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Chagas' disease in Aboriginal and Creole communities from the Gran Chaco Region of Argentina: Seroprevalence and molecular parasitological characterization.

Lucero RH [1,5]; Brusés BL[1]; Cura CI[2] ; Formichelli LB[1]; Juiz, N [2]; Fernández GJ[1]; Bisio, M.[2]; Deluca GD[3] ; Besuschio, S.[2]; Hernández DO[3]; Schijman AG[2]

[1]Area de Biología Molecular, Instituto de Medicina Regional, Universidad Nacional del Nordeste, Resistencia, Argentina.

[2] Laboratorio de Biología Molecular de la Enfermedad de Chagas, Instituto de Ingeniería Genética y Biología Molecular Dr Héctor Torres (INGEBI-CONICET), Buenos Aires, Argentina.

[3] Laboratorio de Aplicaciones Moleculares, Cátedra de Microbiología, Parasitología e Inmunología de la Facultad de Medicina de la UNNE, Resistencia, Argentina.

[4]Consultorio de Chagas, Facultad de Medicina, Universidad Nacional del Nordeste, Corrientes, Argentina.

[5]Cátedra de Medicina III, Area Infectología, Facultad de Medicina, Universidad Nacional del Nordeste, Corrientes, Argentina.

Running title: *T. cruzi* infection in Indigenous and Creole populations.

Corresponding author:

Alejandro Gabriel Schijman

Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (INGEBI-CONICET)

Vuelta de Obligado 2490, (1428) Buenos Aires, Argentina.

E-mail: schijman@dna.uba.ar   Tel / Fax: 54-11-47868578

ABSTRACT

Most indigenous ethnias from Northern Argentina live in rural areas of "the Gran Chaco" region, where *Trypanosoma cruzi* is endemic. Serological and parasitological features have been poorly characterised in Aboriginal populations and scarce information exist regarding relevant *T. cruzi* discrete typing units (DTU) and parasitic loads. This study was focused to characterise *T. cruzi* infection in Qom, Mocoit, Pit´laxá and Wichi ethnias (N=604) and Creole communities (N=257) inhabiting rural villages from two highly endemic provinces of the Argentinean Gran Chaco.

DNA extracted using Hexadecyltrimethyl Ammonium Bromide reagent from peripheral blood samples was used for conventional PCR targeted to parasite kinetoplastid DNA (kDNA) and identification of DTUs using nuclear genomic markers. In kDNA-PCR positive samples from three rural Aboriginal communities of "Monte Impenetrable", minicircle signatures were characterized by Low stringency single primer–PCR and parasitic loads calculated using Real-Time PCR.

Seroprevalence was higher in Aboriginal (47.98%) than in Creole (27.23%) rural communities (Chi square, p= $4.e^{-8}$). A low seroprevalence (4.3%) was detected in a Qom settlement at the suburbs of Resistencia City (Fisher Exact test, p= 2.e-21).The kDNA-PCR positivity was 42.15% in Aboriginal communities and 65.71% in Creole populations (Chi square, p= $5.e^{-4}$). Among Aboriginal communities kDNA-PCR positivity was heterogeneous (Chi square, p= $1.e^{-4}$). Highest kDNA-PCR positivity (79%) was detected in the Qom community of Colonia Aborigen and the lowest PCR positivity in two different surveys at the Wichi community of Misión Nueva Pompeya (33.3% in 2010 and 20.8% in 2014).

TcV (or TcII/V/VI) was predominant in both Aboriginal and Creole communities, in agreement with DTU distribution reported for the region. Besides, two subjects were infected with TcVI, one with TcI and four presented mixed infections of TcV plus TcII/VI. Most minicircle signatures clustered according to their original localities, but in a few cases, signatures from one locality clustered with signatures from other village, suggesting circulation of the same strains in the area. Parasitic loads ranged from undetectable to around 50 parasite equivalents/mL, showing higher values than those generally observed in chronic Chagas disease patients living in urban centres of Argentina. Our findings reveal the persistence of high levels of infection in these neglected populations.

INTRODUCTION

Chagas' disease represents a devastating health and social threat to around eight million infected people in 21 Latin American countries, and is emergent in non-endemic countries introduced by extensive global migrations and perpetuated by means of vertical transmission (Rodrigues Coura and Viñas, 2010). The infection mainly affects rural and neglected populations, such as Aboriginal groups, since they are highly exposed to the risk of vectorial transmission, as a consequence of poverty and lack of sanitized households. Many Creole populations resulting from miscegenation with European colonists and immigrants coexist within these native communities.

In Argentina, the Chagas' disease National Program has achieved important decreases in the rates of parasitic transmission by blood transfusion, vectorial pathway and prenatal care for congenital transmission, especially among non-indigenous communities. Deficient spraying and discontinuity of surveillance favors persistence of highly endemic areas (Zaidenberg, 2014). Aboriginal houses are much more likely to be infested with *Triatoma infestans* than Creoles' houses; indigenous people mostly tend to thatch the roofs and often sleep on the floor, providing the vector with a more primary habitat than just mud walls. Moreover, the lack of timely reporting of re-infestation opens the way for renewed transmission (Dell´ Arciprete et al., 2014). Besides, among indigenous groups, culture and language differences make control efforts more difficult (Dell´Arciprete et al., 2014).

By year 2010, according to the Survey of Indigenous Population of the National Institute of Statistics and Census (INDEC, 2010), there were 955,032 people in Argentina who self-recognised and/or identified themselves as belonging to an indigenous community minority. At the present time, ten well-defined indigenous ethnias are recognized in the country, 90% of them living within "the Gran Chaco Americano" region.

Three different indigenous ethnias inhabit the province of Chaco, namely Qom, spanish-called Toba, that includes 30,000 individuals living in urban and rural areas of central and southeastern plains of the province; Wichis, spanish-called Mataco, that comprises approximately 6,500 individuals living in rural communities in the northwestern corner of the province called "Monte Impenetrable Chaqueño" and Moquoít, spanish-called Mocoví, that includes approximately 6,500 individuals living in the southwestern area of the province. Moreover, in the province of Formosa, the Pilagá, originally named Pit´laxá, are nowadays situated in 19 settlements. Indigenous populations mostly live in rural locations with severe housing constrictions and with limited access to health care services. In many locations there are no medical facilities within or close to the settlements and there is also lack of medical staff trained to understand their cultural characteristics and languages (Petherick, 2010).

Currently, *T. cruzi* is partitioned into six discrete typing units (DTUs) renamed by consensus as TcI–TcVI (Zingales et al., 2009, 2012). Epidemiologically relevant *T. cruzi* DTUs and genetic parasite diversity infecting native populations remain to be determined. Most DTU typing methods have used DNA from cultured parasites, which may underestimate the diversity of natural populations due to subpopulation selection during culturing procedures (Diosque et al., 2003; Macedo et al., 2004). Moreover, parasite cultures from blood samples of chronically infected cases have very low sensitivity. In the last years, sensitive PCR strategies have been developed for typing of *T. cruzi* in clinical samples (Burgos et al., 2007, 2010; Zingales et al., 2012; Monje-Rumi et al., 2015; Cura et al., 2015). This has opened the possibility to deepening our understanding of parasite genetic diversity in native communities.

