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Infected dogs as a risk factor in the transmission of human *Trypanosoma cruzi* infection in western Venezuela

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Abstract

A total of 565 mongrel dogs from rural localities of Venezuela were examined by serological (DAT, IFAT and ELISA) and parasitological tests to address the status of *Trypanosoma cruzi* infection and to evaluate their role in the transmission of the infection to human population. The overall percentage of sero-positive infected dogs shown to be 67.6% (382/565):253 (61.7%) from 47 villages belonging to 8 states located at 4 different geographical regions of western Venezuela and 129 (33.5%) dogs from 48 households located in areas where Chagas disease is endemic. From 101 sampled dogs living in close proximity to 30 acute chagasic patients, 84% expressed specific anti-*T. cruzi* antibodies (Ab) with 12 of them (14%) showing blood circulating parasites (BCP). In these houses a high proportion of sero-positive people (20%) and frequent indoor infestation by triatomine-bugs (70%) was also recorded. The analysis revealed that from the 47 rural villages sampled during the study, 91.5% had the presence of *T. cruzi* sero-positive dogs, ranging from 62% positive localities at the states of Falcon and Cojedes to 100% in the other six studied Venezuelan states. This demonstrates that *T. cruzi*-infected dogs are found throughout all the geographical regions of western Venezuela irrespective of their ecological differences. Molecular typing of *T. cruzi* isolates from infected dogs using ribosomal and mini-exon gene markers, revealed the presence of both *T. cruzi* I and *T. cruzi* II lineages. The coincidence in the circulation of *T. cruzi* II in dog and human populations at the same locality and at the same time is reported and its significance is discussed. The combined serological, parasitological, epidemiological and molecular data is gathered here to call the attention on the presence of infected dogs as a risk factor in the maintenance of *T. cruzi* as a source for infection to humans.

Keywords: Chagas disease; *Trypanosoma cruzi*; Dogs; Transmission; Venezuela

1. Introduction

American trypanosomiasis, or Chagas' disease, is caused by *Trypanosoma cruzi*, a parasite transmitted by blood-feeding triatomine-bugs, blood transfusion, oral ingestion, and/or congenital infection. It courses as an acute or chronic, and frequently fatal infection in human population from the poorest areas of Latin America. Nowadays it is estimated that nearly 100 million people are at risk of infection, over 20 million are already infected and more than 50,000 deaths occur each year due to this disease (WHO, 2005). If these human records sound impressive, what appears to be dramatic is the fact that a higher number of wild and domestic mammals are infected by *T. cruzi* in the same area (Basombrio et

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al., 1993; Diosque et al., 2004; Herrera et al., 2005). Many of these species of mammals have been recognized as important reservoir of *T. cruzi*, especially dogs (Minter, 1976). This animal is a frequent source of blood intake for domestic and peri-domestic triatomine-bugs, which are able to feed preferentially on dogs compared to humans (Gürtler et al., 1996). In addition, it has been demonstrated that infected dogs are more infectious to triatomine-bugs than human, being therefore, considered as a risk factor in the household transmission of the chagasic infection (Zeledon et al., 1973; Gürtler et al., 1993, 1996; Cohen and Gürtler, 2001). On this aspect, estimations carried out on the effect of the infection status of dogs on transmission to children, revealed that the presence of infected dogs in a household may quadruple the risk of infection in infants (Basombrio et al., 1993). In Venezuela, the real prevalence of *T. cruzi*-infection in dogs is unknown. However, Pifano (1973) warned during the last third of the past century that dogs in Venezuela may be an important epidemiological factor because they carry natural infection and constitute a source of infection to the vector triatomine-bugs. The author based his statement on an epidemiological survey carried out from 1960 to 1964 at the Venezuelan north-central region, in which a 36.8% of *T. cruzi*-infected dogs, using xenodiagnosis or hemoculture as methods to detect active blood infection, was revealed.

The present paper deals with the testing for *T. cruzi* infection of 565 dogs from localities of different geographical regions of western Venezuela where Chagas disease is endemic (Añez et al., 2004a). The study involved parasitologic, serologic, and molecular methods aiming to: (a) detect natural *T. cruzi* infection in dogs; (b) estimate the sero-prevalence of *T. cruzi*-infection in dogs; (c) isolate and characterize *T. cruzi* lineages circulating in dogs; and (d) associate serological and molecular data of this dog inspection with previously obtained data on human *T. cruzi* infection in the same area, and even in the same houses.

