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Trypanosoma cruzi DTUs TcV and TcVI are associated to the digestive form of Chagas disease

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Declarations of interest: none
Abstract

The relationship among genetic diversity of *Trypanosoma cruzi* and clinical forms of Chagas disease remain elusive. In order to assess the possible association between different *T. cruzi* Discrete Typing Units (DTUs) and the clinical pictures of the disease, 205 chronic patients from Salta province, Argentina, were analysed. One hundred and twenty-two of these patients were clinically categorized as: cardiac 38.5% (47/122), digestive 15% (18/122), cardio-digestive 16% (20/122) and asymptomatic 30% (37/122). From each patient, blood samples were taken for both, Polymerase Chain Reaction (PCR) targeting kDNA and blood culture analyses. The presence of *T. cruzi* kDNA was detected in 43% (88/205) of the patients. *T. cruzi* DTUs were identified in 74% (65/88) of the kDNA positive patients by PCR-hybridization using specific probes. We detected the presence of DTUs TcI, TcII, TcV and TcVI. Single infections (i.e. presence of only one DTU in the sample) were detected in 38.64% of the samples (34/88), while mixed infections were 35.23% (31/88). TcV was the most prevalent DTU (60.3% - 53/88). The association analyses showed, for the first time to the best of our knowledge, that TcV and TcVI were associated with the digestive form of Chagas Disease (Fisher p=0.0001).

Keywords: Chagas disease, clinical pictures, DTUs, Southern blot, Argentina
1. Introduction

Trypanosoma cruzi is a protozoan parasite which establishes life-long infection in humans and causes Chagas disease. The disease is characterized by a short acute phase, usually asymptomatic, that in the absence of early treatment is followed by a chronic phase, with different clinical forms (Rassi et al., 2010). Approximately 60% of infected individuals are clinically asymptomatic, while around 30% of individuals infected develop the chronic chagasic cardiomyopathy and ~10 - 15% of cases develop digestive megasyndromes, neurological or mixed alterations (Mitelman et al., 2012; Pérez Molina and Molina, 2018).

The distribution of pathogenesis and mortality of Chagas disease varies through countries of Latin America where the disease is endemic (World Health Organization, 2015; Zingales et al., 2012). The causes of this geographical heterogeneity of clinical outcomes, as well as the mechanisms that lead to developing different clinical forms of Chagas disease are uncertain and still remain poorly understood (Lewis et al., 2014; Miles et al., 2003; Pérez-Molina and Molina, 2018; Vago et al., 2000). It has been proposed that the complexity of Chagas disease pathogenesis is probably determined by several different factors, i.e. the genetic diversity of the parasite, the immune status of the host, environmental factors and the interaction among all them (Campbell et al., 2004; Rassi et al., 2010).

T. cruzi is represented by a pool of populations which display great diversity in biological behaviour and genetic variation (Macedo and Segatto, 2010). Based on different molecular markers, T. cruzi is grouped into six genetic lineages (TcI–TcVI), called Discrete Typing Units (DTUs) (Zingales et al., 2009). Four DTUs are mainly present in human infections: TcI, TcII, TcV and TcVI. TcI is the main agent of Chagas disease in north of the Amazon, the Andes, Central America and Mexico, where cardiac forms are common and
digestive syndromes are considered uncommon. On the other hand, TcII, TcV and TcVI are the main agents of Chagas disease in the Southern Cone of South America, where cardiac and digestive forms are present (Pinazo et al., 2014; Zingales et al., 2012). The regional pattern of prevalence of the different DTUs and the different frequencies of clinical forms in different regions of Latin America suggest that there could be a relationship between DTUs and clinical forms of Chagas disease. In Argentina, TcV was found to be the most common DTU in human infections (Cardinal et al., 2008; Corrales et al., 2009; Diez et al., 2008; Diosque et al., 2003; M. M. Monje-Rumi et al., 2015). Studies of T. cruzi genetic diversity in cardiac and immunosuppressed patients from Argentina also showed that TcV was the most frequent DTU (Burgos et al., 2010, 2007; Cura et al., 2012; Diez et al., 2010). However, there are scarce studies regarding T. cruzi diversity in patients with digestive or cardio-digestive forms (Vicco et al., 2012).