In this context, this work aimed to characterize serological and parasitological features of affected Aboriginal and Creole communities residing in rural neglected areas of the Argentinean Gran Chaco.

Materials and Methods

Subjects and samples

Between August 2010 and March 2014, *T. cruzi* infection was searched in a total of 861 individuals inhabiting the Provinces of Chaco and Formosa, in the argentinean Gran Chaco Region. A total of 604 out of 861 individuals belonged to eight Aboriginal communities at a) Province of Chaco: seven rural villages of Colonia Aborigen, N= 35 and Las Hacheras, N= 60 (ethnia Qom); Miraflores, N= 46 (ethnias Wichi and Qom); Misión Nueva Pompeya survey 2010, N= 55 Misión Nueva Pompeya survey 2014, N= 110 (ethnia Wichi, different individuals studied in each survey) and Villa Berthet, N= 115 (ethnia Mocoit) and the urban Mapic settlement in the suburbs of Resistencia city, known as "Gran Resistencia", N= 108 (ethnia Qom) and b) Province of Formosa: rural communities of Estanislao del Campo, N= 53 (ethnia Pit´laxá) and El Potrillo, N= 22 (ethnia Qom) (Figure 1 and Table 1).

Furthermore, 257 out of 861 individuals belonged to Creole rural populations of the Province of Chaco: Concepción del Bermejo (N= 49), Las Breñas (N= 67), Las Garcitas (N= 54) and Lapachito (N=87) (Figure 1 and Table 1).

The study was approved by the bioethical Committee of The Institute of Regional Medicine of the Northeastern National University (UNNE), Resistencia, Chaco and IDACH (Chaco Aboriginal Institute) upon written informed consents of adult individuals or parents/tutors in pediatric cases.

Serological examination

Conventional serological tests were assayed in the Immunological Department of the Institute of Regional Medicine or in the Parasitology School of the Faculty of Biochemistry of the UNNE. Indirect hemagglutination (Chagatest® Wiener Lab, Argentina; cut-off 1:32) and Ig G-ELISA test (*ELISA Chagatest*®, Wiener Lab, Argentina) were performed following the manufacturers´ instructions. In case of discordant findings, a third serological test was carried out using indirect immunofluorescence (Biocientífica, Argentina), following the manufacturers´ instructions. Subjects were considered infected if at least two tests were positive.

Seropositive individuals were notified to the Provincial Public health Chagas program, in coordination with the Health Care program of the UNNE (UNNE-SALUD), for clinical and cardiological examination and treatment with trypanomicidal drugs

following the normative in Argentina. However, these data were not available for the present study. Digestive megasyndromes were not studied.

PCR detection of bloodstream T. cruzi DNA.

PCR based detection of the 330-bp minicircle variable region of parasitic kinetoplastid DNA (kDNA-PCR) was carried out in blood samples of seropositive subjects using primers 121 and 122, as reported (Schijman et al., 2011). Seven hundred µL of peripheral blood were collected in EDTA tubes and stored at -20°C until DNA extraction. The DNA was purified with CTAB (Hexadecyltrimethyl Ammonium Bromide) as previously reported (Escalante, 1997; Lucero, 2007). Quantification of parasitic loads using TaqMan Real Time PCR (qPCR).

Parasitic loads were determined in a group of kDNA-PCR positive samples by means of TaqMan Real Time PCR (qPCR) targeted to a 166-bp segment from *T. cruzi* satellite DNA (SatDNA) using FastStart Universal Probe Master Mix (Roche Diagnostics GmbHCorp, Mannheim, Germany) in a final volume of 20 µL and the following reagents: 0.75 µM of primers Cruzi 1 (5'-ASTCGGCTGATCGTTTTCGA-3') and Cruzi 2 (5'AATTCCTCCAAGCAGCGGATA-3'), and 0.05 µM of specific TaqMan probe Cruzi 3 (5'-Fam-CACACACTGGACACCAA-NFQ-MGB-3'). Thermocycling conditions were a first step of 10 min. at 95ºC followed by 40 cycles at 95ºC for 15 sec. and 58ºC for 1 min. (Ramirez J.C. et al., 2015).

For quantification, a standard curve was prepared from DNA obtained using CTAB reagent from a seronegative blood sample spiked with 10^5 CL Brener parasites/mL. The DNA was mixed with DNA obtained by the same way from a seronegative blood sample in order to create a series of dilutions ranging from 0.1 to 10^4 parasite equivalents in 1 mL of blood (par.eq/mL).

Analytical Validation of DNA extraction and PCR.

DNA extraction with CTAB was validated for conventional PCR using external control panels provided by a referral Laboratory of Molecular Diagnosis at INGEBI-CONICET in the context of an International PCR harmonization study (See LbP/2 in Tables 1 and 2, reported in Schijman et al., 2011).

To validate Sat DNA-qPCR serial dilutions of seronegative EDTA-blood spiked with 0.05 to 10^5 par.eq./mL of cultured CL Brener were constructed. An anticipated linear reportable range (Burd, 2010) was obtained between 0.125 and 10^5 par.eq./mL (Correlation coefficient (r^2) 0.99, Efficiency 1.05).

The integrity of DNA purified with CTAB was evaluated by means of a qPCR targeted to the RNAse P human single copy gene (TaqMan Human RNase P detection reagent, Applied Biosystems, USA) and the resulting Cts were compared to those obtained from GEB samples extracted by a commercial column, considering that Cts≤ 22 indicate adequate integrity of DNA (Ramirez et al., 2015). The RNAse P-PCR cycling conditions involved a first step of 10 min at 95°C followed by 40 cycles at 95°C for 15 sec and 58°C for 1 min. The DNA was obtained using the CTAB reagent in exactly the same way as described for spiked and clinical samples. To validate Sat-DNA qPCR for measurement of parasitic loads, we carried out a recovery study to measure "Trueness" of the assay (Burd, 2010) using proficiency testing samples and compared results from the method under evaluation to the expected reference value. The proficiency samples were constituted by a blinded panel of seronegative EDTA-blood samples spiked with known quantities of Cl Brener, as follows: panel A: three samples containing 1 par.eq/mL; panel B: three samples containing 10 par.eq/ml and panel C: three samples containing 100 par.eq/mL. The panels are similar to the ones used in recent clinical trials with anti-parasitic drugs (Molina et al, 2015). Testing was spread over 2 independent runs, separated by a period of two months using the same reagent lots, as recommended (Burd, 2010).

Genotyping of discrete typing units.

