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Review

Serological Approaches for *Trypanosoma cruzi* Strain Typing

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***Trypanosoma cruzi*, the protozoan agent of Chagas' disease, displays a complex population structure made up of multiple strains showing a diverse ecoepidemiological distribution. Parasite genetic variability may be associated with disease outcome, hence stressing the need to develop methods for *T. cruzi* typing *in vivo*. Serological typing methods that exploit the presence of host antibodies raised against polymorphic parasite antigens emerge as an appealing approach to address this issue. These techniques are robust, simple, cost-effective, and are not curtailed by methodological/biological limitations intrinsic to available genotyping methods. Here, we critically assess the progress towards *T. cruzi* serotyping and discuss the opportunity provided by high-throughput immunomics to improve this field.**

Highlights

Trypanosoma cruzi, the vector-borne protozoan agent of Chagas' disease, displays a complex population structure made up of multiple strains showing diverse genotypic and ecoepidemiological features.

Typing of the infecting strain(s) directly in biological samples may shed new light on *T. cruzi* zoonotic distribution and, more importantly, may facilitate the study of possible associations between parasite genotype and Chagas' disease clinical outcome.

Detailed descriptions of strain-specific antibody profiles or signatures emerge as an appealing approach for *in vivo T. cruzi* strain typing.

High-throughput immunomics provides a unique opportunity to link the increasing ability to catalog variation among *T. cruzi* genomes to the identification of novel polymorphic targets of strain-specific B cell immunity.

Trypanosoma cruzi, an Endemic Threat Going Global

Chagas' disease, caused by the parasitic hemoflagellate *T. cruzi*, is a lifelong, debilitating illness endemic to the Americas, with an estimated toll of 6–7 million people already infected and ~50 000 new cases per year [1]. *T. cruzi* infection primarily occurs when humans are exposed to the contaminated feces of infected hematophagous triatomines that act as vectors. Other documented modes of transmission include blood transfusion, organ transplantation, and congenital transmission. In addition, recent outbreaks of acute and virulent cases were shown to be not strictly vector borne but rather due to accidental ingestion of *T. cruzi*-tainted food and fluids [2]. In recent decades, several factors converged to shift the epidemiological landscape for this disease, which now emerges as a threat to global public health [3]. Despite this alarming situation, the absence of available vaccines, together with the fact that approved drugs show toxicity and variable efficacy, determine that the main control strategy for Chagas' disease still relies on the prevention of parasite transmission.

Biological Implications of *T. cruzi* Genetic Diversity

T. cruzi is an extremely successful zoonotic parasite, showing a broad, diverse, and poorly understood pattern of circulation among at least seven genera of triatomines and a variety of domestic and wild mammals [4]. Adaptations to particular mammal and/or insect species together with ecogeographical barriers, scarce sexual recombination, and iterative population bottlenecks associated with host–vector switching contribute to structuring the population of this parasite [5]. Typing schemes developed with various biochemical and genetic markers converged in the delineation of six major evolutionary lineages or **discrete typing units** (**DTUs**) (see Glossary), termed TcI–TcVI. A potential seventh lineage, Tcbat, and additional divergent sublineages with putative epidemiological and/or clinical significance have been proposed [6,7]. The evolutionary relationships among DTUs have not been fully elucidated, but it is clear that TcI, TcII, TcIII, and TcIV have more ancient origins whereas TcV and TcVI are clusters of hybrid strains, the product of recent and likely independent genetic crosses between TcII and TcIII parentals [5].

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T. cruzi DTUs have distinct, but not exclusive, geographical, ecological, and epidemiological distribution [4,5,8]. Briefly, TcI is by far the most widespread DTU, being found throughout endemic areas in both sylvatic and domestic cycles. In endemic countries located north of the Amazon basin, TcI is prevalent in human infections. TcII is extremely rare in North and Central America, though it prevails in domestic cycles in certain regions of the Southern Cone of South America, particularly in Brazil. TcIII and TcIV are mostly associated with sylvatic transmission cycles. Noteworthy, TcIV has been also increasingly detected as a secondary agent of Chagas' disease in Venezuela [9], and found to be involved in oral transmission to humans [2,10]. TcV and TcVI are mostly restricted to domestic transmission cycles in Southern Cone countries, and particularly in Argentina and Bolivia. Recent findings however suggest that their distribution could be broader than appreciated [11,12]. Despite these epidemiological considerations, it should be emphasized that all six *T. cruzi* DTUs (or seven, including Tcbat) are capable of infecting humans and that there are geographical overlaps and coexistence of distinct DTUs in the same vector and/or mammal host population, as well as in a single individual [5].

