Distribution and pathogenicity of *Trypanosoma cruzi* isolated from peridomestic populations of *Triatoma infestans* and *Triatoma guasayana* from rural Western Argentina

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We assessed the distribution of Trypanosoma cruzi *infection in peridomestic triatomines collected manually at a district-wide scale in rural villages around Olta, Western Argentina, and typed the isolated strains according to their pathogenicity to laboratory mice. Of 1623 triatomines examined, only 14 (0.9%) were infected with* T. cruzi *based on microscopical examination of feces. The prevalence of* T. cruzi *infection was 0.8% in* Triatoma infestans*, 2.3% in* T. guasayana*, and nil in* T. garciabesi, T. platensis, *and* T. eratyrusiformis. *Local transmission occurred in kitchens, store-rooms and goat corrals or nearby, though at very low levels.* T. cruzi *was detected by at least one parasitological method in 11 (79%) of 14 microscope-positive bugs. Hemoculture was the most sensitive method (67%) followed by culture of organ homogenates, histopathology or xenodiagnosis of inoculated suckling mice (55-58%), and culture of microscope-positive bug feces (46%). The evidence suggests that most of the isolated* T. cruzi *strains would be myotropic type III. Our study establishes for the first time that peridomestic, microscope-positive* T. guasayana *nymphs were actually infected with* T. cruzi*, and may be implicated as a putative secondary vector of* T. cruzi *in domestic or peridomestic sites.*

Key words: *Trypanosoma cruzi* - *Triatoma infestans* - *Triatoma guasayana* - *Triatoma garciabesi* - Chagas disease - surveillance - pathogenicity - Argentina

Chagas disease was originally a zoonosis of sylvatic mammals transmitted by triatomine bugs. The process of domestication in Triatominae lead to the simplification of genetic and phenetic characters and may be a generalized current trend (Schofield et al. 1999). *Triatoma infestans*, probably the main vector of *Trypanosoma cruzi*, expresses the extreme of an evolutionary trend toward specialization to a stable habitat. But several species of sylvatic or peridomestic triatomines that are not control targets at present might turn into secondary vectors of *T. cruzi* during or after the elimination of *T. infestans* (Carcavallo & Martínez 1985). In Northern Argentina, *T. sordida*, *T. guasayana*, and *T. garciabesi* may have potential for domestication in the absence of *T. infestans* because they have sylvatic or peridomestic colonies and frequently invade human dwellings (Carcavallo & Martínez 1985, Gürtler et al. 1999). The main peridomestic ecotopes of *T. guasayana* are goat or sheep corrals (Canale et al. 2000), but it is usually associated with bromeliads, *Opuntia* cacti and fallen logs (Wisnivesky-Colli et al. 1997, Noireau et al. 2000). The main peridomestic habitats of *T. garciabesi* are the rugged bark of *Prosopis* trees in which chickens roost and coops (Canale et al. 2000). The peridomestic environment includes key sites for the elimination of *T. infestans*, such as goat or pig corrals and chicken coops (Gürtler et al. 2004), and may be the interface between sylvatic and domestic transmission cycles of *T. cruzi*, where more parasite diversity can be found.

Natural populations of *T. cruzi* are composed of multiple clones distributed into two major phylogenetic lineages that display distinct biological and eco-epidemiological traits (Tibayrenc 1995, Souto et al. 1996, Barnabé et al. 2000). The prevalence of *T. cruzi* infection in domiciliary triatomine populations is closely connected to the risk of human infection and depends on human and animal prevalence rates of infection and host contact rates with vectors (Gürtler et al. 1998, Cohen & Gürtler 2001). In rural Northwestern Argentina, a community-wide spraying of deltamethrin caused a sharp fall in *T. infestans* infection rates with *T. cruzi* from 49 to 4.6% in domestic sites, and from 6 to 1.8% in peridomestic sites on the 5-year period after spraying (Cecere et al. 1999). The prevalence of *T. cruzi* infection ranged from 2.4% in *T. infestans*, 0.7% in *T. guasayana*, to 0.2% in *T. garciabesi*, with nearly two-thirds of the infected bugs being caught in peridomestic sites (Cecere et al. 1999). *T. cruzi* infections in triatomine bugs are usually diagnosed on morphological grounds in unstained fresh fecal preparations examined by light microscopy. Such infections sometimes were cautiously referred to as "*T. cruzi*-like trypanosomes" or "flagellates" because light microscopy has limited sensitivity and specificity (Wisnivesky-Colli et al. 1993, Noireau et al. 2000). Other trypanosomatids, such as *Blastho-*

