1 **Identification of *Trypanosoma cruzi* Discrete Typing Units (DTUs) in Latin-**

2 **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.

Keywords: Chagas disease, *Trypanosoma cruzi*, discrete typing unit, migration, Real-time PCR, sequencing

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 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

83 migrants from Chagas disease endemic countries attending different hospitals in the
84 Barcelona area (Spain), and to assess different molecular typing approaches for the
85 characterization of *T. cruzi* genotypes.
86
87 **2. Material and methods**

88 *2.1. Study population and samples*

89 A total of 75 peripheral blood samples from Latin American migrants who
90 attended seven hospitals in the Barcelona area (Spain) during the period October 2009
91 to February 2014 were included. Selection criteria included patients with request for
92 Chagas disease diagnosis and sufficient sample volume stored to perform the
93 subsequent analysis. Samples were anonymized before being evaluated. Two real-time
94 polymerase chain reaction procedures (qPCR) to detect *T. cruzi* DNA were used and
95 samples yielding positive results in both methods were selected to investigate the
96 parasite DTUs.
97 This study was approved by the Clinical Research Ethics Committee (CEIC) of
98 the Hospital de la Santa Creu i Sant Pau in Barcelona (Project code: IIBSP-CHA-2013-
99 33; CEIC number: 53/2013).
100

101 *2.2. DNA extraction and Real-Time PCR procedures*

102 DNA was extracted from 200 µL of EDTA-blood with the High Pure PCR
103 Template Preparation Kit (Roche, Mannheim, Germany) and eluted in 200 µL of elution
104 buffer (EB) according to the manufacturer's instructions. The extracted DNA was stored
105 at -40ºC until its analysis. Techniques used for the inclusion criteria were two qPCR
106 assays detecting satellite DNA (SatDNA) (primers Cruzi 1 and Cruzi 2, and probe Cruzi
107 3) [17], and kinetoplastid DNA (kDNA) (primers 32F and 148R, and probe 71P) [18] of

108 *T. cruzi*. Five μL of the DNA eluates in a final volume of 20 μL were used in both
109 SatDNA and kDNA qPCR procedures. The amplification was carried out in a Rotor-
110 Gene thermal cycler (RG6000, Corbett & CO, Teddington, United Kingdom). RNAse P
111 human gene (TaqMan RNase P Control Reagents Kit, Applied Biosystems) was
112 included as an internal control of the qPCR amplification and also to evaluate the
113 extracted DNA integrity [19].
114 For quantification, standard curves were built using non-chagasic human blood
115 spiked with cultured epimastigotes of *T. cruzi*. Ten mL of non-infected blood were
116 spiked with *T. cruzi* TcV LLO 52-P39-R1-Cl1 stock, giving a final concentration of 10^5
117 parasite equivalents/mL (par. eq./mL). DNA from spiked blood used to construct the
118 standard curve was extracted in the same way as described above for clinical samples.
119 In order to obtain a panel of samples ranging from 10^5 to 1 par. eq./mL of blood, 1/10
120 serial dilutions of the DNA extracted from the spiked blood in total DNA extractions
121 from non-chagasic individuals were carried out.
122
123 *2.3. T. cruzi DTU characterization*
124 DTU characterization was performed using two sequential flowcharts based on
125 molecular markers in the following order: (i) multiplex real-time PCR using TaqMan
126 probes (MTq-PCR) in which the DTU is resolved after one or two rounds of
127 amplification of the spliced leader intergenic region (SL-IR), 18S-ribosomal DNA
128 (18S), cytochrome oxidase II (COII), and 24Sα-ribosomal DNA (24Sα), as reported
129 [16] (Fig. 1); (ii) a conventional PCR flowchart, which requires at least three
130 independent and sequential amplifications of the nuclear loci SL-IR, 24Sα, and A10
131 fragment (A10), as described elsewhere [20,21] (Fig. 2). The conventional PCR
132 flowchart needs a subsequent electrophoresis in agarose gels in order to visualize the

