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Identification of *Trypanosoma cruzi* Discrete Typing Units (DTUs) in Latin-American migrants in Barcelona (Spain)

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ABSTRACT

Trypanosoma cruzi, the causative agent of Chagas disease, is divided into six Discrete Typing Units (DTUs): TcI–TcVI. We aimed to identify *T. cruzi* DTUs in Latin-American migrants in the Barcelona area (Spain) and to assess different molecular typing approaches for the characterization of *T. cruzi* genotypes. Seventy-five peripheral blood samples were analyzed by two real-time PCR methods (qPCR) based on satellite DNA (SatDNA) and kinetoplastid DNA (kDNA). The 20 samples testing positive in both methods, all belonging to Bolivian individuals, were submitted to DTU characterization using two PCR-based flowcharts: multiplex qPCR using TaqMan probes (MTq-PCR), and conventional PCR. These samples were also studied by sequencing the SatDNA and classified as type I (TcI/III), type II (TcII/IV) and type I/II hybrid (TcV/VI). Ten out of the 20 samples gave positive results in the flowcharts: TcV (5 samples), TcII/V/VI (3) and mixed infections by TcV plus TcII (1) and TcV plus TcII/VI (1). By SatDNA sequencing, we classified the 20 samples, 19 as type I/II and one as type I. The most frequent DTU identified by both flowcharts, and suggested by SatDNA sequencing in the remaining samples with low parasitic loads, TcV, is common in Bolivia and predominant in peripheral blood. The mixed infection by TcV–TcII was detected for the first time simultaneously in Bolivian migrants. PCR-based flowcharts are very useful to characterize DTUs during acute infection. SatDNA sequence analysis cannot discriminate *T. cruzi* populations at the level of a single DTU but it enabled us to increase the number of characterized cases in chronically infected patients.

1. Introduction

Chagas disease is a parasitic infection caused by the flagellated protozoan *Trypanosoma cruzi*. Traditionally linked to rural areas of Central and South America, with approximately 6 million people currently affected [1], the disease has become widespread in Europe and the United States as a consequence of migratory trends [2–4]. Spain is the second country with the largest number of migrants from Latin America after the United States, as well as the European country with the highest Chagas disease burden [5,6].

In the absence of the triatomid vector, *T. cruzi* can be transmitted in non-endemic areas through blood transfusion, organ transplant, congenital transmission, and laboratory accidents [7,8]. Chagas disease occurs in two phases: the acute form, usually without symptoms, and the chronic form, characterized by cardiac or gastrointestinal disorders. However, *T. cruzi*-infected individuals can remain for years or even all their lives in a chronic silent phase of the disease known as the indeterminate form [1,9].

T. cruzi has great genetic diversity and its natural populations are currently divided into six genetic subdivisions, known as Discrete Typing Units (DTUs), which have different geographical distribution: TcI–TcVI [10,11]. The concept of DTUs refers to a set of stocks that are

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genetically more similar to each other than to any other stock, and are identifiable by common genetic, molecular or immunological markers [10,12]. Taxonomic studies have sought to identify associations between DTUs and the clinical presentation of Chagas disease, as well as clarify the geographical distribution of *T. cruzi* genetic subdivisions in endemic regions and transmission cycles [13–15]. There are several methods to characterize *T. cruzi* DTUs, but to date there is no consensus on a genotyping protocol [16].

The aims of this study were to identify *T. cruzi* DTUs in a population of migrants from Chagas disease endemic countries attending different hospitals in the Barcelona area (Spain), and to assess different molecular typing approaches for the characterization of *T. cruzi* genotypes.

2. Material and methods

2.1. Study population and samples

A total of 75 peripheral blood samples from Latin American migrants who attended seven hospitals in the Barcelona area (Spain) during the period October 2009 to February 2014 were included. Selection criteria included patients with request for Chagas disease diagnosis and sufficient sample volume stored to perform the subsequent analysis. Samples were anonymized before being evaluated. Two real-time polymerase chain reaction procedures (qPCR) to detect *T. cruzi* DNA were used and samples yielding positive results in both methods were selected to investigate the parasite DTUs.

