Genotyping of *Trypanosoma cruzi* Sublineage in Human Samples from a North-East Argentina Area by Hybridization with DNA Probes and Specific Polymerase Chain Reaction (PCR)

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**Abstract.** We have evaluated blood samples of chronic and congenital *Trypanosoma cruzi*-infected patients from the city of Reconquista located in the northeast of Argentina where no information was previously obtained about the genotype of infecting parasites. Fourteen samples of congenital and 19 chronic patients were analyzed by hybridization with DNA probes of minicircle hypervariable regions (mHVR). In congenital patients, 50% had single infections with TcIId, 7% single infections with TcIIe, 29% mixed infections with TcIId/e, and 7% had mixed infections with TcIId/b and 7% TcIId/b, respectively. In Chronical patients, 52% had single infections with TcIId, 11% single infections with TcIIe, 26% had mixed infections with TcIIe/d, and 11% had non-identified genotypes. With these samples, we evaluated the minicircle lineage-specific polymerase chain reaction assay (MLS-PCR), which involves a nested PCR to HVR minicircle sequences and we found a correlation with hybridization probes of 96.4% for TcIId and 54.8% for TcIIe.

**INTRODUCTION**

American trypanosomiasis is a zoonosis caused by the protozoan *Trypanosoma cruzi*, extended throughout Latin America, where 16 to 18 million individuals are estimated to be infected and more than 50 million inhabitants are in a health hazard.1 Chagas disease is characterized by a broad range of clinical signs, which may be explained in part by the genetic heterogeneity of *T. cruzi*.2 In fact, a great diversity of *T. cruzi* clones with different biological, immunological, genetic, molecular, and pharmacological response characteristics have been isolated from different sources.3,4 In the last years, virulence and tropism differences have been reported in genetically distant strains.5–8 Furthermore, different *T. cruzi* genotypes have been correlated with different virulence and antibiotic resistance.9–12 These findings indicate that the genotype identification may be relevant to provide a prognosis of disease progression and/or differential treatment decisions based on the infective strain.

Currently, two major lineages are described in *T. cruzi* species, named *T. cruzi I* and *T. cruzi II*, and the *T. cruzi II* is subdivided into five sublineages, *T. cruzi Ia*, Iib, Iic, IID, and Ile.14,15 This classification is mainly based on methods requiring parasite isolation and culture. However, as the isolation of the parasites in cultures or mice could lead to strain selection, only direct characterization from insects and mammalian samples allows strains to be typed without bias.16 On this basis, and given the high sensitivity of kinetoplastid minicircle amplification by polymerase chain reaction (PCR), the polymorphism of minicircle hypervariable regions (mHVRs) has been used in many studies for the direct molecular characterization of parasitic populations from biological samples.5,17–22

Beyond the high variability of mHVR, the comparison of several minicircle regions within and among strains, has allowed identification of some sequences more represented in certain sublineages.23 In a previous work,24 we have described that a predominant minicircle sequence present in TcIId and two others predominant minicircle sequences present in TcIIe, are indeed present in reference strains from other *T. cruzi* lineages and sublineages. However, the amount of these sequences is at least a thousand times more represented in the respective sublineage than in the others. Supported by this fact, we have developed a semiquantitative PCR directly from biological samples, using GrP1 primers, designed by Velázquez and others,24 and primers (GrO-GrP) that amplify O and P sequences,25 to discriminate TcIIe and TcIId sublineages, respectively, and to differentiate them from the other lineages and sublineages.

In this work, we have evaluated this assay by extending the number of reference strains analyzed and testing biological samples by hybridization with a panel of genotype-specific minicircle DNA probes. This new method is highly useful for genotyping directly from blood of infected individuals because it does not require microorganism isolation, avoiding bias in the strain typing. Moreover, it uses kinetoplast DNA PCR products as templates, which is one of the most sensitive assays to detect parasite genetic material in biological samples.21,25–28 On the other hand, this assay requires only a PCR amplification and products are visualized by a simple agarose gel stained with ethidium bromide.

The biological samples analyzed in this work were obtained from acute and chronic chagasic patients from a high endemic region of northeastern Argentina. The current sampling extends epidemiological findings obtained by several groups of Latin America,8,22,26–32 and particularly of Argentina,21,33–36 showing the predominance of TcIId infection in human and mammalian hosts.