133 amplicon sizes (base pairs, bp). Samples already characterized at the level of a single
134 DTU by MTq-PCR were not re-analyzed with the conventional PCR flowchart.
135
136 *2.4. T. cruzi satellite DNA sequencing*
137 A PCR procedure, targeted to the *T. cruzi* SatDNA, was performed in 50 μL
138 reaction volume containing 5 μL of extracted DNA. Final concentrations were: 200 μM
139 of each deoxyribonucleotide triphosphate (dNTP), 1 μM of each primer Cruzi 1 and
140 Cruzi 2 [17], 1.5 U of VWR Taq DNA polymerase (VWR International, Haasrode,
141 Belgium), and 5 μL of 10x Key buffer (supplied with the VWR Taq polymerase).
142 Amplification conditions were as follow: one step of 3 minutes at 94ºC, 44 cycles at
143 94ºC for 45 seconds, 66ºC for 45 seconds and 72ºC for 45 seconds; and a final
144 extension step at 72ºC for 10 minutes. PCR products were detected by agarose gel
145 electrophoresis (2%) stained with ethidium bromide through its observation with
146 ultraviolet light (UV). Amplified fragments were purified with USB ExoSAP-IT PCR
147 Product Clean-up (Affymetrix, Inc. USB Corporation, Cleveland, Ohio, USA), and
148 sequenced (Genomics, Scientific and Technological Centers, Universitat de Barcelona,
149 Spain). Sequences were analyzed using MEGA 6 software
150 (http://www.megasoftware.net) in order to classify them in SatDNA type I, SatDNA
151 type II (or type I/II hybrid according to the position of a set of single nucleotide
152 polymorphisms [SNPs] observed and studied in the SatDNA sequence) [22,23]
153 (Ramírez et al., unpublished results). Sequence data were deposited at GenBank
154 (http://www.ncbi.nlm.nih.gov/genbank) with consecutive accession numbers KX235520
155 to KX235539.
156
157

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 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. (2015) [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

183 already been reported in Bolivian migrants but not simultaneously, as TcV was detected
184 before treatment and TcII after treatment [25]. This is the first time that a mixed
185 infection by TcV plus TcII has been observed in a blood sample of a patient at the same
186 time.

187 With regard to the typification method, the MTq-PCR flowchart characterized
188 five samples: three belonged to TcV and two to TcII/V/VI genotypes. When using the
189 conventional PCR flowchart, two samples were typified as TcV, two as TcII/V/VI, one
190 as TcV plus TcII/VI and the last one as TcV plus TcII (previously identified as
191 TcII/V/VI by the MTq-PCR method). The lesser extent of MTq-PCR to detect mixed
192 infections in comparison with the conventional PCR scheme has been previously
193 observed by Cura et al. (2015) [16].

194 In relation to the *T. cruzi* SatDNA sequencing, it was possible to obtain the 166
195 bp tandem repeated sequences of the 20 samples analyzed. SatDNA based PCR is more
196 sensitive than the PCR assays used for DTU identification, due to the high copy number
197 of the satellite repeats (10^5) present in the genome of the parasite [15,22,27,28]. It is
198 expected to find samples from chronic Chagas disease patients with parasitic loads
199 below the LOD of the PCR typing methods but above that of SatDNA based PCR. The
200 95% of the samples, 19 of them, showed SatDNA type I/II hybrid (see Table 1). The
201 remaining sample (GeneBank accession number KX235536) (5%) had SatDNA type I.
202 Samples of type II were not found [23] (Ramírez et al., unpublished results). This is
203 consistent with the findings obtained with the PCR-based flowcharts.

204 The most widespread and abundant *T. cruzi* DTU in Latin America is TcI, which
205 is associated with human Chagas disease in northern South America but is also
206 occasionally reported in the Southern Cone [29–31]. TcV, as well as TcII and TcVI,
207 seems to be concentrated in central and southern South America and restricted to

208 domestic transmission cycles [11,31,32]. In reference to the remaining DTUs, TcIII is
209 rare in humans and associated with sylvatic cycles and TcIV occurs only sporadically in
210 South America, with the exception of Venezuela [11]. In this study, we identified TcV
211 in most cases, in agreement with previous studies of Bolivian patients in Madrid (Spain)
212 [24,25]. In fact, TcV, together with TcI, are the most common DTUs in domestic cycles
213 in Bolivia [33]. Although we found no TcI by using the *T. cruzi* characterization
214 flowcharts, one of the SatDNA sequences had a type I profile which is related to DTUs
215 TcI and TcIII [23]. Despite the presence of TcIII cannot be ruled-out, chances are that
216 the only SatDNA type I sequence identified belongs to a TcI because of its abundance
217 in the Americas [31]. Other authors have not found any TcIII in their studies of Bolivian
218 patients in Spain [24,25].