This study was approved by the Clinical Research Ethics Committee (CEIC) of the Hospital de la Santa Creu i Sant Pau in Barcelona (Project code: IIBSP-CHA-2013-33; CEIC number: 53/2013).

2.2. DNA extraction and real-time PCR procedures

DNA was extracted from 200 μL of EDTA-blood with the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) and eluted in 200 μL of elution buffer (EB) according to the manufacturer's instructions. The extracted DNA was stored at −40 °C until its analysis. Techniques used for the inclusion criteria were two qPCR assays detecting satellite DNA (SatDNA) (primers Cruzi 1 and Cruzi 2, and probe Cruzi 3) [17], and kinetoplastid DNA (kDNA) (primers 32F and 148R, and probe 71P) [18] of *T. cruzi*. Five μL of the DNA eluates in a final volume of 20 μL were used in both SatDNA and kDNA qPCR procedures. The amplification was carried out in a Rotor-Gene thermal cycler (RG6000, Corbett & CO, Teddington, United Kingdom). RNAse P human gene (TaqMan RNase P Control Reagents Kit, Applied Biosystems) was included as an internal control of the qPCR amplification and also to evaluate the extracted DNA integrity [19].

For quantification, standard curves were built using non-chagasic human blood spiked with cultured epimastigotes of *T. cruzi*. Ten milliliters of non-infected blood were spiked with *T. cruzi* TcV LLO 52-P39-R1-Cl1 stock, giving a final concentration of 10^5 parasite equivalents/mL (par. eq./mL). DNA from spiked blood used to construct the standard curve was extracted in the same way as described above for clinical samples. In order to obtain a panel of samples ranging from 10^5 to 1 par. eq./mL of blood, 1/10 serial dilutions of the DNA extracted from the spiked blood in total DNA extractions from non-chagasic individuals were carried out.

2.3. T. cruzi DTU characterization

DTU characterization was performed using two sequential flowcharts based on molecular markers in the following order: (i) multiplex real-time PCR using TaqMan probes (MTq-PCR) in which the DTU is resolved after one or two rounds of amplification of the Spliced Leader Intergenic Region (SL-IR), 18S-ribosomal DNA (18S), Cytochrome Oxidase II (COII), and 24Sα-ribosomal DNA (24Sα), as reported [16] (Fig. 1); (ii) a conventional PCR flowchart, which requires at least three independent and sequential amplifications of the nuclear loci SL-IR, 24Sα, and A10 fragment (A10), as described elsewhere [20,21] (Fig. 2). The conventional PCR flowchart needs a subsequent electrophoresis in agarose gels in order to visualize the amplicon sizes (base pairs, bp). Samples already characterized at the level of a single DTU by MTq-PCR were not re-analyzed with the conventional PCR flowchart.

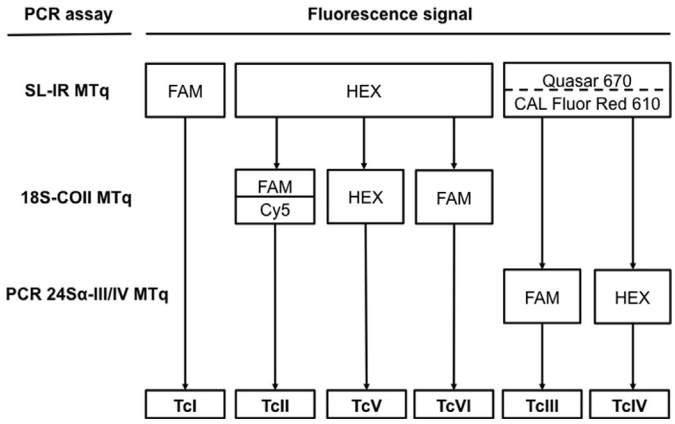

Fig. 1. Multiplex real-time PCR (MTq-PCR) flowchart for identification of *Trypanosoma cruzi* Discrete Typing Units (DTUs). SL-IR: Spliced Leader Intergenic Region; 18S: 18S-ribosomal DNA; COII: Cytochrome Oxidase II; 24Sα: 24Sα-ribosomal DNA. Continuous line indicates the need for reactivity in both FAM and Cy5 fluorescence signals. Dotted line indicates the need for reactivity in at least one out of the two signals (Quasar 670 and CAL Fluor Red 610).
The flowchart was taken from Cura et al. [16].