Parasite DTUs were determined in kDNA-PCR positive DNA samples, using a PCR algorithm targeted to parasite nuclear genes, as described in Burgos et al., 2007. Briefly, (1) spliced leader intergenic region (SL-IR) based PCR was used to distinguish TcI (150 bp), TcII, TcV, and TcVI (157 bp) from TcIII and TcIV (200 bp); (2) heminested SL-IR-I PCR was used to identify TcI (350 bp), and hemi-nested SL-IR-II PCR was used to identify TcII, TcV, and TcVI (300 bp); (3) hemi-nested PCR of the 24S alpha-ribosomal DNA (24 Sα-rDNA) was used to distinguish TcV (125 or 125+ a weak 140 bp fragment) from TcII and TcVI (140 bp); and (4) heminested PCR

targeted to genomic fragment A10 was used to discriminate TcII (580 bp) from TcVI (525 bp).

Analytical sensitivity of each PCR assay is detailed elsewhere (Burgos et al., 2007). Samples that yielded positive results by SL-IR-II PCR but negative by 24S α-rDNA PCR were reported as belonging to the TcII/V/VI group. Those samples that amplified the 140-bp 24S α-rDNA fragment but had negative results of PCR targeted to A10 were reported as belonging to the TcII/VI group. Those samples amplifying a band of 125 bp plus a strong 140 bp fragment after 24S α-rDNA PCR, were interpreted as mixed infections by TcV plus TcII or TcVI, as done before (Burgos et al., 2007).

Minicircle signatures.

Genetic diversity of natural *T.cruzi* populations was explored by Low stringency single primer PCR (LSSP-PCR) (Vago et al., 1996) of the 330 bp amplicons from a group of kDNA-PCR positive samples obtained from Aboriginal patients inhabiting the localities of "Monte Impenetrable Chaqueño". Ten μL of the kDNA-PCR products were run on 1.5% low melting point agarose gels and stained with ethidium bromide. Bands corresponding to 330 bp were cut from the gel, melted and diluted to 1:10 in double distilled water. Three μL of the dilution was used as a template for the LSSP-PCR reaction. LSSP-PCR was performed in 20μL of the final volumen using 150 pmol of the S35 primer, 5U/μL of GoTaq® DNA polymerase (Promega), 2.5 mM of each dNTP, 25mM of MgCl$_2$ and buffer 10X. Amplification was carried out with 5 min of initial denaturing at 94ºC, followed by 40 cycles of 30ºC for 1 min, 72ºC for 1min and 94ºC for 1 min. Finally 30ºC for 1 min and a cycle at 72ºC for 10 min. Five μL of the amplification products were analysed by electrophoresis in 6% polyacrylamide gels and stained with Sybr Green. LSSP-PCR was performed on duplicate for each sample.

Analysis of the bands was performed using Gel documentation and analysis systems (Uvitec. Cambridge, UK). Molecular weight markers were used as reference standards to correct the variations in migration among the different gels. The fragment sizes were calculated using UVIsoft analysis software. A data matrix of 0 and 1 was created on basis of the absence or presence of the bands, respectively. Finally, genetic distance analyses were performed using

FreeTree(http://web.natur.cuni.cz/ ~flegr/programs/freetree.htm). The dendrogram was constructed using TreeView software with 100 bootstrap replicates (http://darwin.zoology.gla.ac.uk/ ~rpage/treeviewx/download.html).

Statistics

Categorical variables were compared using the χ2 test or the Fisher Exact test. Mann-Whitney test was used for non-parametric analyses. Comparison of distribution of parasitic loads among populations was done using Anova, once similarity of variances was demonstrated using the Levene´s test.

P values < 0.05 were considered as significant.

RESULTS

Seroprevalence in Aboriginal and Creole populations.

Eight aboriginal communities were studied; seven reside in rural villages at the Provinces of Chaco and Formosa (496 individuals, Wichi, Pit´laxá, Mocoit and Qom) and one in the urban Mapic settlement of the suburbs of Resistencia city, known as "Gran Resistencia" (108 individuals, Qom) (Figure 1 and Table 1). Furthermore, four rural villages inhabited by Creole populations were included (257 individuals).

Detection of seropositivity for *T. cruzi* allowed diagnosis of Chagas disease in 238 out of 496 rural Aboriginal (47.98%) and in 70 out of 257 Creole tested subjects (27.23%) (Chi square test, $p = 4.e^{-8}$). A higher seroprevalence was detected in those localities sited more distant from urban centres, such as Colonia Aborigen (51.4%), Las Hacheras (56.7%), Miraflores (52.2%) and Misión Nueva Pompeya (survey in 2010, 54.5% and survey in 2014, 43.64%) (Table 1). In contrast, the urban Qom community of Mapic settlement presented a seroprevalence of 4.3%, significantly lower than that found in rural Aboriginal villages (Fisher Exact test, p= 2.e-21) (Table 1).

Molecular detection and characterization of T.cruzi infection:

Molecular studies of *T. cruzi* infection were carried out from 700 µL of frozen EDTA-blood samples using CTAB reagent for DNA extraction (see Materials and Methods). The integrity of DNA extracted by CTAB was evaluated by means of a qPCR assay targeted to the RNAse P human single copy gene in 44 samples from three communities. Median Cts (Q1-Q3) obtained from samples of the different communities were similar: 18.24 (17.66-19.77); 18.91 (18-19.83) and 19.4 (18.55-21.26) in samples from Las Hacheras, Miraflores and Misión Nueva Pompeya (survey 2010), respectively (Mann-Whitney tests, p= ns). These Ct values indicated that the DNA quality was adequate for *T. cruzi* based PCR analyses (Materials and Methods). Then, blood samples from seropositive patients were tested for the presence of parasite DNA using conventional kDNA-PCR (Table 1). Overall kDNA-PCR positivity was 42.15% in Aboriginal communities and 65.71% in Creole populations (Chi- square test, $p = 5.e^{-4}$). Among Creole communities there was no significant difference in PCR positivity (Chi-square test, $p = 0.690$) whereas among Aboriginal communities there was (Chi square test, $p = 1.e^{-4}$). The highest kDNA-PCR positivity (79%) was detected in the Qom community of Colonia Aborigen and the lowest PCR positivities were observed in samples from the Wichi communities of El Potrillo (2/6 seropositives) and Misión Nueva Pompeya (survey in 2010 (10/30 seropositives, 33.3%) and survey in 2014 (10/48 seropositives, 20.8%), (Table 1).

In 54 kDNA-PCR positive patients from three rural communities of Monte Impenetrable, parasitic loads were measured using qPCR (Figure 2 and Table S1). There were no significant differences in the mean parasitic loads among the tested communities: Miraflores: 4.48 par.eq/mL; Las Hacheras: 2.48 par.eq/mL; Misión Nueva Pompeya: survey in 2010, 1.45 par.eq/mL and in 2014, 4.44 par.eq/mL (Anova test, $p = 0.745$; Levene´s test, $p = 0.716$). The trueness of the obtained values was assessed using blinded panels of seronegative blood spiked with CL Brener parasites spanning concentrations in the range of 1 to 100 par.eq/mL (see Materials and Methods). The linear regression analysis gave a correlation coefficient (r^2) of 0.9612, which indicated good correlation between expected and observed parasitic loads (Figure 2).