2. Materials and methods

2.1. Study area

The study on domestic dogs was carried out in 47 villages belonging to 8 different states of western Venezuela where Chagas disease is endemic. The localities were representative of the different regions of this part of the country with their particular characteristics of clime and geographical landscape. This includes localities distributed through Los Llanos region (28) at the states of Barinas (22), Cojedes (3), Portuguesa (2) and Apure (1), located at different altitudes and between 66°21′ and 72°22′W and 6°03′and 10°00′N. In addition, eight localities at the semi-arid region at the state of Falcón (68°14′ and 71°18′W and 10°18′ and 12°11′N) and seven at the Andean region in Trujillo state (70°0′ and 71°0′W and 9°0′ and 10°0′N). At the central-western part of Venezuela four villages were sampled: one at Lara state and three at the state of Yaracuy (68°0′ and 71°0′W and 9°0′ and 11°0′N). Fig. 1 shows the distribution of localities where dogs were sampled.

Most houses in which dogs were sampled were typical rural dwellings built with mud wall and a thatched roof. However, dogs from houses in improved conditions were also sampled. In all cases houses were surrounded by palm trees in which triatomine-bugs infected by *T.* cruzi were present (Añez et al., 2004a).

2.2. Selection of dogs

The study was performed in a total of 565 mongrel dogs from houses located in villages of the above mentioned Venezuelan states. The total number of dogs was sampled during the period of 1995–2005 at different visits to the villages. The states with a fewer number of sampled dogs were those located far away from the laboratory, to which only a visit was done. Two groups of dogs were considered; one comprised of 155 animals living in 48 houses in close proximity with people who were also sampled for Chagas disease infection (347), some of them (30) with acute symptomatic infection and blood circulating parasites reported in previous publications (Añez et al., 1999, 2001, 2004a,b). The second group included 410 dogs from 47 different localities of the above mentioned states where Chagas disease is considered to be endemic. The total group was composed of 65 and 35% male and female dogs, respectively, with a male to female sex ratio of 1.9:1. The mean and standard deviation age of dogs was 2.2 ± 1.7 years (range 2 months to 12 years). In all cases we obtained consent from the dog owners, who also helped us to manipulate the animals during sampling to avoid incidents.

2.3. Sample collection and processing

Samples consisted of 5 ml peripheral blood taken by venipuncture from the dog cephalic or saphenous vein. The collected blood was dispensed into culture tubes and centrifuge tubes under sterile condition using a Bunsen burner connected to a portable gas cylinder. The dog samples were processed for diagnosis using two routine parasitologic methods and three serologic tests. The former included hemoculture using 0.5–1.0 ml of blood in

