Epidemiol. Infect. (2014), **142**, 156–162. © Cambridge University Press 2013
doi:10.1017/S0950268813000757

Trypanosoma cruzi genotyping supports a common source of infection in a school-related oral outbreak of acute Chagas disease in Venezuela

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Received 11 October 2012; Final revision 13 December 2012; Accepted 4 March 2013; first published online 2 April 2013

SUMMARY

Trypanosoma cruzi I, a discrete typing unit (DTU) found in human infections in Venezuela and other countries of the northern region of South America and in Central America, has been recently classified into five intra-DTU genotypes (Ia, Ib, Ic, Id, Ie) based on sequence polymorphisms found in the spliced leader intergenic region. In this paper we report the genotype identification of *T. cruzi* human isolates from one outbreak of acute orally acquired Chagas disease that occurred in a non-endemic region of Venezuela and from *T. cruzi* triatomine and rat isolates captured at a guava juice preparation site which was identified as the presumptive source of infection. The genotyping of all these isolates as TcId supports the view of a common source of infection in this oral Chagas disease outbreak through the ingestion of guava juice. Implications for clinical manifestations and dynamics of transmission cycles are discussed.

Key words: Acute Chagas disease outbreak, DTU genotypes, oral transmission, *Trypanosoma cruzi*.

INTRODUCTION

Chagas disease (CD) is a parasitic illness with an estimated prevalence of eight million infected people in Latin America, although the information currently available does not reflect the true extent of the problem [1]. *Trypanosoma cruzi*, its aetiological agent, is mainly transmitted to humans by different Reduviidae insect vectors or by blood transfusion. Congenital transmission from chronically infected women occurs in 2–8% of pregnancies depending on the endemic area [2, 3]. New epidemiological modes of infection have been detected such as oral transmission and domestic adaptation of wild triatomines. The oral route of transmission is well documented and constitutes a mode of infection that leads to micro-epidemics and outbreaks of a more severe clinical form of acute CD [4–8]. The latter via transmission is currently acquiring relevance due to its association

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Table 1. *Description of* Trypanosoma cruzi *isolates*

Isolate	Geographical origin	Host	Route of infection	DTU
MHOM/VE/2007/1593	Chacao, Caracas	Human	Oral	Id
MHOM/VE/2007/1595	Chacao, Caracas	Human	Oral	Id
MHOM/VE/2007/1598	Chacao, Caracas	Human	Oral	Id
MHOM/VE/1955/PM	Valencia	Human	Vector	Ia/Ib
TGEN/VE/2008/AC1	Altagracia, Caracas	*Panstrongylus geniculatus*		Id
MRAT/VE/2008/RATA6	Altagracia, Caracas	*Rattus rattus*		Id

DTU, Discrete typing unit.

with a high mortality rate. The severity of CD also depends on the genetic diversity of *T. cruzi* and the competence of the host immune system.

In Venezuela, a significant outbreak of acute CD occurred in December 2007 affecting 103 persons at a school in an urban neighbourhood of Caracas where vector transmission had not been reported previously [9]. The infection was associated with *Panstrongylus geniculatus*, a sylvatic triatomine vector that is in a progressive process of domiciliation [10–12] and which had been considered as an inefficient *T. cruzi* transmission vector. The outbreak was suspected of being linked to the ingestion of contaminated guava juice prepared under unsupervised hygienic conditions by a supplier in a distant slum on the western mountain slopes of the city, where triatomines were present. A significant positive correlation was found between ingestion of guava juice and risk of infection [9].

T. cruzi shows a genetic diversity that has been associated with the observed variability of clinical manifestations, geographical distribution and preferential parasite–vector interactions. To characterize the population structure, *T. cruzi* has been subdivided into six discrete typing units (DTUs), namely TcI, TcII, TcIII, TcIV, TcV and TcVI [13]. *T. cruzi* I (TcI) isolates are predominant in Venezuela and other countries of the northern region of South America and in Central America [14–17]. TcI has been mainly associated with low parasitaemia level in peripheral blood and very mild clinical symptoms. However, many symptomatic cases of severe acute CD patients infected with TcI in endemic regions of Venezuela and Colombia have also been described [14, 18, 19]. Five intra-DTU TcI genotypes (Ia, Ib, Ic, Id, Ie) have been described based on sequence polymorphisms found in the mini-exon gene's intergenic region [20–22]. So far, strains associated with documented cases of oral transmission have been characterized as TcII [23] and TcI [24, 25]. In this context we decided to determine the intra-DTU TcI genotypes of *T. cruzi* isolates from the orally transmitted outbreak described above which also allowed us to understand the current circulating infecting strains. Preliminary results revealed a marked genetic homogeneity, with all of the isolates belonging to the TcI lineage.