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crithidia triatomae (Cerisola et al. 1971) and *T. rangeli*, may yield "false positive" results. *B. triatomae* has been found infecting natural populations of Triatominae (e.g. Cecere et al. 1999).

As part of a wider project on the effectiveness of pyrethroid insecticides on peridomestic populations of triatomines in Western Argentina (Gürtler et al. 2004), we sought to assess the distribution of *T. cruzi* infection in peridomestic triatomine populations in an endemic rural district under no vector surveillance; to corroborate that trypanosomes detected by light microscopy were *T. cruzi* using biological criteria, and to type the isolated strains according to their pathogenicity to laboratory mice. The importance of *T. guasayana* and other peridomestic triatomines as secondary vectors of *T. cruzi* was also assessed.

MATERIALS AND METHODS

Study area - The triatomine bugs were collected in rural areas of the Department General Belgrano and Chamical, Province La Rioja, Argentina. The area, located around Olta (30.4ºS, 66.1ºW), is on a semiarid plain with xerophytic vegetation (Gürtler et al. 2004).

Triatomine collection - Six experienced bug collectors searched for triatomine bugs in peridomestic sites of 369 houses in April-May 1999, as described by Gürtler et al. (2004). Timed manual collections in peridomestic sites were conducted using 0.2% tetramethrin for 30 min per house. The collection sites included corrals, pigpens, chicken coops, trees where chickens roosted, piled materials, orchard fences, storerooms, and kitchens. All bugs were examined in the field laboratory to determine numbers by species, stage and collection site as described by Canale et al. (2000), stored and shipped to the laboratory for parasitological examination. Pools of feces from three live or moribund insects of a given species and collection site were examined for flagellate as described. *T. cruzi* and *B. triatomae* (Cerisola et al. 1971) were differentiated on a morphological basis. When a positive pool was detected, the feces of each bug were re-examined individually to identify the infected bug for parasite isolation.

Isolation methods - In brief, feces from each microscope-positive triatomine were cultured and inoculated into suckling mice which were then examined by fresh blood, hemoculture, culture of organ homogenates, serodiagnosis, xenodiagnosis, and histopathology.

Feces culture - Each of the 14 microscope-positive bugs was dissected under sterile conditions to extract the rectal ampoule and prepare an homogenate of feces diluted in 0.4-0.5 ml of Brain Heart Infusion Difco (BHI) (37 g/l). The homogenate was microscopically examined at 400× to determine the number of trypanosomes and then cultured and inoculated into mice. Two scant homogenates were either cultured or inoculated, but not both. Twelve fecal homogenates were inoculated (50-100 µl/tube) into 6 tubes containing 3 ml Nutrient Agar Difco (31 g/l)-0.5 ml defibrinated rabbit blood (Penicillin 200 U/ml-Streptomicin 200 U/ml) as solid slant, and 2 ml BHI-10% fetal calf serum (Bioser, Buenos Aires) as supernatant (Abramo Orrego et al. 1980).

Mice studies - Groups of 2-4 C_3H suckling mice (15-20 days of age, 8 g) kept in separate cages were inoculated intraperitoneally with 0.1 ml of fecal homogenates (about 1-2 x 10^3 to 2 x 10^6 *T. cruzi*/ml) from each positive bug.

Tail blood from each mouse was examined for trypanosomes twice a week. On day 30 post-inoculation, one mouse from each group was studied by xenodiagnosis with 4 *T. infestans* third instar nymphs during 20-25 min; the pooled feces were examined for *T. cruzi* 30 and 60 days post-feeding (Cerisola et al. 1974).

