Infection, Genetics and Evolution 25 (2014) 36–43

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

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

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ARTICLE INFO

Article history:
Received 11 January 2014
Received in revised form 31 March 2014
Accepted 3 April 2014
Available online 13 April 2014

Keywords:
Trypanosoma cruzi
Real-time PCR
Infectiousness
Reservoir

ABSTRACT

The competence of reservoir hosts of vector-borne pathogens is directly linked to its capacity to infect the vector. Domestic dogs and cats are major domestic reservoir hosts of *Trypanosoma cruzi*, and exhibit a much higher infectiousness to triatomines than seropositive humans. We quantified the concentration of *T. cruzi* DNA in the peripheral blood of naturally-infected dogs and cats (a surrogate of intensity of parasitemia), and evaluated its association with infectiousness to the vector in a high-risk area of the Argentinean Chaco. To measure infectiousness, 44 infected dogs and 15 infected cats were each exposed to xenodiagnosis with 10–20 uninfected, laboratory-reared *Triatoma infestans* that blood-fed to repletion and were later individually examined for infection by optical microscopy. Parasite DNA concentration (expressed as equivalent amounts of parasite DNA per mL, Pe/mL) was estimated by real-time PCR amplification of the nuclear satellite DNA. Infectiousness increased steeply with parasite DNA concentration both in dogs and cats. Neither the median parasite load nor the mean infectiousness differed significantly between dogs (8.1 Pe/mL and 48%) and cats (9.7 Pe/mL and 44%), respectively. The infectiousness of dogs was positively and significantly associated with parasite load and an index of the host's body condition, but not with dog's age, parasite discrete typing unit and exposure to infected bugs in a random-effects multiple logistic regression model. Real-time PCR was more sensitive and less time-consuming than xenodiagnosis, and in conjunction with the body condition index, may be used to identify highly infectious hosts and implement novel control strategies.

1. Introduction

Trypanosoma cruzi, the etiologic agent of Chagas disease, has been found infecting approximately 180 species of mammals (WHO, 2002). The competence of reservoir hosts of vector-borne pathogens is directly linked to its capacity to infect the vector. Domestic dogs and cats are major reservoir hosts of *T. cruzi* in the domestic environment throughout the Americas (Cardinal et al., 2008, in press; Crisante et al., 2006; Gürtler et al., 2007; Noireau et al., 2009; Ramírez et al., 2013). They are usually highly infectious to triatomine bugs, and display much higher infectiousness (defined as the proportion of uninfected insects that become infected after blood-feeding once on an infected host) than *T. cruzi*-seropositive humans (Gürtler et al., 2007, 1996). Therefore, dogs and cats frequently constitute the largest source of *T. cruzi* for domestic transmission where *Triatoma infestans* is the main vector species as in the Gran Chaco region (Gürtler et al., 2007).

The chances of a triatomine bug becoming infected with *T. cruzi* after blood-feeding on an infected host mainly depend on the probability of ingesting the parasite (determined by the intensity of parasitemia and bloodmeal size), and its successful establishment and reproduction in the insect gut. The minimum number of *T. cruzi* parasites required to infect a bug through artificial xenodiagnosis was estimated to be one (Moll-Merks et al., 1987). However, the percentage of infected insects could be highly variable and depends on *T. cruzi* Discrete Typing Unit (DTU, i.e., genotypes) and strain, triatomine species and not simply on the

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http://dx.doi.org/10.1016/j.meegid.2014.04.002
1567-1348/

number of trypomastigotes ingested or inoculated (Alvarenga and Leite, 1982; Garcia et al., 2007; de Lana et al., 1998; Neal and Miles, 1977; da Silveira Pinto et al., 2000).

Previous studies that sought to estimate parasitemia in naturally-infected hosts have traditionally relied on direct counting of *T. cruzi* parasites under optical microscopy. However, new technologies have become available for this purpose (i.e., flow citometry, DNA amplification). Real-time or quantitative PCR (qPCR) is useful for the quantification of parasite DNA in different host tissues and its applications have substantially increased in recent years. In Chagas disease, qPCR has been mainly applied to human patients and has been successfully used in the follow-up of treatment outcomes, diagnosis of infection, and to investigate parasite vertical transmission (Bua et al., 2012; Duffy et al., 2009; Moreira et al., 2013; Schijman et al., 2011). Recent studies using qPCR to measure the concentration of *T. cruzi* DNA (i.e., a surrogate of intensity of parasitemia) found that the median bloodstream parasite load of chronic patients was $\leqslant$2 parasite equivalents/mL and differed significantly among countries (de Freitas et al., 2011; Moreira et al., 2013). Higher parasite loads were recorded in chronic patients from Argentina and Colombia (Duffy et al., 2013; Moreira et al., 2013), in patients coinfected with HIV (de Freitas et al., 2011), in seropositive women giving birth to *T. cruzi*-infected newborns (Bua et al., 2012) and in newborns diagnosed by optical microscopy after delivery (Bua et al., 2013).

There is strong evidence on the clustering and heterogeneity in the transmission of pathogens, where 20% of individuals in a population would be responsible for 80% of the transmission (Wilson et al., 2001; Woolhouse et al., 1997; Courtenay et al., 2014). The small fraction of individuals that infect disproportionately more susceptible contacts than most infected individuals became known as "superspreaders" of disease (Stein, 2011). *T. cruzi* infection, although mainly a vector-borne disease, shows that a small fraction of the infected dogs and cats were highly infectious to xenodiagnosis bugs and could be considered "superspreaders" (Gürtler et al., 2007).The infectiousness to the vector *T. infestans* of seropositive dogs was shown to be aggregated (Gürtler et al., 2007) and associated with an index of the host's body condition (i.e., external clinical aspect, ECA) (Petersen et al., 2001), and not associated with *T. cruzi* DTU (Cardinal et al., 2008). Mixed outcomes were registered for age of the dog and exposure to infected bugs (Gürtler et al., 2007, 1996, 1992). The functional relationship between parasitemia and infectiousness to the vector in dogs and cats naturally infected with *T. cruzi* (or any other mammalian nonhuman host) has not been investigated.

In this study we used for the first time a qPCR protocol to quantify the load of *T. cruzi* DNA in the peripheral blood of naturally-infected dogs and cats from a well-defined rural area in the Argentinean Chaco. We also evaluated the association among infectiousness to the vector, bloodstream parasite load and other potential predictors of infectiousness, and identified highly infectious hosts.

2. Materials and methods

2.1. Study area

Field work was conducted in the municipality of Pampa del Indio (26° 2′ 0″ S, 59° 55′ 0″ W), Chaco Province, Argentina. The study area was described elsewhere (Gurevitz et al., 2011). Prior to community-wide residual spraying with pyrethroid insecticides, a cross-sectional survey of all villages revealed that 45.9% of the inhabited houses were infested with *T. infestans* (Gurevitz et al., 2011), and the overall prevalence of *T. cruzi* infection was 27% in bugs, 26% in dogs and 29% in cats in August–December 2008 (Cardinal et al., in press).