METHODS

T. cruzi isolates

The parasite isolates were obtained by haemoculture from peripheral blood of three patients orally infected in an urban outbreak at a school in Caracas, Venezuela, in December 2007 (including the index case); from the faeces of one infected *P. geniculatus* triatomine, and from the blood of a rat captured at the guava juice preparation site which was identified as the source of infection [9]. In addition, a cultured human stock from B. Alarcón de Noya's laboratory, isolate PM [26], as well as Munanta (DTU TcI), Ikiakarora (DTU TcIII) and Guateque (DTU TcVI) strains from M. C. López's laboratory [27] were included (Table 1).

DNA extraction and PCR amplifications

Total DNA extraction from samples was performed by resuspension of parasite pellets in lysis solution (150 mM NaCl, 0·1 M EDTA, 0·1 mg Proteinase K, 0·4% SDS) and incubated for 2 h at 50 °C, followed by phenol-chloroform extractions and RNAse treatment. DNA pellets were resuspended in distilled water and stored at −20 °C. Amplification of spliced leader intergenic region (SL-IR) was performed in a multiplex reaction with primers TC (CCCCCCTCCCAGGCCACACTG), TC1 (GTGTCCGCCACCTCCTTCGGGCC) and TC2 (CCTGCAGGCACACGTGTGTGTG)

https://doi.org/10.1017/S0950268813000757 Published online by Cambridge University Press

[28, 29]. PCR was performed in a final volume of 25 μl containing 100 ng template DNA, 100 pmol of each primer, 2·5 mM of each dNTP, 2·5 U *Taq* polymerase, 2·5 μl of 10× *Taq* polymerase buffer, and 1·5 mM $MgCl_2$. Amplification was performed in a MJR PTC-100 thermocycler (MJ Research, USA) as follows: one step of 1 min denaturation at 94 °C, 27 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final extension step at 72 °C for 5 min. Ribosomal subunit amplification with primers D71 (AAGGTGCGTCG-ACAGTGTGG) and D72 (TTTTCAGAATGGCC-AACAGT) were also performed [28] in a final volume of 25 μl containing 100 ng template DNA, 100 pmol of each primer, 2·5 mM of each dNTP, 2·5 U *Taq* polymerase, 2·5 μl of 10× *Taq* polymerase buffer and 1·5 mM $MgCl_2$. The PCR products were analysed by 1·5% agarose gel electrophoresis for SL-IR amplification and by 12% acrylamide gel electrophoresis for 24Sα amplification. In both cases the DNA was detected by ethidium bromide staining and visualized under ultraviolet light. PCR products of 350 bp and 300 bp for SL-IR amplification were expected for TcI and for TcII, TcV and TcVI, respectively, while no bands were expected for TcIII and TcIV [30]. A product of 110 bp for 24Sα rDNA amplification was expected for TcI, TcIII and TcV, and products of 120 bp and 125 bp, for TcIV and for TcII and TcVI, respectively.

The amplification products for SL-IR were directly sequenced using the dideoxy chain termination method, and also purified from agarose gels using the Qualex kit (Qiagen, Germany) and cloned in p-GEM T vector (Promega, USA), using the blue/white selection with IPTG/X-gal for the identification of transformants. DNA from each one of the three clones was isolated with the SV Minipreps DNA Purification System (Promega) and the two chains sequenced using the dideoxy chain termination method.

DNA sequence and molecular phylogenetic analysis

Multiple sequence alignments were performed using ClustalW2 [31] and edited in BioEdit v. 7.0.9 (Ibis Biosciences, USA).