In the present work, a group of patients from Salta province (Argentina) coursing the chronic phase of Chagas disease were clinically categorized and T. cruzi DTUs circulating in bloodstream of these patients were identified by hybridization assays with specific DNA probes from minicircle hypervariable regions (mHVRs). The possible association of T. cruzi DTUs with the different clinical manifestations of the disease was analysed.

2. Materials and Methods

2.1. Subjects and study area

Patients with chronic Chagas disease were enrolled at San Bernardo Hospital, located in Salta city, Argentina, during the period 2008-2012. The presence of T. cruzi antibodies was tested by Enzyme Linked Immunosorbent Assay, Indirect Haemagglutination and Indirect
Immunofluorescence assays. A patient was considered infected with *T. cruzi* when at least two of the tests were positive, according to public health normative.

2.2. Ethics statement

Before clinical examination and sample collection, patients were asked to participate in the study and signed informed consent was obtained from all the participants. All procedure was approved by the Bioethics Committee of the Faculty of Health Sciences of the National University of Salta, Argentina (RCDN 052-10).

2.3. Clinical examination and exclusion criteria

Clinical evaluation was assessed accurately including physical examination; subjective complaint of frequency and severity of exertional dyspnea; chest X-rays; 12-lead electrocardiograms (ECG); transthoracic echocardiogram and gastrointestinal examinations (esophagogram and Colon X-ray with barium enema opaque examination). Patients with other concomitant pathologies (hypertension, alcoholism, diabetes mellitus, and coronary disease) or any other systemic disease (renal or hepatic) and patients previously treated for Chagas disease were excluded from the study.

Based on clinical records, patients were clinically categorized into four groups: cardiac, digestive, cardio-digestive and asymptomatic presentation. Besides, patients showing cardiac and cardio-digestive forms were further classified into three clinical groups according to Kuschnir classification: Group I: reactive serology, abnormal ECG and chest radiography without cardiac enlargement; Group II: reactive serology, abnormal ECG and chest radiography with cardiac enlargement; and Group III: reactive serology, abnormal ECG and
chest radiography with severe cardiac enlargement (Kuschnir et al., 1985). Patients without complete clinical examination were considered as a separate group.

2.4. Samples

Fifteen millilitres of peripheral blood were obtained by venous puncture. Ten millilitres of blood were used for blood culture; while 5 ml were mixed with an equal volume of a solution of Guanidine 6 M-HCL and 0.2 M EDTA, incubated in water bath at 98 °C for 10 minutes and stored at room temperature until used for DNA extraction.

2.5. Blood cultures

Ten millilitres of heparinized blood were centrifuged (3000 x g, 15 min). The packed red blood cells obtained were washed in liver infusion tryptose (LIT) supplemented with 1% hemin, 20% fetal bovine serum, 100 units/mL of penicillin, and 100 µg/mL of streptomycin and then distributed into five tubes with 2 mL of LIT medium and subsequently incubated at 28 °C. The cultures were monitored with an inverted microscope for 180 days. Negative cultures were discarded. The isolated parasites were maintained by passages in LIT medium at 28 °C. Mass cultures were started in a volume of 2 mL of LIT medium, and additional LIT was gradually added until reaching a final volume of 50 mL. Parasites were harvested by centrifugation (2800 x g, 20 min, 4°C) and washed twice with PBS (Na2HPO410 mM, NaCl 150 mM, pH 7.2). Pellets were stored at -80°C until use.