2.2. Study design

Nested within this larger survey, for current purposes we selected two neighboring villages (10 de Mayo and Las Chuñas, with 60 inhabited houses) showing the highest bug prevalence of infection with *T. cruzi* (61%) at baseline. Within these villages, all the houses with at least one *T. cruzi*-infected *T. infestans* at baseline (38 of 60 houses) were selected to conduct a cross-sectional xenodiagnostic survey of all dogs and cats. A total of 99 (56.3%) dogs and 29 (74.4%) cats were examined by xenodiagnosis to increase the chances of isolating parasites from a large number of individuals. Each head of household was informed on the objectives of the study; an informed oral consent was requested and obtained in all cases.

Blood samples (up to 7 mL) were drawn by venipuncture and allowed to clot at ambient temperature. Each serum was separated after centrifugation at 3000 rpm during 15 min., allocated in triplicate vials and preserved at −20 °C at the field laboratory. Blood samples were also used (2 mL in dogs and 1 mL in cats) for DNA extraction and PCR; these were immediately mixed with an equal volume of guanidine hydrochloride 6 M, EDTA 0.2 M pH 8.00 buffer (GEB), and stored at 4 °C. Serum samples were transported in dry ice to the main laboratory at the end of each survey, whereas GEB samples were kept in a cooler. The host's body condition was used as an index of the nutritional status of dogs as described by Petersen et al. (2001). Each dog was classified as having a good, regular or poor body condition based on the degree of muscle development; external evidence of bone structure, state of fur coat, existence of fatty deposits, and facial expression (Petersen et al., 2001). Only dogs aged 1 year or more were categorized to avoid potential confounders due to growth effects and acute infections (Petersen et al., 2001). Handling and examination of dogs and cats was conducted according to the protocol approved by the 'Dr. Carlos Barclay' Independent Ethical Committee for Clinical Research from Buenos Aires, Argentina (IRB No. 00001678, NIH registered and Protocol No. TW-01-004).

2.3. Xenodiagnosis

Dogs and cats were examined by xenodiagnosis with 10 or 20 uninfected, laboratory-reared fourth-instar nymphs of *T. infestans* exposed to the animal's belly during 20 min, followed by a 10-min re-exposure period if initially most bugs had not blood-fed to repletion (Enriquez et al., 2013a). Each bug was individually examined by optical microscopy (OM) (400×) for *T. cruzi* infection 30 and 60 days after exposure. The infectiousness to the vector of dogs and cats that were seropositive for *T. cruzi* was calculated as the total number of insects infected with *T. cruzi* divided by the total number of insects exposed to the animal and examined for infection at least once (Gürtler et al., 1992). Bug mortality rate at 30 days post-exposure was 3% in both dogs and cats; and bugs exposed to dogs molted after a single blood meal (12%) more frequently than bugs fed on cats (8%), though these differences were not statistically significant (Enriquez et al., 2013b). *T. cruzi*-infected dogs and cats were also classified as having high or low infectiousness to *T. infestans*; the "high infectiousness" category included dogs and cats that were responsible for $\geqslant$80% of all infected triatomines used in the xenodiagnostic tests, and the "low infectiousness" included the remaining seropositive dogs and cats and that were also examined by xenodiagnoses.

2.4. Serodiagnosis

Dogs and cats aged 4 months or more were diagnosed serologically whereas younger animals were examined only by xenodiagnosis because maternally-derived antibodies to *T. cruzi* could

induce a false-positive result. Seventy (70%) dogs and 27 (93%) cats examined by xenodiagnosis were also examined by serodiagnosis. Sera were tested for antibodies to *T. cruzi* by indirect hemagglutination assay (IHA) following the manufacturer's instructions (Wiener Laboratories S.A.I.C., Buenos Aires, Argentina), an in-house enzyme-linked immunosorbent assay (ELISA), and an indirect immunofluorescence test (IFAT) (Ififluor Parasitest Chagas, Laboratorio IFI, Buenos Aires, Argentina). The serodiagnosis methodologies and results of dog and cat populations have been reported elsewhere (Enriquez et al., 2013b). An animal was considered "seropositive" if reactive by at least two assays.

2.5. Molecular analysis

2.5.1. DNA extraction

Guanidine-EDTA blood samples (GEB) of 2 and 4 mL were heated in boiling water for 10 and 15 min, respectively (Britto et al., 1993). Prior to DNA extraction, 200 pg of an internal amplification control DNA (IAC) was added to 400 μL GEB aliquot (Duffy et al., 2009). Total DNA was purified using a commercial kit (DNeasy Blood & Tissue Kit, QIAGEN Sciences, Maryland, USA) and following manufacturer's instructions avoiding the use of proteinase K and the addition of buffer AL, 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. Polymerase chain reaction

DNA from seropositive dogs and cats that were also examined by xenodiagnosis were tested by means of qualitative and qPCR assays. The qualitative PCR assay was targeted to the minicircles of the kinetoplast (kDNA-PCR). The rectal-ampoule samples of 94 OM-negative bugs used in xenodiagnosis of the seropositive dogs was examined by kDNA-PCR to assess the false-negative rate of OM procedures. In addition, we included samples from 16 dogs and 7 cats that resided in the study area and were not infected by *T. cruzi* as determined by ELISA, IHA and xenodiagnosis (i.e., control hosts). The comparison of kDNA-PCR results and xenodiagnosis has been reported elsewhere (Enriquez et al., 2013b).

2.5.3. Real-time PCR assay

The bloodstream parasite load in dogs and cats was quantified by amplifying a *T. cruzi* satellite DNA flanked by the Sat Fw (5′-GCAGTCGGCKGATCGTTTTCG-3′) and Sat Rv (5′-TTCAGRGTTGTTTGGTGTCCAGTG-3′) 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 previously described for human samples (Bua et al., 2012). Samples were run in duplicates using a commercial kit (SYBR GreenER qPCR SuperMix Universal, Invitrogen, Life Technologies, Grand Island, NY, USA) and 8 μL of template DNA in a 20 μL final volume.

The standard calibration curve for IAC was carried out using blood samples of dogs not infected with *T. cruzi* (i.e., dogs residing in non-endemic areas). Samples were spiked with 400 pg, 40 pg and 4 pg of IAC. The IAC was quantified using 0.6 μM of primers, IS Fw (5′-AACCGTCATGGAACAGCACGTAC-3′) and IS Rv (5′-CTAGAACATTGGCTCCCGCAACA-3′) (Duffy et al., 2009) using a commercial kit (Quantitec SYBR Green PCR kit, QIAGEN Sciences, Maryland, USA). After 15 min of pre-incubation at 95 °C, PCR amplification was carried out for 40 cycles (94 °C for 15 s, 59 °C for 30 s and 72 °C for 30 s).

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). Parasite DNA concentration was expressed as equivalent amounts of parasite DNA per mL (Pe/mL). An ABI 7500 thermocycler (Applied Biosystems, Carlsbad, CA, USA) was used in all qPCR amplifications.