RESULTS

In order to characterize the infecting strain that caused the orally acquired CD acute infection, parasites were isolated from three patients from the Caracas outbreak and also from a triatomine and a rat captured at the guava juice preparation site (identified as the source of infection), grown and genotyped. Thus, genomic DNA (gDNA) from the different isolates was purified and PCR performed. As PCR controls, gDNA purified from known *T. cruzi* strains such as Munanta (DTU TcI), Ikiakarora (DTU TcIII) and Guateque (DTU TcVI) were employed as DNA template. As observed in Figure 1*a*, amplification of SL-IR in a multiplex reaction using the TC, TC1 and TC2 primers generated a 350 bp product when the *T. cruzi* gDNAs isolated from the three patients from Caracas (lanes 1–3), and those isolated from the triatomine (lane 5), the rat (lane 6) and control DTU TcI (lane C2) were employed. A single amplification band of 300 bp was generated when DTU TcVI gDNA (lane C3) was employed as DNA template and none when DTU TcIII gDNA (lane C1) was used. These data indicated that all the analysed parasite strains belonged to DTU TcI. This typing was corroborated by the PCR performed with specific primers for 24S amplification which produced amplified bands with the expected size for DTU TcI strains (Fig. 1*b*).

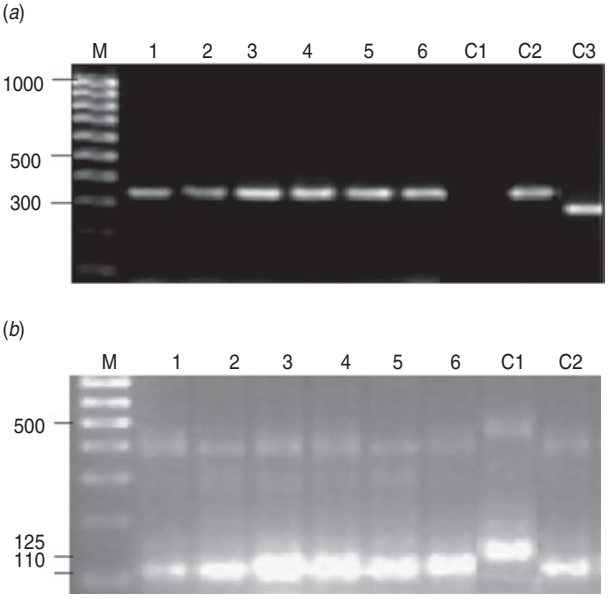

Fig. 1. Gel electrophoresis of PCR products from *T. cruzi* isolates. (*a*) 1·5% Agarose gel electrophoresis of SL-IR amplicons. (*b*) 12% Acrylamide gel electrophoresis of 24Sα amplicons. Lane M, 100 bp DNA ladder (hyperladder IV, Bioline, UK); lanes 1–3, isolates 1593, 1595 and 1598 from patients infected in the Caracas outbreak; lane 4, laboratory strain PM; lane 5, isolate AC1 from an infected *Panstrongylus geniculatus* triatomine captured at the guava juice preparation site; lane 6, isolate RATA 6 from an infected rat captured at the guava juice preparation site; lanes C1, C2, C3, *T. cruzi* control strains Ikiakora (DTU TcIII), Munanta (DTU TcI) and Guateque (DTU TcVI).

