Genotyping of *Trypanosoma cruzi*: Systematic Selection of Assays Allowing Rapid and Accurate Discrimination of All Known Lineages

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Abstract. *Trypanosoma cruzi*, the agent of Chagas disease, can be subdivided into six discrete typing units (DTUs), TcI, TcIIa, TcIIb, TcIIc, TcIIId, or TcIIe, each having distinct epidemiologically important features. Dozens of genetic markers are available to determine the DTU to which a *T. cruzi* isolate belongs, but there is no consensus on which should be used. We selected five assays: three polymerase chain reaction (PCR)-restriction fragment length polymorphisms based on single nucleotide polymorphisms (SNPs) in the *HSP60*, Histone *H1*, and *GPI* loci, and PCR product size polymorphism of the LSU rDNA and mini-exon loci. Each assay was tested for its capacity to differentiate between DTUs using a panel of 48 genetically diverse *T. cruzi* clones. Some markers allowed unequivocal identification of individual DTUs, however, only by using a combination of multiple markers could all six DTUs be resolved. Based upon the results we recommend a triple-assay comprising the LSU rDNA, *HSP60* and *GPI* markers for reliable, rapid, low-cost DTU assignment.

INTRODUCTION

The protozoan parasite *Trypanosoma cruzi*, causative agent of Chagas disease, is harbored by at least 10 million people in Latin America and is estimated to cause ~13,000 deaths per year. *T. cruzi* is endemic across the vast majority of Latin America and into the southern states of the United States, but Chagas disease occurs primarily in areas where human populations come into contact with domiciliated triatomine vector species. Furthermore, blood transfusion and congenital transmission can lead to cases outside Latin America. Control campaigns have resulted in reduced levels of *T. cruzi* transmission across much of the endemic area, yet significant challenges remain. For *T. cruzi*, multilocus genotyping has consistently shown six distinct DTUs, TcI, TcIIa, TcIIb, TcIIc, TcIIId, and TcIIe, each having distinct epidemiologic and evolutionary aspects. Although many typing systems are in use for *T. cruzi*, there is a lack of data regarding comparison of different methods, particularly with respect to the relatively undersampled DTUs TcIIa and TcIIc, which are only occasionally present in domestic settings.

Two of the most commonly used *T. cruzi* genotyping assays exploit sequence variability in the D7 divergent domain of the 24S rRNA locus (LSU rDNA) and in the non-transcribed intergenic region of the SL-RNA (mini-exon) array. This permits discrimination of some of the different lineages by simple visualization of differences in polymerase chain reaction (PCR) product size,9,10,20–22 (Table 2). Using the rDNA target, some lineages are easily genotyped because they give single band profiles, for example, 110 bp for TcI/Iic or 125 bp for TcIIb/Iie. TcIIId strains are typically characterized by the presence of both of these bands, although the larger band can be weak or absent entirely.10,20 Furthermore, TcIIa does not have a single characteristic band size: some isolates, including the reference strain CanIII cl1, give a band smaller than 125 bp, estimated to be either 120 or 117 bp, whereas TcIIa isolates from North America [TcIIat(NA)] seem to be characterized by a 130 bp band.20 Regarding the mini-exon, early studies showed a multiplex PCR assay easily differentiated TcI (350 bp) from TcIIb/Iid/Iie (300 bp).9,10 Using this assay to characterize ZIII isolates (TcIa and TcIc) has proven to be less straightforward; some authors report a lack of amplification,20 whereas others have successfully amplified products of 400 bp for TcIa and 250 bp for TcIc.23,24 Others recommend the use of modified protocols using lineage-specific primers to allow discrimination of ZIII isolates.12,25

A number of PCR-restriction fragment length polymorphism (RFLP) protocols have been described,25,27 but to date, they have only been tested on a limited number of isolates, and it is not clear which are most suitable for standardized, widespread application. We selected three of these assays that, when used in combination, showed the potential to identify
TcI, TcIIa, TcIIb, TcIIc, and a joint TcIIId/TcIIe group. We set out to compare the performance of these three PCR-RFLP assays with the LSU rDNA and mini-exon genotyping assays using a large cohort of T. cruzi clones representing all six DTUs. We show that combining the LSU rDNA assay with two of the PCR-RFLP assays allows the simple, rapid, and low-cost resolution of all known T. cruzi DTUs.