2.5.4. DTU identification

Parasite DTUs were identified by PCR targeted to three genomic markers: spliced-leader (SL) DNA, 24Sα ribosomal RNA genes and A10 marker as described by Burgos et al. (2007). PCR products were analyzed in 2.5% or 3% agarose gels (Invitrogen, USA) and UV visualized after staining with Gel Red (GenBiotech). Identification of DTUs infecting dogs and cats based on isolated parasites was reported elsewhere (Enriquez et al., 2013a). In the current study, additional DTU identifications from seropositive, xenodiagnosis-negative dogs ($n = 6$) and cats ($n = 5$) and from a seropositive, xenodiagnosis-positive dog were attempted directly from GEB or rectal ampoule samples of OM-negative, kDNA-PCR-positive bugs used in xenodiagnostic tests of these animals. These additional dogs and cats belonged to the same villages, and samples were taken at the same time as those included in Enriquez et al. (2013a). DNA was extracted from the bugs' rectal ampoule using DNAzol® (Invitrogen, USA) as described previously (Maffey et al., 2012). DTU identifications were performed introducing the following modifications to avoid unspecific amplifications within the Spliced Leader region. SL-IR I, using primers TC2 and UTCC, was used to identify TcI (475 bp) as follows: one cycle at 94 °C for 3 min, 6 cycles at 62 °C for 40 s, 6 cycles at 60 °C for 40 s, 40 cycles at 58 °C for 40 s and final extension step at 72 °C for 10 min; and SL-IR II, using primers TC1 and UTCC, was used to identify TcII, TcV, and TcVI (425 bp) as follows: one cycle at 94 °C for 3 min, 5 cycles at 68 °C for 40 s, 5 cycles at 66 °C for 40 s, 5 cycles at 64 °C for 40 s, 3 cycles at 62 °C for 40 s, 30 cycles at 60 °C for 40 s and final extension step at 72 °C for 10 min. To enhance *T. cruzi* detection, PCR reactions were carried out at a final volume of 50 μL of reaction mix, with 8 μL of DNA sample as template and using *Taq Platinum* DNA polymerase (Invitrogen, USA). Given that two unspecific bands in the SL-IR I PCR were persistently obtained in GEB samples, the expected amplicon of 475 pb was purified and sequenced to confirm TcI identification.

Incomplete identifications of DTUs were achieved in some cases in which only a 425 bp-band was obtained in the SL-IR II PCR and no bands in the other PCR assays due to low DNA content. These cases were described as TCII/V/VI.

2.6. Sequence analysis of SL-IR I

The PCR-amplified DNA was purified with Qiaquick Gel Extraction kit (Qiagen) and cloned into pGEMT-easy vector following the manufacturer's instructions (Promega, Madison, WI). Recombinant plasmids were screened by digestion with EcoRI (New England Biolabs) and sequenced using T7 and SP6 universal primers by Macrogen (Inc., Seoul, Korea) in an ABI PRISM 3730 DNA Analyzer automated sequencer (Applied Biosystems) and aligned manually or using MEGA 5.1 software (Tamura et al., 2007). Newly reported sequences are available at GenBank under accession numbers KF964667, KF964668 and KF964669. A consensus of forward and reverse sequences was created and a BLAST search was performed in GenBank database to compare sequence identity.

2.7. Data analysis

Agresti–Coull binomial 95% confidence intervals (CI) were used for proportions. The relationship between infectiousness to the vector of dogs and cats infected with *T. cruzi* and potential predictors was analyzed using maximum likelihood multiple logistic regression implemented in Stata (Stata 10.1, Stata Corp, College Station, Texas). Only dogs and cats aged 1 year or more were included in the regression to avoid potential confounders due to

Table 1
Infectiousness to the vector *T. infestans* of dogs and cats infected with *T. cruzi* according to potential risk factors; Pampa del Indio, Chaco, 2008.

Factor	Dogs[a]		Cats[a]	
	Median infectiousness% (first-third quartiles)	OR (95% CI)	Median infectiousness% (first-third quartiles)	OR (95% CI)
Host body condition			–	
Good	16 (5–68)	1	–	–
Regular/poor	64 (32–84)	4.45 (1.24–16.01)	–	–
Parasitemia (Pe/mL)	–	1.02 (1.00–1.04)	–	1.02 (1.00–1.04)
Exposure to infected bugs (abundance of infected-bug captured per 15 min-person per site)				
0	55 (35–75)	1	67 (25–75)	1
1–5	68 (35–84)	0.41 (0.03–6.58)	79 (78–79)	0.21 (0.01–5.17)
>5	20 (5–65)	0.40 (0.02–9.43)	0 (0–50)	0.20 (0.01–6.66)
Age of host	–	1.00 (0.99–1.02)	–	1.18 (0.93–1.48)
DTU				
TcV	30 (16–45)	1	–	–
TcI or TcIII	38 (0–75)	0.58 (0.02–22.42)	–	–
TcVI	62 (30–80)	2.22 (0.45–10.94)	–	–

[a] Three dogs and four cats aged <1 year were not included in the regression.

Table 2
Relationship between host body condition and infectiousness to *T. infestans* of dogs infected with *T. cruzi* aged ⩾1 year; Pampa del Indio, Chaco, 2008.

Host body condition	Percentage of seropositive dogs (No. examined)	Percentage of seropositive dogs with a positive xenodiagnosis (No. examined)	Median infectiousness of xenodiagnosis-positive dogs	Infectious potential index
Good	55 (56)	78 (23)	30	0.13
Regular	50 (50)	93 (25)	63	0.29
Poor	80 (5)	100 (3)	80	0.64
Total	54 (111)	85 (41)	61	0.28

growth effects. Because the bugs used in xenodiagnosis were clustered on individual dogs or cats, observations are not independent. Therefore, the xtmelogit command was used to include random effects that measure the residual effects due to each subject on the probability of bug infection. The dependent variable was the infection status of each bug used in xenodiagnosis. The independent variables were the bloodstream parasite load (a continuous variable, in Pe/mL), body condition (two levels: 0 = good, 1 = regular or poor); age of the host (a continuous variable, in months); exposure to infected bugs, as measured by the abundance of *T. infestans* infected with *T. cruzi* captured per 15 min-person per site in domiciles, kitchens and storerooms (i.e., typical resting sites of dogs and cats), categorized in low, medium and high-risk levels, 0 = 0; 1 = 1–5, and 2 ⩾ 5 bugs, respectively; and identified DTU (0 = TcV, 1 = TcVI; 2 = TcI or TcIII). Identified DTU was excluded as a predictor in cats due to the small sample size. The body condition status was divided into two levels because of the low number of dogs with a poor body condition. Interaction terms were added stepwise and dropped from the final model if not significant at a nominal significance level of 5%. The Wald test examined the hypothesis that all regression coefficients are 0.

To reflect the potential ability of a dog in a given body condition category to infect a bug after feeding to repletion, we calculated an Infectious Potential Index (IPI) for each category (Petersen et al., 2001) as the product of the proportion of seropositive dogs, the proportion of seropositive dogs with a positive xenodiagnosis, and the median infectiousness of seropositive dogs with a positive xenodiagnosis in each category.

