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Detection of *Trypanosoma cruzi* DTUs TcI and TcIV in two outbreaks of orally-transmitted Chagas disease in the Northern region of Brazil

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ABSTRACT

This study describes the laboratory investigation of two acute Chagas disease outbreaks that occurred in the riverside communities of Marimarituba and Cachoeira do Arua, in the Santarem municipality, Para State, located in the Northern region of Brazil, and occurred in March 2016 and August 2017, respectively. The generation of data regarding the diversity of *Trypanosoma cruzi* parasites circulating in the Amazon region is key for understanding the emergence and expansion of Chagas disease. This study aimed to identify *T. cruzi* Discrete Typing Units (DTUs) involved in two outbreaks of acute Chagas disease (ACD) directly from the patient's biological sample. Nested and multiplex PCR targeting the 24Sα (rRNA) and mini-exon genes, respectively, were used to identify *T. cruzi* DTU in blood samples from patients diagnosed with ACD. The samples with positive cPCR were submitted for analysis for *T. cruzi* DTUs, which included 13 samples from the patients with ACD by oral transmission and two samples collected from two newborns of two women with ACD, from Marimarituba and Cachoeira do Arua. The samples were classified as *T. cruzi* TcIV, from Marimarituba's outbreak, and *T. cruzi* TcI, from Cachoeira do Arua's outbreak. The molecular identification of *T. cruzi* may increase understanding of the role of this parasite in Chagas disease's emergence within the Amazon region, contributing to the improvement of the management of this important, but also neglected, disease.

KEYWORDS: Acute Chagas disease. Outbreak. *Trypanosoma cruzi.* Discrete Typing Units.

INTRODUCTION

Chagas disease, caused by the *Trypanosoma cruzi* protozoan, is considered a parasitic disease of high socioeconomic burden in Latin America. *T. cruzi* has a wide genetic diversity. Currently, the parasite is classified into seven DTUs (Discrete Typing Units): *T. cruzi* TcI to VI as well as the newly classified Tcbat[1,2].

According to previous publications, *T. cruzi* DTUs can influence the evolution of the infection, inducing different immune responses in the host, resulting in different clinical forms observed in the chronic Chagas disease (CCD)[3] phase.

T. cruzi TcI, TcIII, and TcIV predominate in the sylvatic transmission cycle. *T. cruzi* TcI has the largest distribution in the Americas, having been detected from the southern United States to South America[4]. In countries such as Mexico, Panama, Venezuela, and Colombia, where *T. cruzi* TcI is the most frequently detected DTU in humans, cases of acute and chronic cardiomyopathy have been described, indicating

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the severity of Chagas disease despite the absence of megasyndromes[4]. *T. cruzi* TcIII and TcIV, usually detected in acute Chagas disease (ACD) outbreaks[5–7], are rarely identified in patients in the CCD phase[8,9].

T. cruzi TcII, TcV, and VI seem largely related to the domestic transmission cycle in South America and have been isolated from patients in the CCD phase, which display a range of clinical presentations from the indeterminate form, the cardiac form and megasyndromes[3,10]. *T. cruzi* TcII is the most frequently identified DTU in patients with CCD, mainly in the Northeastern and Southeastern regions of Brazil[3,4,11]. All *T. cruzi* DTUs, except TcIV, have been reported in congenital transmission, although TcV is the most frequently detected[10].

The Amazon, the largest tropical forest in the world, is the environment where *T. cruzi* TcI, TcIII, and TcIV circulate[12]. The approximation and adaptation of native species of triatomines to the human environment (peridomestic cycle) have been observed as a result of the progressive degradation of the forest, due to extensive activities with agriculture and livestock[12]. Consequently, ACD cases by oral transmission have been systematically reported, especially in the Brazilian Amazon where 3,278 cases of ACD were reported between 2007 and 2020[13]. Most of the cases, 79.7%, occurred in the Para State (Tropical Forest/Amazon Basin) and were associated with family outbreaks related to the ingestion of contaminated food or beverages with *T. cruzi*[13].