MATERIALS AND METHODS

Parasite stocks and extraction of genomic DNA. A panel of 48 T. cruzi biological clones representing all six DTUs was assembled (Table 3). They originate from diverse localities in endemic areas and consist of isolates from sylvatic and domestic transmission cycles; their sources include triatomine vectors, mammal hosts, and infected humans; full details of their origins are given elsewhere. Parasites were cultivated in supplemented RPMI-1640 liquid medium (Sigma, Gillingham, UK) at 28°C as described previously. Total genomic DNA was prepared from logarithmic phase cultures using standard phenol:chloroform protocols or Qiaquick DNA preparation kit (Qiagen, Crawley, UK) according to the manufacturer’s protocols.

PCR product size polymorphism assays. All strains were characterized by PCR amplification of the D7 divergent domain of the 24S rRNA gene (LSU rDNA) and the non-transcribed spacer of the mini-exon gene using standard protocols. Amplification reactions contained 0.2 mmol/L of each dNTP, 1.5 mmol/L MgCl₂, 1 pmol/μL of each primer, 1 Unit of Taq DNA polymerase (Bioline, London, UK) and 10–100 ng gDNA. For the LSU rDNA, PCR primers D71 and D72 were used, and amplifications were performed using an initial denaturation step of 94°C for 3 minutes and then 27 amplification cycles (94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute), followed by a final elongation step at 72°C for 5 minutes. For the mini-exon PCRs, a pool of three primers, TC, TC1, and TC2, was used, and amplifications were performed using an initial denaturation step of 94°C for 3 minutes and then 27 amplification cycles (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds), followed by a final elongation step at 72°C for 5 minutes. LSU rDNA PCR products were separated by gel electrophoresis in 3% agarose gels and mini-exon products in 1.5% gels. For expected product sizes, see Table 2.

PCR-RFLP assays. All strains were characterized by restriction enzyme digestion of PCR products from the amplification of three target loci, as first described by Westenberger and others, with minor modifications to the protocol. The following target/restriction enzyme combinations were used: heat shock protein 60 (HSP60)/EcoRV, histone H1/AlaII, and glucose-6-phosphate isomerase (GPI)/HhaI. Amplification reactions were as above but contained 2 mmol/L MgCl₂, and the primer pairs used were as follows: HSP60_for and HSP60_rev (for HSP60), H1_for and H1_rev (for histone H1), and GPI_for and GPI_rev (for GPI). Amplifications were performed using a touchdown PCR strategy comprising an initial denaturation step of 3 minutes at 94°C, followed by four cycles (94°C for 30 seconds, 64°C for 30 seconds, 72°C for 1 minute), followed by 28 cycles (94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute), and then a final elongation step at 72°C for 10 minutes. PCR products were checked on 1.5% agarose gels and if necessary purified using the Qiaquick gel extraction kit (Qiagen) to remove non-specific products. For restriction enzyme digestion, 10 μL of PCR product (typically ~1 μg) was digested in a reaction containing 0.25 U/μL of the appropriate restriction enzyme, i.e., EcoRV (Promega, Southampton, UK), HhaI (NEB, Hitchin, UK), or AarII (NEB, Hitchin, UK), 100 ng/μL BSA, and 1x quantity of the manufacturer’s recommended reaction buffer in a total volume of 20 μL. The digestion reactions were incubated for 4 hours at 37°C, after which 5 μL of the reaction was used for restriction fragment size analysis using either 1.5% agarose gels (GPI/HhaI) or 3% gels (HSP60/EcoRV and histone H1/AarII). For expected product sizes, see Table 2.

