SHORT COMMUNICATION

Further characterization of *Trypanosoma cruzi* strains through a simple sequence repeat (SSR) of ferredoxin-NADP⁺ reductase 5' UTR

Trypanosoma cruzi displays clonal expansion, and genetic exchanges have contributed to the current complexity of its population (Machado and Ayala, 2001). Several clustering schemes have been proposed to aid the understanding of *T. cruzi* genetic nomenclature, including: zymodemes (Miles *et al.*, 1978), two major phylogenetic lineages (Souto *et al.*, 1996), discrete typing units (Brisse *et al.*, 2000) and haplotype analysis (de Freitas *et al.*, 2006). This clustering scheme shed light on *T. cruzi* population structure and also advanced the analysis of Chagas disease epidemiology. However, except for kDNA or the schizodeme analysis (Morel *et al.*, 1980), no other molecular marker has been successfully deployed to narrow the characterization of particular strains and extend information beyond *T. cruzi* population structure. We have approached this question by looking at the untranslated regions (UTRs) in *T. cruzi* (Brandao, 2006; Brandao and Fernandes, 2006; Brandao and Jiang, 2009). In general, UTRs tend to accumulate mutations at rates higher than the respective open reading frame (ORF) (Makałowski and Boguski, 1998). Thus, molecular clusters for *T. cruzi* strains could be detected by inspecting UTR sequences. We have inspected UTR from low copy number genes such as ferredoxin-NADP⁺ reductase and reasoned that its 5' UTR might be used for this purpose. Ferredoxin-NADP⁺ reductase is present in a wide range of organisms and modulates redox metabolic pathways such as the one involved in electron transfer (Paladini *et al.*, 2009). In *T. cruzi* CL Brener, a search in its

genome sequence (www.tritrypdb.org) shows that ferredoxin-NADP⁺ reductase is single copy, displaying an ORF and 5' UTR of 2004 and 130 bp in length, respectively (according to sequence in GenBank accession no. AY206009). Here, we discuss that it contains two segments that are useful for characterization of strains: a simple sequence repeat (SSR) which allows a narrow analysis at the strain level and an upstream open reading frame (uORF) that identifies two of the major *T. cruzi* populations.

Strains used in this work are described in Santos *et al.* (2002) and were typed by the multiplex PCR based on mini-exon gene (Fernandes *et al.*, 2001). Stocks were maintained in brain heart infusion medium with fetal bovine serum at 28°C. Cells were harvested at 2000 g and DNA was extracted with DNAzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. For some strains, the DNA was extracted from remaining agarose blocks that were prepared for pulsed field gel electrophoresis runnings (not used in this work) according to standard protocols (Canto *et al.*, 1995). An aliquot (1–5 µl) of melted agarose block in TE 1X (Tris-CL 10 mM pH 8.0, 1 mM EDTA) was used directly in the PCR. Oligonucleotides (forward: 5'-CATGCGCTTTGCATGGCTGT-3' and reverse: 5' CGACGGCGATTGTACGCGT-3') were designed based on the 5' upstream region and the ORF of the ferredoxin-NADP⁺ reductase gene (GeneDB systematic name Tc00.104705-3511367.230) to amplify the entire 5' UTR (130 bases) plus 73 bases upstream of the

