Quantification of parasite burden of *Trypanosoma cruzi* and identification of Discrete Typing Units (DTUs) in blood samples of Latin American immigrants residing in Barcelona, Spain

Maykon Tavares de Oliveira, Elena Sulleiro, Aroa Silgado Gimenez, Marta de Lana, Bianca Zingales, João Santana da Silva, J. Antônio Marin-Neto, Israel Molina

1 Department of Infectious Diseases, Universitat Autònoma de Barcelona, Vall d’Hebron University Hospital. PROSICS, Barcelona, Spain, 2 Department of Internal Medicine, Cardiology Division, Medical School of Ribeirão Preto, University of São Paulo (FMRP-USP), Ribeirão Preto, SP, Brazil, 3 Department of Microbiology, Vall d’Hebron University Hospital. Universitat Autònoma de Barcelona PROSICS Barcelona, Spain, 4 School of Pharmacy and Center for Research in Biological Sciences (NUPEB), Federal University of Ouro Preto (UFOP), Ouro Preto, MG, Brazil, 5 Department of Biochemistry, Institute of Chemistry, University of São Paulo (USP), São Paulo, SP, Brazil, 6 Department of Biochemistry and Immunology, Ribeirão Preto Medical School, University of São Paulo (FMRP-USP), Ribeirão Preto, SP, Brazil.

‡ These authors are joint last authors on this work.

Abstract

**Background**

*Trypanosoma cruzi* has a high genetic and biological diversity and has been subdivided into seven genetic lineages, named Tcl-TcVI and TcBat. DTUs Tcl-TcI-TcV and TcVI are agents of ChD in different regions of Latin America. Due to population movements, the disease is an emergent global public health problem. Thus, the aim of this study was to quantify the parasitic load and identify the presence of *T. cruzi* DTUs in 101 Latin American immigrants with chronic ChD, residing in Barcelona, Spain.

**Methodology / Principal findings**

5ml of peripheral blood were collected in guanidine/EDTA from each patient for DNA extraction, quantification of the parasitic load and genotyping. A great variation of the parasitic load of the patients was verified: from 0.001 to 22.2 *T. cruzi* DNA (fg) / Blood DNA (ng). In patients from Bolivia the parasitic load was 3.76±4.43 *T. cruzi* DNA (fg) / Blood DNA (ng) (mean ± SD), in patients of other countries was 0.95±1.38 *T. cruzi* DNA (fg) / Blood DNA (ng). No statistically significant difference was observed in the parasitic load between patients with the indeterminate and cardiac forms of ChD (p = 0.57). Parasite genotyping was performed by multilocus conventional PCR. In patients from Bolivia there was a nearly equal prevalence of DTUs Tcl (27/77), Tcl/TcV/TcVI (26/77), and Tcl/TcVI (22/77). TclV
was detected in only 2 samples (2/77). A higher prevalence of TcII/TcVI (19/24) was verified in patients of other countries, with low prevalence of TcII/TcV/TcVI (4/24) and TcV (1/24).

Conclusions/Significance
In this study, low/medium parasitic load was found in all patients evaluated. Our data corroborate previous conclusions indicating that patients from the Bolivia, living in Spain, are predominantly infected by TcV, and TcVI DTUs. On the other hand, in Non-Bolivians patients TcII/TcVI predominated. Surprisingly, in our cohort of 101 patients no infection by Tcl DTU was observed.

Author summary
Trypanosoma cruzi is divided in seven distinct genetic groups (TcI-TcVI) and TcBat. They can be related to several biological parameters, the main being resistance to specific treatment. Due to the intense migration movements, ChD has become a serious public health problem in Europe. Thus, the work has the important function of identifying the genetic variability of T. cruzi circulating in the European continent, in addition to assessing the parasitic burden present in 101 chronic chagasic patients, residing in Barcelona, Spain. We show differences in the predominance between the infecting DTUs among Bolivian (TcV) and non-Bolivian patients (TcII/TcVI). This is the first study to describe the presence of TcVI genotype in Europe. Although the level of parasite burden is low/medium, it is higher in patients from Bolivia when compared with patients of other countries. The low parasitic burden is a limitation factor for studies aimed at evaluating by qPCR the effects of treating this disease with the drugs available to date, Benznidazole and Nifurtimox, and for clinical trials of new drugs. The information generated in this study should impact planning of more effective public health interventions to improve the health of chagasic patients, control vertical transmission and treatment of ChD.

Introduction
Chagas disease (ChD) is caused by the hemoflagellate protozoan, Trypanosoma cruzi [1]. Approximately 60–70% of the chronic patients have no clinical symptoms (indeterminate form), whereas 30–40% either have or will develop cardiomyopathy, digestive megasymphdromes or both [2]. According to the World Health Organization [3], 6–7 million people are chronically infected with T. cruzi worldwide, and more than 90 million individuals are at risk of infection. T. cruzi is genetically highly diverse and, at present, it has been subdivided into seven genetic lineages or discrete typing units (DTUs), named Tcl to TcVI and TcBat [4,5]. T. cruzi DTUs have distinct, but not exclusive ecological and epidemiological associations [6]. With regard to ChD, DTU Tcl is a major human infection agent in Amazonia, the Andean Region, Central America and Mexico, whereas DTUs TcII, TcV and TcVI are prevalent in patients in the Southern Cone region of South America [6–9].

In recent decades, the population movements from endemic to non-endemic countries have started to create notable changes in the epidemiology of ChD, as T. cruzi has spread worldwide [10,11]. The prevalence of ChD infection in Latin American immigrants living in
Europe is estimated as 4.2%, with the highest prevalence among individuals from Bolivia (18.1%) and Paraguay (5.5%) [12]. Although direct vector transmission cannot occur in the European continent, infected blood transfusion, vertical transmission from mother to fetus and organ transplantation can provide parasite spreading in non-endemic countries [12]. Measures to control vertical transmission have been designed and implemented in some countries in Europe. However, these measures have not been effective [13].

Assessing the *T. cruzi* burden in immigrants from Latin America living in non-endemic countries has important implications for the implementation of medical care, monitoring of vertical transmission, introduction of additional controls for blood banks, training of personnel to diagnose and treat ChD, among others. In this direction, the present investigation aims at evaluating the parasitic load and the genotype of the infecting agent in immigrants from Latin America residing in Barcelona, Spain.