**MATERIALS AND METHODS**

**Reference strains.** Reference strains used in this work were: TcI strains, X10e1l; TcIa strains, CANIHe1l, TcIb strains, TU18 c93, IVV c4, MCV, CBB c13; TcIIc strains, M5631c15.
and P109/2; TcIId strains, JGG, XHCH56; NR cl3, SC43 c11 and MN c2; TcIIe strain, CL Brener and V195 c11.

Patients and blood samples. This study was carried out with 19 chronically and 14 congenitally infected individuals of Reconquista, Santa Fe province, Argentina who were positive by conventional serology enzyme-linked immunosorbent assay (ELISA) and indirect hemaglutination and/or microhematocrit in the congenital cases. Seven milliliters of blood (1 mL in neonates) were mixed with an equal volume of buffer Guanidine HCl/EDTA (6 M/0.2 M), boiled in a water bath for 15 minutes to shear and decatenate the minicircle DNA molecules from the kinetoplast network, and kept at −20°C until used for PCR assays.

Informed consent. Informed consent was obtained from all human adult participants and neonate parents. The project was approved by the Ethical Committee of the Biochemistry Faculty, National University of Litoral, Argentina.

DNA extraction. Samples kept in polypropylene tubes containing guanidine/EDTA lysates were processed using 200 μL aliquots with the phenol-chloroform extraction, ethanol precipitation, and DNA resuspension in 200 μL sterile distilled water. Each set of purifications were performed with the respective positive and negative controls.

Hybridization with specific radioactive probes. All PCR-amplified DNA from blood samples were analyzed by Southern blot assay with a panel of four genotype-specific probes from T. cruzi clones: CBB c13 (TCIIb), NR c13 (TCIIId), sp104 c11 (TCI), and v195 c11 (TCIIe). Because these probes derive from the whole kinetoplast, they are composed of all minicircle sequences present in a parasite clone. Clone sp104 was first isolated from Mepraia spionalis (sylvatic cycle). Meantime, the CBB and NR clones were isolated from chronically infected individuals, and clone v195 from Triatoma infestans (domestic cycle). The probes were radiolabelled with P32 and membranes were explored and analyzed in a Personal Molecular Imager-FX (Bio-Rad, Hercules, CA). Moreover, probes were hybridized against a panel containing minicircle amplicons of T. cruzi clones as described.

Minicircle lineage-specific PCR (MLS-PCR). The assay consists in a nested PCR, in which the first reaction amplifies all mHVRs sequences, according to the classic T. cruzi detection.

Typing of CAN III, M5631, MN, and CL Brener strains. Fifteen strains, belonging to different lineages and sublineages of T. cruzi were analyzed by MLS-PCR using 0.3 pg of the first amplification product as reported in Materials and Methods. Ttyping of CAN III, M5631, MN, and CL Brener strains were performed twice, starting from parasite DNA obtained from collections of two different laboratories. As expected, the belonging to TcIIe sublineage amplified only with Grp1 primers and strains belonging to TcIId sublineages amplified exclusively with GrO and GrP primers, indicating complete matching (Figure 1).

Evaluation of MLS-PCR using reference strains. Fifteen strains, belonging to different lineages and sublineages of T. cruzi were analyzed by MLS-PCR using 0.3 pg of the first amplification product as reported in Materials and Methods. Typing of CAN III, M5631, MN, and CL Brener strains were performed twice, starting from parasite DNA obtained from collections of two different laboratories. As expected, the belonging to TcIIe sublineage amplified only with Grp1 primers and strains belonging to TcIId sublineages amplified exclusively with GrO and GrP primers, indicating complete matching (Figure 1).

Genotyping of human samples by hybridization assay. Until now, the typing of T. cruzi genotypes responsible for Chagas...
disease in northeastern Argentina was not boarded. In this work, we have done a preliminary analysis of genotypes present in this region using minicircle DNA probes and hybridization tests, which is a widely used method to approach this task.11,22,38 These results not only gave information about genotypes present in our region but they allowed us to make a genotyped panel of human samples to test with the MLS-PCR method. From the 14 congenitally infected individuals analyzed, 7 had single infections with TcIId sublineage, 2 with TcIle, 4 mixed infections with TcIId/e, 1 with TcIId/b, and 1 with TcIlb/e. For two congenital patients (2- and 4-month-old samples) were taken: The results for the four samples indicated single infection with TcIId. For the 19 chronic-infected patients, we observed 10 single infections with TcIId, 2 with TcIle, and 5 were mixed infections with TcIId/e. Two samples amplified with S121-122 primers but they were not recognized by any of the four probes. Infections with different genotypes are presented as percentages (Figure 2).