trans-splicing site and 108 bases downstream the initiation codon. The amplification was performed in 50 μ l reaction using reagents at final concentrations as follows: 100 ng DNA, 0.2 μ M each primer (IDT), 200 μ M dNTP (Roche, Mannheim, Germany), 2 mM $MgCl_2$, 1X PCR Buffer minus Mg^{2+} , 1 U non-hot start recombinant *Taq* DNA polymerase (Invitrogen) and deionized water to 50 μ l. The thermal profile consisted of one cycle of 94°C 5 minutes, 30 cycles of 96°C 10 seconds, 55°C 30 seconds, 72°C 30 seconds and one final cycle of 72°C 10 minutes (PTC-100; MJ Research, Waltham MA, USA). The amplified fragments were analyzed by 1.5% agarose gel electrophoresis and UV visualization after ethidium bromide staining. Fragments were cloned using TOPO TA cloning kit (Invitrogen) and recombinant plasmids were purified by Wizard Plus SV Minipreps kit (Promega, Madison, MI, USA) according to the manufacturer's specifications. The sequencing reactions were carried out with ABI PRISM BigDye Terminator Sequencing Reaction Kit (Applied Biosystems, Foster City, CA, USA) using primers forward T7 and reverse SP6. Sequencing fragments were analyzed on DNA capillary sequencer ABI3730. The obtained sequences were edited and aligned using MEGA 4 software (Kumar *et al.*, 2008) with default parameters. The 5' UTR ferredoxin sequence from *T. cruzi* genome reference strain (CL Brener clone), available both in GenBank NCBI and GeneDB (www.genedb.org), was used in the alignment as reference sequence. GenBank accession nos. for this set of sequences are: JN850045, JN850046, JN850047, JN850048, JN850049, JN850050, JN850051, JN850052, JN850053, JN850054, JN850055, JN850056, JN850057, JN850058, JN850059, JN850060, JN850061, JN850062.

It is noteworthy that the ferredoxin-NADP+ reductase 5' UTR shows an uORF. For the strains analyzed here (Tables 1 and 2), this uORF exhibits two

point mutations that specify two groups which match the former *T. cruzi* I and *T. cruzi* II groups clustering [now superseded by the recommended six lineages nomenclature by Zingales *et al.* (2009)]. The predicted length of the 5' UTR is 130 bp and the uORF is located between nucleotide position 89 and 124 as compared to mRNA sequence from CL Brener available in GenBank (accession no. AY206009). The point mutations specific for the two groups in uORF appear at nucleotide positions 97 and 103. Inspection of the segment that precedes the uORF reveals an SSR composed of dinucleotide CA and mononucleotide A. This SSR varies in such a way that we can assign a pattern to a set of 2–4 strains (Table 1). Out of 19 strains, nine patterns are recognized (Table 2). However, this 5' UTR SSR variability does not correlate to the population structure depicted either by the mini-exon gene typing (Fernandes *et al.*, 2001) or the suggested *T. cruzi* nomenclature (Zingales *et al.*, 2009). For example, isolates Y and 4167, which are typed respectively as Tc II and Tc zymodeme III, share the same pattern [(CA)₅(A)₁₂]. Conversely, strains belonging to identical lineages display different patterns, e.g. Dm28c [Tc I, genotype (CA)₃(A)₁₂] and 1502 [Tc I, genotype (CA)₄(A)₁₀].

Two distinct patterns were assigned to CL Brener, one of them extracted from the sequence listed in GenBank-NCBI. As the official genome annotation of the reference clone reports only one copy of ferredoxin-NADP+ reductase gene, probably the sequence deposited in GenBank is either originated from another strain or it is missing in the assembled genome. Based on these nine SSR patterns, we propose using this SSR variability as molecular tool to add another level of information on *T. cruzi* strains. Such a tool should be used after the allocation of each strain into one of the extant lineages or types through the standard molecular markers. Additionally, the presence of an uORF displaying nucleotide substitutions that are group-specific

problem. In most cases, what has happened is simple mislabeling of tubes containing these strains, and fortunately these strains derive from different population and can be easily detected by the current markers, e.g. mini-exon or ribosomal DNA typing. However, when two strains belong to the same population, how can they be distinguished from each other at molecular level? In the past, techniques like the schizodeme analysis allowed a clear distinction between strains and even clones of the same isolate (Morel *et al.*, 1980). However, it is very time-consuming and depends on large amounts of pure kDNA. Other approaches might be used to solve this problem (microsatellite analysis; Macedo *et al.*, 2004), but these techniques are not used routinely by typical labs. Though the 5' UTR segment that we described in this work does not tag specifically a single strain, it may help clear this issue if used in association with markers like ribosomal DNA, mini-exon gene, etc.

In conclusion, the characterization presented here is not a substitute for the current clustering scheme for *T. cruzi* populations. Rather, it is aimed at detecting other level of relatedness among *T. cruzi* strains and might be useful in situations where the current markers do not allow a detailed molecular information of certain strains.

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