RESULTS

LSU rDNA PCR product size polymorphism. All samples were readily genotyped at this locus (Figure 1; Table 3). As expected, all TcI and TcIIc strains gave 110-bp bands and all TcIIb and TcIIe strains gave 125-bp bands. TcIIId strains always had the 110-bp band; usually, the 125-bp band was also present, producing the characteristic double band profile. However, the intensity of the 125-bp band was variable across independent replicates and was sometimes not visible (Figure 1A). CanIII cl1 and three other TcIIa strains gave the expected intermediate-sized product (Figure 1B; Table 3), considered in this study to be 117 bp in accordance with Kawashita and others, rather than 120 bp. One South American TcIIa strain, Saimiri3 cl1, generated the 125-bp normally characteristic for TcIIb/Ice as shown previously. The TcIIa (NA) strains gave 130-bp bands, as found previously for other strains of the same origin.

In summary, genotyping of this locus confirmed its general utility as a discriminatory marker for T. cruzi lineages; however, it does require very small differences in band size (≥5 bp) to be resolved, which can be technically challenging.

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**Table 1**

<table>
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<th>Term</th>
<th>Criterion</th>
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*Group left undefined.

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Low-cost resolution of all known T. cruzi DTUs.
Mini-exon PCR product size polymorphism. All samples were genotyped using PCR amplification of the mini-exon gene (Figure 1C; Table 3). In total, eight isolates were typed as TcIIc (250-bp products), 24 as TcIIb/d/e (300 bp), eleven as TcI (350 bp), and three as TcIIa (400 bp). Compared with the original multiple locus enzyme electrophoresis (MLEE) and/or random amplified polymorphic DNA (RAPD) genotyping, most strains gave product sizes as expected. One strain, Saimiri3 cl1, gave a product estimated to be 380 bp (Figure 1C); a previous study had recorded no amplification for this strain. Another, 10R26, gave no amplification despite repeated attempts, although this strain has previously been reported to contain a TcI-like sequence.

It should be noted that for a number of the strains previously typed as TcIIc (Table 3), the mini-exon exhibited a lack of reproducibility across repeated experiments: the diagnostic 250-bp band was often faint, and multiple non-specific bands were observed more frequently compared with other strains. As an example, profiles showing bands at both 250 and 300 bp were observed for ARMA13 cl1 and SABP19 cl1 (Figure 1C; Table 3). Difficulty in genotyping TcIIc strains by mini-exon PCR is in keeping with previous findings. Nevertheless, in this study, the diagnostic 250-bp band was not observed for any other strains and thus was considered to be indicative of a TcIIc genotype.

The TcIIa strains showed heterogeneity in the size of the mini-exon amplification product and also frequently presented non-specific bands. Besides the two strains already mentioned (10R26 and Saimiri3 cl1), CanIII cl1, 92122102R, and StC10R cl1 did give products of 400 bp, but X10610 cl5 and ERA cl2 produced bands of 350 bp, normally characteristic of TcI.

PCR-RFLP assays. A recent study detailed the development of six PCR-RFLP assays for genotyping T. cruzi lineages. According to the data presented for a panel of 26 T. cruzi isolates (ten of which are also in our panel), there was no single assay that could split the strains into more than three groups of genotypes. However, by combining the data from three of these assays (HSP60 digested with EcoRV, histone H1 digested with AatII, and GPI digested with HhaI), all DTUs except TcIId/IIe would be predicted to have unique multiple assay profiles. To validate the potential of this typing scheme, the three PCR-RFLP assays were applied to all samples.

HSP60. Minimal non-specific PCR products for HSP60 were observed, and this meant that the digestion reaction could be performed directly on PCR products without the need for purification, thus reducing the time needed for this assay. An example of RFLP profiles generated by this assay is shown in Figure 2. The genotype group designations (TcI/IIa/IIb, TcIIc, or TcIId/IIe) were applied to the entire cohort of samples. Across the whole panel of T. cruzi, the genotype assignments showed an exact correlation with those predicted by other genotyping methods (Table 3). Notably, this assay proved to be a reliable method to discriminate TcIIc strains from all other lineages.

