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Chagas disease diagnostic applications: present knowledge and future steps

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Abstract

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, is a life-long and debilitating illness of major significance throughout Latin America, and an emergent threat to global public health. Being a neglected disease, the vast majority of Chagasic patients have limited access to proper diagnosis and treatment, and there is only a marginal investment into R&D for drug and vaccine development. In this context, identification of novel biomarkers able to transcend the current limits of diagnostic methods surfaces as a main priority in Chagas disease applied research. The expectation is that these novel biomarkers will provide reliable, reproducible and accurate results irrespective of the genetic background, infecting parasite strain, stage of disease, and clinical-associated features of Chagasic populations. In addition, they should be able to address other still unmet diagnostic needs, including early detection of congenital *T. cruzi* transmission, rapid assessment of treatment efficiency or failure, indication/prediction of disease progression and direct parasite typification in clinical samples. The lack of access of poor and neglected populations to essential diagnostics also stress the necessity of developing new methods operational in Point-of-Care (PoC) settings. In summary, emergent diagnostic tests integrating these novel and tailored tools should provide a significant impact on the effectiveness of current intervention schemes and on the clinical management of Chagasic patients. In this chapter, we discuss the present knowledge and possible future steps in Chagas disease diagnostic applications, as well as the opportunity provided by recent advances in high-throughput methods for biomarker discovery.

Keywords

Diagnostic applications; Chagas disease; *Trypanosoma cruzi*; biomarker discovery; strain polymorphisms; serotyping

INTRODUCTION

Chagas disease or American Trypanosomiasis, caused by the parasitic protozoan *Trypanosoma cruzi* (Kinetoplastida, Trypanosomatidae), is a life-long, neglected tropical disease, and leading cause of cardiomyopathy in endemic areas (Rassi, Rassi and Marin-

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Neto, 2010). With 8 to 10 million people already infected and up to 120 million individuals at risk of infection, Chagas disease constitutes the most important parasitic disease in Latin America and one of the most common globally (Stanaway and Roth, 2015). Its exact burden is however difficult to assess due to several factors including the widespread geographic distribution of *T. cruzi* vector-borne transmission, the decades-long lag between infection and appearance of symptoms, certain pitfalls of current diagnostic methods, biased prevalence data, and incomplete recognition of Chagas disease-attributable symptoms (Stanaway and Roth, 2015). The most recent estimates indicate that Chagas disease is responsible for ~550,000 disability adjusted life years (DALY), a measure that captures both premature mortality (~12,000 deaths per year) and non-fatal health losses (Stanaway and Roth, 2015). Despite this enormous toll, only two trypanocydal drugs, benznidazole and nifurtimox are currently available for chemotherapy. Both are nitroheterocyclic, oral compounds that require prolonged administration, may display severe adverse effects, cannot be used to treat pregnant women due to their uncertain teratogenic risks and, most importantly, show high efficacy solely if administered at the onset of infection (Carlier and Truyens, 2015; Rassi, Rassi and Marin-Neto, 2010; Viotti et al., 2006). The prospects for the development of an effective vaccine for prophylactic and/or therapeutic purposes, on the other hand are still clouded by substantial scientific and socioeconomic challenges (Beaumier et al., 2016; Bustamante and Tarleton, 2015).

T. cruzi transmission primarily occurs when humans are exposed to the contaminated feces of infected, hematophagous triatomine vectors. Large-scale intervention schemes launched in different regions of Latin America in the 1990’s have successfully shrunk the geographic limits and prevalence of vector-borne parasite transmission, and led to an overall ~40% reduction of disease prevalence (Schofield, Jannin and Salvatella, 2006). However, different ecological and demographic issues converged in the last decades to shift the epidemiological landscape for this disease. For instance, recent outbreaks of acute cases in certain regions from Brazil and Venezuela were not strictly vector-borne but rather due to accidental ingestion of *T. cruzi*-tainted food and fluids (Alarcon de Noya et al., 2010; Segovia et al., 2013). This 'food-borne' transmission mode likely constitutes an ancient epidemiological trait, very important to the zoonotic spreading of the parasite (Gurtler and Cardinal, 2015), and appears to be associated with increased virulence and a higher case-fatality rate in humans (Alarcon de Noya et al., 2010; Segovia et al., 2013). In addition, migratory trends of infected populations from rural areas to urban centers and/or to non-endemic regions along with changes in the eco-geographical distribution of vector populations have led to the gradual urbanization and globalization of Chagas disease, which is now recognized as an emerging worldwide threat to public health (Eisenstein, 2016). Indeed, the risk of acquiring Chagas disease through infected blood transfusion and organ transplantation is becoming a major problem even in areas of non-endemicity, such as the United States, Australia and Europe (Requena-Mendez et al., 2015; Schmunis and Yadon, 2010). Moreover, the congenital route of infection, which constitutes the main transmission mode of *T. cruzi* in non-endemic areas is now estimated to be responsible for 22% of new annual infections in endemic countries with active programs for home vector infestations control (Carlier and Truyens, 2015).