3. Results

3.1. Parasite concentration

A total of 44 seropositive dogs and 14 seropositive cats that had been examined by xenodiagnosis were assayed by qPCR, and additionally, a xenodiagnosis-positive, seronegative cat was also tested. All of these samples were qPCR-positive except one seropositive cat that was negative by xenodiagnosis and kDNA-PCR. All control dogs ($n = 16$) and cats ($n = 7$) that were both seronegative and xenodiagnosis-negative were also negative by kDNA-PCR and qPCR. In two xenodiagnosis-negative, qPCR-positive cats, DNA quantifications could not be normalized because DTU identifications were unsuccessful, therefore they were excluded from further analysis.

The frequency distribution of parasite DNA concentration estimated by qPCR showed a negative binomial distribution in both host populations (Fig. 1). The median bloodstream parasite load was slightly higher in cats (median = 9.7 Pe/mL, first-third quartiles

Table 3
Relationship among qualitative kDNA-PCR, qPCR and parasite load to detect *T. cruzi*-infected dogs and cats with different levels of infectiousness.

Host	Infectiousness[a]	No. positive xenodiagnosis (No. examined)	Mean infectiousness (%)	Median parasite load (*Q*1–*Q*3)	No. positive kDNA (%)	No. positive qPCR (%)
Dogs	High	22 (22)	79	21.3 (7.3–39.3)	22 (100)	22 (100)
	Low	16 (22)	19	2.4 (1.3–12.6)	17 (77)	22 (100)
	Total	38 (44)	48	8.1 (1.5–26.6)	39 (89)	44 (100)
Cats	High	7 (7)	79	96.1(18.1–202.1)	7 (100)	7 (100)
	Low	3 (8)	14	3.0 (2.2–7.4)	6 (75)	7 (88)
	Total	10 (15)	44	9.7 (2.9–96.9)	13 (87)	14 (93)

[a] The "high infectiousness" category included dogs and cats that were responsible for ⩾80% of all infected triatomines used in the xenodiagnostic tests. The "low infectiousness" category included all remaining seropositive dogs and cats examined by xenodiagnosis.

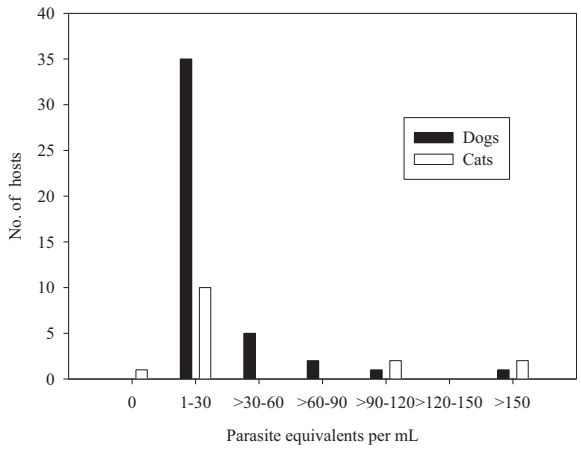

Fig. 1. Frequency distribution of parasite load of *T. cruzi*-infected dogs (black bars) and cats (white bars); Pampa del Indio, Chaco, 2008.

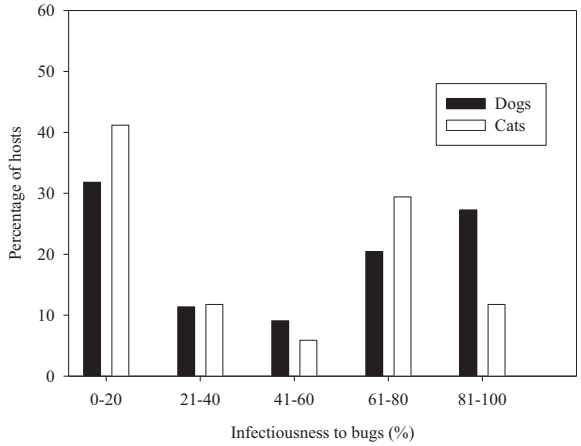

Fig. 2. Frequency distribution of infectiousness to *T. infestans* of *T. cruzi*-infected dogs (black bars) and cats (white bars); Pampa del Indio, Chaco, 2008.

[Q1–Q3] = 2.9–96.9, range 0.0–1101.8) than in dogs (median = 8.1 Pe/mL, Q1–Q3 = 1.5–26.6, range 0.7–214.5), although this difference was not statistically significant (Kruskal–Wallis test, $P = 0.5$). If the two seropositive, xenodiagnosis-negative cats in which DNA quantifications could not be normalized had been infected either with TcI or TcVI (i.e., the extreme cases for normalization), their parasitemia would range between <1 and 10 Pe/mL.

3.2. Infectiousness to the vector and parasite load

Overall, 86% of dogs ($n = 44$) and 64% of cats ($n = 14$) seropositive for *T. cruzi* were positive by xenodiagnosis. The mean infectiousness to *T. infestans* did not differ significantly between dogs (48%, 95% confidence interval [CI] = 33–62%) and cats (44%, 95% CI = 10–63%). Thirteen percent of 94 OM-negative bugs were positive by kDNA-PCR; thus, the corrected infectiousness to the vector would rise to 54%. The frequency distribution of infectiousness showed a bimodal pattern in both host populations (Fig. 2).

The infectiousness of infected dogs and cats increased steeply with parasite load, reaching maximum infectiousness (95%) at 96.9 Pe/mL for cats and 13.0 Pe/mL for dogs (Fig. 3). No clear association was observed between DTU and host infectiousness or bloodstream parasite load (Fig. 3). However, the two cats and the only dog infected with TcI had nil infectiousness despite having 7.4, 9.7 and 20.5 Pe/mL, respectively (Fig. 3) (Appendix). In these three individuals TcI was confirmed by sequence analysis of Spliced Leader region (SL-IR I) (i.e., 98–100% sequence identity).

Random-effects multiple logistic regression model showed that infectiousness to the vector of seropositive dogs was significantly associated with the bloodstream parasite load (OR = 1.02, 95% CI = 1.00–1.04, $P < 0.05$) and body condition (OR = 4.45, 95% CI = 1.24–16.01, $P = 0.02$) (Wald $\chi^2 = 11.76$, $P < 0.01$) (Table 1). No association was found between infectiousness and age of the dog, exposure to infected bugs at the dog's house and DTU. The median parasite load in dogs with poor or regular body condition (9.93 Pe/mL, Q1–Q3 = 3.03–28.15) was approximately 4 times higher than in dogs with good condition (2.67 Pe/mL, Q1–Q3 = 1.38–18.90), but this difference was not statistically significant (Kruskal–Wallis, $P = 0.17$).

The infectiousness of infected cats was significantly associated with parasite load (OR = 1.02, 95% CI = 1.00–1.04, $P = 0.02$) (Wald $\chi^2 = 4.83$, $P = 0.09$) (Table 1). No significant association between infectiousness and age of the cat and exposure to infected bugs was found by random-effects multiple logistic regression analysis.