### Genotyping of patient samples using MLS-PCR assay

To test MLS-PCR directly from human-infected patients, all samples genotyped by hybridization tests were subjected to this nested PCR. In the first PCR, we used generic primers to amplify total mHVR sequence population and the second PCR was carried out using specific primers to detect predominant minicircle sequences of TcIId and TcIle sublineages. In the congenital group, 14 samples from 14 congenitally infected individuals amplified with GrP primers, 10 of which amplified with GrO, which would indicate a minor detection with these primers. From these samples, which specifically amplified for TcIId sublineage two amplified also with GrpI primers, showing TcIId/e mixed infection. On the other hand, only one sample amplified exclusively with GrpI primers, indicating a unique sample belonging to the TcIle sublineage.

In the chronic cases, 12 samples were amplified exclusively with Grp primers. Within these samples, only one was amplified with GrO-specific TcIId primers, and three of them shared a mixed amplification with GrpI TcIle primers. A single TcIle infection was evidenced by a GrpI amplified sample. Finally, three of the samples that were amplified with S121-122 generic primers did not amplify with any pair of specific primers.

Results showed that all the samples (chronic and acute cases) genotyped by hybridization as TcIId, amplified with Grp primers, whereas only some of them amplified with GrO. When we evaluated MLS-PCR sensitivity to amplify O and P sequences, we found that it was related, with high significance ($X^2; P < 0.001$), to the amount of amplified generic PCR product in the first PCR performed with generic primers (Table 1). Taking into account the higher sensitivity of Grp primer and its independence from the amount of the first PCR product, we used only these primers to genotype TcIId strains.

### Comparison between hybridization and MLS-PCR assay

To compare the TcIId and TcIle sublineages discrimination performance of the MLS-PCR assay with the hybridization one, we classified mixed infections with two sublineages as two individual results and single sublineage infection as a single result. The results obtained for each assay were then analyzed by Kappa index. By these criteria, the hybridization results of samples were classified as 27 TcIId (17 single infections and 9 TcIId/e mixed infections plus 1 TcIId/b mixed infection) and 13 TcIle (3 single infections and 9 TcIId/e mixed infections plus 1 TcIId/e mixed infections). The distribution of the number of TcIId, TcIle, and no TcIId/e-infected patients is presented in a contingency table (Table 2). Three patient samples were non-genotyped by hybridization. On the other hand, the MLSPCR results of samples were classified as 28 TcIId (24 single infections and 4 TcIId/e mixed infections) and 7 TcIle (2 single infections and 5 TcIId/e mixed infections). Eight samples were not genotyped by MLS-PCR. The concordance between the hybridization test and MLS-PCR was 96.4% for TcIId sublineages (GrpI primers) and 54.8% for TcIle sublineages (GrpI primers). These results would indicate low sensitivity of GrpI primers to genotype TcIle strains, whereas P primers were highly sensitive to genotype TcIId strains. Although a low sensitivity of GrpI primers was observed, the total concordance of the MLS-PCR assay using both GrP and GrpI primers to

### Table 1

<table>
<thead>
<tr>
<th>DNA template (ng)</th>
<th>Number of samples amplified with O or P primers (n/nt)</th>
<th>Primers O (%)</th>
<th>Primers P (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 56</td>
<td>8/8 (100)</td>
<td>8/8 (100)</td>
<td></td>
</tr>
<tr>
<td>11–56</td>
<td>10/11 (90)</td>
<td></td>
<td>11/11 (100)</td>
</tr>
<tr>
<td>&lt; 11</td>
<td>12/22 (55)</td>
<td>22/22 (100)</td>
<td></td>
</tr>
</tbody>
</table>

