parasites, the following parasites were included in the analysis: *A. caninum* (number of dogs infected with this parasite, n = 26), trematode (possibly *F. hepatica*, n = 7), *Giardia* sp. (n = 5), *Cryptosporidium* sp. (n = 5), *T. gondii* (n = 19) and *N. caninum* (n = 20). These parasites were included because at least 15% of the 32 *T. cruzi*-seropositive dogs examined by xenodiagnosis were exposed to or infected with them. Other parasites found much less frequently (e.g., *Hymenolepis diminuta*, Taenia/*Echinococcus* sp., *Dipylidium caninum*, *Toxocara canis, Spirocerca lupi*, *Trichuris vulpis* and *Entamoeba* sp.) were excluded from these analyses. Bugs from two dogs were excluded from the calculations because all triatomines were dead at the time of examination probably due to exposure to ectoparasiticides. To reduce the number of independent variables because of the limited sample size, three groups of parasites were formed based on the phylogenetic relationship, nutritional requirements, and the location within the host. One group was composed by *A. caninum* and a trematode (i.e., worms); another by *Cryptosporidium* sp. and *Giardia* sp. (i.e., intestinal protozoa), and the third one by *T. gondii* and *N. caninum* (i.e., intracellular protozoa). No dog was free from any of these parasites. Therefore, for comparison we took all dogs negative to each group of parasites as "negative controls". For example, the negative controls for helminths (i.e., *A. caninum* and trematode) were dogs that did not harbor any of these two worms, although they may have been infected with any of the other four parasite groups. Negative controls for the other two groups were established similarly.

The negative control for the group of dogs co-infected with both *A. caninum* and a trematode consisted of six dogs (all of them seropositive to *N. caninum*, three to *T. gondii*, two infected with *Giardia sp*; and one with *Cryptosporidium* sp.). Twenty-two negative dogs to *Cryptosporidium* sp. and *Giardia* sp. had at least a parasite of the remaining four selected for this analysis (i.e., 18 were found infected with *A. caninum*, six with a trematode, 14 seropositive to *N. caninum*, and 13 to *T. gondii*). All four dogs seronegative for *T. gondii* and *N. caninum* showed *A. caninum* infections, and in addition one animal was also co-infected with *Cryptosporidium sp*.

The relationship between infectiousness to the vector of dogs infected with *T. cruzi* and selected predictors was analyzed using multiple logistic regression models implemented in Stata (Stata 12, Stata Corp, College Station, Texas). Only dogs aged 1 year or more were included in the regression to avoid potential confounders due to growth effects. Because the bugs used in xenodiagnosis were clustered on individual dogs, observations are not independent. Therefore, random effects that measure the residual effects due to each subject on the probability of bug infection were included in the model by means of a random intercept. The dependent variable was the infection status of each bug used in xenodiagnosis. Two different models were performed and in both cases the dependent variable was the infection status of each triatomine used in xenodiagnosis. In the first model, which included 32 *T. cruzi*-seropositive dogs, we assessed whether the dependent variable was associated with bloodstream parasite load (a continuous variable, parasite equivalent per mL of peripheral blood, Pe/mL). A second model included 31 seropositive dogs because one dog was not assessed by serology to *T. gondii* and *N. caninum.* This model was performed to evaluate the relationship between infectiousness to the vector and infection with (or exposure to) each of the three groups of parasites taken as dichotomous variables: 1_*A. caninum* &, or trematode; 2_*T. gondii* &, or *N. caninum*; 3_ *Cryptosporidium* sp. &, or *Giardia* sp., with "1 = infected", representing the infection by at least one of the parasites of each group. The age of the dog, a potential confounder, was included in both regressions but excluded from the final models because it was not statistically significant and did not represent a change in the magnitude or direction of the associations found. Body condition was included in the second model with two levels (0 = good, 1 = regular o poor) because of the low number of dogs with a poor body condition.

Interaction terms among the three groups of parasites were added stepwise and dropped from the final model because they did not represent a significant change in the main associations found. The Wald test examined the hypothesis that all regression coefficients were 0. Collinearity among independent variables was assessed in Stata (Stata 12, Stata Corp, College Station, Texas). The Variance Inflation Factor (VIF) was calculated. The values obtained indicate the absence of significant collinearity among variables (mean value obtained VIF = 1.04).