3.3. Body condition and infectiousness

The classification of 111 dogs aged $\geqslant 1$ year showed that 52%, 43% and 5% of them were in a good, regular and poor body condition, respectively (Table 2). The mean (±SD) age (3.0 ± 2.6, 4.0 ± 2.6, and 5.5 ± 6.9 years) and the proportion of female dogs (36%, 39% and 40%, $\chi^2 = 0.2$, df: 2, $P = 0.9$) were similar among categories ranging from good to poor conditions, respectively. The prevalence of *T. cruzi* infection varied between 50% and 80% and was not significantly different among body condition categories ($\chi^2 = 1.7$, df: 2, $P = 0.2$). The median infectiousness increased from 30% in dogs with good condition to 63% and 80% in animals with a regular and poor condition, respectively. Dogs with a poor condition had an infectious potential index (IPI) 2.2 times greater than dogs with a regular condition, and 4.9 times greater than those in a good condition (Table 2). However, "poor" dogs had a very low sample size of 5 versus the total sample size of 111.

3.4. Profile of infected dogs and cats with high or low infectiousness

Nearly half of the dogs (50%) and cats (47%) showed high infectiousness and were responsible of at least 80% of the infected xenodiagnosis bugs. The median parasite load was nearly 10 times greater for dogs with high infectiousness than for dogs with low infectiousness and this difference was statistically significant (Kruskal–Wallis test, $P < 0.01$). For cats this relationship was 32 times greater (Kruskal–Wallis test, $P = 0.02$) (Table 3). Quantitative PCR could not distinguish between highly infectious and non-highly infectious hosts, given that it detected *T. cruzi* DNA in 100% of the seropositive dogs and in 93% of the seropositive cats, whereas kDNA-PCR detected 89% and 87% of them, respectively (Table 3). Four seropositive dogs and one seropositive cat that were both qPCR-positive and kDNA-PCR-negative showed very low infectiousness to the vector (range, 0–5%).

4. Discussion

By means of a real-time quantitative PCR protocol, this study shows that the parasite load detected in the bloodstream of dogs and cats naturally infected with *T. cruzi* was closely associated with their infectiousness to the vector, and on average, was 4–5 times higher than that of chronic Chagas disease patients examined elsewhere (de Freitas et al., 2011; Moreira et al., 2013).

The median bloodstream parasite load was 8.1 Pe/mL in dogs and 9.7 Pe/mL in cats, whereas in chronic patients was $\leqslant 2$ Pe/mL

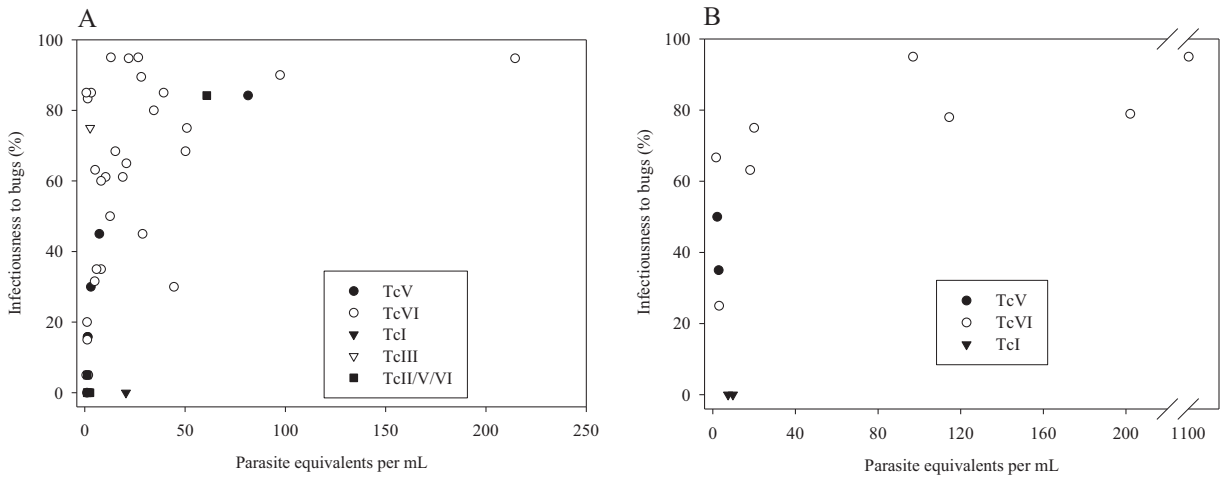

Fig. 3. Distribution of infectiousness to *T. infestans* of *T. cruzi*-infected dogs (A) and cats (B) according to parasite DNA quantification by qPCR and identified DTU; Pampa del Indio, Chaco, 2008. The figure excludes two seropositive cats from which DNA quantifications could not be normalized.

(de Freitas et al., 2011; Moreira et al., 2013). Furthermore, the sensitivity of qPCR in seropositive, chronic patients ranged from 41% to 76% (Duffy et al., 2013; Moreira et al., 2013; Piron et al., 2007), whereas in our study 93–100% of seropositive cats and dogs were qPCR-positive. These substantial differences in parasite load most likely explain the much lower proportion of *T. cruzi*-seropositive humans with a positive xenodiagnosis and their much lower infectiousness to bugs relative to seropositive dogs and cats (Coura et al., 1991; de Freitas et al., 2011; Gürtler et al., 1996).

In our study, the infectiousness of dogs and cats increased non-linearly from 0% to 85% with small increases in bloodstream parasite load. Two direct implications follow from these observations: first, a positive xenodiagnosis can be obtained with very low parasite load (i.e., 0.7 and 1.6 Pe/mL for dogs and cats, respectively), although the ultimate fraction of bugs that become infected may be highly variable. Second, given that 96% of the dogs and 90% of cats had more than 0.7–1.6 Pe/mL, a large proportion of them is expected to be highly infectious to the vector, as our study attests.

The wide variability in the infectiousness of dogs and cats with very low bloodstream parasite load may be related, at least in part, to unrecorded variations in the effective bloodmeal size achieved by the bugs used in xenodiagnosis. The actual infectiousness is expected to be greater than 44–48% because of the limited sensitivity of optical microscopy when the intensity of rectal infections is low (false-negative rate was 13%).

There were a few cases in which the high infectiousness observed could not be explained by the estimated parasite load of the individual host on which the bugs had fed, as in a TcVI-infected dog with a regular body condition and 0.8 Pe/mL which infected 17 of 20 insects. Perhaps the differential origin of the blood samples for qPCR (venous blood) and xenodiagnosis (capillary blood ingested by the bugs) may explain this apparent inconsistency (Ferreira et al., 2007). Parasite DNA concentrations differed between tissues in dogs naturally infected with *Leishmania infantum* (Ferreira et al., 2013). Technical errors in the quantification of bloodstream parasite load may be ruled out because all blood samples from dogs with very low parasite load and large infectiousness were repeated and the initial outcome corroborated (results not shown). Although rather unlikely, we cannot rule out the presence of inhibitors that affected the performance of qPCR despite IAC amplifications were consistently satisfactory. New protocols based on multiplex qPCR amplifying both DNA targets (i.e., DNA of *T. cruzi* and IAC) in the same reaction tube could enhance the reproducibility of qPCR assays (Duffy et al., 2013).