Discrete typing units and minicircle signatures:

A total of 127 kDNA-PCR positive blood samples, 92 from Aboriginal and 35 from Creole communities, were subjected to DTU genotyping using a panel of nuclear

genomic markers, as detailed in Materials and Methods. The DTUs could be determined in 76/92 and 27/35 samples from Aboriginal and Creole populations, respectively. TcV and TcII/V/VI were detected in 43/76 and 30/76 samples from Aboriginal and in 10/27 and 13/27 samples from Creole patients, respectively. Two samples, one from Las Hacheras and one from Las Garcitas were infected with TcVI. In four samples, one from Las Hacheras, one from Miraflores, one from Las Breñas and one from Lapachito, the amplification of two strong amplicons of 125 bp plus 140 bp using 24Sα-rDNA-based PCR were compatible with mixed infections by at least two DTUs: TcV plus TcII or TcVI (V + II/VI in Table 1). Finally, a single sample from a Creole patient of Las Breñas was infected with TcI. TcIII and TcIV were not detected in the studied population.

Minicircle signatures were obtained by LSSP-PCR from the 330bp kDNA-PCR products of blood samples infected with TcV or Tc II/V/VI from aboriginal patients of Las Hacheras, Miraflores and Misión Nueva Pompeya (survey 2010). Specific LSSP-PCR patterns were observed in each community. Figure 3A shows examples of minicircle signatures obtained after gel electrophoresis; diversity in size and number of LSSP amplicons was observed among most samples from the same village (eg lanes 1 to 3 and 11 to 14 from Miraflores, lanes 17 to lane 24 from Misión Nueva Pompeya (survey in 2010); lanes 25 to 28 from Las Hacheras). Some parasite populations typed from patients´ samples of different communities presented similar signatures (eg. Lane 1, M4 from Miraflores and Lane 8, LH231 from Las Hacheras) suggesting circulation of the same populations, which can be explained due to their geographical proximity. This can be observed in the dendrogram of Figure 3B, which shows clusters grouping the majority of samples from each locality except single samples that group in clusters from the neighbouring villages, suggesting circulation of the same strains (Figure 3B, red circles).

Discussion

Despite significant gains in the reduction of Chagas disease burden in many endemic countries, active transmission remains in areas such as the Gran Chaco. High initial vector density, poor housing material, peridomestic infestation, insecticide resistance,

and a lack of systematic insecticide spraying and vector surveillance have previously been incriminated for failure to interrupt and sustain interruption of transmission. We have studied Aboriginal communities that inhabit highly endemic areas for Chagas disease, such as the Monte Impenetrable Chaqueño, and observed high seroprevalences, similar to those reported a decade before: 57% in Chaco and 48% in Formosa (Biancardi et al., 2003; Alonso et al., 2004). An exception was the low seroprevalence found in the Qom community at Mapic settlement located in the surroundings of Resistencia city (4.3%, Table 1), which may be explained because all houses in the settlement have plastered-brick walls with metallic roofs. Previous studies showed that the prevalence of domestic infestation was lower in well plastered houses than in dwellings with walls with cracks. Thus, plastering of walls would be cost-effective in the long-term sustainability of vector control because such houses would be less susceptible to triatomine re-colonization (Gürtler, 2009). Studies by Moretti and coworkers carried out in Wichi and Creole communities from Misión Nueva Pompeya and surrounding rural villages showed very high seroprevalences too (Moretti et al., 2010), more than 10 times higher than the average for Argentina reported by Moncayo and Silveira (2009).

Cultural and educational aspects may account for the impact of *T. cruzi* infection in native populations. Dell´Arciprete and coauthors described that neither the Pit´laxá or Wichi communities perceive the triatomine vector, known as "vinchuca", as a health risk and this perception has consequences on their attitudes with regards to vector surveillance and control. Indeed, household education is considered a generic measure of household socioeconomic status, which is among the main social determinants of health inequalities (WHO, 2010).

In Creole villages, higher seroprevalences were found in Las Garcitas and Las Breñas, although they did not reach the proportions found in most Aboriginal communities. In Concepción del Bermejo, seroprevalence was lower, probably due to succesful vector control achieved in that locality, thanks to a special surveillance program launched by a public-private parternship (http://asp-es.secure-zone.net/v2/index.jsp?id=8177/13194/28297&lng=es).

Gürtler (2009) showed that the household seropositivity to *T. cruzi* in rural villages of Northwest Argentina increased steadily and significantly with decreasing size of

domestic area, a putative index of stable settlement and well-being in Creole communities of the dry Chaco (Gürtler, 2009). A recent sociodemographic study in Pampa del Indio, in the province of Chaco revealed that Qom households likely were more infested than Creoles because of the convergence of multiple factors closely related to structural rural poverty, such as poor housing quality, residential overcrowding, less frequent insecticide use, rather than to direct ethnic or cultural effects facilitating house invasion and colonization by triatomine bugs. After accounting for the effects of other factors with high relative importance, ethnicity per se was a poor predictor of house infestation status (Gaspe et al., 2015).

Seroprevalence studies have been done in indigenous populations from other endemic countries. Samuels and coauthors showed an overall *T. cruzi* seroprevalence of 51.7% in areas of the Bolivian Chaco, that reached 97.1% among participants older than 30 years. In that study, sleeping in a structure with cracks in the walls was associated with infection (Samuels et al., 2012). Chico and coauthors studied 18 sylvatic communities along the Río Napo in Ecuador and found infection in 15 communities, with seroprevalences reaching 18.8% in adults 50 years or older (Chico et al., 1997). Many factors, such as diversity of *T. cruzi* strains circulating in different endemic areas, the use of different parasite antigens in the serological assays tested in each region and diverse susceptibility to infection of different ethnias, might account for these geographical variabilities (Porrás et al., 2015).

According to WHO, development of new methods for diagnosis of infection is one of the ten top priorities in Chagas disease research (WHO, 2015). The CTAB based DNA extraction method that starts from a small volume of frozen EDTA-blood has been appropriate for conventional kDNA-PCR, qPCR for quantification of parasitic loads as well as for direct DTU genotyping. The human RNase P gene based qPCR yield was higher than that obtained in DNA extracts using fiber-glass columns from 300 μL of an initial 10 mL GEB sample (median Ct=21.40, Q1=20.55, Q3=22.68; Ramirez, J.C. et al., 2015).Thus, the method appears suitable for PCR laboratories close to endemic localities, where samples can be collected in EDTA tubes and transported within a few hours to the Molecular Biology facility and stored frozen until DNA extraction. Other advantage of using CTAB is that it does not require solvent

extraction and that interacts with substances than could inhibit the PCR (Escalante et al., 1997).

Overall kDNA-PCR positivity in Creole communities (65.71%) was in agreement with values obtained in other studies of chronic Chagas disease patients (Schijman et al., 2011). In an International study organized by WHO-TDR, the kDNA-based PCR tests with best performance in terms of accuracy gave 63% of positivity in a panel of chronic Chagas disease samples (Schijman et al., 2011) and in the BENEFIT study , kDNA-PCR performed in 1896 chronic Chagas disease patients rendered a positivity of 60.5% at baseline (Morillo et al, 2015).