Initial *T. cruzi* infection in humans is followed by an acute phase, lasting up to 30–60 days. This phase is usually asymptomatic or might present as a nonspecific and self-limiting febrile illness. During the subsequent, chronic phase, parasitemia drops significantly and Chagas' disease evolves into a wide spectrum of pathological symptoms ranging from subclinical to potentially fatal myocardiopathy and/or gastrointestinal mega syndromes [1]. In *in vitro* systems or animal infection models, *T. cruzi* genetic diversity has been partially correlated with clinically relevant phenotypes, such as susceptibility to trypanocidal drugs, tissue distribution, or pathogenesis [13–15]. In patients, however, these kinds of association remain so far circumstantial and controversial. As extensively discussed [5,16], existing studies may have been blurred by several factors including flaws in their design, mixed *T. cruzi* infections, the presence of concurrent pathogens, and genetic/immunological aspects of local human populations. Notwithstanding this, assignment of the *T. cruzi* infecting strain type remains a long-standing research interest in the field, with an expected positive impact on the overall diagnosis and clinical management of Chagas' disease.

T. cruzi Strain Typing: A Crime Scene Investigation

Pioneering studies aimed at typing *T. cruzi* strains were based on biochemical markers [17]. Further advances in typing schemes – based on DNA amplification/restriction/hybridization, karyotyping, sequence-based markers using either a single locus or multiple loci, and amplicon deep sequencing – have greatly improved parasite genotypic resolution [18–21]. However, and due to the scarce parasitemia during the chronic phase of Chagas' disease and the low dosage of some used DNA markers, genotyping methods display suboptimal sensitivity *in vivo*, usually requiring isolation and amplification steps which may bias the parasite population. The frequent occurrence of multistrain infections and the discovery that bloodstream genotypes may differ from those sequestered within tissues [22,23] further complicate this task. Last, but not least, *T. cruzi* genotyping schemes are time- and labor-consuming, costly, and difficult to be implemented in endemic areas, point-of-care sites with limited infrastructure.

In this context, serological typing (serotyping) methods emerge as an appealing alternative. These methods rely on the use of **polymorphic antigens** to detect strain-specific antibody signatures. Serotyping assays are robust, simple, cost-effective, and are not curtailed by relevant methodological and/or biological limitations intrinsic to currently used *T. cruzi* genotyping methods. In favor of their applicability to *T. cruzi*, it was shown that this parasite stimulates robust and persistent humoral immune responses in chronic chagasic patients, regardless of their clinical status [1]. The existence of putatively diagnostic differences in the antigenic constitution of *T. cruzi* strains, on the other hand, is supported by (i) comparative genomic studies showing interstrain variations

Glossary

Discrete typing units (DTUs): sets of stocks of *T. cruzi* that are genetically more similar to one another than to any other stock and are identifiable by common molecular markers.
Pangenome: a collection of the entire gene set and its allelic variants obtained from all strains of a species.
Polymorphic antigen: a molecule that elicits differential and strain-specific immune responses in the infected host. This molecule usually, but not necessarily, shows qualitative and/or quantitative differences among pathogen strains or isolates.
Serotype: a genetically discordant set of antigenic types that could be distinguished within certain of a pathogen's species by means of serologic assays.
TSSA core region: a sequence of the trypomastigote small surface antigen (TSSA) displayed on the trypomastigote outer membrane upon processing of its sorting determinants (signal peptide and GPI-anchoring signal) and subsequent maturation in the secretory pathway.

in the gene dosage, which usually correlates to protein expression yield, and/or in the amino acid sequences of several deduced proteins (see below); (ii) large differences in the overall proteomic profile of distinct strains [24,25]; and (iii) often discordant results of serodiagnostic assays when evaluated on populations of different geographic origin and hence probably infected by different *T. cruzi* strains [26,27].

T. cruzi Serotyping: Progress, Challenges, and Limitations

Serotyping Using Whole Parasites

A method for genotype-specific serodiagnosis of *T. cruzi* infection by means of strain-specific immunity has been recently developed [28]. This technique, named Chagas-Flow ATE, is based on the comparative evaluation of IgG responses towards strains representative of major DTUs associated with human infections (TcI, TcII, and TcVI). To develop this method, authors recorded the independent reactivities of a panel of reference positive sera (obtained from mice experimentally infected with different strains) to three major parasite developmental forms from either the TcI, TcII, or TcVI strain. An algorithm composed of a series of decision trees based on attributes such as target-antigen reactivity and serum dilution/cut-off was then used to integrate these outcomes and classify individual samples. Chagas-Flow ATE demonstrated an excellent performance for diagnosis of single/mixed experimental *T. cruzi* infections [28,29], and potential applicability to direct parasite serotyping in human serum samples [30]. A drawback of this method is the use of whole parasites as target antigens. It is known that complex and undefined mixtures of *T. cruzi* molecules frequently lead to false-positive results when assayed with sera from individuals suffering certain autoimmune diseases and/or infected with coendemic pathogens such as *Leishmania* sp. and *Trypanosoma rangeli* [31]. In the case of the Chagas-Flow ATE method, the presence of such cross-reacting antibodies may alter the magnitude of strain-specific and/or developmental stage-specific signals, thereby affecting the strain typing decision process. Also, shifts in the *T. cruzi* antigenic profile over time under culture conditions [32,33] may entail additional reproducibility issues with the test.