2.4. T. cruzi satellite DNA sequencing

A PCR procedure, targeted to the *T. cruzi* SatDNA, was performed in 50 μL reaction volume containing 5 μL of extracted DNA. Final concentrations were: 200 μM of each deoxyribonucleotide triphosphate (dNTP), 1 μM of each primer Cruzi 1 and Cruzi 2 [17], 1.5 U of VWR Taq DNA polymerase (VWR International, Haasrode, Belgium), and 5 μL of 10× Key buffer (supplied with the VWR Taq polymerase). Amplification conditions were as follow: one step of 3 min at 94 °C, 44 cycles at 94 °C for 45 s, 66 °C for 45 s and 72 °C for 45 s; and a final extension step at 72 °C for 10 min. PCR products were detected by agarose gel electrophoresis (2%) stained with ethidium bromide through its observation with ultraviolet light (UV). Amplified fragments were purified with USB ExoSAP-IT PCR Product Clean-up (Affymetrix, Inc. USB Corporation, Cleveland, Ohio, USA), and sequenced (Genomics, Scientific and Technological Centers, Universitat de Barcelona, Spain). Sequences were analyzed using MEGA 6 software (http://www.megasoftware.net) in order to classify them in SatDNA type I, SatDNA type II (or type I/II hybrid) according to the position of a set of single nucleotide polymorphisms [SNPs] observed and studied in the SatDNA sequence) [22,23] (Ramírez et al., unpublished results). Sequence data were deposited at GenBank (http://www.ncbi.nlm.nih.gov/genbank) with consecutive accession numbers KX235520 to KX235539.

3. Results and discussion

T. cruzi characterization studies are common in endemic countries in contrast to in non-endemic areas such as Europe. In Spain, they are scarce [24,25], and have not included newborns as in the present work.

Seventy-five peripheral blood samples were analyzed. Twenty of them tested positive for *T. cruzi* DNA using both SatDNA and kDNA qPCR methods. They belonged to 14 adults, two children aged 10 and 13, and four newborns to Chagas-infected mothers. All adults were Bolivian, in accordance with the high prevalence of Bolivian people in other studies on Chagas disease in Spain [3,26]. The four newborns and both children were born in Spain but their mothers came from