Kruskal-Wallis test was used to assess the median of *T. cruzi* bloodstream parasite load between groups of dogs. The proportion of anemic dogs with good body condition compared to dogs with regular or poor body condition was analyzed using Fisher's test. A nominal significance level of 5% was considered.

3. Results

3.1. Infectiousness to T. infestans and bloodstream parasite load

Twenty-three (72%) of 32 *T. cruzi*-seropositive dogs were xenodiagnosis-positive. The mean infectiousness of seropositive dogs was 36% (95% CI = 32–40), resulting in 220 triatomines infected with *T. cruzi* out of 619 examined (21 triatomines were not examined because they were dead at 30 days post-exposure). Bug mortality and molting rates at 30 days post-exposure to seropositive dogs were 3% and 12%, respectively, indicating an overall good quality of the xenodiagnostic tests performed, similar to the levels recorded in a previous study (Enriquez et al., 2014). The false-negative rate of microscopical observation was 15% among 54 negative triatomines further examined by kDNA-PCR; these bugs had fed on nine seropositive, xenodiagnosis-negative dogs, five of which turned into xenodiagnosis-positive using kDNA-PCR. Based on GEB samples, two of the nine seropositive, xenodiagnosis-negative dogs were qPCR-positive, and six of them were positive by

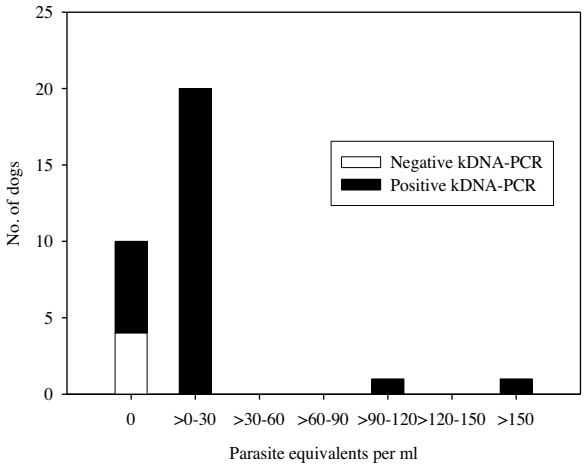

Fig. 1. Frequency distribution of parasite load of *T. cruzi*-seropositive dogs as determined by qPCR. Pampa del Indio and neighboring villages, Chaco, 2013.

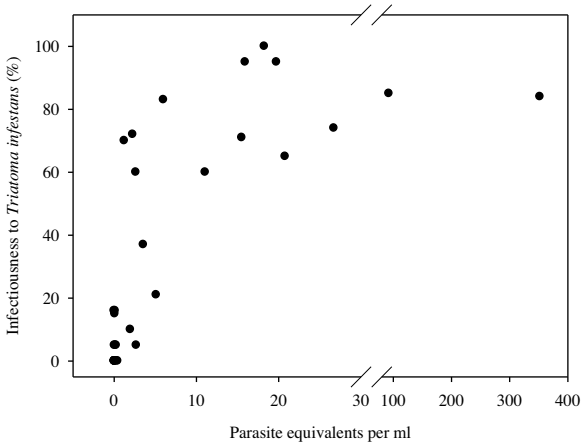

Fig. 2. Distribution of infectiousness to *T. infestans* of *T. cruzi*-seropositive dogs according to bloodstream parasite load. Pampa del Indio and neighboring villages, Chaco, 2013.

kDNA-PCR (Fig. 1). We were able to identify two dogs infected with TcIII and 22 with the group TcII, TcV and/or TcVI. Identification of DTUs was unsuccessful in eight *T. cruzi*-seropositive dogs.

The median parasite load was 1.2 Pe/mL (first-third quartiles [Q1–Q3] = 0.0–15.9; range 0.0–352.2) in *T. cruzi*-seropositive dogs and 4.5 Pe/mL (Q1–Q3 = 0.5–19.7; range 0.0–352.2) in xenodiagnosis-positive dogs (Fig. 1).