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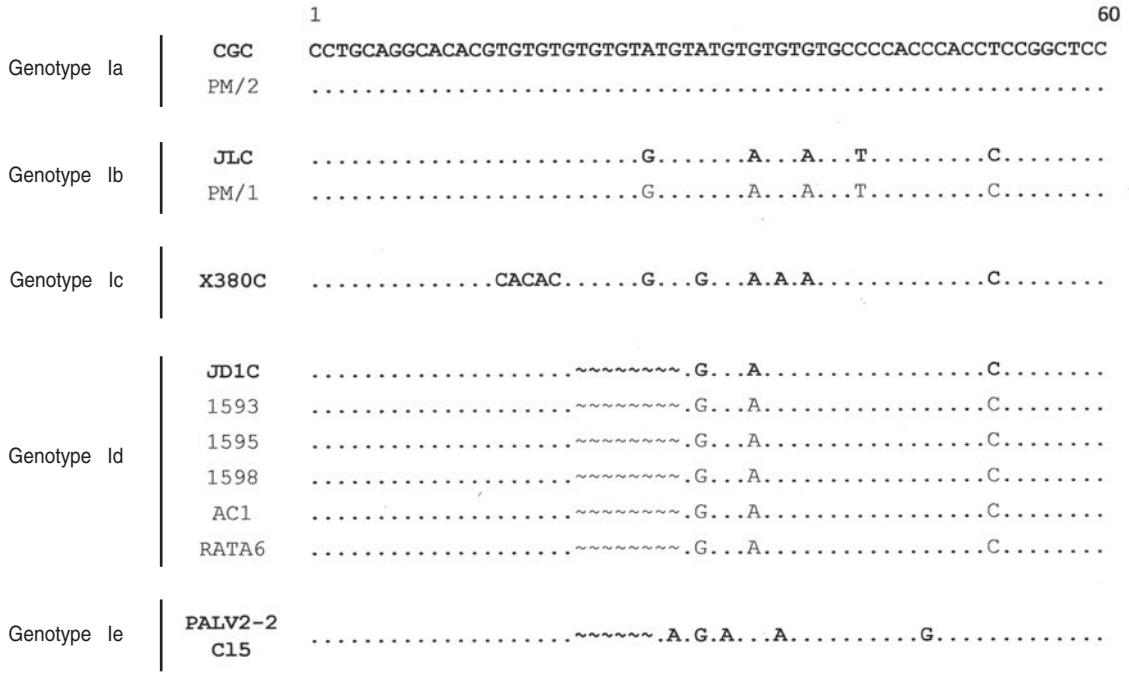

Fig. 2. Multiple alignment of 60-nt region within the SL-IR of the *T. cruzi* I sequences analysed. Partial reference sequences reported in [20, 22] are shown in bold: CGC (AM259467), JLC (AM259468), X380C (AM259472), JD1C (AM259478) and PALV2-2 Cl5 (GQ398812.1). Gaps (~), conserved sites (.).

The SL-IR amplified products were directly sequenced but also cloned into pGEM-T Easy Vector (Promega), in order to reveal the possible existence of mixed TcI SL-IR genotypes. The DNA from different clones was purified and subsequently sequenced. Sequence alignment of the different PCR products (whose electropherograms exhibited no nucleotide ambiguity) and clones corresponding to the isolated parasites in all the patients from Caracas were seen to be identical, indicating that they were all infected by a *T. cruzi* population belonging to a single TcI genotype. The sequences of the different PCR products and clones corresponding to the amplicons generated with the DNA of the parasites isolated from the triatomine and the rat were also identical to those obtained from the parasites isolated from the patients.

Following the intra-DTU TcI classification first proposed by Herrera *et al.* [20] and expanded by Cura *et al.* [22], when the first 60 bp of the SL-IR were aligned with reference sequences, it was observed that all the isolates from the Caracas outbreak presented a gap of 8 nt at position 20–27, as well as other single nucleotide polymorphisms (SNPs), considered as hallmarks of the DTU TcId (Fig. 2). These observations indicated that the triatomine and rat were also infected by a *T. cruzi* population belonging to the same TcI genotype.

On the other hand, a SL-IR sequence divergence was observed in clones corresponding to the laboratory PM strain which was isolated from a patient with vector-acquired CD [26]. These sequences did not have the characteristic 8-nt gap of DTU TcId. Instead they bore the SNP characteristic of the SL-IR sequences from parasites belonging to DTUs TcIa and TcIb (Fig. 2) which was taken as an indication that the laboratory strain was a mixed population of TcIa and TcIb genotypes.

DISCUSSION

In this study we have demonstrated that three patients (including the index case) from an important outbreak of acute CD occurring at a school in an urban neighbourhood of Caracas, and a triatomine and a rat captured at the guava juice preparation site identified as the source of the parasite contamination, were all infected by a *T. cruzi* population belonging to the same TcI genotype. These observations strongly suggest that a single strain was implicated in this outbreak and reinforce the fact that the guava juice preparation site was the source of the infection, identifying the oral route of transmission as a common mechanism of infection [9]. On the other hand, our results indicate that the *T. cruzi* isolate PM, obtained

https://doi.org/10.1017/S0950268813000757 Published online by Cambridge University Press

from a patient with vector-acquired CD [26], was a mixed population of TcIa and TcIb genotypes. Recently, natural mixed populations of TcIa and TcId have been reported in faecal samples of *Triatoma infestans* from Paraguay [22] and in heart, blood and skin chagoma samples from an Argentinean patient [32].