In this scenario, a strong and global partnership aimed to coordinate actions to control parasite transmission is urgently needed. In particular, we need to redouble our efforts to control home vector infestation, to screen blood supplies and to identify and subsequently treat *T. cruzi*-infected people who are still in the early stages of the disease to avoid sequelae, morbidity and economic losses. As a major step towards these goals, we ought to develop novel biomarkers able to overcome the limitations of current diagnostic applications. In this chapter, we critically appraise what has so far been achieved in this area. We also discuss possible ways to proceed in order to address major and still unmet diagnostic demands, and the opportunity provided by recent advances in high-throughput methods (i.e. peptide synthesis technology, genomics, and proteomics) in Chagas disease biomarker discovery.

TRYPANOSOMA CRUZI, AN 'ALL-WHEEL DRIVE' PARASITE

T. cruzi is a promiscuous parasite that traverses a complex life-cycle involving extracellular proliferation and differentiation inside hematophagous insect vectors from different genera, and intracellular proliferation and differentiation in a variety of vertebrate hosts (De Souza, 2002). Host switching, immune pressure as well as constant transition from intracellular to extracellular niches (and *vice versa*) pose significant adaptation challenges, and are concomitantly accompanied by extensive remodeling of different aspects of *T. cruzi* such as intracellular transport, primary metabolism, gene expression profiling and overall cellular architecture (De Souza, 2002). This striking plasticity can be also readily recognized in the diverse genetic, phenotypic and epidemiological features displayed by different strains and field isolates comprised within the *T. cruzi* taxon (Zingales et al., 2012). In this first section, we outline some aspects that underlie the biological flexibility of this 'all-wheel drive' parasite, and that may be relevant in terms of biomarker discovery for Chagas disease diagnostic purposes.

EPIDEMIOLOGICAL FEATURES

Potential *T. cruzi* vectors present a broad geographic distribution (from central Argentina and Chile to southern USA) and include more than 140 species of 'kissing bugs' from the subfamily Triatominae (Hemiptera, Reduviidae). Of these, only a few (i.e. *Triatoma infestans, T. dimidiata, T. brasiliensis, Rhodnius prolixus* and *Pastrongylus megistus*) have adapted to live in domiciliary setting and to blood-feed on humans and/or domestic animals and thus define the 'domestic/peridomestic' cycle of Chagas disease (Gurtler and Cardinal, 2015; Zingales et al., 2012). The 'sylvatic' cycle of *T. cruzi*, on the other hand, is actually an array of poorly understood cycles with different eco-epidemiological properties, each one involving multiple sylvatic and/or synanthropic triatomine species, which in turn feed on a variable range of animals. The latter include a variety of rodents, primates, c arnivores, bats, marsupials (i.e. opossums) and xenarthrans (i.e. armadillos, sloths, anteaters) (Figure 1) (Fernandes et al., 1999; Noireau, Diosque and Jansen, 2009; Zingales et al., 2012). In general terms, and although not yet fully established, all mammals are considered susceptible whereas birds and reptiles are considered refractory to *T. cruzi*. From an epidemiological standpoint, these non-human hosts may play key roles as parasite reservoirs and/or as determinant factors affecting *T. cruzi* transmission dynamics in endemic areas

(Gurtler and Cardinal, 2015; Noireau, Diosque and Jansen, 2009). Importantly, they may also work as complex selective systems leading to the emergence of novel parasite traits (Noireau, Diosque and Jansen, 2009).

Interestingly, distinct though partially overlapping sets of strains circulate in the ‘domestic/peridomestic’ and the ‘sylvatic’ cycles of *T. cruzi* (Gurtler and Cardinal, 2015; Noireau, Diosque and Jansen, 2009; Zingales et al., 2012). In the last decades, environmental alterations and demographic issues converged in favoring the intermingling of the two cycles. This translates into an steady increase of emergent transmission patterns involving 'exotic' *T. cruzi* genotypes, with the possible occurrence of atypical disease physiopathologies (Coura et al., 2002; Zingales et al., 2012).

GENETIC AND PHENOTYPIC VARIABILITY

As revealed for several pathogenic protozoa and fungi, *T. cruzi* displays a basically clonal reproduction mode, with occasional events of genetic exchange leading to the emergence of hybrid genotypes (Messenger and Miles, 2015). These features led to a complex population structure, made up of multiple 'clonal' strains showing remarkable genetic diversity (Tibayrenc and Ayala, 2015). Inter-strain variations may be grasped at the nucleotide level (Ackermann et al., 2012) but also structurally, in terms of dosage/diversification of antigenic gene families (Campo et al., 2004; Cerqueira et al., 2008; Llewellyn et al., 2015; Urban et al., 2011), DNA content and overall genome architecture (Lewis et al., 2009a; Minning et al., 2011; Reis-Cunha et al., 2015; Souza et al., 2011). Importantly, biochemical and genetic typing schemes developed throughout the last decades converged in the delineation of six major *T. cruzi* evolutionary lineages or discrete typing units (DTUs) termed TcI to TcVI, with multiple strains and even cryptic sub-lineages within each DTU (Tibayrenc and Ayala, 2015; Zingales et al., 2012). A potential seventh lineage, termed TcBat, has been recently identified in South and Central American bats (Marcili et al., 2009; Pinto et al., 2012). So far, and although all six (or seven, including TcBat) *T. cruzi* DTUs are capable of infecting humans, certain DTUs such as TcI, TcII, TcV and TcVI are most frequently isolated from clinical samples (Ramirez et al., 2014; Zingales et al., 2012). The reasons for this skewed distribution are unclear, although current evidence suggest that parasite strains detected in patients reflect the principal DTUs circulating among 'domestic/peridomestic' cycles in that geographical area (Messenger, Miles and Bern, 2015).