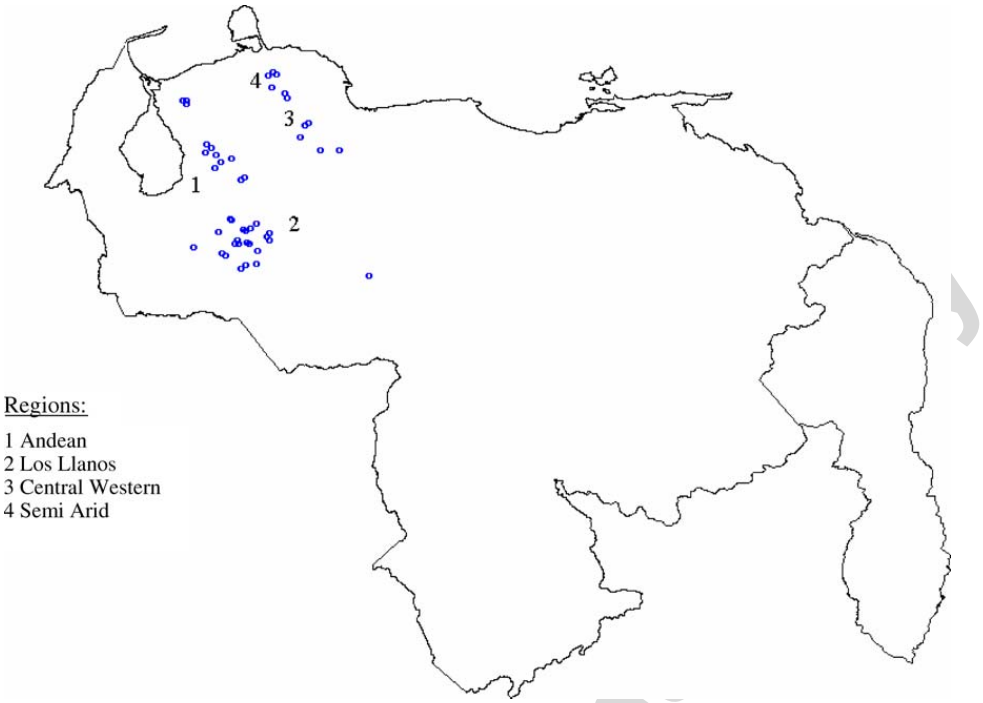

Fig. 1. Distribution of localities per geographical region sampled for *Trypanosoma cruzi* infection in Venezuelan dogs.

NNN culture medium tubes with insect saline as overlay (Añez et al., 1999). Each tube was examined for parasites every 5 days until the 60th day, when discarded if negative. The other method used was the xenodiagnosis using 15–20 IV instar nymphs of *Rhodnius prolixus* from a colony kept at the laboratory. Each insect was examined for parasites each week until the 8th week. Parasites collected from infected bugs were inoculated into laboratory mice to produce parasitemia and then establish the isolate in culture medium for further work. Briefly, we obtained isolates in two ways: directly from blood circulating parasites in infected dogs by hemoculture, and indirectly passing onto mice the metacyclic-forms from infected bugs used in xenodiagnosis, which were then cultured in vitro. The serologic tests used to detect circulating anti-*T. cruzi* antibodies (Ab) in the sampled dogs were a direct agglutination test (DAT) with 2-mercaptoethanol, an indirect immunofluorescence antibody test (IFAT) and an ELISA. Conditions and procedures for serologic tests have been previously reported (Añez et al., 1999, 2001). In all sera samples from examined dogs, titers $\geq$ 1:128 for the DAT and IFAT and an optical absorbance $\geq$ 0.3 for the ELISA were considered positive for infection by *T. cruzi*. The chosen cut-off for DAT and IFAT was decided to be 1:128, which showed the highest degree of agreement when statistically compared with other dilutions, providing a major specificity to the used tests. Dogs were considered as sero-positive when they showed reactivity in at least two of the three serological methods used.

In all cases serological tests were control-validated using *T. rangeli* and *Leishmania* spp sera control from known infected dogs to check reliability and specificity of the used methods. Similarly, dogs from different areas were serological diagnosed for *Leishmania*-infection using the same methods in order to check cross reaction and/or mixed infection by *T. cruzi* and *Leishmania* spp.

2.4. Statistical analysis

The degree of agreement among the diagnostic tests used to detect *T. cruzi*-infection in dogs consisted of the Kappa statistic suggested by Cohen (1960) to estimate the agreement between two observers, and then a generalized one for several observers by Fleiss (1981).

2.5. Genetic typing of T. cruzi isolates from naturally infected dogs

T. cruzi isolates from naturally infected dogs were established and growth under laboratory conditions. DNA of the parasites was extracted using the classical phenol–chloroform method. Isolates were typed through polymerase chain reaction (PCR) amplification as previously reported (Añez et al., 2004b) using: (a) primers D71 (5′-AAG GTG CGT CGA CAG TGT GG-3′) and D72 (5′-TTT TCA GAA TGG CCG AAC AGT-3′) based on the divergent domain of the 24 Sα RNA gene as described by Souto et al. (1996) and (b) primers TC

Table 1
Seropositivity to *Trypanosoma cruzi* in dogs from rural localities of different regions in western Venezuela