Considering the large number of ACD cases reported in Para, the identification of *T. cruzi* DTUs is still scarce. The generation of data on the diversity of *T. cruzi* parasites circulating in the region is essential for understanding the emergence and expansion of Chagas disease. Additionally, it may serve as the foundation for the development of strategies for controlling and managing the disease in the region. This study aimed to identify *T. cruzi* DTUs involved in two outbreaks of ACD directly from the patients' biological samples.

MATERIALS AND METHODS

Area of study

This study is retrospective and describes the laboratory investigation of two ACD outbreaks that occurred in March 2016 and August 2017 in the riverside communities of Marimarituba (2°12'29"S 55°1'14"W) and Cachoeira do Arua (2º38'58"S 55º43'24"W), respectively, in the Santarem municipality, Para State, located in the Northern region of Brazil.

Seventeen patients were observed at the Hospital Municipal de Santarem (HMS) by infectious diseases specialists under an agreement between the Santarem Municipality Health Department and the Center for Tropical Medicine (NUMETROP) of the Department of Infectious and Parasitic Diseases from the Faculty of Medicine of the University of Sao Paulo.

Cases and samples collection

Figure 1 describes the studied cases and the identification process of *T. cruzi*. This investigation, which aimed to characterize *T. cruzi*, was carried out at the Laboratory of Medical Research in Immunology, Hospital das Clinicas, Faculty of Medicine, University of Sao Paulo.

Blood samples were collected and mixed with 6M guanidine HCl plus 0.2M EDTA buffer (pH8) in the Santarem municipality, Para State, and transported to the Laboratory of Medical Research in Immunology, in the Sao Paulo municipality.

DNA extraction

DNA extraction was performed with 200 µL of the sample using the QIAamp™ DNA Mini Kit (Qiagen). The manufacturer's protocol was followed with a modification: the previous treatment with proteinase k and lysis buffer was excluded. The DNA's quantity and purity were determined by the NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA), and then the samples were stored at 20°C.

Conventional PCR (cPCR)

Conventional PCR (cPCR) assays were performed using the S35/S36 primer pair which amplifies a 330-base pair (bp) minicircle sequence from *T. cruzi*. Positive samples were subjected to *T. cruzi* genotyping assays[14].

Genotyping of *T. cruzi*

The nested PCR for the 24Sα ribosomal (rRNA) gene D7 domain was performed with: D75/D76 primers, used in the first round, and D71/D72 primers[15], used in the second round, as previously described[16]. Amplicons of 110-bp are characteristic of *T. cruzi* DTUs TcI/TcIII; 125-bp of TcII/TcVI; 110+125-bp of TcV and 120 or 130-bp of TcIV.

The multiplex PCR for the intergenic region of spliced leader genes (mini-exon gene) was performed according to the method previously described[17]: three oligonucleotides, derived from a hypervariable region of the *T. cruzi* mini-exon repeat, and an oligonucleotide from a specific region