T. cruzi genotypic heterogeneity could also be grasped at the phenotypic level when different biological parameters are studied. These include, for instance, the rate of epimastigote proliferation in the vector midgut (Castro et al., 2012; de Lana et al., 1998; Vieira et al., 2016); and the extent of epimastigote differentiation into metacyclic trypomastigotes, the developmental form that bring the infection into vertebrates (Figure 1) (da Silveira Pinto et al., 2000; de Lana et al., 1998). The molecular basis for these differences are not yet understood but they might be related to the dissimilar resistance capacity of parasite strains to antimicrobial peptides or hemolytic factors and/or to their differential interaction with receptor(s) inside the crop of triatomines (Castro et al., 2012; Gonzalez et al., 2013; Vieira et al., 2016). Importantly, these biological traits define both *T. cruzi* infectivity toward insect vectors and its potential transmissibility to vertebrate hosts (Figure 1). These, together with

differential eco-geographical distribution and certain preference of triatomids for their blood-source are in turn major determinants of Chagas disease epidemiology (Gurtler and Cardinal, 2015; Noireau, Diosque and Jansen, 2009; Zingales et al., 2012).

Parasite genotypic heterogeneity also seems to modulate key aspects of its interaction with vertebrate hosts, including humans. For instance, the capacity of metacyclic trypomastigotes to resist the harsh conditions of the gastric milieu and to invade gastric epithelium following oral infection is largely dependent on the strain-specific glycoprotein composition of their surface coat (Camandaroba, Pinheiro Lima and Andrade, 2002; Hoft et al., 1996; Maeda et al., 2016). In the same line, inter-strain genetic variations underpin a variety of biological traits involved in parasite infectivity and long-term persistence such as antigenic profile, subversion of the immune system, host cell invasion capacity, intracellular growth rate and survival of amastigotes, and sensitivity to anti-Chagasic drugs (Figure 1) (Magalhaes et al., 2015; Martin et al., 2006; Moraes et al., 2014; Mortara et al., 2008; Nagajyothi et al., 2012; Ruiz et al., 1998; Toledo et al., 2003). In contrast, no clear association between a particular *T. cruzi* genotype and an eventual tropism for congenital transmission could be yet established. Even though distinct strains may display subtle differences in their ability to invade trophoblasts or chorionic villi explants *in vitro* (Castillo et al., 2013), genetic profiling experiments have conclusively shown that i) the same set of strains circulate in the bloodstream of transmitting and non-transmitting mothers, and ii) nearly identical *T. cruzi* genetic signatures are recovered from infected infants born to Chagasic mothers coursing concurrent, multi-strain infections (Figure 1) (Burgos et al., 2007; del Puerto et al., 2010; Virreira et al., 2006a). Overall, the actual consensus is that maternal parasite load and human polymorphisms constitute the main risk factors for *T. cruzi* congenital transmission (Bua et al., 2012; Fabbro et al., 2014; Juiz et al., 2016; Kaplinski et al., 2015; Rendell et al., 2015).

Interestingly, certain (though not all) epidemiological studies have shown a partial correlation between the prevalence of particular clinical manifestations of Chagas disease and the genotype of the infecting strain (Andrade, Brodskyn and Andrade, 1983; D'Avila et al., 2009; Luquetti et al., 1986; Macedo and Pena, 1998; Virreira et al., 2006b; Zafra et al., 2011; Zingales et al., 2012). This may be attributed in part to the genetic aspects and immune competence of local human populations (Ayo et al., 2013; Deng et al., 2013; Frade et al., 2013; Luz et al., 2016; Nogueira et al., 2012) and/or to parasite genetic heterogeneities. The latter hypothesis finds support in animal studies (that do not strictly recapitulate Chagas disease-associated physiopathologies), which revealed inter-strain variations in complex phenotypes such as parasitemia, virulence, tissue tropism/distribution and pathogenicity (Figure 1) (Andrade et al., 1999; Andrade, 1990; Camandaroba, Pinheiro Lima and Andrade, 2002; de Souza et al., 1996; Laurent et al., 1997; Monteiro et al., 2013; Revollo et al., 1998; Roellig et al., 2010). However, generalized conclusions are difficult to derive, particularly because these epidemiological studies might have been skewed by a number of intrinsic shortcomings. Briefly, i) they often lacked detailed genetic/clinical information on the studied populations; ii) the infecting genotype has been in some cases inferred based on the prevailing parasite genotypes circulating in the area and not typed directly from patients; iii) patients might have been co-infected with other co-endemic pathogens that impact on the clinical presentation of Chagas disease (Salvador et al., 2016); iv) patients might have been infected with multiple parasite strains, which is usually the case

in endemic areas (Perez, Lymbery and Thompson, 2014); and v) these studies might have been biased due to parasite typing pitfalls (i.e. samples were collected only from peripheral blood, which may not be representative of the situation within affected organs (Manoel-Caetano Fda et al., 2008; Vago et al., 2000)) and/or associations between local parasites and disease, making it difficult to determine whether the absence of a specific strain/DTU in patients with a given disease phenotype is due to parasite factors or to lack of patient exposure to this DTU.