Dogs and cats had similar infectiousness to the vector and this distribution was aggregated, as in studies conducted elsewhere (Gürtler et al., 2007). There was a fraction of highly infectious dogs and cats that may contribute disproportionally to (peri)domestic transmission and persistence of *T. cruzi*. This heterogeneous distribution of parasite burden in host populations is a generalized pattern in macroparasites and microparasites (Wilson et al., 2001; Woolhouse et al., 1997) and may be used for targeted control strategies. *T. cruzi*-infected *Dasypus novemcinctus* armadillos and *Didelphis albiventris* opossums also have high infectiousness (Orozco et al., 2013).

Multiple logistic regression analysis showed that the infectiousness of dogs was associated with the bloodstream parasite load and the body condition of the individual host. The latter is likely reflecting the combined effects of the immune system and the nutritional status of the host (Keusch, 2003; Machado-Coelho et al., 2005; Petersen et al., 2001). Dogs with a regular or poor body condition showed a nearly fourfold higher median parasite load than dogs in good conditions. The former were probably less effective in controlling parasitemia for reasons that remain to be identified.

Exposure to repeated reinfections with *T. cruzi* via inoculations was positively correlated with a slight and transient increase in parasitemia in experimentally infected dogs and mice (Machado et al., 2001; Bustamante et al., 2007). In our study, however, exposure to infected bugs was not associated with increased infectiousness in dogs; previous surveys yielded mixed outcomes on this point (Gürtler et al., 2007, 1992). A possible explanation for these discrepancies is that our metric of exposure in the regression model may not represent in a precise manner the actual likelihood of reinfection the dogs faced throughout its home range in the past. In addition, we found no association between infectiousness and age of dogs (as in one of three previous studies: Gürtler et al., 2007, 1996, 1992) and parasite DTU (Cardinal et al., 2008). Other factors not evaluated here, such as concomitant parasitic infections, stress levels, inoculum size, and genetic background of host and parasite, may eventually mask the effects of age, DTU and reinfections on infectiousness. Moreover, the existence of a polymorphism in the genes involved in the immune response could also affect infectiousness, as in dogs infected with *L. infantum* (Quinnell et al., 2003). In cats, infectiousness was only associated with parasite load. TcVI-infected cats were more infectious that the few ones infected with TcV or TcI, but samples sizes were too small to make any sound conclusion.

The concurrent finding of TcI infections with bloodstream parasite load ranging from 7.4 to 20.5 Pe/mL and nil infectiousness (as determined by optical microscopy) suggest a differential behavior of TcI or a different susceptibility of the vector species involved. Indeed, TcI-infected dogs and cats were rare and could only be identified by means of a highly sensitive kDNA-PCR applied to the rectal-ampoule lysates of xenodiagnostic bugs or GEB samples. The substantial variations in metacyclogenesis and trypomastigote density among triatomine species (Carvalho-Moreira et al., 2003; Loza-Murguía and Noireau, 2010; Perlowagora-Szumlewicz and Moreira, 1994) may explain the nil infectiousness (as measured by optical microscopy) recorded in TcI-infected dogs and cats. However, samples sizes were small and this relationship should be further investigated.

In our study, approximately 50% of the infected dogs and cats were responsible for at least 80% of the infected *T. infestans* used in xenodiagnosis. All of them were positive by qPCR and kDNA-PCR. However, dogs and cats with very low infectiousness to the vector (<20%) were also frequently positive by these techniques. These results suggest that the quantitative estimates of parasite load and not the qualitative outcomes of qPCR or kDNA-PCR would be a more precise tool to identify highly infectious dogs and cats. A wide variability in infectiousness (range, 0–63% for dogs and 35–67% for cats) was observed between 1 and 5 Pe/mL. Courtenay et al. (2014) recently showed that quantitative PCR was needed to identify highly infectious *L. infantum*-infected dogs.

Quantitative PCR was more sensitive and less time-consuming than xenodiagnosis, and alone or in conjunction with the body condition index, may be helpful to identify highly infectious hosts; replace xenodiagnosis (or hemoculture) to assess treatment efficacy or infection status, and contribute to novel control strategies (e.g., targeted treatment or vaccination) that reduce the risks of domestic transmission of *T. cruzi*.

Financial support

This study received financial support from International Development Research Center (Eco-Health Program); the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) to R.E.G.; Agencia Nacional de Promoción Científica y Tecnológica to REG; Agencia Nacional de Promoción Científica y Tecnológica and GlaxoSmithKline to R.E.G., and University of Buenos Aires to R.E.G. J.B,, M.M.O., S.W., A.G.S., R.E.G. and M.V.C. are members of CONICET Researcher's Career.

Acknowledgments

We are grateful to Carolina Cura and Margarita Bisio for laboratory assistance on molecular biology. Romina Piccinali, Marina Leporace, Francisco Petrocco, Laura Tomassone, Leonardo Ceballos, Julián Alvarado-Otegui, Sol Gaspe and Nala for field or laboratory assistance, and to Lucía Maffey, Pilar Fernández, Fernando Garelli, Juan Gurevitz, Yael Provecho, Jimena Gronzo and Carla Cecere for valuable comments. Special thanks to the villagers of Pampa del Indio for kindly welcoming us into their homes and cooperating with the investigation.

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.meegid.2014.04.002.

References

Alvarenga, N.J., Leite, M.A., 1982. Injection of *Trypanosoma cruzi* into the gut of triatomine bugs: number required to infect the vector. Trans. R. Soc. Trop. Med. Hyg. 76, 708–709.

Britto, C., Cardoso, M.A., Wincker, P., Morel, C.M., 1993. A simple protocol for the physical cleavage of *Trypanosoma cruzi* kinetoplast DNA present in blood samples and its use in polymerase chain reaction (PCR)-based diagnosis of chronic Chagas disease. Mem. Inst. Oswaldo Cruz 88, 171–172.

Bua, J., Volta, B.J., Velazquez, E.B., Ruiz, A.M., Rissio, A.M., Cardoni, R.L., 2012. Vertical transmission of *Trypanosoma cruzi* infection: quantification of parasite burden in mothers and their children by parasite DNA amplification. Trans. R. Soc. Trop. Med. Hyg. 106, 623–628.

Bua, J., Volta, B.J., Perrone, A.E., Scollo, K., Velázquez, E.B., Ruiz, A.M., De Rissio, A.M., Cardoni, R.L., 2013. How to improve the early diagnosis of *Trypanosoma cruzi* infection: relationship between validated conventional diagnosis and quantitative DNA amplification in congenitally infected children. PLoS Negl. Trop. Dis. 7, e2476.