* Higher than primers O ($X^2; P < 0.001$).
**Table 2**

Contingency table to analyze the relationship between minicircle lineage-specific polymerase chain reaction (MLS-PCR) and hybridization assay to genotype TcIId and TcIle Trypanosoma cruzi sublineages

<table>
<thead>
<tr>
<th>Probes</th>
<th>TcIId</th>
<th>TcIle</th>
<th>No TcIId/ No TcIle</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>TcIId</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>TcIle</td>
<td>0</td>
<td>7</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>No TcIId/ No TcIle</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>7</td>
<td>8</td>
<td>43</td>
</tr>
</tbody>
</table>

*Kappa index: 0.69.

genotype TcIId, TcIle, or no TcIId/e had a statistical Kappa index of 0.69, indicating a good agreement.

**DISCUSSION**

Human *T. cruzi* infecting strains have begun to be genotyped recently by different groups in different endemic regions of Argentina. Direct genotyping from blood samples is highly difficult due to the poor parasitaemia, mainly in the chronic phase of the infection. Consequently, most of the protocols have resorted to previous parasite isolation in culture medium or mammalian host. However, this approach may produce a selection from the original mixed infecting population, therefore direct PCR from sample would be a valuable method for accurate genotyping. Moreover, as the minicircle PCR amplification from minicircles is one of the most sensitive methods to obtain parasite’s genetic material from biological samples, the use of mHVR can be successfully used to genotype *T. cruzi*.

Although the hybridization and MLS-PCR assays used here are based on the characterization of the same mHVR-PCR product, the last technique uses a second PCR, which identify unique minicircle sequences. This approach could increase the reliability and reproducibility with respect to hybridization with total minicircle sequences as the composition of DNA probes is complex and undefined.

Until now, most of the *T. cruzi* infections studied in mammals and humans from Argentina, TcIId sublineages are highly prevalent, with a lower frequency of TcIle. Keeping in mind these previous results, in this work we have focused on the evaluation of a new assay to characterize these sublineages. For this purpose, three pairs of primers were used. One primer amplifies Grl, a major minicircle sequence in CL-Brener strain of TcIle sublineage, which cover 40% of sequences found in that strain. On the other hand, two pairs of primers amplify predominant sequences in TcIId sublineage, covering 80% of sequences found in that sublineage. In a previous work, using specific probes for two TcIId predominant sequences (O and P), Virreira and others proposed a sub-classification within this sublineage. In this way, TcIId were subdivided in MN-like, Bug2148-like, and TPK-like, if amplified PCR products hybridized mainly with O sequence, mainly with P, or equally with both of them, respectively. Recently, using this criterion the three subgroups of TcIId sublineage were described in northwestern Argentina. In our work, we have amplified these two predominant sequences by PCR in biological samples, however we could not amplify exclusively O sequences, although we have always obtained amplification for single P sequence, or O–P mixed sequences. Therefore, if we correlate our PCR results with Virreira’s classification based on a hybridization test performed with the same oligonucleotids, these results have allowed us to type Bug 2148-like and TPK-like genotypes, although we did not find MN-like genotypes. Indeed, when reference strains were amplified using 0.3 pg of template, SC43 cl1 and MN cl2 were highly amplified with both sets of primers, whereas JGG, XHCH56, and NR cl3, were highly amplified with P primers but not with O primers. As MN cl2 belongs to MNcl2-like and SC43 cl1 belongs to TPK-like (previously classified by Virreira and others), but rendered similar results by MLS-PCR, these results could indicate that the PCR discriminates two variants within TcIId sublineage instead of three.

The difference between the hybridization test performed by Virreira and others and MLS-PCR assay may be a result of a different ratio of O and P sequences in the purified DNA from samples as O sequence obtained from S121-122 generic PCR is less amplified than P sequence according to Table 1. Therefore, probably the amount of O sequences present in DNA template is insufficient to be amplified by means of this amount of template. Because of the higher sensitivity observed in PCR amplifications with P primers, we proposed only the use of these primers to genotype TcIId sublineage by MLS-PCR. Through these criteria a 96.4% of concordance was observed with the hybridization assay.

On the other hand, when TcIle sublineage was genotyped using Grl1 primers, a total concordance was observed when the strain reference panel was evaluated. However, a lower sensitivity was obtained when blood samples were compared with the hybridization test (54.8%). These observations would indicate that the use of Grl1 primers are not enough to type all the samples of this sublineage and other predominant sequences should be added to genotype misclassified samples. Nevertheless, we should not discard that v195 probe specific for TcIle crossreacts with other sublineages.