The kDNA-PCR positivity detected in the Qom community of Colonia Aborigen was higher (79%), but eight of the 15 PCR positive subjects were children. It has been shown that most children harbor higher levels of parasitemia than adults (Burgos et al., 2007; Duffy et al., 2009; Bua et al., 2013).

It is remarkable the low kDNA-PCR positivity obtained in the Wichi population of Misión Nueva Pompeya (Table 1). The fact that similar PCR positivity was obtained from samples collected in two surveys separated by a period of four years, suggests a particular characteristic of these populations rather than technical problems in the collection, transportation and/or processing of samples in the PCR laboratory. Moreover, the RNAse P PCR based analysis in samples from this community gave similar Ct values than those obtained in Miraflores and Las Hacheras, where the DNA-PCR positivities were higher (Table 1). It is tempting to speculate that host factors could play a role in the control of parasite infection, virulence and/or tropism. Characterization of human genetic polymorphism to elucidate its association with infection susceptibility and clinical manifestations has been recently done in other populations infected with *T. cruzi* (Juiz et al., 2015; De Oliveira et al., 2015) and is currently undergone in patients of the ethnia Wichi from this locality (Juiz Natalia and Gonzales, Clara Isabel, personal communications).To our knowledge, this is the first study evaluating parasitic loads in Aboriginal populations. Parasitic loads were estimated from kDNA-PCR positive DNA extracts using a validated qPCR method targeted to satellite DNA (Duffy et al., 2013; Ramirez J.C. et al.; 2015). Similar distribution of parasitic loads was seen in Miraflores, Las Hacheras and in both studies carried out in Misión Nueva Pompeya (Figure 2). A recent study showed that

pre-treated patients residing in non-endemic urban areas from Argentina displayed a median parasitemia of 2.1 (1.18 to 2.78) par.eq/ml using a similar qPCR test (Alvarez et al., 2015) and in argentinean patients admitted to the BENEFIT study, a median parasitemia of 1.93 par. eq/mL was obtained using the same pair of primers in a SYBRGreen Real-Time PCR approach (Moreira et al., 2013). In our study, in Monte Impenetrable, some patients presented higher parasitic loads than those expected for chronic patients, between 10 and 52 par.eq/mL (Table S1). It is tempting to speculate that in chronic Chagas disease patients from these rural areas, higher kDNA-PCR positivity and parasitic loads could indicate the occurrence of events of re-infection. In fact, in Miraflores, microscopic analysis of feces from 512 *T. infestans* bugs collected in 2012 showed that 65% of them were infected with *T. cruzi* (Fernández Gustavo, unpublished results), pointing to a high risk of vectorial transmission in that village. Longitudinal studies of parasitic loads in sequential samples from patients living in rural endemic areas would allow deepen insight in the natural fluctuations of parasitic burden in populations exposed to the risk of re-infection.

There is very scarce information regarding the distribution of DTUs in areas inhabited by Aboriginal communities. In two rural areas of the Argentinean Gran Chaco human blood samples typified by PCR-DNA blotting and hybridization assays with specific DNA probes showed TcV as the most prevalent DTU (30.6%) followed by mixed infections TcV/TcVI (32.6%), (Monje-Rumi et al., 2015). In the Bolivian Chaco, studies in triatomine bugs revealed predominance of TcII/TcV/TcVI followed by TcI, TcIII/TcIV and mixed infections (Perez et al., 2013). Thus, the prevalence of TcV or TcII/V/VI in our setting is in accordance with the above mentioned reports and with other ones carried out previously from cultured isolates in the Gran Chaco Region (Diosque et al., 2003; Cardinal et al., 2008). In contrast to the work by Monje-Rumi et al. (2015) we detected only two patients infected by TcVI and four mixed infections of TcV/TcVI or TcII (Table 1). The use of different molecular typing approaches may account for these differences, which make necessary to reach a consensus for standardization and validation of techniques for molecular genotyping of *T. cruzi*, such as it has been done with respect to conventional and quantitative PCR for monitoring Chagas disease patients under treatment (Schijman et al., 2011; Ramirez J.C. et al., 2015).

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A single sample with TcI was detected, out of 12 typed samples in the Creole village of Las Breñas. TcI had been identified before in patients from the Gran Chaco region (Diosque et al., 2003; Cura et al., 2012). In addition, studies in chronic patients from Chaco who underwent Chagas reactivation due to heart transplantation, allowed identification of TcI strains that were detectable only after immunosuppressive therapy, whereas samples taken from these patients before transplantation were kDNA-PCR negative (Burgos et al., 2010). This may suggest that TcI strains from this region might circulate at very low parasitic loads and thus their detection is infrequent. It could be hypothesized that TcI and perhaps other DTUs could circulate in seropositive patients with very low parasitic loads, such as in those showing positive kDNA-PCR but negative SL-IR and/or 24Sα-rDNA PCR results and in those with negative kDNA-PCR findings. This, in turn could be related to the tissue tropism of TcI strains (Burgos et al., 2010).

Minicircle signatures obtained by LSSP-PCR of *T. cruzi* sequences amplified from infected tissues has been proposed as a useful tool to study the molecular epidemiology of Chagas' disease (Vago et al., 1996; Macedo et al., 2004). We carried out LSSP-PCR from kDNA amplicons obtained in samples infected with the predominant DTU, typed as TcV or TcII/V/VI, and observed infra-DTU diversity that was associated with the geographical origin. Moreover, in Miraflores, LSSP-PCR was done in parasite populations obtained from three infected triatomine bugs (Mv1 to Mv3) collected in a domicile (lanes 4, 5 and 7 in Figure 3A) that clustered with signatures from patients of the same locality (Figure 3A; M17, lane 3; M40, lane 4; Mv1 to Mv3, lanes 5, 6 and 8, respectively). Interestingly, minicircle signatures from parasite populations infecting bugs presented a higher number of fragments than those from human subjects at the same locality, probably representative of a higher degree of diversity (Figure 3). This was also observed using polymorphic microsatellites (Oliveira et al., 1998), suggesting that subpopulation selection occurs in the human host (Macedo et al., 2004).

In a report of the Panamerican Health Association (PAHO, 2008), the Gran Chaco Americano is characterized as a setting in which the impact of control measures for infestation is minimized by its eco-epidemiological characteristics. Its enormous extension and inaccessibility and the low density of population make any operational action difficult to be carried out. These conditions impose the need of special

attention regarding surveillance and control procedures, considering the whole region as an epidemiological unit and not as national areas with political-administrative boundaries. Indeed, these findings reveal a serious situation with high rates of infection and persistent transmission in native populations.

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Legend to Figures

Figure 1

Map showing the Aboriginal and Creole groups in the Provinces of Chaco and Formosa. The localities under study are indicated.

Figure 2.

Parasitic loads measured by qPCR targeted to Satellite DNA (TaqMan Real Time PCR) in three populations of Monte Impenetrable Chaqueño, including two surveys in Misión Nueva Pompeya (different patients) and in external control panels. Panel A: six spiked blood samples containing 1 par.eq/mL; panel B: six spiked blood samples containing 10 par.eq/ml and panel C: six spiked blood samples containing100par.eq/mL. Values are expressed in Log par.eq/ 10 mL blood.