3.2. Relationship between infectiousness and bloodstream parasite load

The infectiousness of *T. cruzi*-seropositive dogs increased sharply with bloodstream parasite load (Fig. 2). The mean infectiousness was 1.4 times higher for dogs in a regular or poor body condition than in those with a good condition (Table 1). Infectiousness was associated positively and marginally significantly with bloodstream parasite load by random-effects multiple logistic regression (OR = 1.02; 95% CI = 1.001–1.033; P = 0.05) (Wald $\chi^2 = 3.83$; P = 0.05).

The median bloodstream parasite load in *T. cruzi*-seropositive dogs with a regular or poor body condition was slightly higher than in seropositive dogs with a good body condition (2.0 Pe/mL; Q1–Q3 = 0.0–15.9 and 1.0 Pe/mL; Q1–Q3 = 0.0–18.3, respectively), although it was not statistically significant (Kruskal-Wallis test, P = 0.67).

3.3. Trematode identification and infection

All trematode eggs were morphologically (i.e., large and width, characteristics of internal content and color) indistinguishable from *F. hepatica*. Their mean length and width were 117 and 63 μm, respectively (Standard Error, SE = 1.6 for both), and were yellowish. These eggs were found in fecal samples from two of the four dogs re-examined for trematode infection in December 2014, evidencing a real infection instead of a pseudo parasitosis. However, no *F. hepatica* DNA could be amplified in these samples by two specific PCR assays, whereas positive controls exhibited the expected bands. Therefore, we cannot conclusively determine whether these were *F. hepatica* infections.

3.4. Infectiousness, body condition and other co-infections

The mean infectiousness of *T. cruzi*-seropositive dogs co-infected with *A. caninum* and a trematode was eight and nine times higher, respectively, than in *T. cruzi*-seropositive dogs without these helminths and these differences were statistically significant (Fig. 3A). Dogs also seropositive to *T. gondii* or *N. caninum* showed a lower infectiousness to the vector than negative controls but it was not statistically significant (Fig. 3B). *T. cruzi*-seropositive dogs co-infected with *Cryptosporidium* sp. showed a mean infectiousness 1.3 times higher than the negative controls, and those co-infected with *Giardia* sp. a 1.5 times lower mean infectiousness; these differences were not statistically significant (Fig. 3C).

Infectiousness to the vector was associated positively and significantly only with co-infections with *A. caninum* and a trematode (OR = 29.0; 95% CI = 3.0–284.4; P < 0.01) (Wald $\chi^2 = 8.4$; P < 0.01). Similar results were obtained in the age-adjusted final model (OR = 27.3; 95% CI = 2.9–257.1; P < 0.01) (Wald $\chi^2 = 9.6$; P < 0.01). No significant association was found between dog infectiousness, body condition or the other co-infections tested (Table 1).

The median parasite load in dogs co-infected with *A. caninum* and a trematode was significantly higher than in dogs not co-infected with these helminths (Kruskal-Wallis test, df = 2, P < 0.01). There were no significant differences between other co-infections and their respective negative controls (Kruskal-Wallis test, df = 2, P = 0.53 for *T. gondii* and *N. caninum*; P = 0.18 for *Cryptosporidium* sp. and *Giardia* sp.) (Fig. 4A–C).

3.5. Profile of dogs infected with T. cruzi and co-infected with A. caninum and a trematode according to body condition and presence of anemia

Twelve out of 25 *T. cruzi*-seropositive dogs were anemic. The proportion of anemic dogs was significantly lower among seropositive dogs in a good body condition (5 of 17 dogs) than in seropositive dogs with a regular or poor body condition (7 of 8 dogs) (Fisher's test, df = 1, P = 0.01).

Within the group of *T. cruzi*-seropositive dogs co-infected with *A. caninum* the proportion of anemic dogs was 2.7 times higher for dogs with regular or poor body condition than for dogs with a good body condition. This ratio was 1.5 for dogs infected with a trematode. No dog showing a good body condition and uninfected with any of these helminths was anemic (Table 2).

There was no significant difference in the mean infectiousness to the vector between anemic (33%, 95% CI = 27–39) and non-anemic *T. cruzi*-seropositive dogs (34%, 95% CI = 28–39), neither was a significant difference in their median parasite load (Kruskal-Wallis test, df = 1, P = 0.55).