Therefore, the PCR assay herein described allows identification of TcIId and TcIle sublineages and TcIId/e mixed infections directly from blood samples. The assay is simpler to do and to standardize than hybridization assay because it uses only PCR amplification technique instead of radioactive or immunolabeled probes. When TcIId genotype was tested, the results were 96.4% coincident with hybridization assay. However, when TcIle genotype was tested, the sensitivity of the new assay was 54.8% compared with hybridization assay. Currently, we are identifying new sets of primers to increase the sensitivity to type TcIle genotype and to classify other genotypes. Because of the high sensitivity and specificity to detect TcIId genotype, the new tool described previously may be highly useful to assess the prevalence of TcIId sublineage directly from blood samples in wide geographic regions, and it would be very useful to support the results obtained by other methods.

When we analyzed the genotyping results of the overall infected samples from the Northeastern Argentina region, we detected TcIId predominance both in children and adults, and in a minor proportion, TcIle. These results are compatible with those obtained from other regions of Argentina. Indeed, PCR-based techniques were recently applied to genotype *T. cruzi* samples from vector, animal, and human samples from Gran Chaco region, Santiago del Estero Province, Argentina, where a predominance of TcIId sublineage was detected in humans, and TcIle in domestic mammals and...
The prevalence of TcIId was also found in congenital cases and their respective mothers, from different regions of Argentina and Bolivia. Similar results were obtained among congenitally infected newborns in northwestern Argentina.

However, a previous work, focused in a restricted endemic area of Chaco Province of Argentina, had reported for the first time the presence of TcIIe associated with dogs, and TcIId associated with human infection. The same sublineage was reported in peripheral blood of chagasic patients co-infected with human immunodeficiency virus (HIV), whereas other sublineages (TcIIb and TcIHe) were found in heart, brain, and skin lesions of these patients. Applying a serological diagnosis method, Di Noia and others showed that 95.4% of confirmed chagasic patients from Argentina were reactive to TSSA-II, a TcII-specific antigen, whereas only 3% of these sera displayed a mixed recognition to TSSA-II and TSSA-I, the latter specific for TcI. None of these patients showed exclusively TcI infection. Because we could not detect TcI in none of our patients our findings are in agreement with this work, although this lineage has been reported in the earliest studies done in our country. Indeed, the first studies using multilocus enzyme electrophoresis, showed that the more abundant (sub) lineages circulating in Argentina were TcI, TcIId, and TcIIb. Overall, TcII was more prevalent in the domestic area, whereas TcI and TcII infections were found in sylvatic mammals. A recent work suggests that TcI may be involved in infections of immunosuppressed patients, since this genotype was found in tissues different from blood. However, the cited work is a case report from a congenital acquired infection and no information about the geographical origin of the mother is available; then epidemiological data cannot be obtained about this patient.

We have also found 30.6% of mixed infections, predominantly TcIId/e, although one TcIIb/d and one TcIIb/e were also detected. It is important to clarify that mixed infections were observed in a minor proportion with MLS-PCR than hybridization assay, which may be a result of the lower detection of TcIIe sublineage by Grp1 primers used as previously discussed.

Interestingly, we found a similar distribution of sublineages in both congenital and chronic patients. Similar results were obtained in northwestern Argentina and in a previous study performed in Bolivia. These results would indicate that congenital transmission is not caused by a specific genotype as was yet suggested by Virreira and others.

In conclusion, our data about T. cruzi genotypes present in the Reconquista region of Santa Fe province, in coincidence with reports of other regions of our country cited previously, support the assumption that human infection by T. cruzi in Argentina is due principally to T. cruzi II. In that context, a correlation between clinical manifestation and the genotypes could be hypothesized as the cardiac sign that Chagas disease is predominant in Santa Fe province and in all of the country of Argentina. This hypothesis would be supported by the correlation of cardiac signs with TcII strains described by other authors, even though more evidences of this assumption should be obtained.

Disclosure: ISM and VG are researchers from the CONICET (Argentina).

References


USE OF PCR TO GENOTYPE *T. CRUZI* FROM BLOOD