Histone H1. The PCR-RFLP of histone H1 was performed for all samples and produced the same three profiles (TcI/IIc, TcIIb, or TcIId/IIe) identified by Westenberger and others (Figure 2; Table 2). Digestion of unpurified PCR products, in some cases, generated some non-specific bands, predominantly one ~320 bp in size. Such bands, however, did not hamper detection of the diagnostic bands. Comparison of the genotype assignments with expectations showed that 45 of...
48 samples in this study gave RFLP profiles consistent with these expectations. Exceptions were Saimiri3 cl1, StC10R cl1, and 92122102R, all of which gave TcI/IIc profiles rather than the expected TcIIa/IId/Ile profile. This single assay reliably resolved DTU TcIIb from all others.

**GPI.** As for HSP90, the digestion reaction for GPI amplification products could be performed directly without the need for purification. All the T. cruzi isolates in this study gave RFLP profiles (Figure 2) that could easily be assigned to the possible genotype groups (TcI/Iic, TcIIa/IId, or TcIIIa/IId) identified by Westenberger and others. Genotypes were consistent with those expected for each DTU based on other markers for 46 of 48 samples (Table 3). Exceptions were StC10R cl1 and 92122102R, both of which gave TcI/IIc profiles rather than the expected TcIIa/IId/Ile profile.

**DISCUSSION**

For many years, MLEE was the method of choice for resolving T. cruzi subgroups. With the advent of direct genetic typing, a range of PCR-based assays capable of delineating T. cruzi subdivisions to varying extents were developed and

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Table 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype (method)*</th>
<th>Mini-exon PCR product size polymorphism</th>
<th>LSU rDNA PCR-RFLP</th>
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| X10/1  | TcI (MLEE)         | TcI†††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††††‌
Typing of the LSU rDNA allowed discrimination of the following groups: TcI/TcIIa, TcIId/TcIIe, and most TcIId samples. 20 There are additional concerns for the resolution of TcIId because the double band profile was detected in this study. This could be a result of differences in experimental conditions or genuine genetic differences between the stocks of the same name that are only 5 bp different in size, which can be technically challenging unless appropriate reference strains are used as standards in each analysis.

High-resolution genetic typing can now be achieved using multilocus sequence typing (MLST)27,28,33 or multilocus microsatellite typing (MLMT),11,13,34 these methods are impractical for differentiating between T. cruzi DTUs. 11,13,34 These methods hold much promise given the low resource requirement. For that objective, PCR-RFLP assays are readily applied. However, there has been a tendency for the number of loci used for typing to be reduced to only two that are widely used (LSU rDNA and mini-exon) or various additional loci that are only used by a small number of laboratories. This creates the problem of reduced discriminatory power and/or difficulty in comparing work in different laboratories using different typing systems. Although high-resolution genetic typing can now be achieved using multilocus sequence typing (MLST)27,28,33 or multilocus microsatellite typing (MLMT),11,13,34 these methods are impractical for simple DTU assignment. For that objective, PCR-RFLP assays hold much promise given the low resource requirement. We selected three PCR-RFLP assays based on data from an analysis of 26 strains showing that they had good potential for differentiating between T. cruzi DTUs.27 We set out to test the performance of these assays for typing a large cohort of cloned T. cruzi isolates and compared them to the commonly used mini-exon and LSU rDNA typing assays.

Typing of the LSU rDNA allowed discrimination of the following groups: TcI/TcIIc, TcIId/TcIIe, and most TcIId samples. Brisse and others20 showed that this marker also allowed resolution of two groups within TcIIa corresponding to strains from North America and South America because of unique profiles for each of these groups. This result was also observed here for four additional samples, strengthening the likelihood that there are conserved differences at this locus between North and South American TcIIa strains, and in keeping with some molecular data indicating a significant divergence.32,35 However, caution is needed when drawing such conclusions, as exemplified by the finding that another TcIIa strain, Saimiri3 cl1, has a TcIIb/TcIIe profile.20 There are additional concerns for the resolution of TcIId because the double band profile that specifies TcIId was not always observed. A single band at this locus is a feature that has also been noted for a small number of other TcIId strains including Sc43 cl1,10,20 for which the double band profile was detected in this study. This could be a result of differences in experimental conditions or genuine genetic differences between the stocks of the same name in the different laboratories. A drawback of this genotyping method is the requirement to distinguish between bands that are only 5 bp different in size, which can be technically challenging unless appropriate reference strains are used as standards in each analysis.