**Materials and methods**

**Study population**

This study included 101 ChD patients who were followed up by the clinical group of Infectious Diseases at Vall d’Hebron University Hospital, Barcelona, Spain, in the period 2015–2019. The patients had two positive serological tests for ChD, according to [3] and positive real-time PCR for *T. cruzi*. Patients were subjected to clinical evaluation consisting of anamnesis, ECG, resting transthoracic echocardiography, chest, esophageal and colon X-ray examination. The patients were classified into different clinical forms of chronic ChD, according to the [14]. Peripheral blood samples (5 mL) were collected and mixed with an equal volume of 6 M Guanidine Hydrochloride / 0.2 M ethylenediaminetetraacetic acid buffer (EDTA) solution, pH 8.0. The Guanidine-EDTA Blood lysates (GEB) were boiled for 15 minutes, incubated at room temperature for 24 h, and stored at 4°C until use [15].

**Ethical clearance**

The study was approved by the Human Research Ethics Committee of the Vall d’Hebron University Hospital. All patients provided written informed consent.

**DNA extraction**

DNA was extracted from 200 μL of GEB samples and eluted with 55 μL of NucliSens easyMAG system (Biomerieux, France), according to the manufacturer’s instructions.

**Parasitic load quantification by qPCR**

The quantitative real-time PCR (qPCR) was performed according to a methodology previously proposed [16], using the multiplex *TaqMan* system targeting the 166 bp region of *T. cruzi* satellite DNA. The qPCR reactions were carried out at 25 μL final volume containing 5 μL DNA from each sample (20 ng/μL), 400 nM of the two primers and 100 nM of the *TaqMan* probe. The Quantitec Multiplex PCR kit (Qiagen, Manchester, United Kingdom) was used and the CFX Real-Time PCR detection system (Bio-Rad, Hercules, CA) used for amplification. The standard curve of the qPCR results was obtained using serial dilutions of 100 ng of DNA extracted from epimastigotes of the strain SO3 cl5 (DTU TcV), with a detection limit of 0.0001 fg, as proposed by [17] and modified by [18]. Positive, negative and reagent internal controls were used in all qPCR reactions.
Genotyping of *Trypanosoma cruzi*

Genotyping of *T. cruzi* in six DTUs (TcI-TcVI) was performed based on multilocus conventional PCR in association with Nested PCR, as described by [15] and modified by [19]. The subsequent identification of genotypes was based on the analysis of the set of profiles of the amplified PCR products presented for each gene target, using the following molecular markers: (i) the intergenic region of the Spliced Leader gene (SL-IRac) using the UTCC and TCac primers; (ii) the intergenic region of Spliced Leader (SL-IR) using TCC, TC1 and TC2 primers; (iii) the variable D7 domain of the 24Sα rRNA gene, with D75, D76 and D71 primers in semi-nested PCR; (iv) the A10 nuclear fragment in semi-nested PCR, with primers Pr1, P6 and Pr3. The PCR systems, gene targets and expected sizes of the amplified products are indicated in Fig 1. In all PCR reactions, DNA control samples from reference strains belonging to the six DTUs and Tcbat were used (Colombiana—TcI; Y—TcII; X109/2—TcIII; CanIII cl1—TcIV; Bug2148 cl1- TcV; CL Brener—TcVI and Tcbat 1994—Tcbat), as well as the negative controls and reagents. All amplification reactions were prepared in a final volume of 30 μL, using 12.5 μL of Mastermix Go Taq Green 2X (Promega, Madison, USA), 5 μL *T. cruzi* extracted DNA, and primers. The PCR cycling conditions were as described [15], using the Thermocycler (G-Storm, model GS 0001). The PCR products were separated by agarose gel electrophoresis (2% or 3% w/v), stained with Syber (Midori Green Advanced DNA Strain, Nippon Genetics Europe GmbH) and viewed on Biorad photo documentation platform (Molecular Imager, Gel DOC XR, Imaging System). Molecular weight markers of 100 bp (Fast Gene Genetics, MWD100) were used to estimate the product size.

**Statistical analysis**

All experiments were performed at least in two technical replicates. Categorical data were expressed as percentages, and continuous data as mean ± standard deviation (SD), or mean interval, according to the normality or nonparametric characteristic of the distribution.
Student’s t-test was used to analyze the significance of statistical differences. Results were deemed as statistically significant when p values were less than 0.05. Analysis was conducted using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

Results

Characteristics of the patients included in this study

This study included 101 patients with chronic ChD, not treated with Benzonidazole or Nifurtimox. All diagnosed by two positive serological tests and positive qPCR for *T. cruzi*, who were followed up at the Infectious Disease Clinic of the Vall d’Hebron University Hospital, Barcelona, in the period between 2015 and 2019. All patients reside in Barcelona and are immigrants from different countries of Latin America: Argentina (~ 8%), Bolivia (~ 77%), Brazil (~ 1%), Ecuador (~ 2%), Honduras (~ 1%), Paraguay (~ 4%), Uruguay (~ 5%) and Venezuela (~ 1%). Two patients were born in Spain, sons of Bolivian immigrants (Table 1). From the patients, 34 (33.7%) were male and 67 (66.3%) females (Table 1). The mean age was 48.2 years (24–80) (Table 1). The indeterminate form of ChD was diagnosed in 53 patients (52.5%) and 48 individuals (47.5%) presented the cardiac form. No patients with the digestive, nervous or mixed clinical forms of ChD were represented in our study population.

Parasitic load

We observed a great variation of the parasitic load in the blood of the 101 patients: from 0.001 to 22.2 *T. cruzi* DNA (fg) / Blood DNA (ng). Regarding the country of origin, the mean ± SD of the parasitic load was 3.76 ± 4.43 *T. cruzi* DNA (fg) / Blood DNA (ng) in the Bolivian group and 0.95 ± 1.38 *T. cruzi* DNA (fg) / Blood DNA (ng) in the non-Bolivian group (Fig 2A). The data were statistically significant with a p value of 0.00029.

No statistically significant difference was observed in the parasitic load between patients with the indeterminate and cardiac forms of ChD (Fig 2B).

*Trypanosoma cruzi* genotyping

In all samples we applied the multilocus conventional PCR to perform the genotyping of the infecting DTUs. However, in samples which had a very low parasitic load we could not obtain amplified products of all the genes necessary for the molecular characterization of *T. cruzi* (Fig 3; Tables 1 and 2). In 27 DNA samples from Bolivian patients (27/77) the products confirmed DTU TcV infection, whereas in two Bolivian patients DTU TcVI was found. 22 Bolivian patients (22/77) had a genetic profile indicating infection by TcII/TcVI DTUs. In the remaining 26 patients from Bolivia (26/77) the amplified products suggested infection with TcII/TcV/TcVI DTUs.