Overall, and although this issue may have major implications for Chagas disease diagnosis and treatment, the existence of particular associations between *T. cruzi* genotype and susceptibility to different clinical presentations on Chagasic patients remains to be addressed (Messenger, Miles and Bern, 2015).

DIAGNOSTIC APPLICATIONS FOR CHAGAS D ISEASE: PRESENT KNOWLEDGE

PARASITOLOGICAL AND CLINICAL METHODS

Upon *T. cruzi* infection, patients undergo the acute phase of Chagas disease, which extends for 40–60 days. Symptoms, if indeed occur, are usually very mild and atypical, thus often misleading its clinical recognition (Rassi, Rassi and Marin-Neto, 2010). In rare cases of vector-borne transmission, a skin nodule (called 'chagoma') or painless prolonged eyelid edema (called the 'Romanha's sign') may indicate the site of parasite inoculation. Due to the patent parasitemia verified at this initial phase, conventional microscopy (i.e. visualization of circulating trypomastigotes in peripheral blood films or buffy coat smears) remains the gold standard for diagnosis, both in acute cases and in newborns that were infected congenitally (Freilij and Altcheh, 1995; Gomes, Lorena and Luquetti, 2009). Either direct tests or concentration tests (i.e. microhematocrit or Strout test) are routinely used for this purpose. These techniques, however present certain limitations in terms of sensitivity (~80–90%), and commonly require highly trained personnel (Table 1) (Freilij and Altcheh, 1995; Gomes, Lorena and Luquetti, 2009).

Following the initial, acute phase, if untreated, patients enter the indeterminate form of the chronic phase that may last for several years or persist indefinitely (Rassi, Rassi and Marin-Neto, 2010). This phase is characterized by the absence of relevant clinical symptoms and very low and intermittent or null parasitemia. During this phase, parasite replication is maintained in check by the elicitation of a strong and parasite-specific B- and T-cell-mediated immunity (Tarleton, 2015), being the latter the most important in terms of controlling the infection. However, elaborate pathogen immune evasion systems (Albareda et al., 2009; Giraldo et al., 2013; Padilla, Simpson and Tarleton, 2009; Paiva et al., 2012; Vasconcelos et al., 2012) and their ability to quickly invade host cells (Mortara et al., 2008; Nagajyothi et al., 2012) turn this immune response only partially effective, and most patients maintain a sub-patent infection for life. *T. cruzi* reactivation in immunocompromised Chagasic patients provides solid support to this hypothesis (Tarleton, 2015). Direct *T. cruzi* detection during the chronic phase requires biological amplification methods, such as hemoculture and xenodiagnosis (Brener, 1962), which are also difficult, expensive, time-

consuming and require special laboratory bio-security conditions. In the case of xenodiagnosis, in addition, it is not applicable to certain patient populations. Most importantly, these methods yield positive results in only a proportion of serologically positive patients, thus limiting their usefulness in diagnosis and/or in monitoring drug efficacy (Table 1).

Up to 20 years after the infection, ~35% of patients develop pathological signs characteristic of Chagas disease such as cardiomyopathy, peripheral nervous system damage or dysfunction of the digestive tract often leading to megaesophagus and/or megacolon (Rassi, Rassi and Marin-Neto, 2010). These pathological changes can be revealed by electrocardiogram clinical diagnosis, X-rays and ultrasound. The nature and relative contribution of the multiple factors involved in this quite broad pathogenic range has been the subject of intense debate (Bonney and Engman, 2015; Machado et al., 2012; Teixeira et al., 2011). The current consensus states that a failure to down-regulate the inflammatory response, which is maintained by parasite persistence in tissues, appears to play a predominant role. Other contributory factors include sex, age and genotypic features of the patient, route of infection, sustained vector exposure, auto-immune responses and the existence of co-infections (Ayo et al., 2013; Bonney and Engman, 2015; Deng et al., 2013; Frade et al., 2013; Luz et al., 2016; Machado et al., 2012; Nogueira et al., 2012; Tarleton, 2015; Teixeira et al., 2011). As mentioned, a possible role of parasite genotype on Chagas disease progression/outcome has been proposed, though further research supported by novel and robust epidemiologic and diagnostic tools, and appropriate animal models are needed to address this issue.

SEROLOGICAL METHODS

Considering that patients seroconvert early upon infection, detection of anti-*T. cruzi* antibodies remains the most effective method for demonstrating direct exposure to the parasite (Gomes, Lorena and Luquetti, 2009). At present, the most widely used serologic methods are Indirect Hemagglutination Assay (IHA), Indirect Immunofluorescence Assay (IFA), and Enzyme-Linked ImmunoSorbent Assay (ELISA) (Table 1). First generation ELISA tests were originally developed using total parasite homogenates or, later on, using biochemically purified, parasite antigenic fractions. Among the latter, TESA (Trypomastigote Excreted/Secreted Antigens) and F2/3 (highly *O*-glycosylated trypomastigote mucins obtained by sequential solvent partitions) demonstrated the highest diagnostic potential (Table 1) (Almeida et al., 1997; Umezawa, Shikanai-Yasuda and Stolf, 1996). In addition to ELISA, multiple techniques including dot blot and Western blot were developed for the evaluation of these reagents.