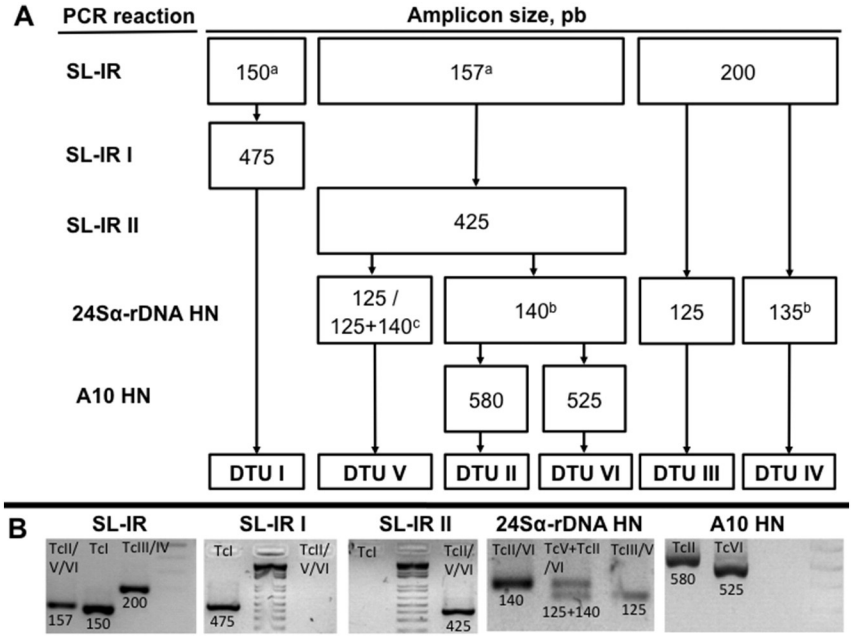

Fig. 2. Conventional Polymerase Chain Reaction (PCR) flowchart for identification of *Trypanosoma cruzi* Discrete Typing Units (DTUs) (A). Amplicon size is indicated in bp (base pairs). SL-IR: Spliced-Leader Intergenic Region; SL-IR I and II: PCR reaction from SL-IR I and II PCRs; 24Sα rDNA HN: heminested amplification of the D7 domain of the 24Sα ribosomal RNA genes; A10 HN: heminested reaction for the A10 fragment. Examples of the PCR products obtained for each DTU by agarose gel electrophoresis in the reactions of the identification flowchart (B). The flowchart was taken and adapted from Burgos et al. [20] and Burgos et al. [21]. [a]Sometimes bands of 150 and 157 bp may be difficult to differentiate and could appear as a single band. [b]Bands of 135 and 140 bp could also be difficult to differentiate. [c]In cases where both 125 and 140 bp 24Sα rDNA HN amplicons are obtained, we interpreted TcV when a band of 125 bp plus a weak 140 bp fragment appeared and mixed infections by TcV plus TcII/VI when a band of 125 bp plus a strong 140 bp fragment were obtained, as done before [56,57].

Bolivia and consequently were congenitally infected. Of the remaining 55 samples, 54 of them tested negative for both qPCR diagnostic methods and the last one was negative for SatDNA qPCR and positive for kDNA qPCR. The discordant sample achieved a parasitic load for kDNA qPCR below the limit of detection (LOD) of the method (0.23 par. eq./mL) [19] and belonged to an Argentinian patient with chronic asymptomatic Chagas disease. These results are consistent with those of Ramírez et al. [19], which reported that kDNA qPCR had higher analytical sensitivity than SatDNA qPCR method.

Ten out of the 20 blood samples selected for the parasite characterization gave positive results in the DTU identification flowcharts (Table 1). Five of these were identified as belonging to a particular *T. cruzi* DTU, in all cases TcV. In other three patients, the characterization gave a TcII/V/VI profile, since the 24Sα yielded undetectable results. The remaining two samples presented mixed infections, one by TcV plus TcII/VI and the other one by TcV plus TcII, discriminated using A10 genomic fragment (Fig. 1–Supplementary material). The combination of TcV plus TcII has already been reported in Bolivian migrants but not simultaneously, as TcV was detected before treatment and TcII after treatment [25]. This is the first time that a mixed infection by TcV plus TcII has been observed in a blood sample of a patient at the same time.

With regard to the typification method, the MTq-PCR flowchart characterized five samples: three belonged to TcV and two to TcII/V/VI genotypes. When using the conventional PCR flowchart, two samples were typified as TcV, two as TcII/V/VI, one as TcV plus TcII/VI and the last one as TcV plus TcII (previously identified as TcII/V/VI by the MTq-PCR method). The lesser extent of MTq-PCR to detect mixed infections in comparison with the conventional PCR scheme has been previously observed by Cura et al. [16].

In relation to the *T. cruzi* SatDNA sequencing, it was possible to obtain the 166 bp tandem repeated sequences of the 20 samples analyzed. SatDNA based PCR is more sensitive than the PCR assays used for DTU identification, due to the high copy number of the satellite repeats (10^5) present in the genome of the parasite [15,22,27,28]. It is expected to find samples from chronic Chagas disease patients with parasitic loads below the LOD of the PCR typing methods but above that of SatDNA based PCR. The 95% of the samples, 19 of them, showed SatDNA type I/II hybrid (see Table 1). The remaining sample (GenBank accession number KX235536) (5%) had SatDNA type I. Samples of type II were not found [23] (Ramírez et al., unpublished results). This is consistent with the findings obtained with the PCR-based flowcharts.