Serotyping Using TSSA

The trypomastigote small surface antigen (TSSA) was the first identified *T. cruzi* antigen with serotyping potential [34]. TSSA is a surface adhesion molecule, with mucin-like features [35], involved in the initial interaction of the trypomastigote with the target cell [36,37]. Epitope mapping studies led to the identification of a broad (~40 residues long), antigenic region encompassing most of the **TSSA core region** [34,38,39] (Figure 1). TSSA alleles display a number of diagnostic polymorphisms that allow their classification into four 'isoforms', each one corresponding to an ancestral DTU (TcI to TcIV) [34,40]. The majority of these polymorphisms accumulate within the TSSA core region (Figure 1) and have a major impact on its antigenic properties [34,38,39]. Overall, four major TSSA **serotypes** could be defined: TSSAI (TcI), TSSAII (TcII), TSSAIII (TcIII), and TSSAIV (TcIV) (Figure 1). Being hybrids, TcV/TcVI genomes code for both TSSAII and TSSAIII isoforms.

Currently available *T. cruzi* genomes were reanalyzed here using the CL Brener strain (TcVI) TSSAII coding sequence as query (TcCLB.507511.91). Two nonsyntenic TSSA loci could be defined: a TSSA-tandem locus and a TSSA single-copy locus. The former bears up to 14 identical or nearly identical TSSA gene copies arranged in a head-to-tail tandem, and it is found in all strains analyzed so far (Figure 2). Strains from hybrid DTUs (at least for TcVI) seem to have retained solely TSSAII copies at this tandem locus (Figure 2). The TSSA single-copy locus, on the other hand, is restricted to TcVI (and likely also TcV) strains (Figure 2), suggesting that it may have emerged upon their hybridization. This locus codes for a single TSSAIII allele, with a still undetermined level of expression. Two possible 'TSSA surface configurations', displaying