The non-Bolivian patients (24/101) were infected by DTUs TcV (Argentina), TcII/TcVI (Argentina, Ecuador, Honduras, Paraguay, Spain and Venezuela) and TcII/TcV/TcVI (Argentina, Brazil, Uruguay and Paraguay) (Tables 1 and 2).

Discussion

*T. cruzi* is composed of heterogeneous subpopulations that circulate in both domestic and wild cycles [20], and this diversity can be observed at the morphological [1,21], biological [22], antigenic [23] and at a genetic level [24,25]. Moreover, the parasite species are currently subdivided into seven distinct genetic groups (DTUs TcI–TcVI), and the Tcbat [4], with the additional fact that each DTU has its own characteristics [5]. In order to better understand the
Table 1. General information of patients involved in the study and criteria for genotyping of *Trypanosoma cruzi*.

| SAMPLE CODE | AGE (years) | GENDER | COUNTRY OF ORIGIN | CLINICAL FORM | PARASITE LOAD (T. cruzi DNA (fg) / Blood DNA (ng)) | TARGET GENES | DTU’S |
|-------------|-------------|--------|-------------------|---------------|--------------------------------------------------|--------------|-------|
|             |             |        |                   |               | SLIR-ac SL-IR I and II 245s rDNA                  |              |       |
| 1           | 32          | F      | Bolivia           | Cardiac       | 10.10 157bp 300bp 125bp Neg                       | TcV          |       |
| 2           | 39          | F      | Bolivia           | Indeterminate | 2.49 157bp 300bp 125bp Neg                       | TcII/TcV/TcVI |       |
| 3           | 27          | F      | Bolivia           | Indeterminate | 6.74 157bp 300bp 125bp Neg                       | TcV          |       |
| 4           | 43          | M      | Bolivia           | Cardiac       | 6.75 157bp 300bp 125bp Neg                       | TcV          |       |
| 5           | 43          | M      | Bolivia           | Indeterminate | 1.88 157bp 300bp 125bp Neg                       | TcV          |       |
| 6           | 65          | F      | Bolivia           | Cardiac       | 1.52 157bp 300bp 125bp Neg                       | TcV          |       |
| 7           | 49          | F      | Bolivia           | Cardiac       | 6.21 157bp 300bp Neg                            | TcII/TcV/TcVI |       |
| 8           | 68          | F      | Bolivia           | Cardiac       | 9.36 157bp 300bp 125bp Neg                       | TcV          |       |
| 9           | 39          | M      | Bolivia           | Indeterminate | 1.41 157bp 300bp 125bp Neg                       | TcV          |       |
| 10          | 60          | F      | Bolivia           | Indeterminate | 1.81 157bp 300bp 125bp Neg                       | TcV          |       |
| 11          | 41          | M      | Bolivia           | Indeterminate | 4.57 157bp 300bp 125bp Neg                       | TcV          |       |
| 12          | 39          | M      | Bolivia           | Indeterminate | 13.31 157bp 300bp 125bp Neg                      | TcV          |       |
| 13          | 43          | M      | Bolivia           | Cardiac       | 1.29 157bp 300bp 125bp Neg                       | TcV          |       |
| 14          | 46          | M      | Bolivia           | Indeterminate | 3.21 157bp 300bp 125bp Neg                       | TcV          |       |
| 15          | 43          | F      | Bolivia           | Indeterminate | 1.34 157bp 300bp Neg                            | TcII/TcV/TcVI |       |
| 16          | 38          | F      | Bolivia           | Cardiac       | 3.98 157bp 300bp 125bp Neg                       | TcV          |       |
| 17          | 56          | F      | Bolivia           | Indeterminate | 7.97 157bp 300bp 125bp Neg                       | TcV          |       |
| 18          | 67          | F      | Bolivia           | Indeterminate | 1.00 157bp 300bp 125bp Neg                       | TcV          |       |
| 19          | 59          | F      | Bolivia           | Cardiac       | 4.22 157bp 300bp 140bp 525Pb                     | TcVI         |       |
| 20          | 35          | M      | Bolivia           | Indeterminate | 0.31 157bp 300bp 125bp Neg                       | TcV          |       |
| 21          | 44          | F      | Bolivia           | Indeterminate | 0.98 157bp 300bp 125bp Neg                       | TcV          |       |
| 22          | 56          | F      | Bolivia           | Indeterminate | 1.70 157bp 300bp 125bp Neg                       | TcV          |       |
| 23          | 54          | F      | Bolivia           | Cardiac       | 0.39 157bp 300bp Neg                            | TcII/TcV/TcVI |       |
| 24          | 28          | M      | Bolivia           | Indeterminate | 3.58 157bp 300bp 125bp Neg                       | TcV          |       |
| 25          | 27          | F      | Bolivia           | Indeterminate | 8.97 157bp 300bp 125bp Neg                       | TcV          |       |
| 26          | 26          | M      | Bolivia           | Indeterminate | 6.65 157bp 300bp Neg                            | TcV          |       |
| 27          | 39          | F      | Bolivia           | Indeterminate | 2.70 157bp 300bp 125bp Neg                       | TcV          |       |
| 28          | 37          | F      | Bolivia           | Indeterminate | 5.78 157bp 300bp 125bp Neg                       | TcV          |       |
| 29          | 56          | F      | Bolivia           | Indeterminate | 8.56 157bp 300bp 125bp Neg                       | TcV          |       |
| 30          | 60          | F      | Bolivia           | Cardiac       | 6.86 157bp 300bp 125bp Neg                       | TcV          |       |
| 31          | 33          | M      | Bolivia           | Indeterminate | 3.10 157bp 300bp 125bp 525Pb                     | TcVI         |       |
| 32          | 62          | F      | Bolivia           | Cardiac       | 0.10 157bp 300bp Neg                            | TcII/TcV/TcVI |       |
| 33          | 44          | F      | Bolivia           | Cardiac       | 4.81 157bp 300bp Neg                            | TcII/TcV/TcVI |       |
| 34          | 45          | F      | Bolivia           | Cardiac       | 12.94 157bp 300bp Neg                           | TcII/TcV/TcVI |       |
| 35          | 47          | M      | Bolivia           | Indeterminate | 7.92 157bp 300bp Neg                            | TcII/TcV/TcVI |       |