Table 1
Infectiousness to *T. infestans* of *T. cruzi*-seropositive dogs (n) according to potential risk factors adjusted by age; Pampa del Indio and neighboring villages, Chaco, 2013.

Potential risk factors[a]	n[b]	No. of dogs	Mean infectiousness (95% CI)	OR (95% CI)	P
Age	31	31	–	1.0 (1.0–1.1)	0.28
A. caninum &, or trematode	31	25	45 (41–50)	27.3 (2.9–463.2)	<0.01
T. gondii &, or *N. caninum*[c]	31	28	32 (28–36)	0.1 (<0.1–1.7)	0.12
Cryptosporidium sp. &, or *Giardia* sp.	31	10	33 (29–37)	1.9 (0.2–17.2)	0.57
Body condition	31				
Good		22	33 (29–37)	1	–
Regular/Poor		9	45 (38–52)	3.0 (0.4–25.6)	0.31

[a] Two-way interaction terms were not significant except for *A. caninum* &, or trematode × *T. gondii* &, or *N. caninum*, which was finally omitted due to collinearity.
[b] One *T. cruzi*-seropositive dog in a good body condition was excluded from the analysis because it was not assessed serologically for *T. gondii* and *N. caninum*.
[c] Only exposure to these parasites was assessed.

Table 2
Infectiousness to *T. infestans* and bloodstream parasite load in dogs seropositive to *T. cruzi* according to co-infections with *A. caninum* and a trematode categorized by host body condition. Pampa del Indio and neighboring villages, Chaco, 2013.

Parasite coinfection[a]	Body condition	No. dogs examined for anemia (Total)[b]	Percentage of anemic dogs	Mean infectiousness% (95% CI)	Median parasite load (Q1–Q3)
A. caninum	Good	15 (19)	33	33 (27–39)	3.6 (0.4–15.9)
	Regular-Poor	5 (6)	80	38 (28–49)	2.0 (1.2–7.2)
	Total	20 (25)	45	34 (29–49)	2.8 (0.4–15.5)
Trematode	Good	3 (3)	67	45 (33–58)	3.6 (0.4–15.9)
	Regular-Poor	3 (4)	100	57 (45–70)	1.2 (0.0–18.3)
	Total	6 (7)	83	51 (42–60)	2.4 (0.4–15.9)
Negative[c]	Good	2 (4)	0	5 (0–11)	0.0 (0.0–0.0)
	Regular-Poor	2 (2)	100	9 (0–19)	0.1 (0.0–0.2)
	Total	4 (6)	50	6 (0–8)	0.0 (0.0–0.0)

[a] Each co-infecting helminth was tested independently.
[b] Seven *T. cruzi*-seropositive dogs with no hematological results were excluded from the table.
[c] "Negative" refers to *T. cruzi*-seropositive dogs negative to *A. caninum* and trematode.

No significant differences were found between categories of body condition in dogs co-infected with *A. caninum* and a trematode in both the mean infectiousness and the median parasite load (Kruskal-Wallis test, df = 1, P > 0.9 in both cases) (Table 2).

4. Discussion

This cross-sectional study recorded a positive association between infectiousness of *T. cruzi*-seropositive dogs and co-infection with at least two worms, *A. caninum* and a trematode (possibly *F. hepatica*). The *T. cruzi* bloodstream parasite load of dogs infected with either of both helminths was also higher than in dogs without these co-infections. Furthermore, infectiousness to the vector was positively associated with bloodstream parasite load as documented before (Enriquez et al., 2014).

Although restricted by the rather limited number of *T. cruzi*-infected dogs detected, our results support the hypothesis that certain worms may have a synergistic interaction with phylogenetically unrelated parasites such as protozoa (Mwangi et al., 2006; Boel et al., 2010). Other hematophagous parasites identified in stool samples from these dogs (although in low frequency and excluded from the analysis, such as *Spirocerca lupi* and *Trichuris vulpis*) may also be positively associated with infectiousness to the vector and parasite load. Interestingly, some *T. cruzi*-seropositive dogs infected with *S. lupi* and *T. vulpis* were also co-infected with *A. caninum* and a trematode.