In the late 1980's, the advent of recombinant DNA technology allowed the generation of parasite DNA or cDNA expression libraries, which were then coupled to high-throughput screenings using serum samples from Chagasic patients or experimentally infected animals. These approaches provided the first glimpses into the genomic make up of this parasite and, most importantly, led to a 'golden era' of *T. cruzi* antigen discovery. Indeed, by using these methods several of the immunodominant *T. cruzi* antigens that are still in use were identified and characterized (Burns et al., 1992; Cotrim et al., 1990; Hoft et al., 1989; Houghton et al.,

1999; Ibanez, Affranchino and Frasch, 1987; Ibanez et al., 1988; Lafaille et al., 1989; Levin et al., 1989). For a complete list of these antigens (henceforth the 'parental repertoire'), see Table 1. The fact that many of them emerged from independent screenings carried out in different laboratories further supported their diagnostic potential but, unfortunately also led to a confusing nomenclature that still persist (Table 1) (reviewed in (da Silveira, Umezawa and Luquetti, 2001; Frasch et al., 1991)). Some of the antigens included in the *T. cruzi* 'parental repertoire' were extensively validated, in certain cases by way of multicenter trials (Caballero et al., 2007; Reithinger et al., 2010), and set the stage for the development of second-generation Chagas disease diagnostic methods. A variety of antigen expression procedures (i.e. using full-length or partially deleted recombinant, fusion proteins or synthetic peptides functionalized in different ways) and antigen display strategies (i.e. using mono- or multi-epitopic molecules) were evaluated before them being incorporated either individually or in defined mixtures into in-house and/or commercial kits (Godsel et al., 1995; Hernandez et al., 2010; Houghton et al., 2000; Krieger et al., 1992; Longhi et al., 2012; Oelemann et al., 1999; Pastini et al., 1994). Some of these tests have shown very good performances, and were thus extensively used in basic research and/or as confirmatory tests in clinical practice up to this day.

The major advantage of recombinant antigens-based tests is that they minimize the extent of specificity problems. As previously shown, sera from individuals with other co-endemic infections such as leishmaniasis and/or afflicted of certain autoimmune disorders cross-react with crude preparations of *T. cruzi* antigens (Araujo, 1986; Gomes, Lorena and Luquetti, 2009; Schnaidman et al., 1986). On the down side, they present lower sensitivity than whole parasite-based techniques. In addition, it should be said that the proven success of this 'parental repertoire', along with a shift in R&D priorities converged in curbing the enthusiasm for subsequent large-scale antigen-discovery efforts for Chagas disease. Indeed, most of the additional antigens/biomarkers with moderate-to-excellent diagnostic performance that emerged in the last decades were identified either as by-products of basic hypothesis-driven research (Buchovsky et al., 2001; Di Noia et al., 2002; Martinez et al., 1991; Saborio et al., 1990) or using low-to-medium throughput antigen expression/synthesis approaches (see below). Most importantly, just a few of these novel antigens have been incorporated into the already existing diagnostic application platforms.

New generation tests displaying potentially improved accuracy such as the Chemoluminescent Microparticle ImmunoAssay (CMIA) and its improved version, Architect Chagas (both from Abbott Laboratories, Wiesbaden, Germany) (Abras et al., 2016; Praast et al., 2011), or the Multi-cruzi test (InfYnity Biomarkers, Lyon, France) (Granjon et al., 2016) have been recently developed. Confirming the above mentioned trend, both use a large panel of *T. cruzi* antigens belonging to the 'parental repertoire' -those discovered in the 80's-, which in the case of the Multi-cruzi test is supplemented with TSSA (Trypomastigote Small Surface Antigen (Di Noia et al., 2002)). Despite the virtual lack of antigen innovation, these tests incorporate a large degree of automation and highly sensitive detection systems (Abras et al., 2016) or major technical improvements, such as a multiplexed printing method inside ELISA microplates (Granjon et al., 2016).

Overall, currently available serodiagnostic tests are simple, affordable and display quite good results in terms of large-scale population diagnosis. However, they all present certain minor concerns with regards to their reproducibility, reliability, specificity (those using whole parasites) and sensitivity (those using parasite fractions and/or recombinant antigens) which, in turn affect their overall performance. In addition, and likely due to their biased antigenic composition, they also show suboptimal performance with particular Chagasic populations (i.e. acute and/or congenital infections) (Gomes, Lorena and Luquetti, 2009), and often discordant results between assays depending on the geographic origin of the patients (Caballero et al., 2007; Guzman-Gomez et al., 2015). The latter may be attributed to variations in the local human immune responses and/or, as stated above, to differences in the antigenic constitution of *T. cruzi* DTUs that prevail in different endemic areas. Importantly, even after successive improvements, none of the available tests emerged as the ‘gold standard’, i.e. able to show ~100% specificity and sensitivity. In this context, current guidelines developed by the World Health Organization advice the use of at least two serological tests based on different principles for reaching a 'conclusive' diagnosis. In the case of ambiguous or discordant results, a third technique should be conducted. These guidelines thereby increase the cost of diagnosis and risk of patient ‘loss’, particularly in endemic areas. Most importantly, these diagnostic limitations delay the initiation of chemotherapy, thus limiting its efficacy.