Figure 3

Minicircle signatures from natural *T.cruzi* populations infecting aboriginal populations of Northeastern Argentina.

A: Minicircle signatures obtained by LSSP-PCR from bloodstream TcV parasite populations from Miraflores (Mn); Misión Nueva Pompeya (MNPn) and Las Hacheras (LHn). Lanes: 1, M4; 2, M17; 3, M40; 4, Mv1; 5, Mv2; 6, MW marker; 7, Mv3; 8, LH231; 9, LH232; 10, LH culture isolate; 11, M43; 12, M107; 13, M58; 14, M49; 15, MW marker; 16, M47; 17, MNP255; 18, MNP262; 19, MNP265; 20, MNP266; 21 MW marker; 22, MNP270; 23, MNP283; 24, MNP287; 25, LH200; 26, LH201; 27, LH210; 28, LH211; 29, MW marker. Mv1 to 3 represent parasitic populations from three triatomine fecal samples collected from a house in Miraflores.

B: Dendrogram from LSSP-PCR based signatures shown in A. Bootstraps are indicated in the nodes and red circles show minicircle signatures from samples of a certain village that cluster with samples from other village.

PROVINCE OF FORMOSA

El Potrillo

Estanislao del Campo

Wichi

Pit´laxá

PROVINCE OF CHACO

Misión Nueva Pompeya

Las Hacheras

Nueva Pompeya

Las Hacheras

Miraflores

Miraflores

Colonia Aborigen

Wichi

Qom

Mocoitm

Colonia Aborigen

Mapic

Barrio Mapic

Villa Berthet

Villa Berthet

ABORIGINAL

C. del Bermejo

Las Breñas

CREOLE

Las Garcitas

Lapachito

Fig. 1

25
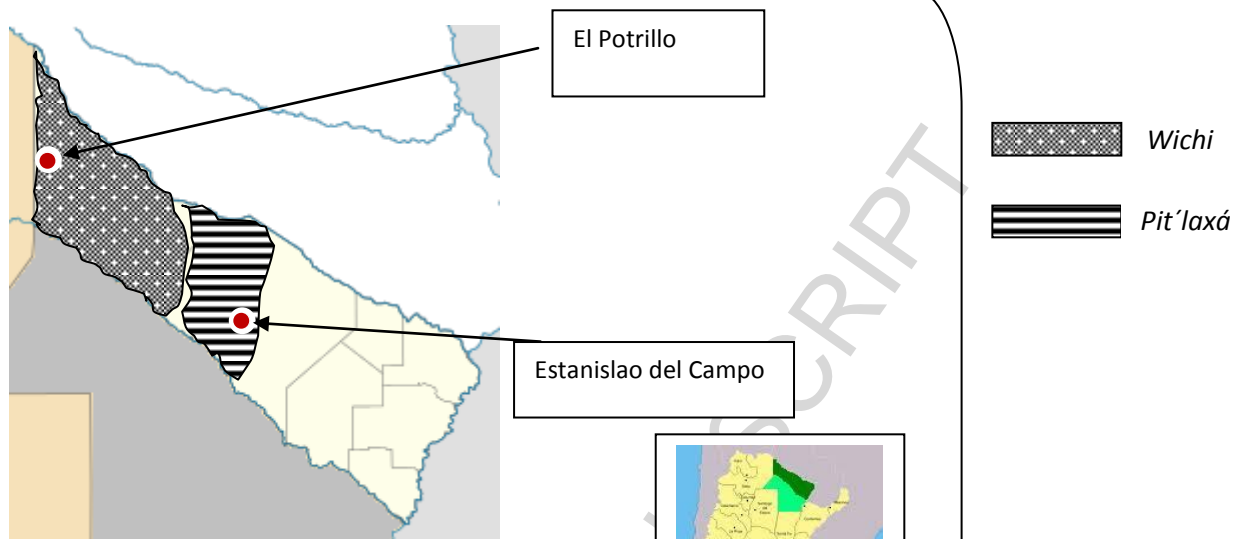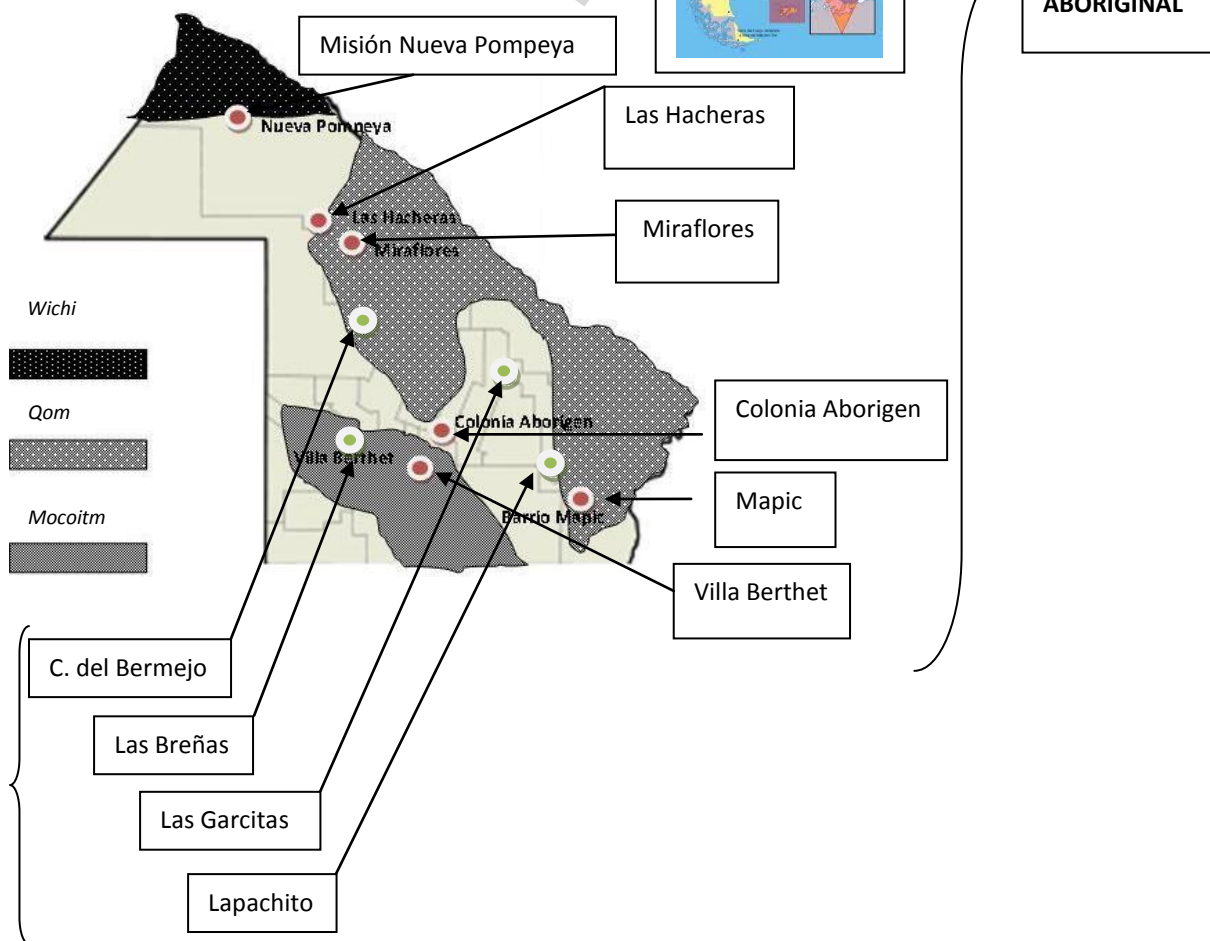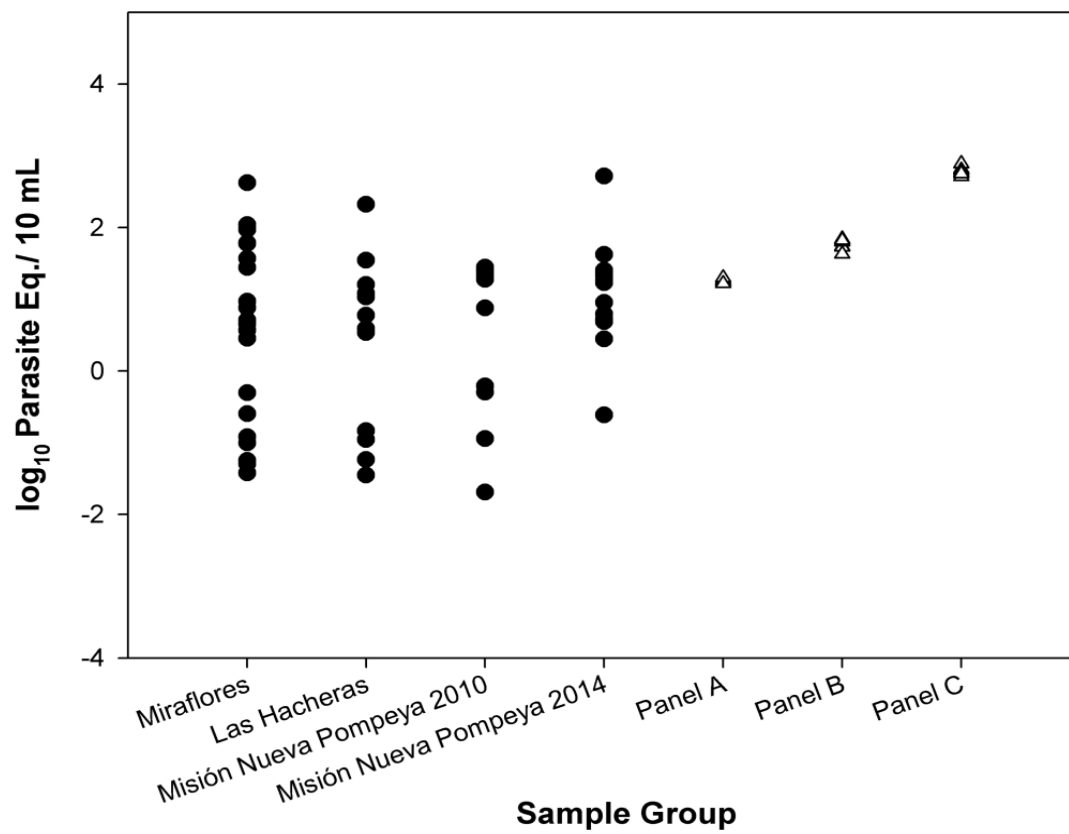
Fig. 2