The most widespread and abundant *T. cruzi* DTU in Latin America is TcI, which is associated with human Chagas disease in northern South America but is also occasionally reported in the Southern Cone [29–31]. TcV, as well as TcII and TcVI, seems to be concentrated in central and southern South America and restricted to domestic transmission cycles [11,31,32]. In reference to the remaining DTUs, TcIII is rare in humans and associated with sylvatic cycles and TcIV occurs only sporadically in South America, with the exception of Venezuela [11]. In this study, we identified TcV in most cases, in agreement with previous studies of Bolivian patients in Madrid (Spain) [24,25]. In fact, TcV, together with TcI, are the most common DTUs in domestic cycles in Bolivia [33]. Although we found no TcI by using the *T. cruzi* characterization flowcharts, one of the SatDNA sequences had a type I profile which is related to DTUs TcI and TcIII [23]. Despite the presence of TcIII cannot be ruled-out, chances are that the only SatDNA type I sequence identified belongs to a TcI because of its abundance in the Americas [31]. Other authors have not found any TcIII in their studies of Bolivian patients in Spain [24,25].

Another point to consider is the fact that 19 out of the 20 sequences obtained a profile SatDNA I/II hybrid associated to DTUs TcV–TcVI. In this case, these sequences are most likely to come from TcV, predominant in Bolivia [33] and, moreover, five out of the ten samples with positive results in the flowcharts were characterized as TcV. We have also found the presence of TcV plus TcII and TcV plus TcII/VI indicating mixed infections, which have been described as frequent in *T. cruzi* including a combination of different DTUs [33–35]. In Spain, they were detected in Bolivian patients with chronic Chagas disease in the 15% of the samples [25]. As mentioned above, TcII and TcVI are associated with domestic cycles in the Southern Cone [11]. Thus, this type of combined infections cannot be discarded in the rest of the samples studied.

Table 1
Results obtained for the 20 blood samples of Bolivian patients included in the study.

Year	Sex (M, F)	Age (d, m, y)	Symptoms (A, C, D)	Treatment[a] (yes, no)	SatDNA qPCR			kDNA qPCR			DTU		SatDNA sequence type
					Ct	Par. eq./mL[b]	Log_{10} par. eq./10 mL	Ct	Par. eq./mL[c]	Log_{10} par. eq./10 mL	MTq-PCR	Conv. PCR	
2013	F	1m	A	No	18.1	937.7	4.0	17.6	300.7	3.5	TcV	NP	I/II
2010*	M	10m	A	No	19.0	529.4	3.7	15.3	1686.0	4.2	NP	TcV + II/VI	I/II
2009	M	21d	A	No	20.6	136.2	3.1	20.7	32.3	2.5	TcV	NP	I/II
2009	F	7m	A	No	23.1	20.2	2.3	21.7	15.2	2.2	TcV	NP	I/II
2011	M	38y	A	No	24.6	10.5	2.0	23.9	2.6	1.4	TcII/V/VI	TcV + II	I/II
2010	F	32y	A	No	25.7	3.3	1.5	27.2	NQ	–	TcII/V/VI	–	I/II
2011	M	29y	A	No	26.0	4.1	1.6	24.3	2.0	1.3	–	–	I/II
2010	M	45y	A	No	26.7	2.0	1.3	25.1	1.2	1.1	–	TcII/V/VI	I/II
2010*	M	10y	A	No	27.2	1.5	1.2	23.5	3.8	1.6	–	TcV	I/II
2011	M	36y	A	No	27.6	NQ	–	31.4	NQ	–	–	–	I/II
2010	M	37y	C	No	28.1	NQ	–	29.5	NQ	–	–	–	I/II
2011	M	43y	A	No	28.2	NQ	–	22.9	5.8	1.8	–	TcV	I/II
2010	F	34y	A	Yes	28.3	NQ	–	25.1	1.2	1.1	–	–	I/II
2012	F	26y	A	No	29.2	NQ	–	28.1	NQ	–	–	–	I/II
2009	F	39y	D	Yes	29.6	NQ	–	28.5	NQ	–	–	–	I/II
2014	F	45y	A	No	30.2	NQ	–	28.0	NQ	–	–	–	I/II
2009	F	30y	D	Yes	30.5	NQ	–	28.5	NQ	–	–	–	I
2012	F	30y	D	Yes	30.5	NQ	–	30.3	NQ	–	–	–	I/II
2010	M	Adult**	A	No	33.0	NQ	–	29.6	NQ	–	–	TcII/V/VI	I/II
2014	M	13y	A	No	35.2	NQ	–	35.7	NQ	–	–	–	I/II