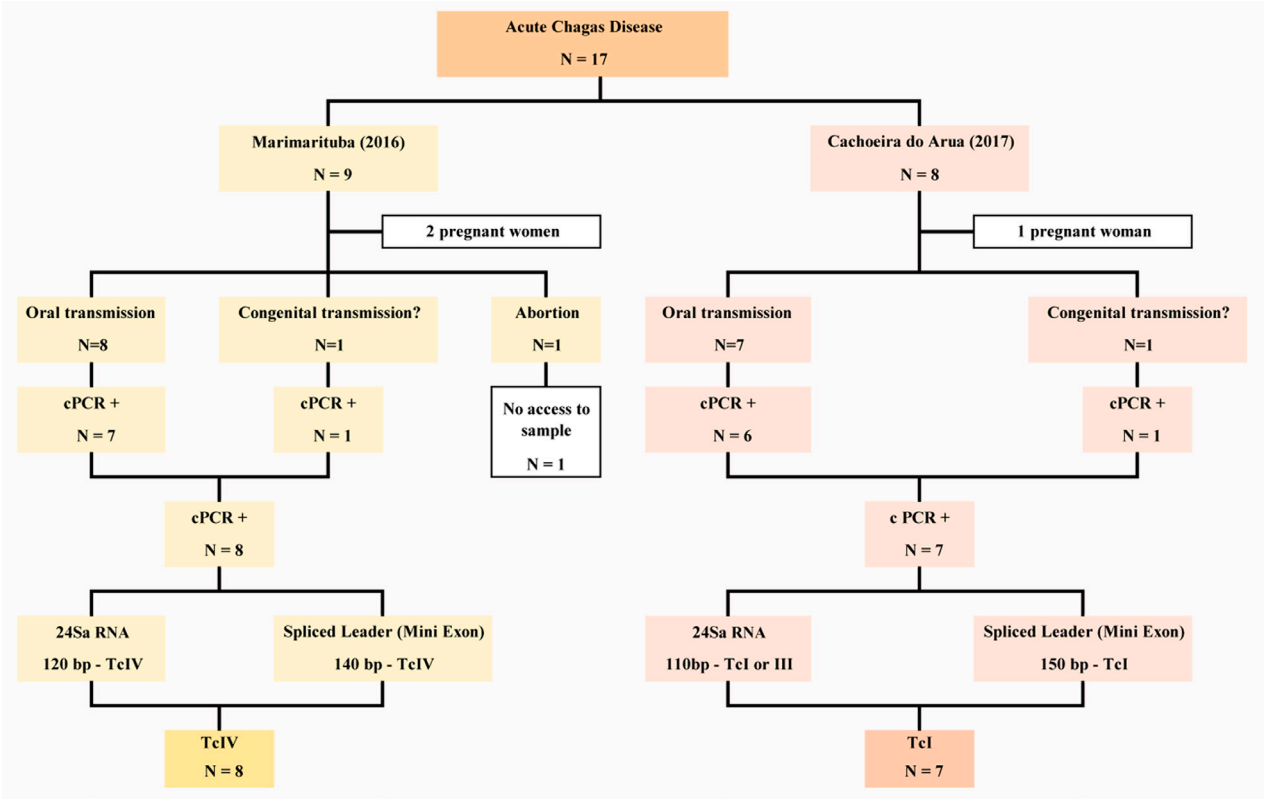

Figure 1 - Distribution of analyzed cases according to outbreak, cPCR results and *T. cruzi* DTUs.

of the *T. rangeli* non-transcribed spacer were used as upstream primers. A common oligonucleotide, designed in the most conserved part of the mini-exon, was used as the downstream primer. The mini-exon PCR assay was performed after the 24Sα ribosomal (rRNA) to discriminate *T. cruzi* TcI/TcIII and to detect *T. rangeli*. Amplicons of 200-bp are characteristic of TcI; 250-bp; 150-bp of TcIII, as well as TcIV and *T. rangeli* which generated an amplicon of 10-bp.

Ethics statement

The study was cleared by the Ethical Review Board of the Faculty of Medicine of the University of Sao Paulo (approval Nº 2.728.843). The procedures were conducted in compliance with the ethical standards of the committee. Patients diagnosed with CD were treated according to the guidelines of the Brazilian Health Ministry.

RESULTS

This study describes the laboratory investigation of two ACD outbreaks that occurred in the riverside communities of Marimarituba and Cachoeira do Arua, in the Santarem municipality, Para State, located in the Northern region of Brazil, in March 2016 and August 2017, respectively. Of the 15 patients confirmed with ACD, 8 lived in Marimarituba and 7 lived in Cachoeira do Arua, and they reported the symptoms after having consumed fruit juices collected from typical palm trees of the region: bacaba (*Oenocarpus bacaba*) and pataua (*Oenocarpus bataua*).

Two pregnant patients were followed up, and their pregnancies lasted until full term. The child born to the patient from Marimarituba presented a positive direct examination and cPCR for *T. cruzi*. The child born to the patient from Cachoeira do Arua was diagnosed by cPCR.

The description of the 17 cases is summarized in Table 1 and Figure 1.

Only the samples with a positive cPCR were submitted for analysis for *T. cruzi* DTUs, which included 13 samples from the patients with ACD by oral transmission and 2 samples collected from the two children born to the two women with ACD, from Marimarituba and Cachoeira do Arua.