Region of western Venezuela	Venezuelan State	Number of localities with infected dog/total localities studied (%)	Number of examined dog (%)	Number of sero-positive dog (%)
Los Llanos	Apure	1/1 (100)	9 (2.2)	6 (66.6)
	Barinas	22/22 (100)	144 (35.1)	124 (86.1)
	Cojedes	2/3 (66)	22 (5.4)	11 (50.0)
	Portuguesa	2/2 (100)	12 (2.9)	11 (91.6)
Semi-arid	Falcon	5/8 (62.5)	45 (10.9)	18 (40.0)
Andean	Trujillo	7/7 (100)	97 (23.6)	44 (45.3)
Central-western	Lara	1/1 (100)	8 (1.9)	4 (50.0)
	Yaracuy	3/3 (100)	73 (17.8)	35 (47.9)
Total	8	43/47 (91.5)	410	253 (61.7)

(5′-CCC CCC TCC CAG GCC ACA CTG-3′), TC1 (5′-GTG TCC GCC ACC TCC TTC GGG CC-3′), and TC2 (5′-CCT GCA GGC ACA CGT GTG TGT G-3′) for amplification of an intergenic region of the mini-exon gene as described by Souto et al. (1996). The PCR amplified products were separated by electrophoresis in 3% agarose gels stained with ethidium bromide.

3. Results

3.1. Seroprevalence of T. cruzi infection in dogs of rural localities from different regions in western Venezuela

From a total of 410 dogs serologically examined to detect the status of *T. cruzi* infection, 253 (61.7%) revealed positive results in at least two of the three sero-tests, and were considered as sero-positive. The analysis of the seropositivity to *T. cruzi* obtained in dogs from different regions of western Venezuela revealed a high proportion of infection in Los Llanos where 81.3% (152/187) of infected dogs was detected, with a range from 50 to 91.6%. In addition, quite similar relative proportions of 48, 45, and 40% of infected dogs were detected in rural localities of the central-western, Andean, and semi-arid regions, respectively.

In 43 out of the 47 (91.5%) localities sampled the presence of sero-positive dogs for *T. cruzi* was detected. The relative proportion of localities with sero-positive dogs ranged from about 60% of localities in Falcon (5/8) and Cojedes (2/3) states to 100% in all the sampled localities of the other six studied states. It is worth calling that the state of Barinas with the major number of sampled localities (22) showed sero-positive animals in all of them. Similarly, the states of Trujillo and Portuguesa, with previous report of high sero-prevalence of *T. cruzi* in humans (Añez et al., 2004a), also showed positive animals in all the sampled localities. Details on the states comprising the study regions, the number of localities sampled in each of the states, the amount of sampled dogs and the proportion of resulted sero-positive, are shown in Table 1.

3.2. Detection of T. cruzi-infection in dogs living in close proximity to people sampled for Chagas disease

Sero-parasitological examination carried out in 155 dogs from 48 houses in which people were sampled for detection of Chagas disease, revealed 129 (83.2%) sero-positives with 13 (10%) of them bearing *T. cruzi* active infection. From these, 101 dogs belong to 30 households in which acute cases of Chagas disease have been previously diagnosed. In this case 84.2% of the dogs (85/101) resulted sero-positive and 14% of them (12/85) had blood circulating parasites (BCP), detected by hemoculture and/or xenodiagnosis. It is relevant to note that 20.3% of the people living in those houses were sero-positive to *T. cruzi*, and in 70% of the households people informed of indoor triatomine-bugs. Dogs from houses where non-chagasic cases were previously detected, also showed BCP although in 2.3% of them (Table 2). In this group of dogs, however, the seropositivity was quite similar to that observed in dogs living close to acute cases. In these houses people also showed a relatively high seroprevalence (15%) and, in addition, indoor triatomine-bugs were detected in a 38%. Details of the results obtained at the two types of sample houses are shown in Table 2.