Hemoculture - Immediately after xenodiagnosis and under anesthesia, heparinized blood was extracted by cardiac puncture and inoculated into 2-3 tubes per mouse without prior centrifugation. On days 45-65 post-inoculation, the remainder mice of each group were processed for sero-diagnosis, hemoculture and histopathology.

Organ culture - Heart, liver and spleen samples were homogenated and cultured separately in nutrient agar as before. A total of 12-20 culture tubes from each microscope-positive bug was stored at 28ºC and 50% relative humidity and examined at 400× twice a month for 4-5 months. Small volumes of BHI were added as needed. The supernatant of negative or weakly growing cultures were transferred every 1-2 months into new tubes.

Store at liquid nitrogen - Cultured parasites were centrifuged, the pellet dilluted in fetal calf serum-RPMI 80% , ice DMSO- RPMI 80% was added and immediately frozen in liquid nitrogen.

Serology - Plasma was tested by an enzyme-linked immunosorbent assay (ELISA) as described previously (Carlomagno et al. 1987). Sera from parasitemic and non infected mice were used as controls. Absorbance values greater than or equal to 0.2 at 490 nm were considered reactive.

Anatomopathological studies - Mice inoculated with parasites derived from 7 microscope-positive *T. infestans* and one *T. guasayana* were subjected to histopathological studies. Samples of organs were embedded in paraffin and stained with hematoxilin-eosin and Masson's trichrome as described by Carlomagno et al. (1987). The intensity of myocardium lesions was graded from 0 (null) to 4 (especially severe acute myocarditis).

T. cruzi reinfection in mice - C3H suckling mice were infected with blood trypomastigotes (at least 1 *T. cruzi*/ 5 µl blood) of a mouse previously inoculated with feces of *T. guasayana*.

Characterization of strains - Mortality and parasitemia profiles, pathogenicity and tisular tropism were evaluated to characterize the isolates according to Andrade (1974).

RESULTS

A total of 5251 *T. infestans*, 379 *T. guasayana*, 95 *T. garciabesi*, 6 *T. platensis* and 2 *T. eratyrusiformis* was collected from 1748 peridomestic sites inspected. Of the 1623 live or moribund triatomines examined for infection, only 14 (0.9%) bugs were infected with *T. cruzi* based on microscopical observation of feces. Ten (71%) of the infected triatomines came from different houses and sites,

which included trees with or without chickens roosting, store-rooms, kitchens and goat corrals. The percentage of *T. cruzi* infection was three times larger in *T. guasayana* (2.3% of 86 bugs) than in *T. infestans* (0.8% of 1481 bugs) and included 2.3% (2 of 86) and 0.8% (5 of 651) of infected nymphs in each species, respectively. No infected bug was detected among 48 *T. garciabesi*, 6 *T. platensis*, and 2 *T. eratyrusiformis*.

T. cruzi was detected by at least one parasitological method in 11 (79%) of 14 microscope-positive *T. infestans* and *T. guasayana* (Table I). *T. cruzi* was isolated more often by hemoculture (67%) than by culture of organ homogenates or xenodiagnosis of inoculated mice (55-58%), or culture of bug feces (46%) or fresh blood examination (33%). A total of 10 (83%) sets of culture tubes (each set originated from one individual bug) remained uncontaminated during the 4-5 month follow-up.

T. cruzi was detected by at least one parasitological method in 10 (83%) positive *T. infestans*, and 8 (67%) isolates were obtained (Table II). Three (27%) groups of mice only showed very low parasitemia (less than 5 parasites per 5 µl of blood), and other three were parasitemia-negative and xenodiagnosis-positive. Four (67%) groups of mice tested by ELISA between 23 and 65 days post-inoculation were seroreactive for *T. cruzi* at serum dilutions from 50 to 1600. All of the seroreactive mice were organ-, hemo-culture- or histopathology-positive for *T. cruzi*. In the two groups of seronegative mice, the Los Bordos strain was amastigote-positive by histopathology whereas the San Felipe-Olta strain was hemoculture-positive.