M, male; F, female; d, days; m, months; y, years; A, asymptomatic; C, cardiac; D, digestive; NP, not performed; NQ, non-quantifiable; Ct, cycle threshold; Par. eq./mL, parasite equivalents in 1 mL of blood; Log_{10} par. eq./10 mL, logarithmic values of parasite equivalents in 10 mL of blood; qPCR, quantitative polymerase chain reaction; SatDNA, satellite DNA; kDNA, kinetoplastid DNA; DTU, discrete typing unit; MTq-PCR, multiplex real-time PCR using TaqMan probes flowchart; Conv. PCR, conventional PCR flowchart.

* Siblings.

** Age unknown.

[a] Treatment with Benznidazole finished.

[b] Limit of quantification (LOQ) for SatDNA qPCR: 1.53 par. eq./mL [15].

[c] LOQ for kDNA qPCR: 0.9 par. eq./mL [19].

In the peripheral blood of patients TcV is described as predominant [21,36,37]. The absence or low presence of other *T. cruzi* genotypes in this sample may be due to their location in tissues and a low parasitic load in circulating blood [21,38–40]. It has been shown that *T. cruzi* I patients from the southern cone usually present low parasitemias, respect to patients infected by TcV [21]. Studies of cardiac explants of Argentinian patients have also demonstrated that TcI can also cause cardiopathies in the Southern Cone of Latin America [21], where this kind of disorder is known to be produced by TcII, TcV and TcVI [30]. Indeed, TcI was more common in cardiac explants than TcV, which was mainly detectable in culture isolates from peripheral blood [36]. This tropism of *T. cruzi* genotypes could partly explain the TcV findings and absence or minority presence of TcI in our samples.

On the other hand, TcI has been detected in the bloodstream of patients from the Southern Cone under conditions of immunosuppression, due to organ transplantation or human immunodeficiency virus (HIV) co-infection [21,41,42]. It has therefore been proposed that in conditions of immunocompetence, these TcI strains may display low parasitic loads, which impedes their detection in peripheral blood [21]. In fact, patient parasite populations characterized as TcII/V/VI showed a mean parasitic load of 0.8 or 0.4 log_{10} par. eq./10 mL by Sat-DNA qPCR or kDNA qPCR, respectively; *T. cruzi* populations characterized as TcV gave a mean value of 2.1 log_{10} par. eq./10 mL using SatDNA qPCR and 2.3 log_{10} par. eq./10 mL by kDNA qPCR; finally, populations characterized as mixed infections of TcV plus TcII and TcV plus TcII/VI showed a mean parasitic load of 2.8 log_{10} par. eq./10 mL using both SatDNA and kDNA qPCR methods (see Table 1).