2.6. DNA extraction and PCR amplification
DNA was obtained from 200µL of the blood/guanidine HCl-EDTA mixture of each patient and from parasite pellets of axenic cultures. A conventional phenol-chlorophorm-isoamyllic protocol for DNA extraction was used. Samples were conserved at -20 ºC until used for amplification of parasite DNA. Parasite detection in blood samples was performed by specific Polymerase Chain Reaction (PCR) amplification of the 330 bp fragment corresponding to the hypervariable regions of the parasite kDNA minicircles (mHVRs). Each sample was tested in duplicate and appropriate controls were used to rule out possible DNA contamination. PCR was carried out in a total volume of 50 µL reaction mix containing: 1X GoTaq amplification buffer, 3mM MgCl2 solution, 0.2mM dNTP, 1.25 U of GoTaq DNA polymerase (Promega, USA), 0.5 µM of kDNA specific primer 121 (5’-AAATAATGTACGGG(T/G)GAGATGCATGA-3’) and 122 (5’-GGTTGATTGGGTGGTGTGAATATA-3’) and 7.5 µL of template DNA. Amplification was performed in a MJR PTC-100 thermocycler (MJ Research, Watertown, MA, USA). Cycling parameters were: one step of 3 min at 94°C; two cycles at 97.5°C for 1 min and 64°C for 3 min, followed by 33 cycles of 1 min at 94°C followed by 1 min at 64°C with a final extension at 72°C for 10 min. Ten microliters of the PCR reaction were visualized in a 2% agarose gel stained with ethidium bromide.

2.7. T. cruzi DTU identification

The kDNA from blood and culture samples was used as target to determine the infecting T. cruzi DTU by PCR-DNA blotting and hybridizations assays with five specific mHVR non-radioactive probes from T. cruzi clones: X10cl1 (TcI), Tu18cl2 (TcII), M5631cl5 (TcIII), LL0555R3cl2 (TcV) and Cl-Brener (TcVI) as described by Monje-Rumi et al. (2015).
Briefly, Southern blot analysis was performed using 10 µL of each 121-122 PCR product. Samples were subjected to electrophoresis and transferred to Hybond N + nylon membranes (Roche Diagnostics, Suiza). The membranes were pre-hybridized for at least 30 minutes at 42°C and individually hybridized with the generated probes. Labelling of the probe and DNA hybridization were performed according to the protocol supplied with the PCR-DIG DNA-labelling and detection kit (Roche Applied Science, Alemania).

2.8. Statistical analysis

The Fisher exact test was used to determine the significance of associations between parasite DTUs and clinical manifestations of the disease. A p-value <0.05 was considered significant. GraphPad Prism version 5.0 (GraphPad Software, San Diego, California, USA) was used to plot the results.

3. Results

3.1. Clinical forms distribution in patients infected with T. cruzi

A total of 205 chronic patients (87 men and 118 women) with a mean age of 49.2 years (range 18-84 years) were evaluated. Clinical examination was completed in 59.5% (122/205) of the enrolled patients; while 40.5% (83/205) had an incomplete clinical examination. Patients with complete clinical examination were categorized into four groups according to clinical forms of the disease: cardiac 38.5% (47/122), digestive 15% (18/122), cardio-digestive 16% (20/122) and asymptomatic 30% (37/122) forms (Table 1).

Fifty-one patients with cardiac involvement (47 cardiac patients and 4 patients with incomplete digestive examination) were categorized according to the Kuschnir classification.
as: Group I, 59% (30/51); Group II, 25% (13/51); and Group III, 16% (8/51). In addition, in 20 patients showing cardio-digestive clinical form, the severity of the cardiomyopathy was classified as: Group I, 40% (8/20); Group II, 45% (9/20); and Group III, 15% (3/20). On the other hand, the digestive involvement of all 38 patients with digestive or cardio-digestive forms was diagnosed as megacolon. In 28% (5/18) of patients who presented exclusively the digestive form of the disease, sigmoid colon enlargement was also observed.

3.2. High prevalence of TcV and mixed infections

*T. cruzi* kDNA was detected in 43% (88/205) of patients. PCR-hybridization with specific probes allows identification of infecting DTUs in 74% (65/88) of the amplified kDNA samples. Single infections (i.e. presence of only one DTU in the sample) were detected in 38.64% (34/88); 35.23% (31/88) showed mixed infections (i.e. more than one DTU in the sample); while no hybridization to the employed probes was observed in 26.14% (23/88). TcV showed the highest frequency (60.3% - 53/88) among the studied samples, both in single (25 samples) as in mixed infections (28 samples). Frequencies of each DTU are shown in Figure 1.