In general, histopathology of the *T. cruzi* strains isolated from *T. infestans* revealed myocardium and skeletal muscle involvement at 1-2 months post-inoculation. Low mortality at the first month, low parasitemia and virulence suggest that these strains belonged to type III. The Bajo di Lucca strain induced moderate to very severe pathologic changes in all organs, and was the only one to produce heavy mortality at 28-30 days post-inoculation. The myocardium had a very severe and intense inflammatory process, associated to a moderate number of amastigote nests. The liver had a moderate inflammation with no amastigotes found, whereas hollow viscera showed slight inflammation with scarce amastigote nests. El Simbolar strain induced very severe myocarditis, with numerous amastigote nests (grade 4) (Fig. 1a); moderate inflammation in the liver but not lesions in hollow visceras. The Los Bordos strain induced a light inflammatory infiltration, with amastigote nests only in heart and striated muscle. The La Aguadita and Tala Verde strains 1-2 induced light to moderate inflammatory infiltrations but no amastigotes were seen (Table II, Fig. 1d).

T. cruzi was recovered from only one of the two positive *T. guasayana* (Table II). Mice inoculated with feces from this *T. guasayana* had low levels of parasitemia (< 4 parasites per slide) during most of the follow-up and positive xenodiagnosis at 30 days post-inoculation. Both the inoculated and reinoculated mice were seroreactive for *T. cruzi*. The *T. guasayana*-derived strain from El Simbolar induced a light to moderate cardiac inflammatory process (Fig. 1b,c), with some amastigote nests. Striated muscle, liver and hollow viscera presented a light or moderate diffuse inflammatory process, but amastigotes were either scarce or absent. Mice reinoculated with this strain showed a very similar parasitologic and histophatologic pattern as the inoculated mice.

DISCUSSION

Our study shows that peridomestic triatomines from rural Western Argentina were actually infected with mostly myotropic *T. cruzi* strains, though at very low infection rates, and peridomestic *T. guasayana* was three times more infected with *T. cruzi* than *T. infestans*.

Most (83%) of the microscopically detected trypanosomes proved to be *T. cruzi* according to standard biological criteria (Hoare 1972). In addition to being morphologically indistinguishable from *T. cruzi*, the trypanosomes were successfully cultured in specific media, induced a detectable serological response to *T. cruzi*, and were pathogenic to laboratory mice. The infected mice were also infective to *T. infestans* and reinoculated mice, allowing the continuity of infection. To our knowledge, this may be the first study that applies these criteria to establish that positive peridomestic triatomines, particularly *T. guasayana*, were actually infected with *T. cruzi*.

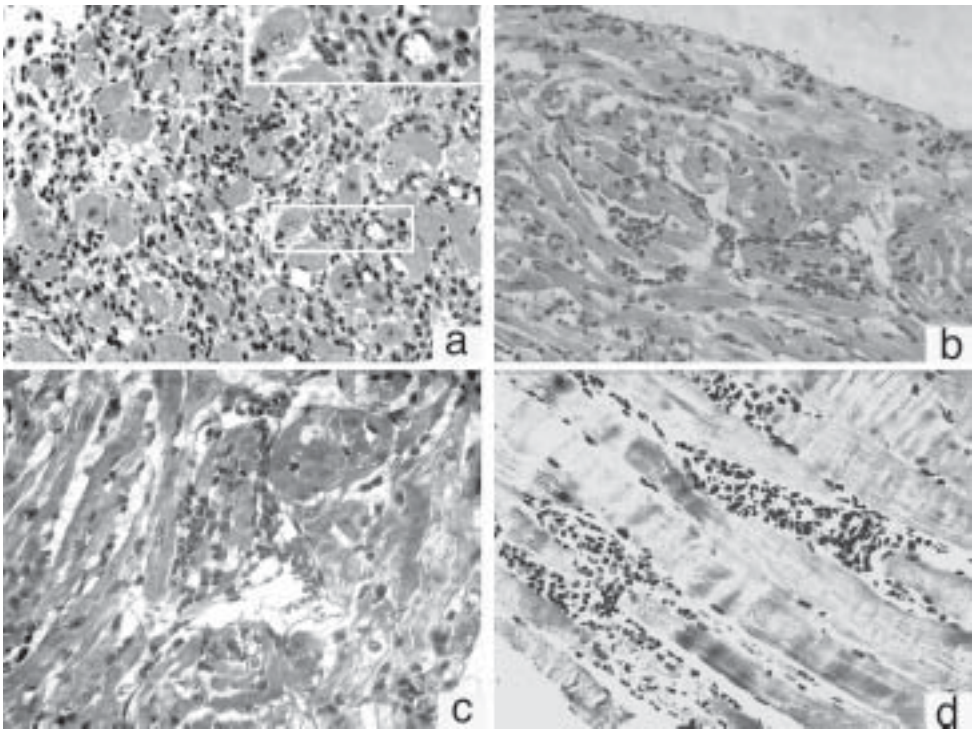
Parasites were isolated from two-thirds of the infected bugs. Isolation of *T. cruzi* from bug feces has not been favored in the past because of fungal and bacterial contaminations, but here only two of 13 cultures of *T. infestans*

