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# Analysis of molecular diversity of the *Trypanosoma cruzi* trypomastigote small surface antigen reveals novel epitopes, evidence of positive selection and potential implications for lineage-specific serology ☆

Tapan Bhattacharyya [a,*], Jessica Brooks [a], Matthew Yeo [a], Hernan J. Carrasco [b], Michael D. Lewis [a], Martin S. Llewellyn [a], Michael A. Miles [a]

[a] *Pathogen Molecular Biology Unit, Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK*
[b] *Instituto de Medicina Tropical, Facultad de Medicina, Universidad Central de Venezuela, Caracas, Venezuela*

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ABSTRACT

Chagas disease, marked by life-long chronic infection with *Trypanosoma cruzi*, remains a major parasitic disease in Latin America. Genetically heterogeneous, *T. cruzi* is divided into six discrete typing units (DTUs), most recently grouped as TcI–VI. The trypomastigote small surface antigen (TSSA) of *T. cruzi* has been described as the only known serological marker to identify infection by TcII–VI, as distinct from TcI. Here, by comparative analysis of a cohort of 25 reference strains representing all the known DTUs, we show that *TSSA* intra-specific diversity is greater than previously reported. Furthermore, TcIII and IV *TSSA* PCR products are, contrary to expectation, both digested by PvuII, revealing a more nuanced genotyping pattern. Amino acid sequence diversity reveals that the TSSA epitope considered to be serologically characteristic of TcII–VI is restricted to TcII, V and VI, but not of III or IV, and that the diagnostic peptide described as TcI-specific shares key features with TcIII and IV. Notably, *TSSA* sequences inferred greater phylogenetic affinities of TcIII and IV to TcI than to TcII, V or VI. A high ratio of non-synonymous to synonymous nucleotide substitutions ($\omega = 1.233$) indicates that the TSSA gene has been under positive selection pressure. The demonstration of lineage-specific epitopes within TcII–VI has implications for sero-epidemiological studies of Chagas disease based on this antigen.



## 1. Introduction

Chagas disease, caused by infection with the zoonotic protozoan *Trypanosoma cruzi* and transmitted by triatomine bugs, remains a major parasitic disease in the Americas, infecting at least eight million people (www.who.int). Chronic human infection can lead to debilitation and death by cardiac and/or intestinal complications, with a disease burden of ~0.7 million disability-adjusted life years (DALYs) and ~14,000 deaths annually (Hotez et al., 2009). *Trypanosoma cruzi* has in recent years been genotypically divided into the six intra-species lineages (discrete typing units, DTUs) TcI and TcII a–e (Brisse et al., 2000), now renamed TcI–VI (Zingales et al., 2009), with TcV and VI derived from hybridisation between TcII and III (Machado and Ayala, 2001; Gaunt et al., 2003; Westenberger et al., 2005). The geographical distribution of the DTUs is complex: TcI is found as far north as the USA; in contrast TcII, V and VI predominate in the southern cone countries, although human TcII infection has been reported in Colombia (Zafra et al., 2008). The different genotypes may also be associated with varying disease symptoms and natural transmission cycles (sylvatic or domestic). TcI is linked to chagasic cardiomyopathy in Central and northern South America, whereas TcII, V and VI in the southern cone correlate with cardiomyopathy and the presence of chagasic megasyndromes of the colon and oesophagus (Prata, 2001). TcIII and IV, predominantly found in sylvatic transmission cycles, are also capable of human infection. Yeo et al. (2005) proposed that, broadly, opossums are the natural hosts of TcI, armadillos hosts of several lineages of TcII–VI, and that their respective arboreal and terrestrial ecologies explain intra-specific diversity of *T. cruzi* in South America and the separate origins for Chagas disease in the northern and southern parts of that continent. Despite recent successes in disrupting vector-borne transmission in southern cone countries (Schofield et al., 2006) and the launch of the World Health Organization (WHO) Global Network for Chagas Elimination (www.who.int/mediacentre/news/releases/2007/pr36/en/), Chagas disease remains a major public health issue in Latin America (Reithinger, 2009).