Nine isolates were obtained by hemoculture. DNA from these isolates was examined by using the PCR-hybridization approach. No mixed infections were detected: 6 isolates were TcV and the remaining 3 isolates were TcII. Noteworthy, for those same patients, direct identification by PCR-hybridization from blood samples showed that most of them (8/9) had mixed infections (Table 2).

3.3. TcV and/or TcVI are associated to digestive syndromes
The possible association between the infecting strains and the pathological outcome of Chagas Disease, was assessed considering 60 patients with complete clinical examination. The DTUs identified in these patients are shown in Table 3. No associations between DTUs and clinical manifestations were observed when the defined groups (cardiac, digestive, cardio-digestive and asymptomatic) were analysed. Interestingly, when we compared digestive (i.e. digestive plus cardio-digestive) vs. non-digestive groups (i.e. cardiac plus asymptomatic), we found that only samples positive for TcV and/or TcVI were associated with digestive syndromes. Instead, samples negative for TcV and TcV (i.e. samples that did not hybridize with any probe and a TcI-single infection) corresponded only to non-digestive patients (Fisher test, p=0.001, Figure 2).

4. Discussion

The determining factors of different clinical forms of Chagas disease are still unknown. The possible association between parasite genetic diversity and the pathological outcome of Chagas disease has been suspected for a long time (Campbell et al., 2004; Miles et al., 2009); however, reports on this subject show contrasting results (Apt et al., 2015; D’Ávila et al., 2009; Del Puerto et al., 2010; Díaz et al., 2015; Diez et al., 2010; Martinez-Perez et al., 2016; Ramírez et al., 2010; Rodrigues-dos-Santos et al., 2018). Most of these works found no statistical correlation between clinical forms and the parasite’s genetic diversity (Apt et al., 2015; D’Ávila et al., 2009; Del Puerto et al., 2010; Díaz et al., 2015; Diez et al., 2010; Martínez-Perez et al., 2016; Rodrigues-dos-Santos et al., 2018). On the other hand, association of Chagas cardiomiopathy to TcI (Colombia) (Ramírez et al., 2010) and to TcI/TcII (Chile) were reported (Muñoz-San Martín et al., 2018). Here, we identified the infecting T. cruzi
DTUs in a group of patients in chronic phase of Chagas disease showing different clinical forms (cardiac, cardio-digestive, digestive and indeterminate). We found a statistically significant association between the presence of TcV and/or TcVI and the digestive form of the disease. To the best of our knowledge, this is the first report of statistically supported association between DTUs and Chagas disease with digestive pathology.

We found that 26.14% (23/88) of the samples did not hybridize with any of the designed probes. The intra-DTU reactivity of mHVR probes varies according to the genetic diversity of each DTU (Veas et al., 1991). In this sense, mHVR probes of both genetically homogeneous TcV and TcVI lineages are DTU-specific and they should detect all (or almost all) PCR-positive samples infected by such lineages. In contrast, TcI and TcII show a high genetic intra-DTU diversity (Baptista et al., 2014; Llewellyn et al., 2009; Tomasini et al., 2011). It was demonstrated that mHVR probes from TcI clones hybridize only against genetically close genotypes (Bosseno et al., 2000; Veas et al., 1991, 1990). Moreover, in a recent study we show that shared mHVR classes between two TcI strains was only 17.5%; and between two TcII strains was 7.1%. Conversely, the two TcV strains examined in the mentioned study shared 97.3% of its mHVR classes (Rusman et al., 2019). Taking into account that (i) TcI, TcII, TcV and TcVI are the DTUs described in human infections in Argentina (Cura et al., 2012; Lucero et al., 2016)(and also the DTUs identified in the present work); and (ii) TcI and TcII show a higher genetic diversity than TcV and TcVI; we consider that those samples that did not hybridize with the used probes probably correspond to infections due to TcI and/or TcII. Interestingly, samples that did not hybridize with any probe corresponded only to no-digestive patients (i.e. cardiac and asymptomatic forms of the disease). This is in accordance with the geographic distribution pattern of digestive syndromes.
and DTUs in America where non-TcV-TcVI DTUs are mainly distributed in the south cone where digestive syndromes are more prevalent (Zingales et al., 2012).