(Continued)
Table 1. (Continued)

| SAMPLE CODE | AGE (years) | GENDER | COUNTRY OF ORIGIN | CLINICAL FORM | PARASITE LOAD (T. cruzi DNA (fg) / Blood DNA (ng)) | TARGET GENES | DTU’S |
|-------------|-------------|--------|-------------------|---------------|--------------------------------------------------|--------------|-------|
| 36          | 53          | F      | Bolivia           | Cardiac       | 1.93                                              | SLIR-ac       | TcV   |
| 37          | 71          | F      | Bolivia           | Cardiac       | 2.24                                              | SLIR-1 and II| TcII/TcV/TcVI |
| 38          | 56          | F      | Bolivia           | Indeterminate | 5.30                                              | SL-IR I and II| TcV   |
| 39          | 64          | F      | Bolivia           | Indeterminate | 2.94                                              | 125bp        | TcII/TcV/TcVI |
| 40          | 64          | M      | Bolivia           | Indeterminate | 8.97                                              | 300bp        | TcII/TcV/TcVI |
| 41          | 44          | M      | Bolivia           | Cardiac       | 5.05                                              | 125bp        | TcII/TcV/TcVI |
| 42          | 80          | F      | Bolivia           | Cardiac       | 20.87                                             | 300bp        | TcII/TcV/TcVI |
| 43          | 36          | M      | Bolivia           | Indeterminate | 1.87                                              | 300bp        | TcII/TcV/TcVI |
| 44          | 68          | M      | Bolivia           | Indeterminate | 1.85                                              | 300bp        | TcII/TcV/TcVI |
| 45          | 62          | M      | Bolivia           | Cardiac       | 22.20                                             | 300bp        | TcII/TcV/TcVI |
| 46          | 42          | F      | Bolivia           | Indeterminate | 10.70                                             | 300bp        | TcII/TcV/TcVI |
| 47          | 48          | M      | Bolivia           | Indeterminate | 6.14                                              | 300bp        | TcII/TcV/TcVI |
| 48          | 53          | F      | Bolivia           | Cardiac       | 0.26                                              | 300bp        | TcII/TcV/TcVI |
| 49          | 43          | F      | Bolivia           | Cardiac       | 0.98                                              | 300bp        | TcII/TcV/TcVI |
| 50          | 45          | F      | Bolivia           | Cardiac       | 0.79                                              | 300bp        | TcII/TcV/TcVI |
| 51          | 51          | F      | Bolivia           | Indeterminate | 1.02                                              | 300bp        | TcII/TcV/TcVI |
| 52          | 43          | F      | Bolivia           | Cardiac       | 1.58                                              | 300bp        | TcII/TcV/TcVI |
| 53          | 50          | F      | Bolivia           | Indeterminate | 0.84                                              | 300bp        | TcII/TcV/TcVI |
| 54          | 43          | F      | Bolivia           | Cardiac       | 0.18                                              | 300bp        | TcII/TcV/TcVI |
| 55          | 39          | F      | Bolivia           | Indeterminate | 0.11                                              | 300bp        | TcII/TcV/TcVI |
| 56          | 49          | M      | Bolivia           | Indeterminate | 0.08                                              | 300bp        | TcII/TcV/TcVI |
| 57          | 37          | F      | Bolivia           | Indeterminate | 0.001                                             | 300bp        | TcII/TcV/TcVI |
| 58          | 49          | M      | Bolivia           | Indeterminate | 0.69                                              | 300bp        | TcII/TcV/TcVI |
| 59          | 70          | F      | Bolivia           | Cardiac       | 0.11                                              | 300bp        | TcII/TcV/TcVI |
| 60          | 47          | M      | Bolivia           | Indeterminate | 5.89                                              | 300bp        | TcII/TcV/TcVI |
| 61          | 57          | M      | Bolivia           | Indeterminate | 0.67                                              | 300bp        | TcII/TcV/TcVI |
| 62          | 42          | F      | Bolivia           | Indeterminate | 7.40                                              | 300bp        | TcII/TcV/TcVI |
| 63          | 46          | F      | Bolivia           | Cardiac       | 0.36                                              | 300bp        | TcII/TcV/TcVI |
| 64          | 42          | F      | Bolivia           | Indeterminate | 4.05                                              | 300bp        | TcII/TcV/TcVI |
| 65          | 70          | F      | Bolivia           | Cardiac       | 2.24                                              | 300bp        | TcII/TcV/TcVI |
| 66          | 75          | F      | Bolivia           | Cardiac       | 0.22                                              | 300bp        | TcII/TcV/TcVI |
| 67          | 39          | F      | Bolivia           | Indeterminate | 0.05                                              | 300bp        | TcII/TcV/TcVI |
| 68          | 45          | F      | Bolivia           | Indeterminate | 0.36                                              | 300bp        | TcII/TcV/TcVI |
| 69          | 69          | F      | Bolivia           | Indeterminate | 0.10                                              | 300bp        | TcII/TcV/TcVI |

(Continued)
disease in each geographical region, it is important to study the molecular epidemiology of this parasite, which is naturally related to the main biological characteristics that have already been mentioned.

Currently immigration from Latin American countries to Europe has increased, especially in southern European countries such as Spain and Italy [26]. Since a considerable proportion