TABLE I

Isolation of *Trypanosoma cruzi* from microscope-positive *Triatoma infestans* and *T. guasayana* captured manually at peridomestic sites in May 1999 - Olta and neighboring rural villages, central Argentina

Host	Detection method	Nr of positive bugs examined	Nr positive	(%)
Inoculation of mice	Hemoculture	12	8	67
	Organ culture	12	7	58
	Hispathology [a]	7	4	57
	Serodiagnosis	9	5	56
	Xenodiagnosis	11	6	55
	Fresh blood	12	4	33
Triatomine bug	Culture of feces	13	6	46
Total by at least one method		14	11	79

a: finding of *T. cruzi* amastigotes



Histopathological findings in mice inoculated with feces of *Triatoma infestans* and *T. guasayana* infected with *Trypanosoma cruzi*. Severe acute miocarditis in a mouse inoculated with feces of a IV instar nymph of *T. infestans* captured in El Simbolar. Insert: nests of amastigotes (a). Moderate acute miocarditis in a mouse infected with feces of a V instar nymph of *T. guasayana* from El Simbolar (b: 20×, c: 40×). Acute miositis in a mouse infected with feces of a V instar nymph of *T. infestans* found in Tala Verde (d).

feces presented fungal contaminations. In both cases, all isolation attempts were negative but histopathological studies revealed *T. cruzi* amastigotes and light lesions. In addition, seroreactivity for *T. cruzi* failed twice to reveal mouse infections in which parasites were subsequently isolated. Mice infected with Los Bordos strain were not seroreactive at 65 days post-inoculation though histopathological findings were positive.

Suckling mice inoculated with *T. infestans* and *T. guasayana* feces displayed serological, parasitological, and pathological findings compatible with *T. cruzi*. All strains isolated from *T. infestans* and the one from *T. guasayana* apparently were myotropic type III, with slow parasite multiplication, low virulence, no mortality during the first month of infection, and pathogenicity especially affecting skeletal muscles and myocardium (Andrade 1974, Andrade 2000).

Moderate lesions and scarce amastigote nests were detected in mice inoculated with *T. guasayana*-derived *T. cruzi*, whereas more severe lesions were produced by some strains isolated from *T. infestans*. Most mice inoculated with feces from *T. infestans* and *T. guasayana* developed very low parasitemia and suffered low mortality, probably mostly caused by low infecting doses, as the field-collected triatomines usually had a low intensity of infection on arrival to the laboratory.

Mice infected with *T. guasayana*-derived parasites showed a pattern similar to acute Chagas disease. Among all the trypanosomatids detected in humans or mammals in the Americas, *T. cruzi* is the only agent causing tissue pathology. *T. rangeli* is not pathogenic to humans, and so far has not been detected in Argentina (D'Alessandro & Del Prado 1977). The different methods herein used confirm that the trypanosomatids isolated from *T. guasayana* were *T. cruzi* beyond any doubt.