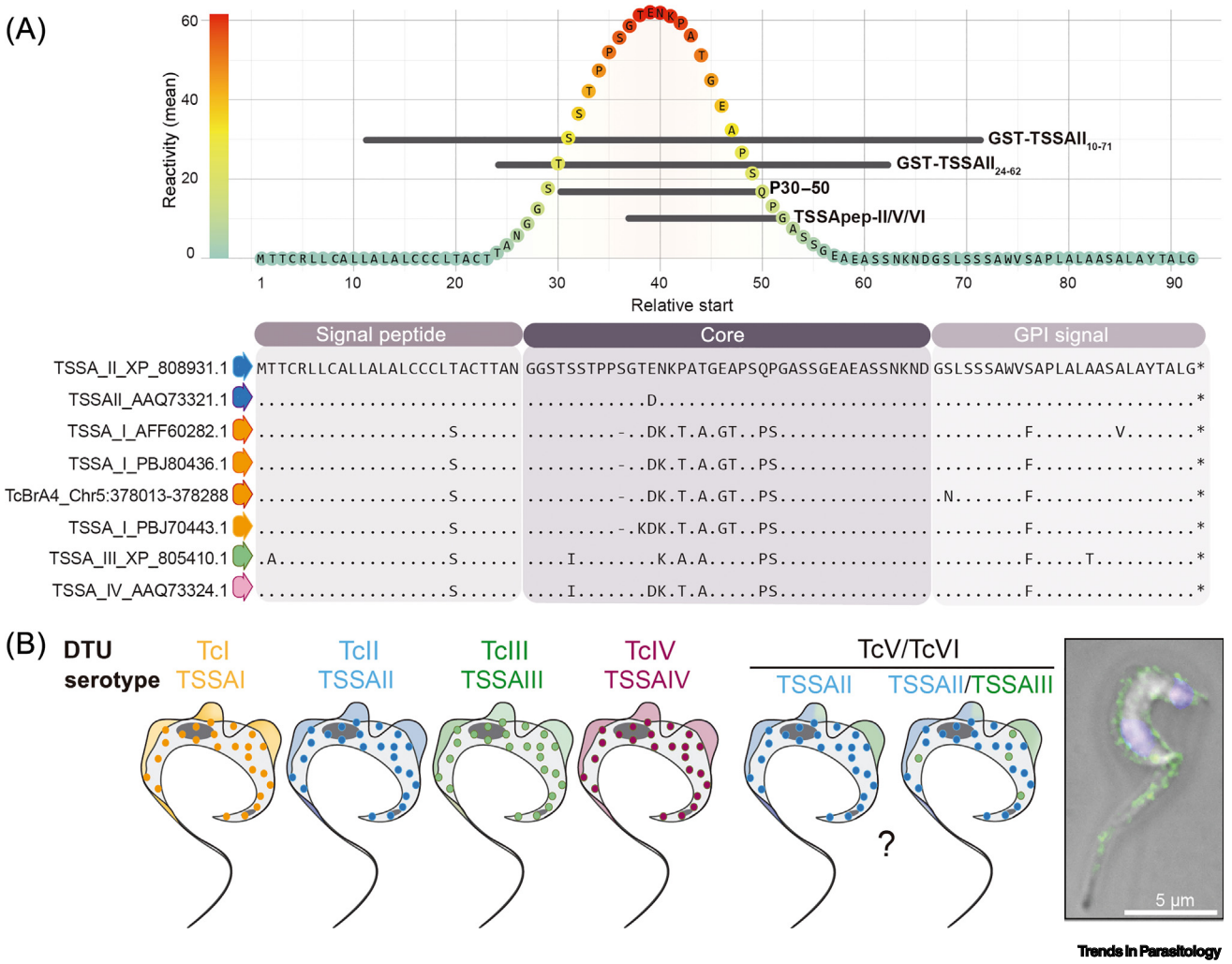

Figure 1. Antigenic and Molecular Features of the Trypomastigote Small Surface Antigen (TSSA). (A) Microarrays displaying completely overlapped 15mer peptides spanning the sequence of CL Brener (TcVI) TSSAII (XP_808931.1) were probed with purified IgG from human chronic Chagas' disease subjects [39]. Partial sequences derived from XP_808931.1 evaluated in seroepidemiologic studies are superimposed. Below, a schematic illustration shows polymorphisms among the predicted amino acid sequences of TSSA alleles (as obtained from TriTrypDB). XP_808931.1 was used as reference and the different structural regions (signal peptide, central and antigenic core, and GPI-anchoring signal) are indicated as defined in [34]. Allele symbols match those in Figure 2 and were colorized according to the *Trypanosoma cruzi* discrete typing unit (DTU) to which they belong (blue, TcII; orange, TcI; green, TcIII; magenta, TcIV). (B) Schematic representation of the 'TSSA surface configuration' and TSSA serotype for trypomastigotes from each DTU (denoted as above, with hybrid lineages colorized in blue and green). Note two possible TSSA surface configurations for hybrid strains (one displaying minor amounts of the TSSAIII isoform in green, along with the most prevalent and phenotypically dominant TSSAII isoform in blue). A representative image showing the patchy distribution of TSSAII on the membrane of *T. cruzi* CL Brener trypomastigotes, typical of parasite surface molecules [85], is shown in the inset to the right.

or not minor amounts of the TSSAIII isoform, may be envisaged for hybrid lineages (Figure 1). TSSAII variants encoded by the TSSA-tandem locus turn out to be prevalent and phenotypically dominant, thus defining an apparent TSSAII serotype for hybrid strains (Figure 1).

Recombinant, glutathione S-transferase (GST)-fusion proteins encompassing residues 10 to 70–71 of TSSAI and TSSAII sequences (GST-TSSAII$_{10–71}$, Figure 1) were initially explored as serotyping tools [34]. This survey, together with others carried out with a shorter TSSAII recombinant protein (GST-TSSAII$_{24–62}$, Figure 1) revealed a consistent prevalence of ~80–95% for TSSAII and <5% for TSSAI in chronic chagasic patients from Southern Cone countries [23,31,41–46]. Further mapping