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

229 In the peripheral blood of patients TcV is described as predominant [21,36,37].
230 The absence or low presence of other *T. cruzi* genotypes in this sample may be due to
231 their location in tissues and a low parasitic load in circulating blood [21,38–40]. It has
232 been shown that *T. cruzi* I patients from the southern cone usually present low

233 parasitemias, respect to patients infected by TcV [21]. Studies of cardiac explants of
234 Argentinian patients have also demonstrated that TcI can also cause cardiopathies in the
235 Southern Cone of Latin America [21], where this kind of disorder is known to be
236 produced by TcII, TcV and TcVI [30]. Indeed, TcI was more common in cardiac
237 explants than TcV, which was mainly detectable in culture isolates from peripheral
238 blood [36]. This tropism of *T. cruzi* genotypes could partly explain the TcV findings
239 and absence or minority presence of TcI in our samples.

240 On the other hand, TcI has been detected in the bloodstream of patients from the
241 Southern Cone under conditions of immunosuppression, due to organ transplantation or
242 human immunodeficiency virus (HIV) co-infection [21,41,42]. It has therefore been
243 proposed that in conditions of immunocompetence, these TcI strains may display low
244 parasitic loads, which impedes their detection in peripheral blood [21]. In fact, patient
245 parasite populations characterized as TcII/V/VI showed a mean parasitic load of 0.8 or
246 0.4 $\log_{10}$ par. eq./10 mL by Sat-DNA qPCR or kDNA qPCR, respectively; *T. cruzi*
247 populations characterized as TcV gave a mean value of 2.1 $\log_{10}$ par. eq./10 mL using
248 SatDNA qPCR and 2.3 $\log_{10}$ par. eq./10 mL by kDNA qPCR; finally, populations
249 characterized as mixed infections of TcV plus TcII and TcV plus TcII/VI showed a
250 mean parasitic load of 2.8 $\log_{10}$ par. eq./10 mL using both SatDNA and kDNA qPCR
251 methods (see Table 1).

252 Five out of six neonates and pediatric samples could be characterized, four of
253 them corresponding to children under 10 months old and with the highest parasitic load
254 (Table 1). Congenitally infected neonates frequently harbor a high parasitic burden [43],
255 which facilitates DTU typing compared to the low parasitemia frequent in chronic
256 Chagas disease adults [16,44]. However, in some instances, there are newborns with
257 lower parasitemia and it seems to be related to several factors: the amount of parasites

258 transmitted from the mother, the virulence of the parasite strains, the stage of gestation
259 when occurs the transplacental transmission of the parasite and the fetal capacity to
260 control the infection [44–46]. Actually, congenital infection of *T. cruzi* results from the
261 interaction between parasites, pregnant women, placenta and fetuses as reported Carlier
262 and Truyens (2015) [46]. More recently, Juiz et al. (2016) [47] confirmed the placenta
263 as a key organ for the parasite transmission by describing the association of human
264 polymorphisms in placentally expressed genes with the susceptibility to congenital *T.*
265 *cruzi* infection. Four out of the five samples were identified as TcV, in agreement with
266 the results obtained in previous studies [43,44,48], and the fifth was a mixed infection
267 of TcV plus TcII/VI. Other authors have reported that mixed infections are frequent in
268 newborns and younger patients with early infection in Bolivia and Chile [33,49].