3.3. Agreement among serological methods used to evaluate T. cruzi-infected dogs

Statistical analysis showed that the diagnostic methods used in the present work resulted very reliable judg-

Table 2
Trypanosoma cruzi-infection in dogs living in close proximity to chagasic people

Type of sampled houses	Number of sampled houses	Number of examined dogs	Number of sero-positive dogs (%)	Number of dog with BCP* (%)	Number of people/seropositive to *T. cruzi* (%)	Number of houses with indoor triatomine-bugs (%)
With acute chagasic patients history	30	101	85 (84.2)	12 (14)	187/38 (20.3)	21 (70)
With unknown chagasic history	18	54	44 (81.5)	1 (2.3)	160/24 (15)	7 (38)
Total	48	155	129 (83.2)	13 (10)	347/62 (17.8)	28 (58)

* BCP—blood circulating parasites. For description see text.

ing by the high level of concordance among the serological tests and between them and the two parasitological methods. When we compared the serological methods, the major degree of agreement was detected between IFAT and ELISA with a $k = 0.973$, and k values of 0.876 between DAT and the previous two, indicating a high degree of agreement among the three sero-tests. Similarly, a high correspondence was also observed between parasitological and serological results. For instance, IFAT and ELISA tests classified as positive 100% of those dogs which resulted positive to *T. cruzi* using xenodiagnosis or hemoculture. In relation to the comparison with *T. rangeli* and/or *Leishmania* infection in the examined dogs, no cross reaction was detected when using the dilution 1:128 as the cut-off for DAT and IFAT serological tests. In some cases, dogs with positive serology to *T. cruzi* also showed sero-positive results to *Leishmania braziliensis*, but they also revealed positive results in their corresponding *Leishmania (Viannia)* specific PCR hybridization assay, demonstrating mixed infections (this information will be considered elsewhere). Regarding the mixed infection *T. cruzi–T. rangeli*, a general proportion of 45% was detected, indicating circulation of both parasites at the same area (details will be considered elsewhere).

3.4. Molecular typing of *T. cruzi* isolates in naturally infected dogs

Blood circulating parasites from 5 out of the 13 dogs naturally infected with *T. cruzi* were established in vitro and used for genetic typing of the lineages *T. cruzi* I and *T. cruzi* II. PCR amplification of DNA using primers to detect two markers, the ribosomal 24 Sα-rDNA and mini-exon genes, allowed us to differentiate between the two *T. cruzi* lineages. An isolate obtained from an infected dog in Trujillo state and identified as MCAN/Ve/2000/RS-00, was typed as *T. cruzi*-lineage I, showing DNA bands of 110 and 350 bp for ribosomal and mini-exon genes, respectively (Fig. 2). The other four isolates from infected dogs sampled in Barinas state, Venezuela (MCAN/Ve/1999/BN-99; MCAN/Ve/2000/T-00; MCAN/Ve/2000/A-00; and MCAN/Ve/2001/R-01) showed concordant results with DNA bands of 125 and 300 bp for ribosomal and mini-exon genes, respectively, and were accordingly typed as *T. cruzi*-lineage II (Fig. 2).