MOLECULAR METHODS

In the late 1980's, DNA-based molecular methods emerged as an appealing alternative for the diagnosis of Chagas disease, mainly to overcome the low sensitivity of direct parasitological approaches (Moser, Kirchhoff and Donelson, 1989; Sturm et al., 1989). The introduction of the recently developed polymerase chain reaction (PCR) assay, in particular, held promises of high sensitivity, specificity, and high-throughput potential. The first targets for PCR amplification were focused on the major molecular component of the mitochondrial DNA (also known as kinetoplast DNA or kDNA). In *T. cruzi* and other kinetoplastid parasites, this component is present in the form of circular DNA of short length (mini-circles), which add up to several thousand copies per cell (Simpson, 1986). Later on, a large repertoire of kDNA or nuclear DNA targets including single- or multi-copy genes and satellite sequences, as well as different multi-target strategies have been explored (Schijman et al., 2011). More recently, a parasite concentration approach based on short and stable RNA aptamers was developed to facilitate PCR-based detection methods, and proposed as a potential alternative tool in monitoring parasite load in Chagasic patients (Nagarkatti et al., 2012; Nagarkatti et al., 2014).

Major challenges for the clinical implementation of PCR-based techniques derive from a number of technical factors such as source (i.e. cord blood, umbilical tissue, placental, tissue), volume, conservation and transportation of the samples, and underlying molecular biology protocols (i.e. DNA isolation, purification, pre-treatment, thermo-cycling conditions, etc.) (Schijman et al., 2011). In addition, the reproducibility and overall performance of PCR-based methods is significantly affected by the fluctuations in parasitemia that characterize the chronic phase of Chagas disease, and by inter-DTU variations in dosage and/or sequence of the targets of amplification (Lewis et al., 2009b).

Despite these issues, several PCR- and, more recently, quantitative real-time PCR (qPCR)-based procedures have been developed. These allowed detection and quantification of parasite DNA from clinical samples with variable levels of reliability, complexity, selectivity and analytical sensitivity (Duffy et al., 2009; Duffy et al., 2013; Piron et al., 2007; Qvarnstrom et al., 2012; Valadares et al., 2008). In a recent work, a multicentre trial optimized and evaluated in parallel two last generation qPCR methods targeting either satellite or kDNA targets (Ramirez et al., 2015). These methods were tested using a large and heterogeneous panel of blood samples from acute and chronic patients either asymptomatic or showing different clinical manifestations, and infected through different transmission modes (Ramirez et al., 2015). Though highly specific and reproducible, both methods showed clinical sensitivities of ~80%, which is still not good enough for their application as confirmatory testing of blood donors or in clinical settings. In addition, it is worth noting that DNA-based molecular methods are expensive and difficult to be implemented in endemic areas, e.g. in PoC sites with limited infrastructure (Table 1).

DIAGNOSTIC APPLICATIONS FOR CHAGAS D ISEASE: PENDING ISSUES

As described above, current diagnostic methods and particularly those based on serology are highly accurate in detecting most of *T. cruzi* infections in humans. However, there are some clinical and/or epidemiological situations, discussed in this section, in which their performance is significantly hampered by methodological and/or biological issues.

EARLY DIAGNOSIS OF CONGENITAL TRANSMISSION

According to epidemiological data, maternal-fetal transmission occurs in an average of 5% of infected mothers in endemic areas, thus leading to ~15,000 congenital cases per year (Carlier and Truyens, 2015). Diagnosis in newborns is commonly based on the microscopic observation of trypomastigotes in peripheral and/or umbilical cord blood, which is more effective by the microhematocrit concentration technique (Freilij and Altcheh, 1995). Considering the usually high parasitemia at the initial stage, PCR-based analyses provide a valuable supporting tool to detect infection and to evaluate treatment outcome in these patients (Table 1) (Duffy et al., 2009; Russomando et al., 1998; Schijman et al., 2003). Standard serodiagnostic methods, though routinely used have low positive predictive value until 8–9 months after birth due to passive transfer of maternal IgG antibodies (Freilij and Altcheh, 1995). In this context, identification of novel antigens recognized by fetal IgM or IgA appeared as an appealing approach. However, and despite initial promising results (Antas et al., 2000; Betonico et al., 1999; Corral, Altcheh and Freilij, 1998; Lorca et al., 1995), these efforts have been discontinued due to the high number of false-negatives (Blanco et al., 2000; Freilij and Altcheh, 1995; Gomes, Lorena and Luquetti, 2009). It should be noted though, that a serious, high-content and unbiased screening for congenital IgM specificities have not been yet undertaken.