| SAMPLE CODE | AGE (years) | GENDER | COUNTRY OF ORIGIN | CLINICAL FORM | PARASITE LOAD (T. cruzi DNA (fg) / Blood DNA (ng)) | GENOTYPING CRITERION | TARGET GENES | DTU'S |
|-------------|-------------|--------|-------------------|---------------|-------------------------------------------------|----------------------|-------------|-------|
| 70          | 33          | F      | Bolivia           | Cardiac       | 1.23                                            | SLR-ac               | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 71          | 66          | F      | Bolivia           | Cardiac       | 0.001                                          | SL-IR I and II       | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 72          | 37          | F      | Bolivia           | Cardiac       | 0.13                                           | 24S rDNA             | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 73          | 60          | F      | Bolivia           | Cardiac       | 0.04                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 74          | 35          | F      | Bolivia           | Cardiac       | 0.47                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 75          | 45          | F      | Bolivia           | Cardiac       | 3.20                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 76          | 52          | M      | Bolivia           | Indeterminate | 1.48                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 77          | 67          | F      | Bolivia           | Cardiac       | 0.43                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 78          | 42          | F      | Argentina         | Cardiac       | 1.46                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 79          | 59          | M      | Uruguay           | Indeterminate | 4.66                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 80          | 33          | F      | Argentina         | Indeterminate | 4.67                                           |                     | 157bp       | 300bp | 125bp | Neg   | TcV        |
| 81          | 24          | M      | Paraguay          | Indeterminate | 0.10                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 82          | 54          | F      | Brazil            | Cardiac       | 0.17                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 83          | 73          | F      | Paraguay          | Cardiac       | 2.41                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 84          | 63          | M      | Honduras          | Indeterminate | 1.25                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 85          | 28          | M      | Uruguay           | Cardiac       | 0.87                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 86          | 47          | M      | Uruguay           | Indeterminate | 0.23                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 87          | 42          | F      | Spain             | Cardiac       | 0.78                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 88          | 40          | F      | Paraguay          | Cardiac       | 0.001                                          |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 89          | 69          | M      | Paraguay          | Indeterminate | 1.42                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 90          | 47          | M      | Uruguay           | Indeterminate | 2.81                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 91          | 42          | F      | Spain             | Cardiac       | 0.001                                          |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 92          | 40          | F      | Argentina         | Indeterminate | 0.17                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 93          | 39          | F      | Argentina         | Cardiac       | 0.001                                          |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 94          | 32          | F      | Uruguay           | Indeterminate | 0.004                                          |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 95          | 43          | M      | Ecuador           | Cardiac       | 0.48                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 96          | 46          | M      | Argentina         | Indeterminate | 0.47                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 97          | 43          | F      | Argentina         | Cardiac       | 0.001                                          |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 98          | 42          | M      | Ecuador           | Cardiac       | 0.74                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 99          | 35          | F      | Venezuela         | Cardiac       | 0.001                                          |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 100         | 64          | F      | Argentina         | Cardiac       | 0.001                                          |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |
| 101         | 43          | M      | Argentina         | Cardiac       | 0.08                                           |                     | 157bp       | 300bp | 140bp | Neg   | TcII/TcVI   |

F = Female, M = Male; bp = Base Pair; Neg = Negative; fg = Femtogram; ng = Nanogram; TcI, TcII, TcV and TcVI = T. cruzi genetic group; DTU’s = Discrete Typing Units.

https://doi.org/10.1371/journal.pntd.0008311.t001
of Latin American immigrants may be infected with *T. cruzi*, the epidemiology of ChD, originally endemic in Latin America, has changed considerably [10,27]. As a consequence, the number of reported cases of ChD with or without cardiac involvement has increased dramatically in recent years, especially in Spain, Italy and Switzerland [28,29].

In the present study we evaluated the parasitic load in the peripheral blood of 101 individuals serologically and real-time PCR positive for ChD, residing in Barcelona, Spain. Most of the patients were from Bolivia (77%). Patients from countries of the Southern Cone (Argentina, Brazil, Paraguay and Uruguay), northern South America (Ecuador and Venezuela) and Central America (Honduras) were also included. A wide variation of the parasitic load was observed among the patients and in most of them parasitemia was low / medium. Interestingly, the mean ± SD of the parasitic load of the Bolivian group (3.76 ± 4.43 *T. cruzi* DNA (fg) / Blood DNA (ng)) was higher than that of the group of patients from other countries (0.95 ± 1.38 *T. cruzi* DNA (fg) / Blood DNA (ng)).

In support to our conclusions, several studies employing quantitative real-time PCR (qPCR) have reported low / medium parasitic load values in chronic ChD patients of different countries of Latin America [30–33].

To investigate the impact of transfusion-acquired *T. cruzi* infection, [34] investigated blood donors who originated from Chagas-endemic areas and resided in the Mallorca Islands (Spain). Seropositivity for ChD was found in 23 (1.9%) of 1,201 donors and *T. cruzi* DNA with less than 1 parasite equivalent / mL was detected in the peripheral blood of 60.86% (14 of 23). Of the 14 patients in which circulating *T. cruzi* DNA was detected, 10 were from Bolivia, 3 from Argentina and 1 from Venezuela.

Higher parasitic load ranging from 1.43–11.14 parasite equivalents/mL (median 2.54) was reported in 65 chronic ChD patients from different regions of Brazil [15]. In a study similar to ours, the authors characterized the infectious DTU in 28 patients. They verified the prevalence of TcVI, TcII and mixed infection TcVI + TcII. When *T. cruzi* genotypes were compared with the parasite load, more elevated parasite loads were observed in patients infected by TcII (median of 7.56 par. Eq./mL) in comparison to patients infected by TcVI (median of 2.35 par. Eq./mL) [15].

In the present study we observed that patients from Bolivia (~77%) showed nearly equal prevalence of infections by TcV, TcII/TcVI and TcII/TcV/TcVI genotypes. In contrast, TcII/
TcVI prevailed in patients from Argentina (~8%), Paraguay (~ 4%) and Uruguay (~ 5%). Of note, TcIII and TcIV were not identified in any patient, nor was TcI.

A previous study [35] also characterized the infecting DTU in peripheral blood samples of 10 migrants from Bolivia who attended hospitals in the Barcelona area. In agreement with our

Table 2. Genotyping of Trypanosoma cruzi from peripheral blood of chronic chagasic immigrant patients.

| DTU’S      | NUMBER OF PATIENTS | GEOGRAPHICAL ORIGIN                                      |
|------------|--------------------|----------------------------------------------------------|
| TcV        | 28                 | Bolivia and Argentina                                     |
| TcVI       | 2                  | Bolivia                                                  |
| TcII/TcVI  | 41                 | Argentina, Bolivia, Ecuador, Honduras, Paraguay, Spain, Uruguay and Venezuela |
| TcII/TcV/TcVI | 30             | Argentina, Bolivia, Brazil, Paraguay and Uruguay. |

https://doi.org/10.1371/journal.pntd.0008311.t002
observations, in five samples TcV was identified; in three samples a TcII/V/VI profile was obtained and in the remaining two samples mixed infections TcV plus TcII/VI and TcV plus TcII was reported.

The DTUs infecting Latin American migrants attending a reference Clinic in Madrid was also defined [36]. As in our cohort, patients from Bolivia predominated (~90%). Overall, the most common DTU found was TcV (55.2%), followed by TcIV (16.2%), TcII (9.5%) and TcI (3.8%).