According to current molecular evidence, TcI is one of the parental DTUs in the hybridization events that, separated by periods of clonal evolution, explain the genetic diversity of the extant *T. cruzi* populations. In Venezuela and Colombia, TcI isolates have been associated not only with low parasitaemia and very mild clinical symptoms, but also with severe acute symptomatic cases and death [14, 19, 20]. TcI has a notable intra-DTU diversity, recently classified into five genotypes (Ia, Ib, Ic, Id, Ie) [20–22], although it could not be definitely associated with the diversity of TcI biological characteristics observed, i.e. transmission cycles, host preferences, clinical manifestations or geographical distribution [33, 34]. In Colombia, an association was found for TcId with sylvatic populations of *Rhodnius prolixus* and wild reservoirs [21]. We here report its association with sylvatic populations of *P. geniculatus*. The close contact with infected sylvatic species might explain oral outbreaks in areas without a history of vector transmission. In fact, Caracas is a densely populated urban area currently considered non-endemic for CD, since active vector transmission had not been reported previously. However, a local sylvatic triatomine vector, *P. geniculatus*, is present in the surrounding mountain area where the nocturnal incursion of sylvatic infected triatomine bugs has allowed the opportunity of easy contamination of unprotected food and beverages with *T. cruzi* [9].

A multilocus microsatellite typing of *T. cruzi* DTU I [16] showed a sylvatic TcI genotype predominance in the Venezuelan adult intradomiciliary triatomine sampled, including *Triatoma maculata, P. geniculatus*, and *R. prolixus*. The TcI sylvatic genotype was different from the domestic one observed in TcI strains from infected humans sampled across the country. In Colombia, TcId has also been mainly associated with sylvatic triatomines and reservoir populations suggesting, in both countries, a possible and progressive introduction of a sylvatic sub-DTU I in the domestic environment, independent of the transmission route of the parasite or the species of the triatomine vector.

In a broader study TcId was also found in a variety of peridomestic, domestic and sylvatic triatomines, as well as in wild reservoirs and in human samples from South America [22]. These data are also consistent with the epidemiological changes that are taking place with regard to CD transmission since *P. geniculatus* had been considered as an inefficient *T. cruzi* transmission vector.

A preferential cardiac tropism for TcId has been suggested [32]. This tropism was consistent with the clinical manifestations of patients from the Caracas outbreak, where in 61 (59%) of the 103 confirmed cases, one or more abnormalities were observed on ECG recordings, 75% were symptomatic, 20·3% required hospitalization and a child aged 5 years died of acute chagasic myocarditis [9].

This report shows that *T. cruzi* isolates from sylvatic vectors and reservoirs, as well as isolates from humans infected in an orally acquired CD outbreak in a non-endemic region of Venezuela belong to a same *T. cruzi* I genotype, TcId. The similarity in the parasite isolates from three patients of the urban oral outbreak and the triatomine and the rat captured at the guava juice preparation site, allows us to suggest that the source of infection was common for these persons and the vector.

Although further analysis with more isolates is needed, this work contributes to the knowledge and possible association between the oral route of transmission, the severity of the disease and the genotypes of *T. cruzi* isolates, providing evidence that DTU TcId can successfully infect humans by the oral route.

ACKNOWLEDGEMENTS

This work received financial support from Sección de Inmunología, Instituto de Medicina Tropical, Universidad Central de Venezuela; Fondo Nacional de Ciencia y Tecnología (travelling expenses of Z.D. for a research cooperation with Spain, grant no. G-2005000387), (T.A., grant no. 2006000216); Plan Nacional I+D+i, MINECO, Spain (M.C.T. and M.C.L., grant nos. BFU2010-1670 and SAF2012-35777); FEDER (M.C.T. and M.C.L). M. C. Thomas, M. C. López, O. Noya and B. Alarcón de Noya are members of the NHEPACHA network (Latin American Network for Chagas disease) and PAICET network (Tri-Continental Altantic Platform for Research and Control of Tropical Diseases).

DECLARATION OF INTEREST

None.

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