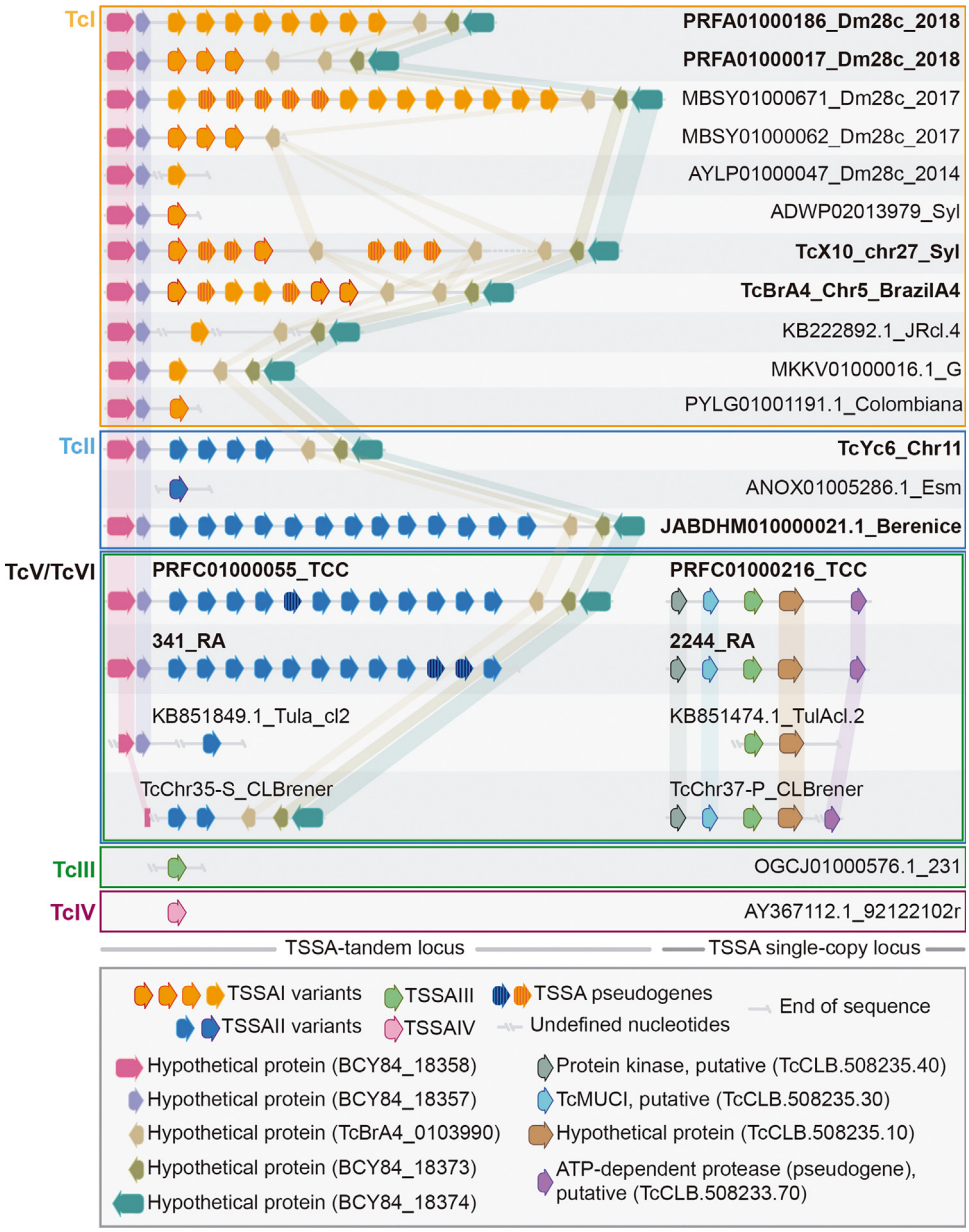

Figure 2. Genomic Organization of the Trypomastigote Small Surface Antigen (TSSA) in *Trypanosoma cruzi*. Schematic representation of genomic scaffolds containing TSSA sequences from different discrete typing units (DTUs) (colorized as in Figure 1). We identified two TSSA loci (TSSA-tandem locus and TSSA single-copy locus). Only those contigs in which at least one synteny block flanking TSSA copies was identified, or those carrying variant TSSA sequences (ANOX01005286.1, TcII; OGCJ01000576.1, TcIII; AY367112.1, TcIV) were included. For Dm28c (TcI), both TSSA haplotypes could be identified (MBSY01000671/MBSY01000062 and PRFA01000186/PRFA01000017). The connecting blocks between the scaffolds highlight the homology between TSSA flanking genes and are colorized matching them. Annotated genes are indicated by arrow symbols, orientated according to their sense of transcription. The accession numbers of the annotated sequences (as obtained from TriTrypDB or NCBI) are indicated and separated from the corresponding strain using underscores; those obtained using third-generation sequencing technologies are marked in bold.

studies [38] allowed the definition of a minimal region in TSSAII, from residue 30 to 50, able to recapitulate its overall serodiagnostic performance (p30–50, Figure 1). Figures determined by these assays were congruent with the clear preponderance of TcII/TcV/TcVI strains in domestic cycles in such endemic areas [23,47–50]. Moreover, samples analyzed in parallel by genotypic and TSSA-based serologic assays showed a good concordance between both methods [41–43]. In patients from Mexico, Colombia, and Venezuela, and also congruent with genotypic data, the estimated prevalence of TSSAII dropped to ~30–40% [41]. Despite the acknowledged dominance of TcI in such countries, recognition of TSSAI was also very low (~25%, see later) [41].