*Trypanosoma cruzi* mucins – surface glycoproteins with a high level of O-glycosylation at serine or threonine amino acid residues

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the Accession Nos. GU059921–GU059937 and GU075671–GU075678.

* Corresponding author. Tel.: +44 207 927 2319; fax: +44 207 636 8739.
*E-mail address:* Tapan.Bhattacharyya@lshtm.ac.uk (T. Bhattacharyya).

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– comprise a large (>800) gene family (El-Sayed et al., 2005) believed to play a key role in host immune evasion and in maintaining infection (Buscaglia et al., 2006). The majority of mucin genes belong to the multi-gene sub-families TcMUC I and TcMUC II. Di Noia et al. (2002) assigned a bi-allelic single-copy gene to a third mucin family (TcMUC III), the two alleles of which were reported to correspond in distribution with lineages TcI and TcII–VI, respectively. Antisera raised against a recombinant protein product of this gene from strain CL Brener (TcVI) identified the native form expressed on the surface of cell-derived trypomastigotes (equivalent to bloodstream form trypomastigotes from mammalian hosts). The protein was thus named the trypomastigote small surface antigen (TSSA). They also reported: (i) PvuII digestion of a *TSSA* PCR product occurred only for TcI, not TcII–VI; (ii) the major antibody (Ab)-recognition epitope was restricted to a 10-amino acid region in TcII–VI (41-KPATGEAPSQ-50) where TcI versus TcII–VI differences clustered, with no immunological cross-reactivity between TSSA-I and TSSA-II isoforms. In a survey of chagasic patient sera from Argentina, Brazil and Chile, anti-TSSA antibodies were attributable only to the TcII–VI isoform, leading to the proposition that TSSA is the first serological marker to identify a *T. cruzi* lineage in human infection and that TcII–VI, not TcI, is the cause of Chagas disease. However, the Di Noia et al. (2002) study was based on a genotypically narrow range of *T. cruzi* strains, including those grossly categorised as TcII–VI, and on sera from a limited geographical area, as was later acknowledged (Buscaglia et al., 2006). Furthermore, TcI is known to cause severe and fatal Chagas disease with myocarditis in Venezuela (Miles et al., 1981a; Añez et al., 2004) and north-eastern Brazil (Teixeira et al., 2006).

Here, we determine the nucleotide (nt) and predicted amino acid sequence of a fragment of the *TSSA* gene across a panel of 25 *T. cruzi* strains representing a geographical and genotypic range that encompasses all described DTUs. A greater diversity is revealed than previously reported, including in the PvuII digest pattern, and in the region spanning the reported Ab-recognition epitope. The epitope considered specific for TcII–VI is shown to identify only TcII, V and VI. In addition, the peptide described as TcI-specific shares key features with TcIII and IV. We demonstrate that the bi-allelic description of the *TSSA* gene is not sufficient when all DTUs are examined, and that on the restricted basis of this gene, TcIII and IV share greater inferred phylogenetic affinities with TcI than with TcII, V and VI. The identification of lineage-specific epitopes within TcII–VI indicates a revised potential differential serology for epidemiological surveys of *T. cruzi* using this antigen.

## 2. Materials and methods

Table 1 lists the *T. cruzi* strains used in this study and their origins, all of which were biological clones. The PCR primers T5/ATG/E and EMT5/A, as described in Di Noia et al. (2002), were used to amplify an approximately 190 bp region of the *TSSA* gene from genomic DNA across the panel of strains listed in Table 1. Reactions comprised of 1× $NH_4$ reaction buffer, 1.5 mM $MgCl_2$ (Bioline, UK), 40 μM dNTPs (NEB, UK), 10 pmol of each primer and 1 U BioTaq DNA polymerase (Bioline). Amplification conditions were: one cycle at 94 °C for 3 min; 25 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s; and one cycle at 72 °C for 10 min. PCR products were digested using 2 U PvuII (Promega, UK) and separated by electrophoresis on 2.5% agarose gels (Bioline). DNA sequencing was achieved using a BigDye® Terminator v3.1 RR-100 kit (Applied Biosystems, UK) according to standard protocols. Sequence alignment was performed using BioEdit software (Hall, 1999), phylogenetic analysis using MEGA4 software (Tamura et al., 2007), and non-synonymous/synonymous nt substitutions per site ratios ($dN/dS$; $\omega$) were calculated using SNAP software (Korber, 2000) employing the method of Nei and Gojobori (1986) with a Jukes-Cantor correction (http://hcv.lanl.gov/content/sequence/SNAP/SNAP.html).