We found mixed infections (more than one DTU in a single patient) in 35.23% (31/88) of the studied samples, which is in agreement with previous studies in patients coursing the chronic phase of Chagas disease (Bontempi et al., 2016; Del Puerto et al., 2010; Martinez-Perez et al., 2016; M. Monje-Rumi et al., 2015). In a previous work, several assumed single TcV infections resulted to be mixed infections after treatment (Martinez-Perez et al., 2016). Burgos and collaborators (2010) described the detection of TcI in heart explant of a heart-transplanted patient; while, TcV parasites were found in this patient’s blood and skin during post-transplant reactivation. Here, the hemocultures of nine patients with mixed infection, allowed the identification of only one DTU (Table 2), suggesting a possible selection of parasitic subpopulations during the isolation process. Our results, and previous reports of other authors, suggest that mixed infections are more frequent than previously reported and may be related to incomplete premunition of the primary infections (Tomasini et al., 2017).

The most frequent T. cruzi lineage found among the studied patients was TcV. This result is in agreement with several epidemiological studies carried out in different regions of Argentina, Bolivia and Chile that showed TcV as the most prevalent DTU in human infections from the Southern-cone (Apt et al., 2015; Cura et al., 2012; Del Puerto et al., 2010; Diez et al., 2010; Macchiaverna et al., 2018; M. M. Monje-Rumi et al., 2015; Zingales, 2018).

5. Conclusions

More studies are needed in order to clearly understand the determining factors of the clinical forms of Chagas disease, which surely are a multifactorial phenomenon. However, the
results presented here suggest that patients infected with TcV and/or TcVI will have a higher risk of developing the digestive form of the disease; and show that identification of the infecting *T. cruzi* DTUs could help to determine the risk of future digestive complications in patients.

**Acknowledgements**

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Captions

**Figure 1. Venn diagram showing DTU frequencies found in patients.** The number of each DTU found both in single and mixed infections is shown.

**Figure 2. Association between the detected DTUs and the different clinical manifestations.** TcV-TcVI positive: samples in which at least TcV or TcVI lineages were detected in single or mixed infection. TcV-TcVI negative samples that did not hybridize with any probe including one TcI-single infection: Digestive = digestive + cardio-digestive. Non-digestive: cardiac + asymptomatic
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https://doi.org/10.1016/S0140-6736(17)31612-4


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https://doi.org/10.1371/journal.pntd.0000899


https://doi.org/10.1016/S0140-6736(10)60061-X


https://doi.org/10.1371/journal.pntd.0006939


https://doi.org/10.1016/j.meegid.2010.10.020


Trop. 184, 38–52. https://doi.org/10.1016/j.actatropica.2017.09.017


Table 1. Clinical manifestation and DNA parasite detection by PCR in chronic chagasic patients