Marimarituba

The outbreak in Marimarituba happened between March and April 2016. Eight patients between 2 and 56 years old (62.5% female) were diagnosed with ACD. A two-year-old child died (index case) and this case was published in 2019[16].

Table 1 - Description of the two ACD outbreaks that occurred in March 2016 and August 2017 at Marimarituba and Cachoeira do Arua.

Location	Marimarituba – PA 2° 12' 29" S 55° 1' 14" W	Cachoeira do Arua – PA 2º 38' 58" S 55º 43' 24" W
Year	2016	2017
Orally infected patients (N)	8	7
Age range	2 to 56	4 to 48
Female	63.50%	52.10%
ACD Diagnosis		
Thick drop	7	7
Serological + Epidemiology + Symptoms	1	
Deaths	1	0
Ingested beverage	Bacaba (*Oenocarpus bacaba*)	Pataua (*Oenocarpus bataua*)
Pregnant	2	1
Born alive	1	1
PCR S35/36 +		
Oral outbreak	7	6
Possible congenital	1	1
PCR genotyping assays		
24Sα ribosomal (rRNA) gene D7 domain	120-bp	110-bp
Intergenic region of spliced leader genes	150-bp	350-bp
DTU	TcIV	TcI

According to the patients' reports, the estimated date of ingestion of bacaba wine from the same origin happened between March 8th and March 15th, 2016. The patients showed no signs of inoculation.

Among the seven confirmed cases of the Marimarituba outbreak, one patient with a negative direct test was cPCR positive. Another patient with a positive direct test was cPCR negative. In this case, the PCR sample was collected on the fifth day of treatment with benznidazole.

Two women were pregnant at the time of diagnosis: in one case there was a miscarriage in the 15th week of pregnancy, nine weeks after the ACD diagnosis; in the other pregnant woman, spontaneous vaginal labor occurred during the period of ACD diagnosis. The child's diagnosis occurred three months after birth by thick drop, as described in a previous publication[18].

All patients were treated with benznidazole at 5 mg/kg/day soon after the diagnosis, except for the two pregnant women, of which one was treated after delivery and the other after abortion. The child was also submitted to therapy following the diagnosis.

Four cases had an adverse event: two patients presented with dermopathy, and one 6-year-old child presented with nausea secondary to medication use. The treatment was not interrupted despite the adverse events. There was a 75-year-old adult patient who developed peripheral neuropathy at the end of treatment.

All eight samples, including the child that was born, presented the 24Sα reaction 120-bp and mini-exon 150-bp, corresponding to *T. cruzi* TcIV (Figure 2).

Cachoeira do Arua

The second outbreak of ACD happened in Cachoeira do Arua, in August–September 2017. The diagnosis of ACD was confirmed by a thick drop in seven patients. Similar to the outbreak described above, the patients included a child and a pregnant woman in the 26th week of pregnancy. The ages ranged between 4 and 48 years old, and 57. 1% were women. Three patients reported the ingestion of pataua juice from the same origin on August 21st, 2017, and the symptom onset started five to seven days after consumption, with no signs of inoculation upon physical examination.

Six patients were treated with benznidazole at 5 to 10 mg/kg/day soon after diagnosis. The pregnant woman started the treatment after term delivery, 3 months and 15 days after the diagnosis. The child was diagnosed three months after birth.

The sample, whose cPCR was negative for the detection of *T. cruzi*, was collected from the pregnant woman after