The mean infectiousness to the vector of *T. cruzi*-seropositive dogs at the same time seropositive to *T. gondii* or *N. caninum* was nearly half than that registered in the negative control dogs, thus suggesting an antagonistic association which was not statistically significant. Absence of true negative controls (i.e., dogs only infected with *T. cruzi*) is a limitation of this study that may mask the association between other parasites and *T. cruzi* parasitemia and infectiousness. For example, nearly 40% of *T. cruzi* and *T. gondii* or *N. caninum*-seropositive dogs were not co-infected with *A. caninum* or the trematode, and the mean infectiousness to the vector of this small number of dogs was about eight times lower (5%, 95% CI = 1–14%; 4%, 95% CI = 1–9%, respectively) than the mean infectiousness including dogs co-infected with *A. caninum* or the trematode. Another limitation of this study is related to the method used for the diagnosis of *T. gondii* and *N. caninum* (i.e., antibody detection) which only determines exposure to these parasites and not a true infection.

The sequence and time elapsed between infections may also influence the outcome of the interaction between parasites (Graham, 2002; Jackson et al., 2006; Telfer et al., 2008). Cross-sectional studies cannot determine the sequence and time period between infections and it is a limitation of our study. Longitudinal or interventional studies are more appropriate to examine the presence of interactions between parasite species (Telfer et al., 2008; Fenton et al., 2014). Therefore, we plan to experimentally manipulate helminthic infections to further test the current results.

The mechanism by which co-infections with *A. caninum* and a trematode modify infectiousness to the vector and *T. cruzi* parasite load could be immunomodulation and/or deterioration of dog health, with an expected decrease of the immune response. The hypothesized interaction between parasites not competing for nutrients or for localization in the host would be through the host immune system (Cox, 2001). Two studies on rodents experimentally infected with *Leishmania major* and co-infected with a fluke (*Schistosoma mansoni*) or a nematode (*Litomosoides sigmodontis*) reported a slower progression of the disease caused by *Leishmania* than in the control group, and higher parasite load for *L. major* in rodents co-infected with flukes (La Flamme et al., 2002; Lamb et al., 2005). In *Leishmania* infections, Th1 is the most important protective immune response, as in *T. cruzi*, but it is also respon-