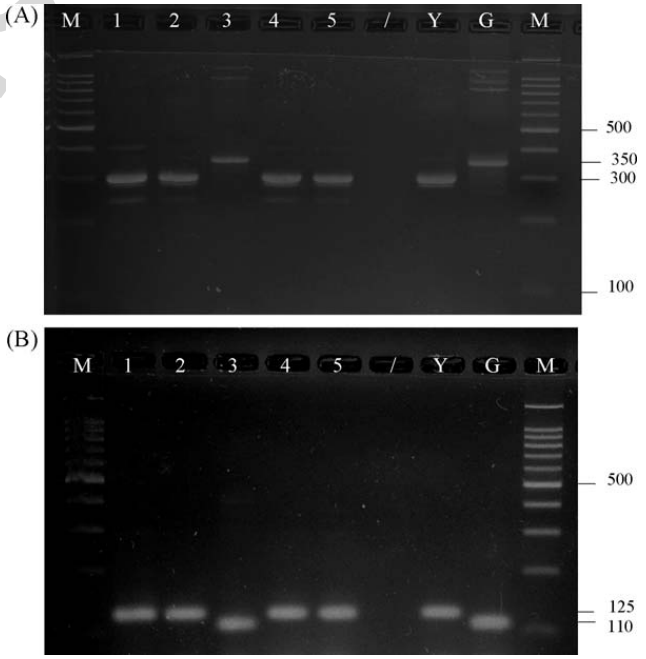

Fig. 2. *Trypanosoma cruzi* typing using agarose gel (3%) electrophoresis of PCR products generated by amplification of DNA from Venezuelan dog isolates: (A) amplification of mini-exon gene sequences and (B) amplification of 24 Sα-rDNA sequences. DNA from the reference strains Y and G for *T. cruzi* II and *T. cruzi* I, respectively, were used as positive control. M: molecular marker (bp). (1) MCAN/Ve/1999/BN-99 (lineage II); (2) MCAN/Ve/2000/T-00 (lineage II); (3) MCAN/Ve/2000/RS-00 (lineage I); (4) MCAN/Ve/2000/A-00 (lineage II) and (5) MCAN/Ve/2001/R-01 (lineage II).

4. Discussion

In the present study a total of 565 dogs, 410 from 47 rural localities of different geographical region of western Venezuela and 155 living in close proximity to chagasic people in endemic areas, were serologically examined to detect specific anti-*T. cruzi* circulating antibodies (Abs). Serological methods previously used by us in surveys of patients (Añez et al., 1999) were standardized for dog serology. From the total sera tested, 382 (67.6%) resulted sero-positive according to the established criteria (two out of three reactive sera-tests). Due to the high general sero-prevalence detected in the examined dogs, we were able to quantify the relative proportion of infected animals per locality, as a first step to understand how the infection was distributed in this part of the Venezuelan geography. The obtained information may give some clues on the amount of infection in Venezuelan dogs in localities of those regions and whether it should be consider a potential risk for *T. cruzi* transmission to human. Surprisingly, in 91.4% (43/47) of the sampled localities dogs were diagnosed as sero-reactive, indicating previous contact to *T. cruzi*. The analysis of the detection of infected dogs at any locality, revealed 100% of infection in the Andean (7) and central-western (4) regions; 96% in localities of Los Llanos region (28) and 62.5% in those of the semi-arid region (8). These results demonstrate that *T. cruzi*-infected dogs are found throughout all the geographical regions of western Venezuela irrespective of their ecological or climatic differences, judging by the relatively high proportion of localities with infected animals. Taken together, in all Venezuelan regions investigated 253 out of 410 dogs (61.7%) examined from 47 rural localities, presented Abs against *T. cruzi* when evaluated through standardized serological methods. Prevalence ranged from 40% at the semi-arid region of Falcon state to 81% at Los Llanos region. It is relevant to note that in localities of the states of Barinas and Portuguesa, two well known endemic areas for Chagas disease in Venezuela (Añez et al., 1999, 2004a), high proportions of reactive dogs were detected with values of 86 and 91%, respectively. Therefore, results obtained lead us to conclude that dogs in rural localities of Venezuela may be consider an important domestic reservoir likewise in other countries (Zeledon, 1974; Wisniveski-Collie et al., 1985; Cohen and Gürtler, 2001; Beard et al., 2003; Reithinger et al., 2005). The fact that almost all the studied localities had infected dogs beside the high proportion of infection detected in both population of dogs and humans, allow us to consider this animal as an important factor in the maintenance of *T. cruzi* as a source for infection to human in the study area, assuming that the nearest the reservoir the more successful the transmission. To validate the latter statement, we examined 101 dogs from 30 households where acute chagasic patients were previously diagnosed. From these 101 dogs, 85 (84.2%) resulted sero-positive for anti-*T. cruzi* specific Abs. In addition, in 12 dogs (14%) BCP were detected either by xenodiagnosis or hemoculture, indicating active *T. cruzi* infection, which correspond to the human acute phase of Chagas disease. Interestingly, one of the infected dogs had a sudden death after a hard running exercise as referred by its owner. In any case, according to data from this study, *T. cruzi* infection in dogs appears to be very high in comparison to human infection, considering that in highly endemic areas acute cases have been estimated in about 3 patients per 1000 inhabitants (Añez et al., 1999). To explain this, we consider that the more frequent infection of dogs, as compared to the observed cases in humans, may be due to a more efficient mechanism of infection in these animals. Dogs can be infected both by contaminating feces from infected bugs, deposited on their skin after bug feeding, with infective metacyclic trypomastigotes penetrating via insect bite site through scratching and by ingesting infected triatomine-bugs either indoor or at the peridomestic area, a fact previously reported by others (Zeledon, 1981). To consistently corroborate dog infection by vector transmission, we enquired on the presence of triatomine-bugs on the referred houses, finding that bugs were present indoor in 70% and their inhabitants, who resulted with 20.3% of positive sero-reaction, were able to recognize domiciliary infestation by these insects and its association with dogs. Similarly, observations made in houses without chagasic history of human acute cases, revealed 81.5% of sero-reactive dogs with 2.3% of them with BCP, which should indicate that infected dogs associated with the presence of triatomine-bugs may be an important risk factor for domiciliary human transmission of *T. cruzi*. These results support previous studies demonstrating that bug population size and vector infection rates increase with the number or proportion of infected dogs per household (Gürtler et al., 1998). A supporting fact that allows us to incriminate dogs as a reservoir and a potential risk factor in the transmission of *T. cruzi*-infection to human is the observation that an infected dog bearing BCP was able to infect four children after the animal suffered an accident, contaminating them when helping the bleeding dog.