Five out of six neonates and pediatric samples could be characterized, four of them corresponding to children under 10 months old and with the highest parasitic load (Table 1). Congenitally infected neonates frequently harbor a high parasitic burden [43], which facilitates DTU typing compared to the low parasitemia frequent in chronic Chagas disease adults [16,44]. However, in some instances, there are newborns with lower parasitemia and it seems to be related to several factors: the amount of parasites transmitted from the mother, the virulence of the parasite strains, the stage of gestation when occurs the transplacental transmission of the parasite and the fetal capacity to control the infection [44–46]. Actually, congenital infection of *T. cruzi* results from the interaction between parasites, pregnant women, placenta and fetuses as reported Carlier and Truyens [46]. More recently, Juiz et al. [47] confirmed the placenta as a key organ for the parasite transmission by describing the association of human polymorphisms in placentally expressed genes with the susceptibility to congenital *T. cruzi* infection. Four out of the five samples were identified as TcV, in agreement with the results obtained in previous studies [43,44,48], and the fifth was a mixed infection of TcV plus TcII/VI. Other authors have reported that mixed infections are frequent in newborns and younger patients with early infection in Bolivia and Chile [33,49].

Congenital Chagas disease is the main responsible for autochthonous *T. cruzi* infection in non-endemic countries [46]. Several studies showed that there is a clear correlation between the *T. cruzi* lineages found in the mother and in the congenitally infected newborn and they are also similar to those detected in the local population [20,46, 48,50,51]. However, differences between the DTUs affecting the mother and the infant can be also observed in case of mixed infections [46]. Higher parasitemia in pregnant women without vector-borne exposure is suspected and it favors vertical transmission [52]. Thus, it can be expected that TcV could be the prevailing DTU of autochthonous cases in the Barcelona area if infected women of reproductive age are not treated [53,54] although congenital cases caused by TcII and TcVI could not be ruled out.

Limiting factors for DTU identification flowcharts of the study, together with the parasitic load, are the low number of tested samples and the fact that only peripheral blood samples were analyzed, since it was not reasonable to obtain cardiac and/or digestive tissue biopsies in this group of patients. Another possible explanation for the negative results of some samples could be the quality of the extracted DNA due to the long-term storage panel. Nevertheless, the results reported in this study did not show a relationship between the storage time of DNA samples and successful DTU identification. Samples were collected

from patients during the period October 2009 to February 2014 and the collection dates of the 10 samples with positive results in the DTU identification flowcharts ranged from November 2009 to January 2013. Actually, all samples were detectable for RNAse P human gene with a Ct values ranged from 20.4 to 24.4, which indicates a good quality of the DNA regardless of whether they could be genotyped or not.

The MTq-PCR flowchart has multiple advantages: reduction of carry-over contamination, amenability to quantification and automation for kit production, fast determination and easy interpretation of results [16]. On the other hand, the conventional PCR flowchart requires multiple sequential reactions but it allowed us to identify parasite genotypes in specimens with lower parasitic loads than the MTq-PCR flowchart. Both methods are useful to determine *T. cruzi* DTUs in cultured stocks and also in samples of patients with acute or congenital infection but their sensitivity decreases when they are used in blood samples from patients in the chronic stage of the disease.

The molecular characterization of *T. cruzi* based on the SatDNA has the benefit that it can be applied with low parasitic loads. Indeed, SatDNA is a widely used parasitic target for the molecular diagnosis of *T. cruzi* infection and its good performance has been proved [55]. Although the method has the limitation that it cannot classify *T. cruzi* populations at the level of a single DTU, it enables us to increase the number of characterized cases when compared to the flowcharts, at least in the form of SatDNA type I, II and I/II hybrid. Further studies in this field are needed.

4. Conclusions

The most identified DTU was TcV, one of the most common genotypes in Bolivia and predominant in peripheral blood samples. A mixed infection by TcV plus TcII was detected for the first time simultaneously in Bolivian migrants. The 95% of the samples analyzed showed SatDNA type I/II hybrid sequences and only a sample had SatDNA type I sequence, probably belonging to a TcI population.

Identification flowcharts are very useful to characterize DTUs in *T. cruzi* natural populations during acute infection but not sensitive enough for analysis of patients with low parasitic loads. SatDNA sequence analysis cannot discriminate *T. cruzi* populations at the level of a single DTU but it enabled us to increase the number of characterized cases in chronically infected patients.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parint.2016.12.003.

Conflict of interest

The authors declare no conflicts of interest.

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