The mini-exon marker reliably discriminated DTU TcI, TcIIa, and TcIIe. For example, TcIIa strains gave a number of different profiles, which in some cases would lead
to incorrect DTU assignment if this marker was used alone. One reason for the variable TcIIC and TcIIA profiles is the presence of insertions and/or deletions within the target locus, influencing the efficiency of primer binding.\textsuperscript{20} Such indels have been characterized in two TcIIC isolates and a TcIIA isolate,\textsuperscript{25} but the data presented here suggest that these may not be preserved features within and/or between these sublineages. Furthermore the mini-exon locus exhibits significant secondary-structure,\textsuperscript{27} which could also adversely affect amplification in some cases. Although the mini-exon assay is clearly inferior to the others tested here in terms of its reliability, direct sequencing of the SL-rRNA locus does have potential for the characterization of intra-DTU diversity, as, for example, shown by a recent study of TcI strains in Colombia.\textsuperscript{28}

The three PCR-RFLP assays are clearly useful additions to the repertoire of available \textit{T. cruzi} genotyping protocols. The data presented here are mostly consistent with expectations based on the original study.\textsuperscript{27} If each assay is considered separately, there are two cases where unique profiles that are specific to a single DTU were observed. First, the \textit{HSP60/EcoRV} assay reliably discriminated TcIIc strains and second, the histone \textit{H1/AatII} assay generated TcIIb-specific profiles. The application in combination of the three RFLP markers assessed in this study proved to reliably discriminate all strains into the four non-hybrid DTUs and a fifth combined TcIIb/IIC hybrid group, agreeing with the results from the analysis of Westenberger and others\textsuperscript{27} of 26 strains. However, exceptions to this general rule occurred in the case of the histone \textit{H1/AatII} assay for TcIIa(NA) strains and Saimiri3 c11 and also with the \textit{GPI/IHaI} assay, again with TcIIa(NA) samples. These discrepancies, caused by point mutations in a relevant restriction site, may or may not reflect a more substantial overall divergence between such North American strains and other members of TcIIa; full sequencing of the target loci will be needed to resolve this question. Overall, these assays were simple to perform and, although they require an extra experimental step and additional reagents compared with the mini-exon or LSU rRNA assays, they seem to be less subject to equivocal results. On the other hand, only LSU rDNA is capable of separating TcIIb and TcIIC samples.

None of the individual markers tested here allowed complete DTU resolution, and in any case, reliance on a single marker would be inadvisable because of the consequent loss of resolution and the potential influence of genetic exchange on some lineages. Using a combination of multiple assays, therefore, permits more reliable DTU assignment. Brisse and others\textsuperscript{20} proposed a multiple assay system based on a combination of mini-exon, LSU rDNA and 18S rRNA (SSU rDNA) PCR product size polymorphism assays. Although this strategy does permit assignment into each of the six DTUs, the mini-exon assay seems to lack reproducibility in some cases, and several of the assignments depend on the absence, rather than the presence, of bands, which is inadvisable.

The data presented here show that the application of another combination of markers, including PCR-RFLPs, can achieve the same level of resolution with all assignments depending on the presence of specific band sizes. Only the LSU rDNA marker and the mini-exon are able to distinguish TcIIa(NA) from TcI, so one of these assays should be included. The results of this study lead to a strong preference for LSU rDNA because it allows separation of TcIIb from TcIIc and is much more reliable for typing of TcIIa and TcIIC. Follow-up with RFLP of \textit{HSP60} could resolve all six DTUs. However, each of the RFLPs relies on the presence or absence of either one or two SNPs, which may be affected by mutations in as yet untested strains. It would therefore seem sensible to include a second PCR-RFLP assay in addition to \textit{HSP60/EcoRV}. The \textit{GPI/IHaI} assay has two advantages over histone \textit{H1/AatII}; first, the absence of non-specific bands, and second, a larger, and therefore more easily visible, smallest digestion product.