As an alternative approach to discover surrogate markers of congenital infection, the group of Dr. Frasch in Argentina undertook the identification of *T. cruzi* antigens exclusively and/or preferentially recognized by fetal IgGs. This approach relied on the parallel evaluation of paired mother/newborn serum samples against a multiplexed antigen panel

followed by the comparison between signatures of recognition. Using this strategy, they identified SAPA (Shed Acute-Phase Antigen) and, to a lesser extent, Ag13/TcD as suitable markers to detect congenital transmission (Table 1) (Reyes et al., 1990). Both antigens belonged to the above mentioned *T. cruzi* 'parental repertoire' and, interestingly, both (particularly SAPA) had been previously found to display a biased, though not exclusive recognition towards acute infection sera (Affranchino et al., 1989). This is consistent with the assumption that congenital *T. cruzi* infection constitutes in fact an acute infection in newborns (Carlier and Truyens, 2015).

Further research disclosed that SAPA is a repetitive sequence displaying a complex antigenic structure (Alvarez et al., 2001) and involved in improving the pharmacokinetics of *trans*-sialidase, a major *T. cruzi* virulence factor (Buscaglia et al., 1999; Dc-Rubin and Schenkman, 2012; Frasch, 2000). Up to this day, SAPA remains the only available serological marker of early infection and has been widely used to diagnose recently-acquired vector-borne infections and congenitally transmitted cases (Mallimaci et al., 2010; Russomando et al., 2010; Volta et al., 2015).

More sophisticated, laborious and difficult to interpret methods were also developed. These include testing of the newborn sample by immunoblot against the TESA fraction (Table 1) (Umezawa et al., 1996) and, more recently, the Chunap (Chagas urine nanoparticle) test (Castro-Sesquen et al., 2014). In the latter method, instead of *T. cruzi*-specific antibodies, active infection is indirectly assessed by the capture and concentration of *T. cruzi* TESA antigens from patient's urine, which are then revealed using a monoclonal antibody directed to a parasite lipophosphopeptideglycan, included in the test (Castro-Sesquen et al., 2014). When evaluated on children living in endemic areas and tested at the peak of parasitemia (~1-month old) Chunap was able to accurately diagnose congenital infections (Castro-Sesquen et al., 2014), and is now being evaluated for other diagnostic applications (Castro-Sesquen et al., 2016).

On a final note, it should be stressed that given the high efficacy of trypanocidal drugs in infected newborns, their rapid diagnosis and subsequent treatment is essential. Intensive screening for distinctive immune responses (i.e. those based on IgM and/or IgA antibodies) and/or for specific molecular signatures that may translate into surrogate biomarkers for early detection of congenital *T. cruzi* transmission should be thus considered a top-ranking priority in Chagas disease applied research.

RAPID ASSESSMENT OF THERAPY EFFICACY

Overall, the best chemotherapy results have been achieved in acute or early chronic infections. However, even in children the cure rate is up to 62% at 2 years of follow-up, although this figure may vary according to population and geographical location (Ribeiro et al., 2009). This variability may be attributed in part to differences in the prevalence of human-associated genotypes across endemic areas. Indeed, substantial inter-strain variations in drug resistance have been ascertained both in *in vitro* systems and in animal infection models (Figure 1) (Moraes et al., 2014; Toledo et al., 2003).

During the chronic stage, the efficacy of drug treatment is lower and variable; and is also difficult to assess because most studies used different treatment regimens and outcome evaluation methods (variable assays, frequency and duration of follow-up) (for a recent review, see (Pinazo et al., 2014)). Even in successful treatments, the gold standard for evaluating drug efficacy, which relies on consistent negative results using conventional parasitological and whole parasite-based serological tests, may take years to decades to assess, thus stressing the necessity of novel and improved therapeutic response markers.

Several therapeutic studies support the usefulness of PCR-based strategies to evaluate treatment outcome in congenital or acute cases of Chagas disease. Moreover, PCR-based methods may also assist, though with sub-optimal performance in cases of lower parasitemias, in evaluating chemotherapy in chronic cases of Chagas disease (Britto et al., 1995; Galvao et al., 2003; Munoz et al., 2013; Russomando et al., 1998; Schijman et al., 2003). In particular, PCR-based methods seem to be a rapid indicator of the parasite's susceptibility to drugs, allowing early therapy modification in cases of resistance or reactivation of Chagasic infection (Lages-Silva et al., 2002; Pinazo et al., 2014; Schijman et al., 2000).