The scenario of the distribution of T. cruzi DTUs in ChD patients in countries of North, Central and South America has been outlined [5], based on data of [9] who surveyed articles in which approximately 6,400 DTUs were classified according to their geographical origin and hosts.

Our data regarding DTUs infecting migrants from Latin American countries residing in Barcelona follows the pattern of the geographic distribution of DTUs in the countries of origin. Two aspects stand out: To the best of our knowledge, this is the first study to describe the presence of TcVI genotype in the European continent in Bolivian patients with chronic ChD. TcI DTU was not found in any sample analyzed.

TcVI is highly related to the domestic cycle of ChD in some regions of the Southern Cone [6]. It is involved in human infections in the Chaco region in Northern Argentina; in Chile [37,38], and Brazil, more specifically in an outbreak of oral transmission in Santa Catarina state [39] and in endemic disease area in Minas Gerais state [40].

TcI DTU has a wide geographical distribution. TcI isolates are prevalent in patients from North America (Mexico and the United States); countries of Central America and northern South America (Colombia and Venezuela). Human TcI are abundant in Chile and the Brazilian Amazonia [5]. The fact that we did not find TcI in our cohort most probably is due to the low representativeness of individuals from Honduras and Venezuela or to the low abundance of this DTU in the sample.

We attempted to look for a possible association between the genotype of the parasite and the clinical presentation of ChD in the chronic phase. But, as discussed previously [5] we found none. We also found no correlation between the level of the parasite load and the infecting DTU.

Thus, knowing the parasite load and genetic variability of T. cruzi in chronic immigrant patients may be crucial to understanding the public health implications of ChD in European countries. Enhancing this understanding can allow for appropriate conception and planning of more effective public health interventions to improve the health of immigrants and control vertical transmission, which is a serious problem in European today.

Conclusions

The data of this study corroborate previous reports indicating the prevalence of patients from Bolivia among the Latin American immigrants residing in Barcelona. We show differences in the infecting DTUs between Bolivian and non-Bolivian patients. This is the first study to describe the presence of TcVI genotype in European continent. Although the level of parasite burden is low / medium in the patients, it is higher in patients from Bolivia as compared with patients of other countries. The information generated in this study should impact planning of more effective public health interventions to improve the health of immigrants, control vertical transmission and treatment of ChD.

Acknowledgments

We thank Pilar Alcubilla for her technical support.
Author Contributions

Conceptualization: Maykon Tavares de Oliveira, Elena Sulleiro, Aroa Silgado Gimenez, Israel Molina.

Data curation: Maykon Tavares de Oliveira, Elena Sulleiro, Israel Molina.

Formal analysis: Maykon Tavares de Oliveira.

Funding acquisition: Maykon Tavares de Oliveira, J. Antônio Marin-Neto.

Investigation: Maykon Tavares de Oliveira, Marta de Lana, Bianca Zingales, João Santana da Silva.

Methodology: Maykon Tavares de Oliveira, Elena Sulleiro, Aroa Silgado Gimenez.

Project administration: J. Antônio Marin-Neto, Israel Molina.

Resources: Maykon Tavares de Oliveira, Elena Sulleiro, Marta de Lana, Bianca Zingales, Israel Molina.

Supervision: J. Antônio Marin-Neto, Israel Molina.

Validation: Maykon Tavares de Oliveira.

Visualization: Maykon Tavares de Oliveira, Elena Sulleiro, Aroa Silgado Gimenez, Marta de Lana, Bianca Zingales, J. Antônio Marin-Neto, Israel Molina.

Writing – original draft: Maykon Tavares de Oliveira.

Writing – review & editing: Maykon Tavares de Oliveira, Marta de Lana, Bianca Zingales, João Santana da Silva, J. Antônio Marin-Neto, Israel Molina.

References

1. Chagas C. Nova tripanozomiase humana. Estudos sobre a morfologia e o ciclo evolutivo do Schizotrypanum cruzi n. gen., n. sp., agente etiológico de nova entidade morbida do homem. Mem Inst Oswaldo Cruz. 1909; 159–218.

2. Marin-Neto JA, Rassi A Jr, Maciel BC, Simoes MV, Schmidt A 2010. Chagas heart disease. In Yusuf S, Cairns JA, Camm AJ, Fallen EL, Gersh BJ (eds.), Evidence-based cardiology, 3rd ed., BMJ Books, London, p. 823–841.

3. WHO—Chagas disease (American trypanosomiasis). Fact sheet N˚340. Updated March 2018.

4. Zingales B, Andrade SG, Briones MRS, Campbell DA, Chiarini E, Fernandes O, Guhl F, Lages-Silva E, Macedo AM, Machado CR, Miles MA, Romanha AJ, Sturm NR, Tibayrenc M, Schijman AG. A new consensus for Trypanosoma cruzi intraspecific nomenclature: second revision meeting recommends TcI to TcVI. Mem Inst Oswaldo Cruz. 2009; 104: 1051–1054. https://doi.org/10.1590/S0074-02762009000700021 PMID: 20027478

5. Zingales B. Trypanosoma cruzi genetic diversity: Something new for something known about Chagas disease manifestations, serodiagnosis and drug sensitivity. Acta Trop. 2018 Aug; 184:38–52. https://doi.org/10.1016/j.actatropica.2017.09.017 Epub 2017 Sep 21. PMID: 28941731

6. Zingales B, Miles AM, Campbell AD, Tibayrenc M, Macedo AM, Teixeira MMG, Schijman AG, Llewellyn SM, Lages-Silva E, Machado RC, Andrade SG, Sturm NR. The revised Trypanosoma cruzi subspecific nomenclature: Rationale, epidemiological relevance and research applications. Infect Genet Evol. 2012; 12:420–53. https://doi.org/10.1016/j.meegid.2011.12.009 PMID: 22226704

7. Miles MA, Llewellyn MS, Lewis MD, Yeo M, Baleela R, Fitzpatrick S, Gaunt MW, Mauricio IL 2009. The molecular epidemiology and phylogeography of Trypanosoma cruzi and parallel research on Leishmania: looking back and to the future. Parasitology 136: 1509–1528.