Burgos, J.M., Altcheh, J., Bisio, M., Duffy, T., Valdares, H.M.S., Seidenstein, M.E., Piccinali, R., Freitas, J.M., Levin, M.J., Machi, L., Macedo, A.M., Freilij, H., Schijman, A.G., 2007. Direct molecular profiling of minicircle signatures and lineages of *Trypanosoma cruzi* bloodstream populations causing congenital Chagas disease. Int. J. Parasitol. 37, 1319–1327.

Bustamante, J.M., Novarese, M., Héctor, W., Rivarola, H.W., Lo Presti, M.S., Fernández, A.R., Enders, J.E., Fretes, R., Paglini-Oliva, P.A., 2007. Reinfections and *Trypanosoma cruzi* strains can determine the prognosis of the chronic chagasic cardiopathy in mice. Parasitol. Res. 100, 1407–1410.

Cardinal, M.V., Lauricella, M.A., Ceballos, A.L., Lanati, L., Marcet, P.L., Levin, M.J., Kitron, U., Gürtler, R.E., Schijman, A.G., 2008. Molecular epidemiology of domestic and sylvatic *Trypanosoma cruzi* infection in rural northwestern Argentina. Int. J. Parasitol. 38, 1533–1543.

Cardinal, M.V., Orozco, M.M., Enriquez, G.F., Ceballos, L.A., Gaspe, M.S., Alvarado-Otegui, J.A., Gurevitz, J.M., Kitron, U., Gürtler, R.E., 2014. Heterogeneities in the ecoepidemiology of Trypanosoma cruzi infection in rural communities of the Argentinean Chaco. Am. J. Trop. Med. Hyg., in press.

Carvalho-Moreira, C.J., Spata, M.C., Coura, J.R., Garcia, E.S., Azambuja, P., Gonzalez, M.S., Mello, C.B., 2003. In vivo and in vitro metacyclogenesis of two strains of *Trypanosoma cruzi* in the vectors *Triatoma pseudomaculata* and *Rhodnius neglectus*: short/long-term and comparative study. Exp. Parasitol. 103, 102–111.

Coura, J.R., de Abreu, L.L., Willcox, H.P., Petana, W., 1991. Evaluation of the xenodiagnosis of chronic Chagas patients infected ten years or over in an area where transmission has been interrupted – Iguatama and Pains, West Minas Gerais State, Brazil. Mem. Inst. Oswaldo Cruz 86, 395–398.

Courtenay, O., Carson, C., Calvo-Bado, L., Garcez, L.M., Quinnell, R.J., 2014. Heterogeneities in *Leishmania infantum* infection: using skin parasite burdens to identify highly infectious dogs. PLoS Negl. Trop. Dis. 8, e2583.

Crisante, G., Rojas, A., Teixeira, M.M.G., Añez, N., 2006. Infected dogs as a risk factor in the transmission of human *Trypanosoma cruzi* infection in western Venezuela. Acta Trop. 98, 247–254.

da Silveira Pinto, A., de Lana, M., Britto, C., Bastrenta, B., Tibayrenc, M., 2000. Experimental *Trypanosoma cruzi* biclonal infection in *Triatoma infestans*: detection of distinct clonal genotypes using kinetoplast DNA probes. Int. J. Parasitol. 30, 843–848.

de Freitas, V.L., da Silva, S.C., Sartori, A.M., Bezerra, R.C., Westphalen, E.V., Molina, T.D., Teixeira, A.R., Ibrahim, K.Y., Shikanai-Yasuda, M.A., 2011. Real-time PCR in HIV/*Trypanosoma cruzi* coinfection with and without Chagas disease reactivation: association with HIV viral load and CD4 level. PLoS Negl. Trop. Dis. 5, e1277.

de Lana, M., da Silveira Pinto, A., Barnabé, C., Quesney, V., Noël, S., Tibayrenc, M., 1998. *Trypanosoma cruzi*: compared vectorial transmissibility of three major clonal genotypes by *Triatoma infestans*. Exp. Parasitol. 90, 20–25.

Duffy, T., Bisio, M., Altcheh, J., Burgos, J.M., Diez, M., Levin, M.J., Favaloro, R.R., Freilij, H., Schijman, A.G., 2009. Accurate real-time PCR strategy for monitoring bloodstream parasitic loads in Chagas disease patients. PLoS Negl. Trop. Dis. 3, e419.

Duffy, T., Cura, C.I., Ramirez, J.C., Abate, T., Cayo, N.M., Parrado, R., Bello, Z.D., Velazquez, E., Muñoz-Calderon, A., Juiz, N.A., Basile, J., Garcia, L., Riarte, A., Nasser, J.R., Ocampo, S.B., Yadon, Z.E., Torrico, F., de Noya, B.A., Ribeiro, I., Schijman, A.G., 2013. Analytical performance of a multiplex real-time PCR assay using TaqMan probes for quantification of *Trypanosoma cruzi* satellite DNA in blood samples. PLoS Negl. Trop. Dis. 7, e2000.

Enriquez, G.F., Cardinal, M.V., Orozco, M.M., Lanatti, L., Schijman, A.G., Gürtler, R.E., 2013a. Discrete typing units of *Trypanosoma cruzi* identified in rural dogs and cats in the humid Argentinean Chaco. Parasitology 12, 1–6.

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

Ferreira, R.A., Lazzari, C.R., Lorenzo, M.G., Pereira, M.H., 2007. Do haematophagous bugs assess skin surface to detect blood vessel? PLoS One 2, e932.

Ferreira, S. de A., Almeida, G.G., Silva, S. de O., Vogas, G.P., Fujiwara, R.T., de Andrade, A.S., Melo, M.N., 2013. Nasal, oral and ear swabs for canine visceral leishmaniasis diagnosis: new practical approaches for detection of *Leishmania infantum* DNA. PLoS Negl. Trop. 7, e2150.

Garcia, E.S., Ratcliffe, N.A., Whitten, M.M., Gonzalez, M.S., Azambuja, P., 2007. Exploring the role of insect host factors in the dynamics of *Trypanosoma cruzi–Rhodnius prolixus* interactions. J. Insect Physiol. 53, 11–21.

Gurevitz, J.M., Gaspe, M.S., Enriquez, G.F., Vassena, C., Alvarado-Otegui, J.A., Provecho, Y.M., Mougabure-Cueto, G., Picollo, M.I., Kitron, U., Gürtler, R.E., 2011. Unexpected pyrethroid resistance and control failures of Chagas disease vector in Argentina. J. Med. Entomol. 49, 1379–1386.

Gürtler, R.E., Petersen, R.M., Rubel, D.N., Lauricella, M.A., Wisnivesky-Colli, C., 1992. Infectivity to the vector *Triatoma infestans* of dogs infected with *Trypanosoma cruzi* in north-west Argentina. Ann. Trop. Med. Parasitol. 86, 111–119.

Gürtler, R.E., Cecere, M.C., Castañera, M.B., Canale, D., Lauricella, M.A., Chuit, R., Cohen, J.E., Segura, E.L., 1996. Probability of infection with *Trypanosoma cruzi* of the vector *Triatoma infestans* fed on infected humans and dogs in northwest Argentina. Am. J. Trop. Med. Hyg. 55, 24–31.