A

B

Fig. 3

TABLE 1. Serological, kDNA-PCR and DTU findings in Aboriginal and Creole communities from Northeastern Argentina.

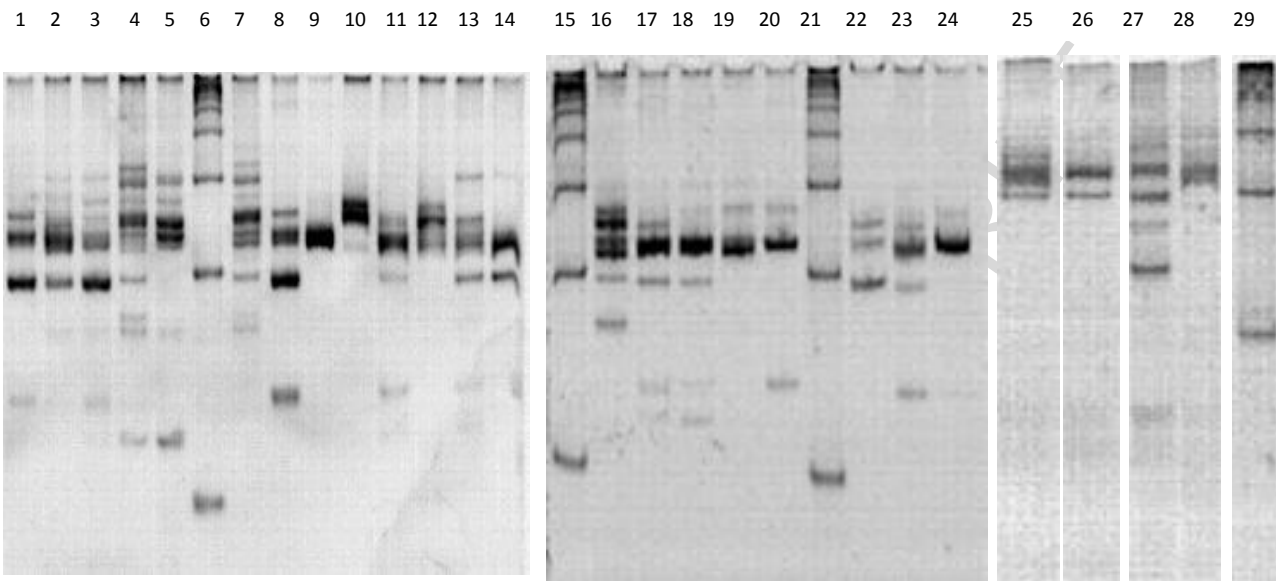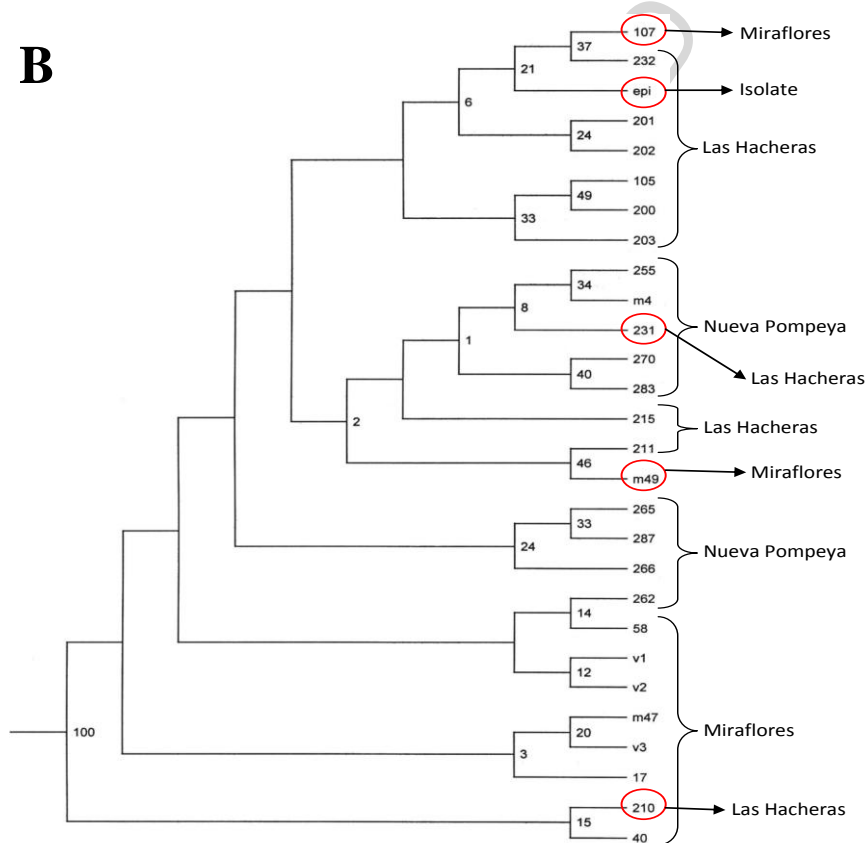| Locality / ethnia Latitude / Longitude	Number Subjects	Age (years)	Seropositive cases N (%)	kDNA -PCR positive cases N (%)	Number of DTU typed / kDNA- PCR +	DTU (N)
E. del Campo/ Pit´laxá -25.05/-60.1	53	15-67 Median 31 Media: 34.3 SD 14.42	25 (47.2)	9 (36)	2/9	II/V/VI (2)
El Potrillo/ Qom -26.18 /-58.18	22	6-59 Median 18 Media : 24 SD 15.54	6 (27.3)	2(33)	2/2	V (2)
Mapic Settlement/ Gran Resistencia Qom -27.44 /-58.98	108	1-60 Median 11 Media: 14.3 SD 11.53	4(3.7)	2(50)	2/2	V (1) II/V/VI (1)
Cnia. Aborígen/ Qom -26.46 /-60.025	35	1-55 Median 16 Media:21.74 SD 16.84	19 (54.3)	15 (79)	15/15	V (10) II/V/VI (5)
V. Berthet/ Mocoit -27.28 /-60.41	115	1-62 Median 26 Media: 27 SD 16,65	52 (45.2)	18 (34.6)	18/18	V (11) II/V/VI (7)
Miraflores/ Qom-Wichi -25.44/60.88	46	17-78 Median 40 Media :40.5 SD 17.65	24 (52.2)	14 (58.3)	12/14	V (8) II/V/VI (3) V+II/VI (1)
Las Hacheras/ Qom -25.38/-60.98	60	20-76 Median 33.5 Media: 36.4 SD 21.18	34 (56.7)	22 (64.7)	16/22	V (6) VI (1) II/V/VI (8) V+ II/VI (1)
Misión Nueva Pompey (August 2010) Wichi	55	28-60 Median 32	30 (54.5)	10 (33.3)	9/10	V (5) II/V/VI (4)

-25.44/-60.88		Media: 35.6 SD 20.67				
Misión Nueva Pompey (March 2014) Wichi -25.44 /-60.88	110	7-72 Median 28 Media 31.0 SD 13.3	48 (43.64)	10 (20.83)	ND	ND
Concepcion del Bermejo Creole -26.60 /-60.94	49	1-56 Median 12 Media 6,88 SD 14.96	8 (16.32)	6 (75)	5/6	V (2) II/V/VI (3)
Las Garcitas Creole -26.45 / 60.013	54	2-53 Median 14 Media 7.46 SD 14.60	21 (38.9)	14 (66.6)	6/6	V (2) II/V/VI (3) VI (1)
Lapachito Creole -27.15 /-59.38	87	2-59 Median 11 Media 11.78 SD 14.33	10 (11.49)	5 (50)	4/5	V (1) II/V/VI (2) V+ II/VI (1)
Las Breñas Creole -27.08 /-61.08	67	8-93 Median 32 Media 26.46 SD 18.89	31 (46)	21 (68)	12/16	V (5) II/V/VI (5) V+ II/VI (1) I (1)

Graphical abstract

El Potrillo: 27.3% seropositives, 33% kDNA PCR positives, DTU: V

Estanislao del Campo: 47.2% seropositives, 36% kDNA PCR positives, DTU: II/V/VI

Wichi

Pit'laxá

Seropositivity > 50%

Seropositivity 30- 50%

Seropositivity 10- 30%

Seropositivity < 10%

Nva. Pompeya 2010: 54.5% seropositives, 33.3% kDNA PCR positives, DTU: V; II/V/VI

Nva. Pompeya 2014: 43.6% seropositives, 20.83% kDNA PCR positives , DTU: ND

Las Hacheras: 56.7% seropositives, 64.7% kDNA PCR positives, DTU: V;VI; II/V/VI; V+II/VI

Miraflores: 52.2% seropositives, 58.3% kDNA PCR positives, DTU: V;II/V/VI; V+II/VI

C. Aborigen: 54.3% seropositives, 79% kDNA PCR positives, DTU: V; II/V/VI

Mapic: 3.7% seropositives, 50% kDNA PCR positives, DTU: V;II/V/VI

V. Berthet: 45.2% seropositives, 34.6% kDNA PCR positives, DTU: V; II/V/VI

Wichi

Toba

Mocoví

C. del Bermejo: 16.32% seropositives, 75% kDNA PCR positives, DTU: V; II/V/VI

Las Garcitas: 38.9% seropositives, 66.6% kDNA PCR positives, DTU: V; II/V/VI;VI

Las Breñas: 46% seropositives, 68% kDNA PCR positives, DTU: V; II/V/VI;V+II/VI; I

Lapachito: 11.49% seropositives, 50% kDNA PCR positives, DTU: V; II/V/VI; V+II/VI

HIGHLIGHTS

- Infection by Trypanosoma cruzi was searched in Qom, Mocoit, Pit´laxá and Wichi ethnias and Creole communities inhabiting rural villages from two highly endemic provinces of the Argentinean Gran Chaco.

- Seroprevalence was high in Aboriginal and Creole rural communities. Low seroprevalence was detected in a urban Qom settlement in the region.

- PCR targeted to minicircle sequences show high variability in PCR positivity among different Aboriginal communities.

- Parasitic loads showed similar distribution in rural villages of the Monte Impenetrable Chaqueño, and revealed cases with higher parasitemias than those expected for argentinean chronic Chagas disease patients of urban centers, suggesting the occurrence of re-infections.

- Discrete typing units were identified directly from blood samples showing TcV as the predominant DTU followed by TcVI, TcI and mixed infections.