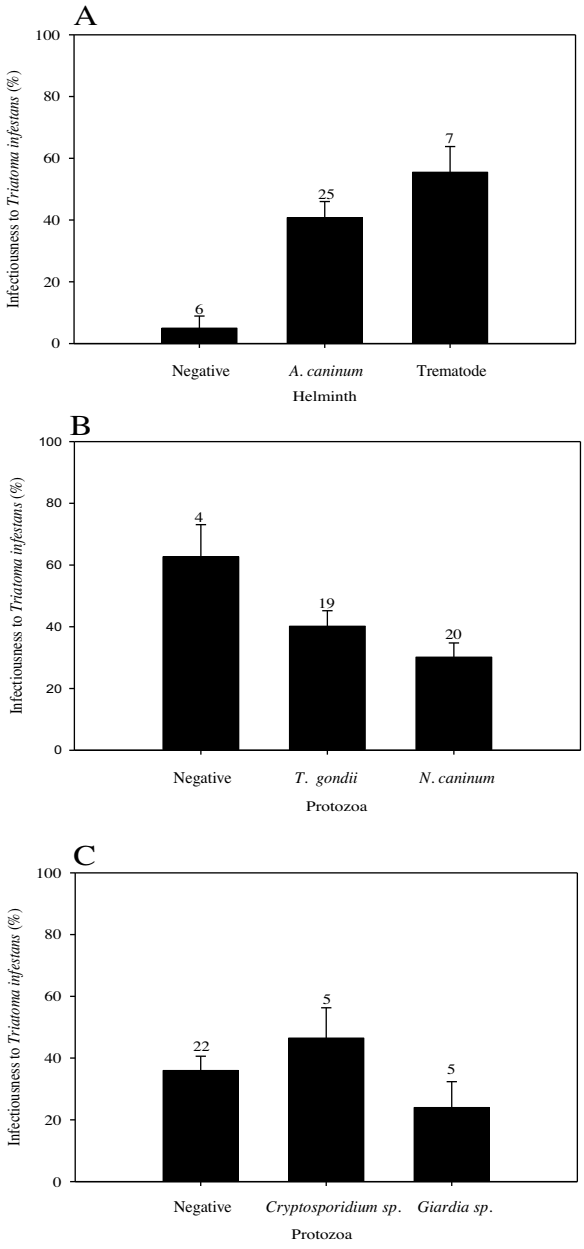

Fig. 3. Mean infectiousness to *T. infestans* of *T. cruzi*-seropositive dogs co-infected with (or exposed to) other parasite species. Pampa del Indio and neighboring villages, Chaco, 2013. (A) Only includes *A. caninum* and a trematode (possibly *F. hepatica*). (B) Only includes *T. gondii* and *N. caninum* (C) only includes *Cryptosporidium* sp. and *Giardia* sp. The "Negative" category corresponds to "negative controls" for each group of parasites. The whiskers above each bar represent the 95% confidence interval, and the number of dogs in each case is also indicated.

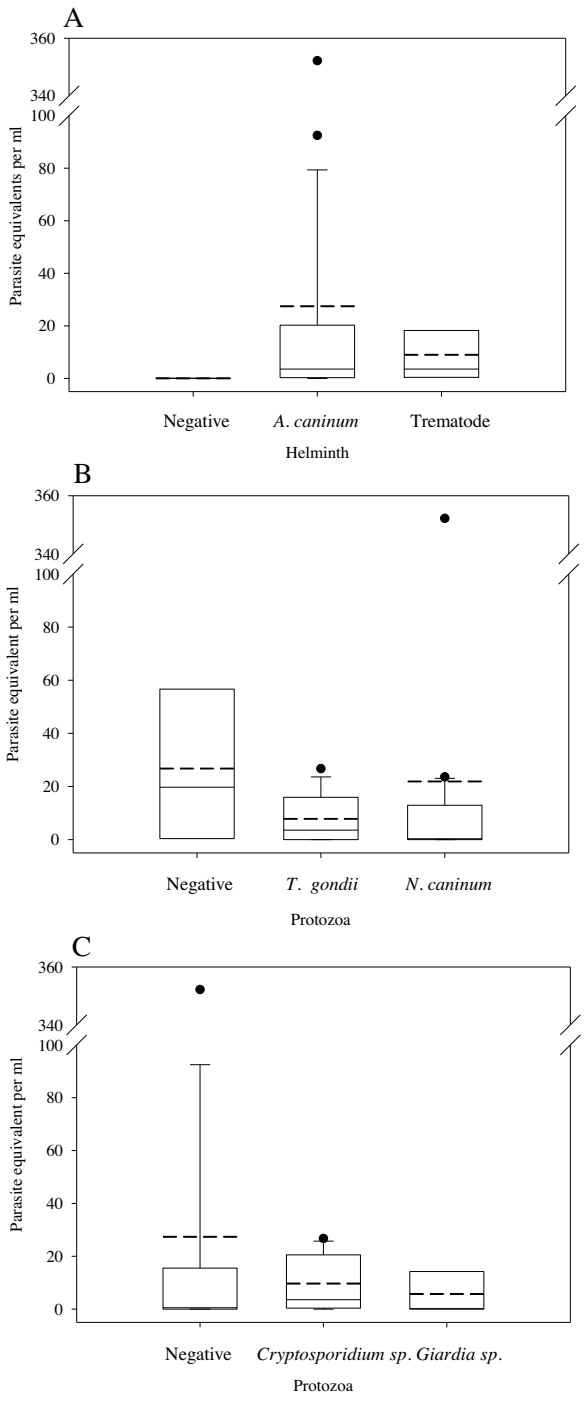

Fig. 4. Distribution of parasite load among *T. cruzi*-seropositive dogs co-infected with (or exposed to) other parasite species. Pampa del Indio and neighboring villages, Chaco, 2013. (A) Only includes *A. caninum* and a trematode (possibly *F. hepatica*). (B) Only includes *T. gondii* and *N. caninum* (C) only includes *Cryptosporidium* sp. and *Giardia* sp. The "Negative" category corresponds to "negative controls" for each group of parasites. The horizontal dash bar indicates the mean of parasite equivalent per ml and the black horizontal bar indicates the median. Boxes represent the Q1 and Q3 quartiles, the minimum and maximum parasite load values is indicated by the lines extending from the boxes, and black circles represent outliers.

sible, at least in part, for the observed pathology. Both studies found that rodents with a previous helminthic infection had a lower Th1 specific immune response than the control group (as measured by cytokine levels and cells corresponding to each type of immune response), suggesting that the interactions between parasite species were related to immunomodulation.