## 3. Results

### 3.1. Alignment of TSSA gene fragment DNA sequences

Fig. 1A shows the nt polymorphisms within and between DTUs for the *TSSA* gene fragment across the panel of strains described in

**Table 1**
Panel of biological clones of *Trypanosoma cruzi* strains used in this trypomastigote small surface antigen (TSSA) comparison. Previous nomenclature for TcII–VI[IIa–e] is shown in brackets.

| Discrete typing unit | Strain | Origin | Host | Reference |
|---|---|---|---|---|
| TcI | Sylvio X10/1 | Belém, Brazil | *Homo sapiens* | Miles et al. (1978) |
| | Cutia cl1 | Espiritu Santo, Brazil | *Dasyprocta agouti* | Brenière et al. (1998) |
| | Sp104 cl1 | Region IV, Chile | *Triatoma spinolai* | Brenière et al. (1991) |
| | P209 cl93 | Sucre, Bolivia | *H. sapiens* | Brenière et al. (1998) |
| | OPS21 cl11 | Cojedes, Venezuela | *H. sapiens* | Brisse et al. (2000) |
| | 92101601P cl1 | Georgia, USA | *Didelphis marsupialis* | Barnabé et al. (2001) |
| TcII[IIb] | TU18 cl93 | Tupiza, Bolivia | *Triatoma infestans* | Brenière et al. (1998) |
| | CBB cl3 | Region IV, Chile | *H. sapiens* | Brenière et al. (1991) |
| | Mas cl1 | Brasilia, Brazil | *H. sapiens* | Brisse et al. (2000) |
| | IVV cl4 | Region IV, Chile | *H. sapiens* | Brenière et al. (1998) |
| | Esm cl3 | São Felipe, Brazil | *H. sapiens* | Miles et al. (1977) |
| TcIII[IIc] | M5631 cl5 | Selva Terra, Brazil | *Dasypus novemcinctus* | Miles et al. (1981b) |
| | M6241 cl6 | Belém, Brazil | *H. sapiens* | Tibayrenc and Ayala (1988) |
| | CM17 | Meta, Colombia | *Dasypus* sp. | Brisse et al. (2000) |
| | X109/2 | Makthlawaiya, Paraguay | *Canis familiaris* | Chapman et al. (1984) |
| TcIV[IIa] | CanIII cl1 | Belém, Brazil | *H. sapiens* | Miles et al. (1978) |
| | 92122102R | Georgia, USA | *Procyon lotor* | Sturm et al. (2003) |
| | Dog Theis | Oklahoma, USA | *Canis familiaris* | Barnabé et al. (2001) |
| TcV[IId] | MN cl2 | Region IV, Chile | *H. sapiens* | Brisse et al. (2000) |
| | Bug 2148 cl1 | Rio Grande do Sul, Brazil | *T. infestans* | Souto et al. (1996) |
| | SO3 cl5 | Potosi, Bolivia | *T. infestans* | Brenière et al. (1991) |
| | SC43 cl1 | Santa Cruz, Bolivia | *T. infestans* | Tibayrenc and Miles (1983) |
| TcVI[IIe] | CL Brener | Rio Grande do Sul, Brazil | *T. infestans* | Brisse et al. (1998) |
| | P63 cl1 | Makthlawaiya, Paraguay | *T. infestans* | Chapman et al. (1984) |
| | Tula cl2 | Tulahuen, Chile | *H. sapiens* | Tibayrenc and Ayala (1988) |