Our district-wide study confirms that in rural semiarid Argentina, peridomestic triatomine populations have very low rates of *T. cruzi* infection. Microscopical examination of triatomine feces may have underestimated the true bug infection rates to some extent, as suggested by paired comparisons with the polymerase chain reaction (Breniere et al. 1995). Interestingly, peridomestic *T. guasayana* had a non significative three times larger infection rate than *T. infestans* despite being less abundant. *T. guasayana* has been considered a potential risk for humans because of its widespread geographic range and larger capacity to endure low temperatures than *T. infestans* (Carcavallo & Martinez 1985). More recently in Northwestern Argentina, *T. guasayana* frequently invaded homes and bit people (Gürtler et al. 1999); it was found infected with *T. cruzi* (Cecere et al. 1999) and significantly associated with transmission to native dogs during the surveillance phase (Castañera et al. 1998). *T. guasayana* thus appears implicated as a putative secondary vector of *T. cruzi* in domestic or peridomestic sites under certain eco-epidemiological scenarios.

Some of the peridomestic triatomines infected with *T. cruzi* were collected in kitchens or store-rooms. As these

TABLE II

Parasitological, serological, and histopathological results for each microscopically-positive *Triatoma infestans* and *T. guasayana* captured manually at peridomestic sites in May 1999 - Olta and neighboring rural villages, central Argentina

	Bugs		Mice									
									Myocardium		Other organs [b]	
Triatomines [a] (Location)	Collection site	Feces culture	Mortality	Para-sitemia	Hemo-culture	Organ culture	Xeno-diagnosis	Sero-reactivity	Infiltrates	Amastigotes	Infiltrates	Amastigotes
T. infestans male (Bajo di Lucca)	Tree	Yes	3/4	No	Yes	Yes	No data	No data	Very severe	Yes	Moderate	Yes
T. infestans IV instar (El Simbolar)	Kitchen	No	0/1	Yes	Yes	Yes	No	Yes	Very severe	Yes	Moderate	No
T. infestans male (Los Bordos)	Goat corral	No	0/4	No	No	No	No	No	Light	Yes	Light	Yes
T. infestans female (San Felipe-Olta)	Tree with chickens	No data	0/3	No	Yes	No	No	No	No data	No data	Light/ moderate	No
T. infestans V instar (La Aguadita 1)	Store-room	Yes	0/3	No	Yes	Yes	Yes	Yes	Light	No	Light	No
T. infestans V instar (La Aguadita 2)	Store-room	Yes	0/3	No	Yes	Yes	Yes	Yes	Moderate	No	Light/ moderate	No
T. infestans III instar (Tala Verde 1)	No data	No	0/2	Yes	Yes	Yes	Yes	Yes	Moderate	No	Light/ moderate	No
T. infestans V instar (Tala Verde 2)	No data	Yes	0/4	Yes	Yes	Yes	Yes	No data	Light	No	Light/ moderate	No
T. infestans adult (Tala Verde 3)	No data	No	0/3	No	No	No	Yes	No data	No data	No data	No data	No data
T. guasayana V instar (El Simbolar)	Goat corral	Yes	0/7	Yes	Yes	Yes	Yes	Yes	Light/ moderate	Yes	Light	Yes

a: not shown: 2 *T. infestans* (from Esquina Norte and El Virque) and 1 *T. guasayana* (from El Simbolar) in which *T. cruzi* was not isolated, and 1 feces culture-positive *T. infestans* (from La Aguadita) not inoculated in mice; *b*: includes liver, spleen and hollow viscera; subinoculated mice showed a very similar pattern.

are resting sites of dogs or cats and both are highly infective to bugs (Gürtler et al. 1998), these were the likely sources of infections. However, other infected adults and nymphs were captured from goat or pig corrals and chicken coops, but goats and pigs only exceptionally have been infected with *T. cruzi* (Pinto Dias 2000) and chickens are refractory to infection. Light-trapping collections of large instars of *T. infestans* and *T. guasayana* suggested active walking dispersal from potential sources located about 50 m away (Vazquez-Prokopec et al. 2004). Therefore, the finding of two infected nymphs of *T. guasayana* in goat corrals located 10-40 m away from bedrooms suggests that local transmission occurred there or nearby, though at very low levels and from as yet unidentified sources. Ongoing work is directed to establish whether these infections are linked to the domestic or sylvatic transmission cycle of *T. cruzi* by means of molecular typing techniques.

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