With regard to the association between health condition and helminth infections, alterations in hematologic parameters causing anemia were found associated with infections with *A. caninum* in dogs and with *F. hepatica* in sheep (Dracz et al., 2014; Matanović et al., 2007). In particular, high loads of *A. caninum* may cause severe anemia and death in dogs (Bowman et al., 2004; Heukelbach and Feldmeier, 2008). Our study found that *T. cruzi*-seropositive dogs with a regular or poor body condition included a significantly higher proportion of anemic dogs. Indeed, the prevalence of anemic dogs was higher for dogs co-infected with a trematode but not in dogs co-infected with *A. caninum*. Anemia in dogs may be caused by multiple factors including the historical exposure to, course of infection, and parasite load of these or other parasites, given that

the local dogs were rarely dewormed. Further studies are necessary to test whether immunomodulation and/or health status of the dogs were involved in the interactions between *T. cruzi* and both helminths.

A drawback of cross-sectional studies is that spurious associations might occur. In our study, other factors such as *T. cruzi* and host genotype may also contribute to the observed infectiousness and parasite load heterogeneity. For example, in *Leishmania infantum*-infected dogs the risk of being parasite positive (i.e., with patent parasitemia), was associated with MHC (major histocompatibility complex) class II polymorphism (Quinnell et al., 2003). Castillo-Neyra et al. (2016) observed that intrinsic factors at the individual level have a role in infectiousness variability in experimentally-infected guinea pigs. Other factors such as dose of infection and re-infection events could further contribute to heterogeneity in infectiousness (Wilson et al., 2001; Gürtler et al., 2007).

Another limitation of the present work was the failure to conclusively identify the species of trematode infecting the study dogs. So far, no record of *F. hepatica* infection in dogs was found in the literature, though finding of *F. hepatica* eggs in dog feces was reported in an endemic area in Peru (Marcos et al., 2006). In this case parasite identification only relied on eggs morphology, and ingestion of raw viscera was suggested as the source of these eggs rather than a true infection. Trematode eggs were also found in 4% of study dogs in Serra do Cipó National Park area (Brazil) though the species was not identified (Costa Santos et al., 2012). Other trematodes already described in dogs were unlikely. *Alaria* eggs (maximum length <62 μm), although described in carnivores of the Chaco Province (Argentina) are half the length of the observed eggs (Rigonatto et al., 2000). *Paragonimus*, a trematode genus with a broad geographical distribution including South America, was also ruled out given that its southernmost records are from Colombia and Venezuela (Uruburu et al., 2008).

One of the strengths of this study was the use of a reliable, analytically and clinically validated molecular tool (i.e., SatDNAqPCR) for the quantification of *T. cruzi* DNA (Bua et al., 2012; Enriquez et al., 2014). The nuclear satellite target did not show significant differences with kinetoplast DNA target in a recent report where the same human samples were quantified by both set of primers (kDNAqPCR and SatDNAqPCR) (Álvarez et al., 2015).

Although more studies are necessary to unveil the mechanisms involved in the observed pattern, our findings have implications for the epidemiology and control of *T. cruzi*. Firstly, the high frequency of poly-parasitism observed in the study dog population could support the development of innovative strategies for the control of Chagas disease and other zoonosis cost-effectively. Moreover, given *T. cruzi* vaccine boost immune response to suppress parasitemia (Basombrio et al., 1993; Aparicio-Burgos et al., 2001; Pérez-Brandan and Basombrío, 2012), helminth immunomodulation (Elias et al., 2006; Metenou et al., 2011) could diminish vaccine efficacy. Further investigations of the interspecies interactions herein described are needed to assess the potential impact of co-infections on candidate vaccines against *T. cruzi*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2016.04.042.

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