Building on these previous data, a *T. cruzi* lineage-specific serological test was developed from a set of TSSA-derived peptides (termed TSSApep-I, -II/V/VI, -III, and -IV) [51,52]). When evaluated on chagasic patients, results were in line with those described above, though the estimated prevalence for TSSApep-II/V/VI was ~70% in Argentina, Brazil, and Bolivia, and ~10–20% in northern South American countries [51,52]. Disregarding differences between study populations, the lower sensitivity of TSSApep-II/V/VI as compared to recombinant TSSAII proteins may well be attributed to the fact that this peptide solely spans residues 37 to 52 (Figure 1), hence lacking some critical antigenic determinants [38]. When assayed in a Bolivian population of 121 chronic patients stratified by Chagas' disease-associated cardiomyopathy, TSSApep-II/V/VI reactivity correlated with severity of clinical findings, suggesting it may be explored as a disease prognosis biomarker [51,52]. Surveys conducted using this set of TSSA-derived peptides also indicated that TcIV strains circulate at low frequency in domestic cycles from Venezuela and Colombia, which is in agreement with current genotypic data [5,8].

The proven diagnostic power of TSSApep-II/V/VI stimulated the development of an immunochromatographic diagnostic test, bearing this peptide immobilized on a nitrocellulose membrane and Protein G conjugate for broad detection of specific antibodies. This test was successfully evaluated for the assignment of *T. cruzi* lineage infections in humans and in a range of domestic and wild mammals, thereby leading to numerous insights into parasite ecoepidemiology [52–55].

Taken together, these data suggest that TSSA has the potential to predict the *T. cruzi* strain type on biological samples with near DTU-level accuracy. Despite this, the resolution and specificity of TSSA-based serotyping assays need to be improved. Intensive peptide-mapping approaches that dissect the epitopic mesh in the TSSA antigenic core [38] and a better resolution of the variety of TSSA sequences encoded by TcIII and TcIV DTU [5] are required to partially address these issues. Assessing the performance of the TSSA serotyping method for the diagnosis of mixed *T. cruzi* infections is also required. In addition, exhaustive screening for antibodies specific to TSSAIII may aid in defining the TSSA serotype of hybrid strains (Figure 1), and, in turn, in the discrimination between TcII- and TcV/TcVI-caused infections.

Major current challenges of TSSA-based serotypification include the discrimination between TcV- and TcVI-caused infections, which is a caveat common to most *T. cruzi* genotyping methods [18–21], and the accurate identification of TcI-infected individuals. Regarding the latter aspect, the fact that TSSAI shows poor intrinsic immunogenicity [36] likely explains its very low seroprevalence across endemic areas, even in those with established dominance of TcI strains [41,44,51]. Interestingly, heterologous expression of a recombinant *O*-glycosylated TSSAI in *Leishmania tarentolae* displayed enhanced recognition by chagasic sera from northern South American countries as compared to its deglycosylated counterpart [56].

Coming-of-Age of *T. cruzi* Serotyping

Because of the *T. cruzi* genetic diversity and complex antigenic constitution, it would be extremely unlikely that TSSA or any given single molecule can distinguish parasite strain types reliably. Rather, large-scale analysis of polymorphic antigens to detect strain-specific antibody signatures seems to be the way forward. Indeed, sequence analyses followed by B-cell epitope prediction on deduced proteins derived from CL Brener allelic pairs has been explored as a novel strategy to identify polymorphic antigens able to serodiagnose the parasite DTU in experimental infections [57]. On the other hand, our recent exploration via peptide microarrays of just a fraction (~7%) of the *T. cruzi* deduced proteome allowed for the identification and characterization of thousands of novel disease-specific sequences [39,58–60], indicating that a vast majority of the parasite's antigenic repertoire remains uncharacterized. Together, these findings stress the need for more comprehensive screening of parasite genomic sequences and infected host antibody repertoires to improve and expand the *T. cruzi* serotyping tools.

Recent advances in microarray technology and high-throughput immunomics are enabling the study of B-cell responses at an unprecedented, proteome-wide scale [61]. As exemplified by studies in other infectious diseases [62–64], high-throughput approaches using protein and peptide arrays rapidly led to the discovery of strain-specific serological profiles or signatures that can be next exploited in simplified serological typing assays. In *Plasmodium falciparum*, protein microarrays have been successfully used to assay human malaria seroreactivity towards hundreds to low thousands of antigens [64,65] and to study the dynamics of these immune responses.

Also coming of age is *T. cruzi* genomics, now with a reasonable number of genomes sequenced using third-generation technologies based on long reads [66–69]. Furthermore, the most recent additions to this list include two genomes with their assemblies guided by proximity ligation mapping [70]. These technological advances led to an improved resolution of structural genome features, including the delineation of highly diverse and immunogenic *T. cruzi* protein families [66,67,70]. Recent comparative genomics exercises show that the genetic variation between strains is mostly derived from the more fluid, repetitive or disruptive chromosomal compartments composed of members of these large gene families [66,70].