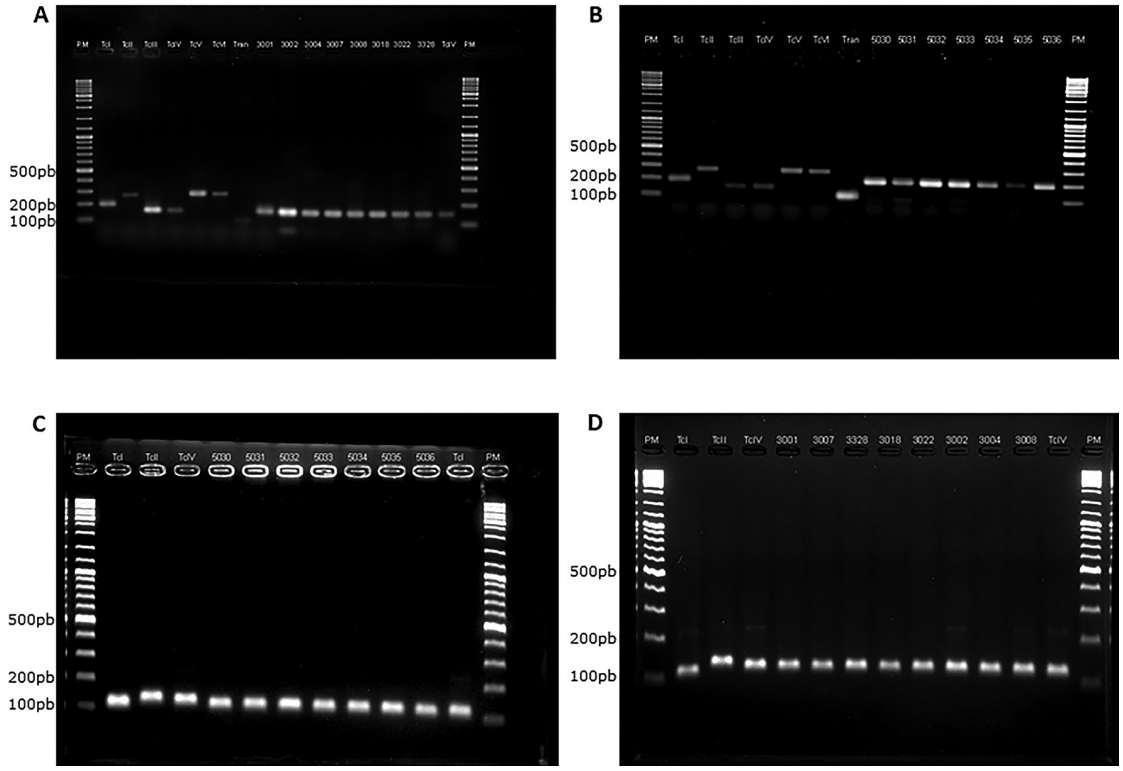

Figure 2 - Genotyping patterns of *T. cruzi* sequences: SL-IR (A and B) and 24Sα rRNA (C and D). Reference strains (GeneBank accession code): TcI – JR cl4 (HQ604893.1), 110-bp; TcII – Y (AF301912), 125-bp; TcIII – MT3663 (AF303060) 110-bp; TcIV – JJ (AY491761), 120-bp; TcV – Bertha (FJ555614), 125 and 110-bp and TcVI – CLBrener (AF245383), 125-bp. Marimarituba's samples (A and C): 3001, 3002, 3004, 3007, 3008, 3018, 3022, 3328. Cachoeira do Arua's samples (B and D): 5030 to 5036. MM: 100-bp Molecular Weight Marker.

delivery and after treatment with benznidazole. Two patients had dermopathy secondary to medication use, although the treatment was not suspended. In this outbreak, there were no deaths. In this study, the *T. cruzi* DTU classification was TcI, including for the child that was born during the outbreak. The PCR target 24Sα rDNA resulted in 110-bp, and the PCR mini-exon had a 140-bp product (Figure 2).

DISCUSSION

Despite the large reduction in the incidence of ACD cases by vectorial transmission, as a consequence of the implementation of elimination programs for *Triatoma infestans,* total control of transmission is impossible due to the sylvatic cycle of triatomines and parasites. The control of the disease is even more challenging in an ecosystem such as the Amazon.

In this region, the natural transmission cycles of *T. cruzi* are complex, owing to the fact that they involve several routes, as well as the considerable diversity of infected vectors and wild mammals. *T. cruzi* DTUs TcI, TcIII, and TcIV are implicated in the sylvatic transmission cycle of this region[3,4].

The occurrence of ACD linked to the ingestion of contaminated food has been reported systematically in the last 15 years, especially in the Amazon region, as well as vectorial transmission related to accidental human exposure to the wild cycle[13].