<table>
<thead>
<tr>
<th>Group of Patients</th>
<th>Cardiac involvement</th>
<th>Digestive involvement</th>
<th>Number of patients</th>
<th>Men</th>
<th>Women</th>
<th>Age mean ± SD</th>
<th>Positive PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac</td>
<td>+</td>
<td>-</td>
<td>47</td>
<td>22</td>
<td>25</td>
<td>49.47 ± 11.85</td>
<td>23 (49)</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td>10</td>
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<td></td>
<td>13</td>
</tr>
<tr>
<td>Digestive</td>
<td>-</td>
<td>+</td>
<td>18</td>
<td>6</td>
<td>12</td>
<td>44.67 ± 10.82</td>
<td>6 (33)</td>
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<td>4</td>
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<tr>
<td>Cardio-digestive</td>
<td>+</td>
<td>+</td>
<td>20</td>
<td>8</td>
<td>12</td>
<td>52.85 ± 12.57</td>
<td>11 (55)</td>
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<td>5</td>
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<tr>
<td>Asymptomatic</td>
<td>-</td>
<td>-</td>
<td>37</td>
<td>9</td>
<td>28</td>
<td>51.86 ± 12.07</td>
<td>16 (43)</td>
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<tr>
<td>Incomplete</td>
<td>+</td>
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<td>4</td>
<td>4</td>
<td>-</td>
<td>53.75 ± 4.65</td>
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<td>NE</td>
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<td>6</td>
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<td>49.45 ± 11.96</td>
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<tr>
<td><strong>Total</strong></td>
<td>205</td>
<td>87</td>
<td>118</td>
<td>49.92 ± 11.85</td>
<td>88 (43)</td>
<td>41</td>
<td>47</td>
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*NE: not examined: The subjects had no test of: chest X-rays, 12 lead electrocardiograms (ECG), severity of exertional dyspnea, esophagogram and / or Colon X-ray with barium opaque enema examination.
Table 2. Detection *T. cruzi* DTUs in blood culture and blood sample from nine patients with chronic Chagas Disease.

<table>
<thead>
<tr>
<th>Code Patients</th>
<th>Age</th>
<th>Sex</th>
<th>Clinical manifestation</th>
<th>Hybridization with specific probes</th>
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<tr>
<td>ARSTHS19</td>
<td>56</td>
<td>Men</td>
<td>Cardiac II</td>
<td>TcII</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>TcI/TcII/TcV/TcVI</td>
</tr>
<tr>
<td>ARSTHS20</td>
<td>32</td>
<td>Women</td>
<td>ND</td>
<td>TcV</td>
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<td></td>
<td></td>
<td>TcV</td>
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<td>54</td>
<td>Women</td>
<td>Cardiac I</td>
<td>TcV</td>
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<td>TcV/TcVI</td>
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<td>ARSTHS36</td>
<td>22</td>
<td>Women</td>
<td>Digestive</td>
<td>TcV</td>
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<td>TcII/TcVI</td>
</tr>
<tr>
<td>ARSTHS38</td>
<td>51</td>
<td>Men</td>
<td>Cardio-digestive II</td>
<td>TcV</td>
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<td>TcII/TcV</td>
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<tr>
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<td>Men</td>
<td>ND</td>
<td>TcV</td>
</tr>
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<td>TcV/TcVI</td>
</tr>
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<td>Women</td>
<td>Cardio-digestive III</td>
<td>TcV</td>
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<td>TcV/TcVI</td>
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<td>Women</td>
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<td>TcII/TcV</td>
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<td>ND</td>
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<td>TcII/TcV</td>
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ND*: Without clinical examination.
### Table 3. Clinical manifestation and DTU identification in patients with complete clinical examination

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<tr>
<th></th>
<th>TcI</th>
<th>TcV</th>
<th>TcVI</th>
<th>TcII/TcV</th>
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<th>TcV/TcVI</th>
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<th>TcII/TcV/TcVI/TcVI</th>
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<tr>
<td><strong>Digestive</strong></td>
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<td>-</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<td>-</td>
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<td>1</td>
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<td>6</td>
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<td>1</td>
<td>7</td>
<td>19</td>
</tr>
</tbody>
</table>

|                  | 1   | 17  | 4    | 6        | 3         | 8        | 2            | 1                   | 2  | 16    | 60    |
Highlights

- TcV and/or TcVI infections were statistically associated to digestive syndromes
- All the possible clinical forms of Chagas Disease were found
- Mixed infections (more than one DTU) were frequent in the studied patients.
Authors contributions

Monje-Rumi MM: Investigation; Formal analysis
Floridia-Yapur N: Investigation; Formal analysis; Writing - Original Draft
Zago MP: Investigation
Ragone PG: Formal analysis; Writing - Original Draft
Pérez Brandán CM: Investigation
Nuñez S: Resources
Barrientos, N: Resources
Tomasini N: Formal analysis; Writing - Review & Editing
Diosque P: Conceptualization; Founding acquisition; Writing - Review & Editing
Figure 1