8. Guhl F, Ramírez JD 2011. Trypanosoma cruzi I diversity: towards the need of genetic subdivision? Acta Trop. 119: 1–4.

9. Brenière S.F., Waleckx E., Bamabé C., 2016. Over six thousand Trypanosoma cruzi strains classified into discrete typing units (DTUs): attempt at an inventory. PLoS Negl. Trop. Dis. 10, e0004792.
10. Basile L, Jansa JM, Carlier Y, Salamanca DD, Angeheben A (2011). Chagas disease in European countries: the challenge of a surveillance system. Euro Surveill 16.

11. Requena-Méndez A, Aldasoro E, De Lazzari E, Sicuri E, Brown M, Moore DA, Gascon J, Muñoz J. Prevalence of Chagas disease in Latin-American migrants living in Europe: a systematic review and meta-analysis. PLoS Negl Trop Dis. 2015 Feb 13; 9(2): e0003540. https://doi.org/10.1371/journal.pntd.0003540 eCollection 2015 Feb. PMID: 25680190

12. Pérez-Molina JA, Molina I. Chagas disease. Lancet. 2018 Jan 6; 391(10115):82–94. https://doi.org/10.1016/S0140-6736(17)31612-4 Epub 2017 Jun 30.

13. Munoz J, Coll O, Juncosa T, Verges M, Del Pino M, (2009) Prevalence and vertical transmission of Trypanosoma cruzi infection among pregnant Latin American women attending 2 maternity clinics in Barcelona, Spain. Clin Infect Dis 48: 1736–1740. https://doi.org/10.1086/599223 PMID: 19438393

14. Dias JC, Ramos AN Jr, Gontijo ED, Luquetti A, Shikanai-Yasuda MA, Coura JR, Torres RM, Melo JR, Almeida EA, Oliveira W Jr, Silveira AC, Rezende JM, Pinto FS, Ferreira AW, Russi A, Fragata AA Filho, Sousa AS, Correia D Filho, Jansen AM, Andrade GM, Britto CF, Pinto AY, Russi A Jr, Campos DE, Abad-Franch F, Santos SE, Chiarle E, Hasslocher-Moreno AM, Moreira EF, Marques DS, Silva EL, Marin-Neto JA, Galvão LM, Xavier SS, Valente SA, Carvalho NB, Cardoso AV, Silva RA, Costa VM, Vivaldini SM, Oliveira SM, Valente VD, Lima MM, Alves RV (Brazilian Consensus on Chagas Disease, 2015). Epidemiol Serv Saude. 2016 Jun;25(sp3):7–86. https://doi.org/10.5123/S1679-4974201600000002 PMID: 27869914

15. Rodrigues-Dos-Santos I, Molo MF1, de Castro L. Hasslocher-Moreno AM, do Brasil PEA, Silvestre de Sousa A, Britto C, Moreira OC. Exploring the parasite load and molecular diversity of Trypanosoma cruzi in patients with chronic Chagas disease from different regions of Brazil. PLoS Negl Trop Dis. 2018 Nov 12; 12(11):e0006939. https://doi.org/10.1371/journal.pntd.0006939 PMID: 30418976

16. Piron M, Fisa R, Casamitjana N, López-Chejade P, Puig L, Vergés M, Gascon J, Gómez i Prat J, Portús M. Sauleda S. Development of a real-time PCR assay for Trypanosoma cruzi detection in blood samples. Acta Trop. 2007 Sep; 103(3):195–200. Epub 2007 Jun 23.

17. Cummings KL, Tarleton RL. Rapid quantitation of Trypanosoma cruzi in host tissue by real-time PCR. Mol Biochem Parasitol. 2003 Jun; 129(1):53–9.

18. Silva MC, Davoli-Ferreira M, Medina TS, Sesti-Costa R, Silva GK, Lopes CD, Cardozo LE, Gava FN, Lyroni K, Dias FC, Frade AF, Baron M, Nakaya H, Figueiredo F, Alves-Filho JC, Cunha FQ, Tsatsanis C, Chevillard C, Cunha-Neto E, Hirsch E, Silva JS, Cunha TM. Canonical PI3K signaling in myeloid cells restricts Trypanosoma cruzi infection and dampens chagasic myocarditis. Nat Commun. 2018 Apr 17; 9(1):1513. https://doi.org/10.1038/s41467-018-03986-3 PMID: 29966415

19. da Cruz Moreira Otacílio; Ramirez Juan Carlos. Genotyping of Trypanosoma cruzi from Clinical Samples by Multilocus Conventional PCR. In: Karina Andrea Gómez; Carlos Andrés Buscaglia. (Org.). Methods in Molecular Biology. 1ed.: Springer New York, 2019, v. 1955, p. 227–238.

20. Tibayrenc M, Ward P, Moya A, Ayala FJ. Natural populations of Trypanosoma cruzi, the agent of Chagas disease, have a complex multiclonal structure. Proc NatAcad. Sci. 1986; 83:115–119.

21. Brener Z, Chiari E. (Morphological variations observed in different strains of Trypanosoma cruzi), the agent of Chagas disease, have a complex multiclonal structure. Proc NatAcad. Sci. 1986; 83:115–119.

22. Andrade SG. Caracterização de cepas de Trypanosoma cruzi isoladas no Recôncavo Baiano. Rev Inst Med Trop Sao Paulo. 1963 Sep-Oct; 5:220–4.

23. Maguire JH, Hoff R, Sleigh AC, Mott KE, Ramos NB, Sherlock IA. An outbreak of Chagas’ disease in southwestern Bahia, Brazil. Am J Trop Med Hyg. 1986 Sep; 35(5):931–6.

24. Macedo AM, Machado CR, Oliveira RP, Penha SD. Trypanosoma cruzi: genetic structure of populations and relevance of genetic variability to the pathogenesis of Chagas disease. Mem Inst Oswaldo Cruz. 2004; 99:1–12. https://doi.org/10.1590/S0074-027620040000100001 PMID: 15057339

25. Tibayrenc M, Ayala FJ. The population genetics of Trypanosoma cruzi revisited in the light of the predominant clonal evolution model. Acta Trop. 2015 Nov; 151:156–65. https://doi.org/10.1016/j.actatropica.2015.06.008 PMID: 26188332

26. Rechel B, Miladovszy P, Ingleby D, Mackenbach JP, Mckee M (2013) Migration and health in an increasingly diverse Europe. Lancet 381: 1235–1245. https://doi.org/10.1016/S0140-6736(12)62086-8 PMID: 23541058