The combination of these maturing fields, *T. cruzi* genomics and high-throughput immunomics, now provide a path for exploration and discovery of novel antigens with strain-discrimination capability. One possible strategy would start by designing microarrays with overlapped peptides spanning natural variants derived from the *T. cruzi* **pangenome**. Once the microarray is produced, the experiments performed are basically highly multiplexed ELISA tests, where the peptides are incubated with appropriate serum samples or antibodies collected thereof (see below). This process results in a table of each peptide and a number related to ‘how strong’ its reactivity was. Data is then processed, possibly reconstructing the original proteins, and used to draw conclusions about the retrieved antigens: prevalence, specificity, identity, and features of identified epitopes and, most importantly, lineage discrimination capacity (Figure 3). When using peptide arrays as a discovery platform, most of the information about spatial configuration of the original proteins is lost, hence only linear epitopes are discovered and characterized. To also discover conformational epitopes, protein microarrays, where whole proteins or independently folding domains are placed on the solid support, can be used [71]. However, protein microarrays have some limitations, such as their lower capacity (in comparison with peptide arrays) and the challenge of achieving a microarray with a collection of properly folded proteins in the first place.

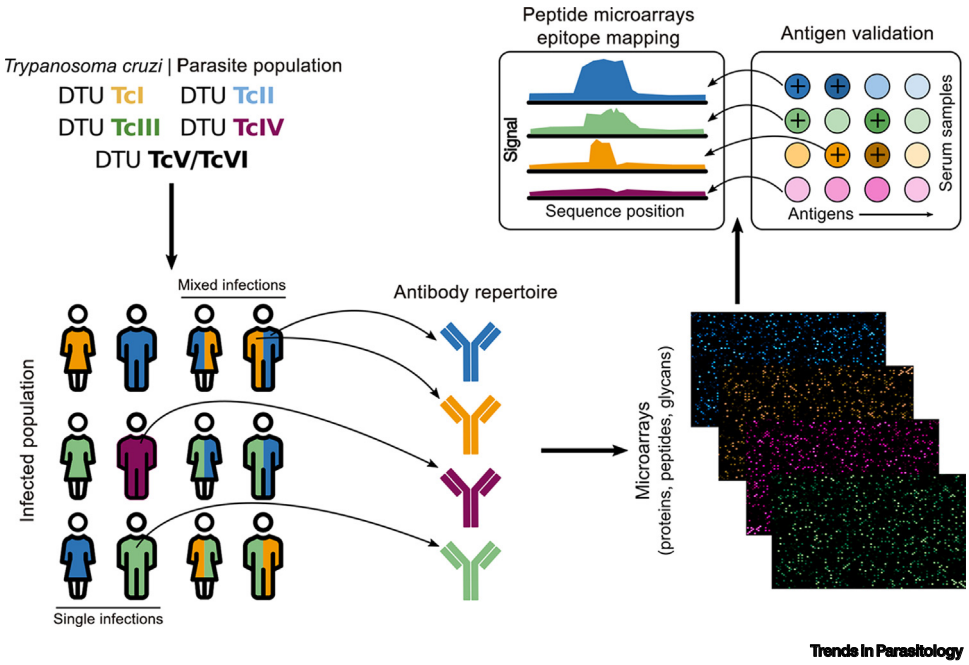

Figure 3. Schematic View of a Possible Strategy for Discovery and Validation of *Trypanosoma cruzi* Polymorphic Antigens. Microarrays (displaying peptides, proteins, glycopeptides, or glycans) can be screened with antibodies purified from individuals infected with different *T. cruzi* discrete typing units (DTUs). Parasites (top left) were colorized according to the DTU to which they belong, as in previous figures (orange, TcI; blue, TcII; green, TcIII; blue and green, hybrid lineages). The same color code is used to represent a population of Chagas' disease patients (bottom left) depicting hypothetical single or mixed infections. Antibody (serum) samples can be used to assay microarrays containing parasite molecules (bottom right). Several rounds of assays can be performed at this stage – in which identified polymorphic candidate antigens are displayed on additional microarrays for serologic validation. When using *T. cruzi*-derived peptide microarrays (top right), precise epitope mappings can be obtained from the seroreactivity of individual overlapping peptides [39]. If protein microarrays are used, only the antigen is validated (the epitope may remain unmapped).