Regarding the genetic typing of the five isolates recovered from dogs in the present work (four typed as *T. cruzi* II and one as *T. cruzi* I), the scarce number of isolates is not enough to reach any conclusion on the predominance of any *T. cruzi* lineages circulating in

the dog studied population. However, the analysis of the distribution of each of the dog typed isolates revealed a coincidence with the human typed isolates in at least two of them. This coincidence of circulation of *T. cruzi* II was detected between the dog isolates MCAN/Ve/2000/T-00 and MCAN/Ve/2000/A-00, and the human isolates MHOM/Ve/2000/AC and MHOM/Ve/2000/RM, which were obtained simultaneously during the same sampling at the localities of San Silvestre and Potreritos, state of Barinas. The former were isolated from two dogs of 5 months (T-00) and 3 months (A-00) old, and the latter from two asymptomatic children of 11 years (AC) and 6 years (RM) old, whose clinical picture, clinical condition, cardiac evaluation, and typing of the isolated parasites have been previously reported (Añez et al., 2004b). The two other dog isolates characterized as *T. cruzi* II (MCAN/Ve/1999/BN-99 and MCAN/Ve/2001/R-01) were obtained at the localities of Santa Lucia and Santa Ines, State of Barinas, Venezuela, places where we previously detected and characterized the two human isolates MHOM/Ve/1998/7-98 and MHOM/Ve/1995/8-95 as *T. cruzi* I (Añez et al., 2004b). Our findings may suggest that both *T. cruzi* I and *T. cruzi* II circulate at the same endemic localities of western Venezuela and both of them appear to be responsible for human and dog infections with variable clinical profiles, corroborating our previous observation in human cases (Añez et al., 2004b). The present information may result controversial to other workers and probably more investigation is needed to establish the relationships between the *T. cruzi* lineages of Venezuelan isolates and the clinical prognostic value for the parasite infection in areas where Chagas disease is endemic. However, what may not be ignored is the observed difference in behavior among the molecular typed Venezuelan isolates and those typed in other Latin-American countries. The fact that we have found in Venezuela the same *T. cruzi* lineages circulating in dogs and human in the same endemic area, do not support previous statements from other workers referring preferential association of *T. cruzi* genotypes with sylvatic (*T. cruzi* I) or domestic (*T. cruzi* II) cycles of transmission (Zingales et al., 1998, 1999). These results obtained in dogs, in conjunction with the predominance of *T. cruzi* lineage I in a large number of acute chagasic patients (Añez et al., 2004b) and those from triatomine-bugs and wild reservoirs (Añez et al., in preparation), lead us to conclude that *T. cruzi* I and *T. cruzi* II circulate in Venezuela irrespective of the geographical regions and/or endemic areas and that both of the lineages are responsible for the human clinical pictures of Chagas disease and for the relatively high prevalence of the American trypanosomiasis in dogs. The above findings, beside the results obtained on the prevalence and distribution of infection in the dog studied population, suggests the presence of infected dogs as a potential risk factor in the transmission of *T. cruzi* to human in Venezuela, which must be taken into consideration when Chagas disease control programs have to be implemented.

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