27. Gascon J, Bern C, Pinazo MJ. Chagas disease in Spain, the United States and other non- endemic countries. Acta Trop. 2010 Jul-Aug; 115(1–2):22–7. https://doi.org/10.1016/j.actatropica.2009.07.019 Epub 2009 Jul 29. PMID: 19646412

28. Jackson Y, Gélat L, Wolff H, Holst M, Mauris A, Tardin A, Sztajzel J, Besse V, Loutan L, Gaspoz JM, Jannin J, Albajar Vinas P, Luquetti A, Chappuis F. Prevalence, clinical staging and risk for blood-borne transmission of Chagas disease among Latin American migrants in Geneva, Switzerland. PLoS Negl Trop Dis. 2010 Feb 2; 4(2):e592. https://doi.org/10.1371/journal.pntd.0000592 PMID: 20126397
29. Coura JR, Viñas PA. Chagas disease: a new worldwide challenge. Nature. 2010 Jun 24; 465(7301): S6–7. https://doi.org/10.1038/nature09221 PMID: 20571554

30. Duffy T, Cura CI, Ramírez JC, Abate T, Cayo NM, Parrado R, Bello ZD, Velázquez E, Muñoz-Calderon A, Juiz NA, Basile J, García L, Riarte A, Nasser JR, Yacono ZD, Torrico F, de Noya BA, Ribeiro I, Schijman AG. Analytical performance of a multiplex Real-Time PCR assay using TaqMan probes for quantification of *Trypanosoma cruzi* satellite DNA in blood samples. PLoS Negl Trop Dis. 2013; 7(1): e2000. https://doi.org/10.1371/journal.pntd.0002000 PMID: 23350002

31. Moreira OC, Ramírez JD, Velañez E, Melo MF, Lima-Ferreira C, Guli F, Sosa-Estani S, Marin-Neto JA, Morillo CA, Britto C. Towards the establishment of a consensus real-time qPCR to monitor *Trypanosoma cruzi* parasitemia in patients with chronic Chagas disease cardiomyopathy: a substudy from the BENEFIT trial. Acta Trop. 2013 Jan; 125(1):23–31. https://doi.org/10.1016/j.actatropica.2012.08.020 PMID: 22982466

32. Hernández C, Cucunubá Z, Flórez C, Olivera M, Valencia C, Zambrano P, León C, Ramírez JD. Molecular Diagnosis of Chagas Disease in Colombia: Parasitic Loads and Discrete Typing Units in Patients from Acute and Chronic Phases. PLoS Negl Trop Dis. 2016 Sep 20; 10(9):e0004997. https://doi.org/10.1371/journal.pntd.0004997 PMID: 27648938

33. D’Ávila DA, Galvão LMC, Sousa GR, Britto C, Moreira OC, Chiari E. Monitoring the parasite load in chronic Chagas disease patients: comparison between blood culture and quantitative real time PCR. PLoS One. 2018 Nov 29; 13(11): e0208133. https://doi.org/10.1371/journal.pone.0208133 PMID: 30496249

34. Cancino-Fauré B, Fisa R, Riera C, Bula I, Girona-Llobera E, Jimenez-Marco T. Evidence of meaningful levels of *Trypanosoma cruzi* in platelet concentrates from seropositive blood donors. Transfusion. 2015 Jun; 55(6):1249–55. https://doi.org/10.1111/trf.12989 Epub 2015 Feb 13. PMID: 25683267

35. Abras A, Galván M, Muñoz C, Juiz NA, Ramírez JC, Cura CI, Tebar S, Fernández-Arévalo A, Pinaño MJ, de la Torre L, Posada E, Navarro F, Espinal P, Ballart C, Portús M, Gascón J, Schijman AG. Identification of *Trypanosoma cruzi* Discrete Typing Units (DTU’s) in Latin-American migrants in Barcelona (Spain). Parasitol Int. 2017 Apr; 66(2):83–88. https://doi.org/10.1016/j.parint.2016.12.003 Epub 2016 Dec 7. PMID: 27940065

36. Martínez-Perez A, Poveda C, Ramírez JD, Norman F, Gironés N, Guli F, Monge-Maillo B, Fresno M, López-Vélez R. Prevalence of *Trypanosoma cruzi*’s Discrete Typing Units in a cohort of Latin American migrants in Spain. Acta Trop. 2016 May; 157:145–50. https://doi.org/10.1016/j.actatropica.2016.01.032 Epub 2016 Feb 2. PMID: 26851167

37. Valdares HM, Pimenta JR, de Freitas JM, Duffy T, Bartholomeu DC, Oliveira Rde P, Chiari E, Moreira MC, Filho GB, Schijman AG, Franco GR, Machado CR, Pena SD, Macedo AM. Genetic profiling of *Trypanosoma cruzi* directly in infected tissues using nested PCR of polymorphic microsatellites. Int J Parasitol. 2007; 37:839–50. https://doi.org/10.1016/j.ijpara.2007.10.017 PMID: 18154957

38. Burgos JM, Diez M, Vigliano C, Bisio M, Riso M, Duffy T, Cura C, Brusses B, Favaloro L, Leguilzamon MS, Lucero RH, Laguens R, Levin MJ, Favaloro R, Schijman AG. Molecular identification of *Trypanosoma cruzi* discrete typing units in end-stage chronic Chagas heart disease and reactivation after heart transplantation. Clin Infect Dis. 2010; 51:485–95. https://doi.org/10.1086/655680 PMID: 20645859

39. Andrade SG, Campos RF, Steindel M, Guerreiro ML, Magalhães JB, Almeida MC, Reis JN, Santos VC, Valdares HM, Reis MG, Macedo AM. Biological, biochemical and molecular features of *Trypanosoma cruzi* strains isolated from patients infected through oral transmission during a 2005 outbreak in the state of Santa Catarina, Brazil: its correspondence with the new *T. cruzi* Taxonomy Consensus (2009). Mem Inst Oswaldo Cruz. 2011 Dec; 106(8):948–56.

40. Oliveira MT, de Assis GF, Oliveira e Silva JC, Machado EM, da Silva GN, Veloso VM, Macedo AM, Márten HR, de Lana M. *Trypanosoma cruzi* Discrete Typing Units (TcI and TcVI) in samples of patients from two municipalities of the Jequitinhonha Valley, MG, Brazil, using two molecular typing strategies. Parasit Vectors. 2015 Oct 31; 8:568. https://doi.org/10.1186/s13071-015-1161-2 PMID: 26520576