In this report, two outbreaks were described and, in both, individuals from the same family shared the consumption of artisanal beverages and were diagnosed with ACD, without any signs of inoculation upon physical examination. Oral transmission probably represents the main mechanism of parasite dispersion between vectors and wild animals. In humans, the oral transmission of Chagas disease is considered when there is more than one acute case of the disease related to the ingestion of suspected food[19].

T. cruzi DTU identification was done directly from venous blood samples in cases of a positive cPCR. In most reports, the parasites were isolated from the blood of patients, either by blood culture or xenodiagnosis, and subsequently replicated in cultures[3]. Although the isolation and cultivation of the parasite increase the sensitivity of molecular assays applied to the classification of *T. cruzi* DTUs, there are certain restrictions: the rate of parasite recovery is low, and the process can select subpopulations that do not necessarily reveal the *T. cruzi* DTUs responsible for infecting the patient[3].

In the study's genotyping, the finding of *T. cruzi* TcI in Cachoeira do Arua's patients and *T. cruzi* TcIV in Marimarituba's reflect the DTUs that circulate in the region's wild cycle.

T. cruzi TcI, in addition to the strong association with *Didelphis*, has also been isolated together with *T. cruzi* TcIV from non-human primates and vectors of the genus *Rhodnius* spp., whose main nest is the palm tree[5]. Six percent of human infections were caused by *T. cruzi* TcIV, predominantly in the Amazon region, and related to outbreaks of oral transmission. *T. cruzi* TcI has been detected both in the wild and domestic cycle of *T. cruzi*. This DTU has the highest incidence in the Americas, with approximately 60% of all documented typing processes. It is responsible for approximately 30% of human infections and has been observed in both the acute and chronic phases of Chagas disease[4].

Despite the identification of different DTUs, i.e., *T. cruzi* TcIV in the Marimarituba outbreak, in which one of the patients died, and *T. cruzi* TcI in Cachoeira do Arua without the occurrence of deaths, it is not possible to attribute the fatal outcome to *T. cruzi* TcIV. Although several studies have attempted to correlate the implications of the different DTUs with the clinical course of Chagas disease, no conclusive evidence has been found so far[3,10].

T. cruzi TcI, in addition to being associated with ACD cases, was also shown to be the main cause of chronic infection in patients from Manaus municipality, Amazonas State[20]. With the exception of the JJ strain, isolated from a patient with CCD in Barcelos municipality, Amazonas State, there are no more reports of the identification of this DTU at this stage of the disease in Brazil[5]. Another report identified *T. cruzi* TcIV in three patients with CCD in Ecuador, with one patient presenting the indeterminate form, and two patients with the cardiac form and megasyndromes[9].

Two possible congenital transmissions are reported in the present paper: *T. cruzi* TcIV, which has not yet been related to vertical transmission, and TcI. It should be noted that the congenital form of CD is considered acute, therefore, of compulsory notification, although regrettably there is no specific national surveillance policy for exposed pregnant women and children, and even infected non-pregnant women of childbearing age in Brazil[13].

The Clinical Protocol and Therapeutic Guidelines (PCDT, in the Portuguese acronym) for Chagas disease from the Ministry of Health were issued as recently as 2018, containing a recommendation for prenatal screening for pregnant women in risk groups[13]. CD is considered a neglected disease that affects people living on farms, rural workers, riverine populations, quilombolas, and indigenous people, who have limited access to health and education services[13].

CONCLUSION

This study allowed us to identify orally transmitted *T. cruzi* DTUs involved in two ACD outbreaks directly from the patient's biological sample, providing more data on the diversity of *T. cruzi* in the region. The complementing of field research with molecular methods of parasitic characterization can help us to understand the role of the parasite in the emergence of CD in the Amazon region, contributing to the improvement of the management of this important, albeit neglected disease.

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AUTHORS' CONTRIBUTIONS

VLTF, MRP and HRE: conceptualization, investigation, formal analysis and writing; EYSN and CAF: carried out the molecular assays and the initial analyses; JGPLA and OCPB: designed the data collection instruments, collected data and carried out the initial analyses; FOSF and MHL: coordinated and supervised data collection and wrote and critically reviewed the manuscript. All authors reviewed and approved the final manuscript as submitted and agreed to be accountable for all aspects of the study.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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