In this study, the combination of LSU rDNA PCR with \textit{HSP60/EcoRV} and \textit{GPI/IHaI} PCR-RFLPs reliably determined the “correct” DTU for 45 of the 48 cloned isolates. The first exception was that the two TcIIa strains from North America gave TcIIb/IIC-type RFLP profiles, although they could still be identified by the characteristic 130bp LSU rDNA PCR product. Second, strain Saimiri3 c11, which has been typed as TcIIa by MLEE and RAPD,\textsuperscript{19} had atypical profiles for several of the single-locus genotyping assays used here. The existence of a minority of strains that do not fit comfortably into the DTU concept, should, however, be viewed as an interesting feature of the species rather than an inadequacy of a genotyping system that works most of the time. Complications caused by the existence of such rare isolates or others from as yet unsampled populations are likely to be unavoidable without the application of more markers. Observation of unexpected multilocus genotypes could indicate as yet undiscovered lineages or recombinant strains that warrant further study, for instance by MLST. MLST has not only allowed the identification of recombinant in \textit{T. cruzi}\textsuperscript{22,23} but also in other eukaryotic pathogens, including \textit{Leishmania} spp.,\textsuperscript{39,40} \textit{Giardia lamblia},\textsuperscript{41} \textit{Toxoplasma gondii},\textsuperscript{42} and \textit{Candida} spp.\textsuperscript{43,44} Nevertheless, as a tool for simple DTU assignment, our data show that a triple-assay comprising LSU rDNA, \textit{HSP60/EcoRV}, and \textit{GPI/IHaI} (Figure 3) represents a good compromise of type-ability of most strains, adequate discriminatory power, reproducibility, and cost, as well as minimal sample material and time requirements.

Further development of multiple locus PCR-RFLP systems will require testing of many more isolates to prove the reliability of each target/enzyme combination. Testing of additional targets is clearly warranted, particularly of ones capable of discriminating between TcIIb and TcIIc and any that unequivocally identify TcIIa. Details of other RFLP markers capable of similar levels of resolution were published during the course of this study, including an assay targeted to the \textit{GP72} gene using the restriction enzyme \textit{TaqI}, which does seem to provide discrimination between TcIIb and TcIIe.\textsuperscript{19} These authors also proved the potential of using PCR-RFLPs to detect diversity of \textit{T. cruzi} in both clinical and field samples.

In this study aimed at validating genotyping assays, we used a panel of cloned, laboratory cultivated strains. The utility of these assays in practice, however, is subject to the complication of mixed infections, which are well documented in both vectors and mammal hosts, including \textit{humans}.\textsuperscript{45-47} Depending on the strains present in such cases, mixed genotype profiles could be observed. For example, mixtures of TcIIb and TcIIc would generate TcIIb/IIC profiles for the RFLP assays tested here. It may also be necessary to distinguish between \textit{T. cruzi} and \textit{Trypanosoma rangeli}, and this can be done either morphologically, or genetically using an assay that exploits PCR product size differences in the large subunit rRNA gene (LSU rDNA).\textsuperscript{48} Furthermore, if there is reason to suspect an isolate belongs to the closely related, but host-restricted subspecies
Trypanosoma cruzi marinkellei, it can be identified by its unique 135-bp band for the LSU rDNA PCR assay.\textsuperscript{20}

The strains that are used for testing of typing systems need to be carefully considered. Although there are abundant isolates described from domestic transmission cycles, the diversity of T. cruzi in sylvatic settings is less well understood. Indeed, the predominantly sylvatic DTUs TcIIa and TcIIc are often poorly represented in many types of study, including those aimed at characterizing genetic markers. Reducing this sample bias is also particularly important because the success of control strategies targeted at domestic transmission means that the epidemiology of Chagas disease is changing, and adventitious transmission of T. cruzi from sylvatic sources (i.e., TcI, TcIIa, and TcIIc) is seen as increasingly important.\textsuperscript{2} This is exemplified by cases of acute Chagas disease caused by enzootic transmission in the Brazilian Amazon, which may become increasingly frequent as migration more often brings humans into contact with sylvatic vectors of T. cruzi.\textsuperscript{6} Reliable and reproducible genotyping protocols will aid characterization of new isolates and should contribute to a coordinated research effort across multiple disciplines.

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REFERENCES