In both cases, good-quality genome sequences are essential to design and produce these arrays. An alternative to proteome-derived peptides is the use of random sequences. These can be displayed as synthetic peptides coupled to a solid support or on the surface of biological platforms [72]. A key advantage of this approach is that it is not designed to accommodate any given pathogen or disease, such that the same library is universally appropriate. The sequence bound by an antibody is presumably not the cognate epitope but rather a structural mimic. Indeed, because the potential diversity of peptide sequences is larger than the sequence space explored in these arrays a broad range of mimicry is afforded [72]. As for the downside of random sequence approaches, it is often difficult (and sometimes impossible) to assign the reactive sequence to the cognate parasite antigen, thus precluding its downstream biological characterization [72]. Notwithstanding this, the feasibility of random sequences-based assays for the profiling of different immune responses, including those elicited in chagasic patients, has been extensively demonstrated [72–76]. A hybrid strategy between scanning the limited number of coding sequences derived from *T. cruzi* genomes and the virtually infinite source of random sequences is the generation of residue variants. In such a randomization process, using parasite-encoded proteins as a starting point effectively reduces the sequence space that needs to be explored. Different techniques can be used to generate and explore synthetic peptide variants (e.g., alanine scans or full-residue scans) or protein variants through shotgun mutagenesis [77].

Whatever the strategy used to screen and analyze the *T. cruzi* antigenic landscape, the most promising selected candidates can be produced and assayed for downstream validation using an extensive panel of appropriate samples. These serological assays can be carried out in a number of formats, including lower scale protein or peptide microarrays [39,62], antigen-coated bead-based flow cytometry applications [78], or customized ELISA tests [58]. One major limitation for the whole process of discovery of *T. cruzi* polymorphic antigens is the absence of reference serum standards to calibrate the screening/validation assays. In this sense, the recent call for a Patient Registry for Chagas' disease could be an excellent opportunity to channel these needs [79].

Final candidates can be integrated in a serotyping test that should be able to (i) discriminate between *T. cruzi*-infected and noninfected individuals; (ii) assign the infecting strain, at least with DTU-level accuracy; and (iii) differentiate between single or mixed infections. Ideally, the test has to be also easy to assay and interpret, versatile (able to evaluate serum samples from multiple species), and adaptable to a point-of-care platform, to be deployed in field studies.

A Bittersweet Symphony: *T. cruzi* Serotyping Using Glycan Antigens

In contrast to bacterial species, where serotyping is often mostly based on glycan antigens, in *T. cruzi* there is a lack of well validated antigenic glycans. Considering the unique features of protein glycosylation pathways in *T. cruzi,* and the huge functional and diagnostic significance of carbohydrates on the biology of this organism [80,81], it could be hypothesized that parasite-derived glycans and/or glycopeptide microarrays may also serve as a starting point for the discovery of serotyping reagents. The few validated *T. cruzi* glycoantigens (e.g., α-Gal epitope) are not, however, appealing for serotyping purposes as they are common to all parasite genotypes and also to a number of pathogens [82]. One recent exception is the glycosylated TSSA variant described above [56]. This is a nice example where a poorly seroreactive antigen variant (nonglycosylated, recombinant TSSAl) can be enhanced by glycosylation.

Concluding Remarks

Antibodies are unique among biomarkers in their ability to identify individuals with past exposure to a wide array of pathogens: viruses, bacteria, fungi, protozoa, and helminths. The exquisite resolution of these molecules allows in principle for the distinction not only of the infectious agent but also of different strains or antigenic subtypes defined within a microbial taxon. Serotyping could be a rapid, sensitive, cost-effective, and relatively noninvasive alternative to stringent pathogen genotyping in humans. These techniques have extensive experimental support and numerous applications in clinical microbiology and vaccine development [83,84]. Although current *T. cruzi* serotyping approaches offer robust results, they do possess significant limitations that warrant further investigation (see Outstanding Questions), particularly on the identification and validation of parasite polymorphic antigens. In this context, high-throughput immunomic approaches may provide the opportunity to link the increasing ability to catalog variation among *T. cruzi* genomes to the identification of novel targets of strain-specific B-cell immunity.

Development of novel and robust *T. cruzi* serotyping schemes and/or tools will shed new light onto the complex and fluctuating association of parasite genotypes with mammal/vector hosts, biomes, or habitats. Most importantly, they will also facilitate the unraveling of possible relationships between parasite genetic variability and clinical features, a major issue in Chagas' disease applied research. This, in turn, may lead to a better management of *T. cruzi*-infected individuals to improve prognostic outcomes. Once an association between *T. cruzi* strain and disease phenotype is clearly established, experiments can be designed to determine its underlying molecular basis, which might ultimately pave the way to novel and much needed targets of intervention.

Outstanding Questions

What would be the real impact of the accurate identification of the infecting strain on *T. cruzi* ecoepidemiological studies and on the clinical management of Chagas' disease?

What would be the desired level of resolution for a *T. cruzi* strain typing method – distinction of parasite DTU or discrimination among strains or sublineages belonging to the same DTU?

What are the advantages of serology-based typing methods over molecular genotyping in Chagas' disease?

What is the best strategy for the discovery of *T. cruzi* polymorphic antigens? What are the main challenges in this field? What tools are expected to help in this process?

What are the main characteristics that a *T. cruzi* serotyping test should fulfill?

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