269 Congenital Chagas disease is the main responsible for autochthonous *T. cruzi*
270 infection in non-endemic countries [46]. Several studies showed that there is a clear
271 correlation between the *T. cruzi* lineages found in the mother and in the congenitally
272 infected newborn and they are also similar to those detected in the local population
273 [20,46,48,50,51]. However, differences between the DTUs affecting the mother and the
274 infant can be also observed in case of mixed infections [46]. Higher parasitemia in
275 pregnant women without vector-borne exposure is suspected and it favors vertical
276 transmission [52]. Thus, it can be expected that TcV could be the prevailing DTU of
277 autochthonous cases in the Barcelona area if infected women of reproductive age are
278 not treated [53,54] although congenital cases caused by TcII and TcVI could not be
279 ruled out.

280 Limiting factors for DTU identification flowcharts of the study, together with
281 the parasitic load, are the low number of tested samples and the fact that only peripheral
282 blood samples were analyzed, since it was not reasonable to obtain cardiac and/or

283 digestive tissue biopsies in this group of patients. Another possible explanation for the
284 negative results of some samples could be the quality of the extracted DNA due to the
285 long-term storage panel. Nevertheless, the results reported in this study did not show a
286 relationship between the storage time of DNA samples and successful DTU
287 identification. Samples were collected from patients during the period October 2009 to
288 February 2014 and the collection dates of the 10 samples with positive results in the
289 DTU identification flowcharts ranged from November 2009 to January 2013. Actually,
290 all samples were detectable for RNAse P human gene with a Ct values ranged from 20.4
291 to 24.4, which indicates a good quality of the DNA regardless of whether they could be
292 genotyped or not.

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

302 The molecular characterization of *T. cruzi* based on the SatDNA has the benefit
303 that it can be applied with low parasitic loads. Indeed, SatDNA is a widely used
304 parasitic target for the molecular diagnosis of *T. cruzi* infection and its good
305 performance has been proved [55]. Although the method has the limitation that it cannot
306 classify *T. cruzi* populations at the level of a single DTU, it enables us to increase the
307 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.

Conflict of interest

The authors declare no conflicts of interest.

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602 **Legend to Figures**

603 Fig. 1. Multiplex real-time PCR (MTq-PCR) flowchart for identification of
604 *Trypanosoma cruzi* discrete typing units (DTUs). SL-IR: spliced leader intergenic
605 region; 18S: 18S-ribosomal DNA; COII: cytochrome oxidase II; 24Sα: 24Sα-ribosomal
606 DNA. Continuous line indicates the need for reactivity in both FAM and Cy5
607 fluorescence signals. Dotted line indicates the need for reactivity in at least one out of
608 the two signals (Quasar 670 and CAL Fluor Red 610). The flowchart was taken from
609 Cura et al. (2015) [16].

610

611 Fig. 2. Conventional polymerase chain reaction (PCR) flowchart for identification of
612 *Trypanosoma cruzi* discrete typing units (DTUs) (A). Amplicon size is indicated in bp
613 (base pairs). SL-IR: spliced-leader intergenic region; SL-IR I and II: PCR reaction from
614 SL-IR I and II PCRs; 24Sα rDNA HN: heminested amplification of the D7 domain of
615 the 24Sα ribosomal RNA genes; A10 HN: heminested reaction for the A10 fragment.
616 Examples of the PCR products obtained for each DTU by agarose gel electrophoresis in
617 the reactions of the identification flowchart (B). The flowchart was taken and adapted
618 from Burgos et al. (2007) [20] and Burgos et al. (2010) [21]. [a]Sometimes bands of 150
619 and 157 bp may be difficult to differentiate and could appear as a single band. [b]Bands of
620 135 and 140 bp could also be difficult to differentiate. [c]In cases where both 125 and 140
621 bp 24Sα rDNA HN amplicons are obtained, we interpreted TcV when a band of 125 bp
622 plus a weak 140 bp fragment appeared and mixed infections by TcV plus TcII/VI when
623 a band of 125 bp plus a strong 140 bp fragment were obtained, as done before [56,57].

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627 **Supplementary material**

628 Fig. 1. Mixed infections of TcV plus TcII and TcV plus TcII/VI discriminated using
629 24Sα-ribosomal DNA HN (A) and A10 fragment HN (B) in an agarose gel
630 electrophoresis. NTC: no-template control; 24Sα rDNA HN: heminested amplification
631 of the D7 domain of the 24Sα ribosomal RNA genes; A10 HN: heminested reaction for
632 the A10 fragment.