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

Keusch, G.T., 2003. The history of nutrition: malnutrition, infection and immunity. J. Nutr. 133, S336–S340.

Loza-Murguía, M., Noireau, F., 2010. Vectorial capacity of *Triatoma guasayana* (Wygodzinsky&Abalos) (Hemiptera: Reduviidae) compared with two other species of epidemic importance. Neotrop. Entomol. 39, 799–809.

Machado, E.M.M., Fernandes, A.J., Murta, S.M.F., Vitor, R.W.A., Camilo Jr., D.J., Pinheiro, S.W., Lopes, E.R., Adad, S.J., Romanha, A.J., Pinto Dias, J.C., 2001. A study of experimental reinfection by *Trypanosoma cruzi* in dogs. Am. J. Trop. Med. Hyg. 65, 958–965.

Machado-Coelho, G., Caiaffa, W., Genaro, O., Magalhães, P., Mayrink, W., 2005. Risk factors for mucosal manifestation of American cutaneous leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 99, 55–61.

Maffey, L., Cardinal, M.V., Ordóñez-Krasnowski, P.C., Lanati, L.A., Lauricella, M.A., Schijman, A.G., Gürtler, R.E., 2012. Direct molecular identification of *Trypanosoma cruzi* discrete typing units in domestic and peridomestic *Triatoma infestans* and *Triatoma sordida* from the Argentine Chaco. Parasitology 139, 1570–1579.

Moll-Merks, C., Werner, H., Dönges, J., 1987. Suitability of in-vitro xenodiagnosis: development of *Trypanosoma cruzi* in *Triatoma infestans* depending on larval stage of bugs and number of trypomastigotes taken during in-vitro blood meal. Zentralbl. Bakteriol. Mikrobiol. Hyg. 268, 74–82.

Moreira, O.C., Ramírez, J.D., Velázquez, E., Melo, M.F., Lima-Ferreira, C., Guhl, F., Sosa-Estani, S., Marin-Neto, J.A., Morillo, C.A., Britto, C., 2013. Towards the establishment of a consensus real-time qPCR to monitor *Trypanosoma cruzi* parasitemia in patients with chronic Chagas disease cardiomyopathy: a substudy from the BENEFIT trial. Acta Trop. 125, 23–31.

Neal, R.A., Miles, R.A., 1977. The number of trypomastigotes of *Trypanosoma cruzi* required to infect *Rhodnius prolixus*. Rev. Inst. Med. Trop. Sao Paulo 19, 177–181.

Noireau, F., Diosque, P., Jansen, A.M., 2009. *Trypanosoma cruzi*: adaptation to its vectors and its hosts. Vet. Res. 40 (2), 26.

Orozco, M.M., Enriquez, G.F., Alvarado-Otegui, J.A., Cardinal, M.V., Schijman, A.G., Kitron, U., Gürtler, R.E., 2013. New sylvatic hosts of *Trypanosoma cruzi* and their reservoir competence in the humid Chaco of Argentina: a longitudinal study. Am. J. Trop. Med. Hyg. 88, 872–882.

Perlowagora-Szumlewicz, A., Moreira, C.J., 1994. In vivo differentiation of *Trypanosoma cruzi* – 1. Experimental evidence of the influence of vector species on metacyclogenesis. Mem. Inst. Oswaldo Cruz 89, 603–618.

Petersen, R.M., Gürtler, R.E., Cecere, M.C., Rubel, D.N., Hansen, D., Lauricella, M.A., Carlomagno, M., 2001. Association between nutritional indicators of dogs seroractive for *Trypanosoma cruzi* in a rural area of northwestern Argentina. Parasitol. Res. 87, 208–214.

Piron, M., Fisa, R., Casamitjana, N., López-Chejade, P., Puig, L., Vergés, M., Gascón, J., Gómez i Prat, J., Portús, M., Sauleda, S., 2007. Development of a real-time PCR assay for *Trypanosoma cruzi* detection in blood samples. Acta Trop. 103, 195–200.

Quinnell, R.J., Kennedy, L.J., Barnes, A., Courtenay, O., Dye, C., Garcez, L.M., Shaw, M.A., Carter, S.D., Thomson, W., Ollier, W.E.R., 2003. Susceptibility to visceral leishmaniasis in the domestic dog is associated with MHC class II polymorphism. Immunogenetics 55, 23–28.

Ramírez, J.D., Turriago, B., Tapia-Calle, G., Guhl, F., 2013. Understanding the role of dogs (*Canis lupus familiaris*) in the transmission dynamics of *Trypanosoma cruzi* genotypes in Colombia. Vet. Parasitol. 196, 216–219.

Schijman, A.G., Bisio, M., Orellana, L., Sued, M., Duffy, T., Mejia Jaramillo, A.M., Cura, Auter, F., Veron, V., Qvarnstrom, Y., Deborggraeve, S., Hijar, G., Zulantay, I., Lucero, R.H., Velazquez, E., Tellez, T., Sanchez Leon, Z., Galvão, L., Nolder, D., Monje Rumi, M., Levi, J.E., Ramirez, J.D., Zorrilla, P., Flores, M., Jercic, M.I., Crisante, G., Añez, N., De Castro, A.M., Gonzalez, C.I., Acosta Viana, K., Yachelini, P., Torrico, F., Robello, C., Diosque, P., Triana Chavez, O., Aznar, C., Russomando, G., Büscher, P., Assal, A., Guhl, F., Sosa Estani, S., Da Silva, A., Britto, C., Luquetti, A., Ladzins, J., 2011. International study to evaluate PCR methods for detection of *Trypanosoma cruzi* DNA in blood samples from Chagas disease patients. PLoS Negl. Trop. Dis. 5, e931.

Stein, R.A., 2011. Super-spreaders in infectious diseases. Int. J. Infect. Dis. 15, 510–513.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2007. MEGA 5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

World Health Organization (WHO), 2002. Control of Chagas Disease: Second Report of the WHO Expert Committee. World Health Organization, Geneva (57–58).

Wilson, K., Bjørnstad, O.N., Dobson, A.P., Merler, S., Poglayen, G., Randolph, S.E., Read, A.F., Skoping, A., 2001. Heterogeneities in macroparasite infections: patterns and processes. In: Hudson, P.J., Rizzoli, A., Grenfell, B.T., Heesterbeek, H., Dobson, A.P. (Eds.), The Ecology of Wildlife Diseases. Oxford University Press, Oxford, UK, pp. 6–44.

Woolhouse, M.E., Dye, C., Etard, J.F., Smith, T., Charlwood, J.D., Garnett, G.P., Hagan, P., Hii, J.L., Ndhlovu, P.D., Quinnell, R.J., Watts, C.H., Chandiwana, S.K., Anderson, R.M., 1997. Heterogeneities in the transmission of infectious agents: implications for the design of control programs. Proc. Natl. Acad. Sci. 94, 338–342.