In 1982, Drs. Krettli and Brener in Brazil demonstrated the existence of a special type of antibodies, which they termed 'antibodies against living blood forms', that were absent in parasitological cured hosts early after drug treatment (Krettli and Brener, 1982). These antibodies were shown to participate in resistance against *T. cruzi* and were solely detectable by complement-mediated lysis (CoML) assays (Krettli and Brener, 1982). Upon these findings, the authors proposed the inclusion of the CoML technique in the context of cure criteria. Indeed, the CoML technique offered an important contribution for post-therapeutic monitoring of Chagas disease in clinical trials, mostly by significantly reducing the required follow-up periods (Table 1) (Galvao et al., 1993). Numerous improvements in terms of the sensitivity and operational safety of the CoML method have been later on introduced (Alessio et al., 2014; Martins-Filho et al., 2002; Vitelli-Avelar et al., 2007). Most importantly, this concept of 'non-conventional' serological responses set the basis for multiple screenings looking for parasite molecules that could be the target of antibody responses showing different qualitative, quantitative and/or kinetic properties. Several potential biomarkers have been thereby identified and evaluated for their post-therapeutic application (Andrade et al., 2004; Fernandez-Villegas et al., 2011; Gazzinelli et al., 1993; Laucella et al., 2009; Machado-de-Assis et al., 2012; Sanchez Negrette et al., 2008; Silva et al., 2002). They included different parasite fractions (i.e. TESA, F2/3), individual recombinant antigens belonging to the 'parental repertoire' such as F29/FCaBP/Tc-24/Tc-28/1F8 (Sosa Estani et al., 1998), and other molecules such as TSSA (our unpublished results) (Table 1). Overall, the best results were obtained with the F2/3 fraction (Table 1) (Andrade et al., 2004; de Andrade et al., 1996). Interestingly, antibodies directed to α-galactosyl-containing epitopes (i.e. the main antigenic determinant in the F2/3 fraction), were later on shown to induce trypomastigote lysis independently of complement (Pereira-Chioccola et al., 2000).

More recently, the group of Dr. Tarleton explored the use of a multi-pronged approach based on the simultaneous evaluation of *T. cruzi*-specific B- and T-cell responses as an alternative

way of measuring treatment efficacy (Alvarez et al., 2016; Laucella et al., 2009). By doing so, both a decline in the frequency of IFNγ-producing T cells and in antibody titers measured by a previously developed recombinant multiplex serological assay (Cooley et al., 2008) were observed shortly after benznidazole treatment and were thus proposed as surrogate markers for refining the post-therapeutic cure criterion (Albareda and Laucella, 2015; Alvarez et al., 2016; Laucella et al., 2009). Identification of novel biomarkers for early evaluation of anti-trypanocidal drug efficacy will fasten and improve the assessment of current chemotherapy treatments in clinical trials and, importantly, will be instrumental for the development of novel and improved treatments.

INDICATION/PREDICTION OF CHAGAS DISEASE PROGRESSION

As mentioned, Chagas disease evolves into a wide range of pathological symptoms, ranging from subclinical to potentially fatal megasyndromes (Rassi, Rassi and Marin-Neto, 2010). Identification of diagnostic and/or predictive biomarkers of disease progression would therefore represent a major achievement towards improving the clinical management of Chagasic patients. Some authors proposed serological alternatives to tackle this issue, such as quantifying the antibody titers to Ag1/JL7/FRA/H49 to distinguish between Chagas disease-associated cardiac pathology and other non-Chagasic cardiological dysfunctions (Table 1) (Abraham and Derk, 2015; Bhattacharyya et al., 2014; Kaplan et al., 1997; Levin and Hoebeke, 2008; Thomas et al., 2012). On the other hand, antibodies to another 'parental repertoire' antigen, termed JL5, were shown to cross-recognize endogenous β1-adrenergic and M2 muscarinic receptors. Such 'auto-antibodies' were shown to be associated with arrhythmogenic anomalies, which may contribute to cardiac alterations in Chagasic patients (Kaplan et al., 1997), and were thus proposed to be included in the context of disease prognosis (Table 1). More recently, a certain correlation between serological responses to TSSA and electrocardiogram abnormalities was also noted (Table 1) (Bhattacharyya et al., 2014).

Alternatively, several groups attempted to identify biochemical markers of cardiac damage and/or inflammation such as TNF-α (Talvani et al., 2004a), angiotensin-converting enzyme 2 (ACE2) (Wang et al., 2010), Brain and Atrial Natriuretic Peptides (BNP and ANP, respectively) (Garcia-Alvarez et al., 2010; Heringer-Walther et al., 2005; Ribeiro et al., 2002) as candidates for disease prognosis (Table 1). In particular, the concentration of BNP and ANP in serum were systematically studied as markers of heart damage in Chagasic patients (Garcia-Alvarez et al., 2010; Heringer-Walther et al., 2005; Ribeiro et al., 2002) since they had been previously related with cardiovascular diseases (Wang et al., 2006; Wondergem et al., 2001). Increased concentrations of BNP and ANP strongly correlated with the severity of Chagas-associated cardiac damage, being BNP more sensitive than ANP (Fernandes et al., 2007; Heringer-Walther et al., 2005; Talvani et al., 2004b). Upon these findings, the authors proposed that BNP could be measured periodically in asymptomatic patients as screening test to detect incipient ventricular dysfunction (Heringer-Walther et al., 2005). In the same line, higher levels of plasma ACE2, that catalyzes the conversion from angiotensin II to angiotensin 1–7 (Keidar, Kaplan and Gamliel-Lazarovich, 2007), were shown to correlate with clinical severity and worsening echocardiographic parameters in Chagasis patients (Wang et al., 2010). More importantly, given that the combined

determination of BNP concentration and ACE2 activity had better positive predicted value than when separately analyzed, authors encouraged the use both markers to predict fatal outcomes (Wang et al., 2010).

In the same line, the role of endothelin-1 (ET-1) as a prognostic marker for *T. cruzi*-induced pathogenesis has also been extensively studied, though in animal models. ET-1 is a vasoactive peptide synthesized by many cell types including cardiac myocytes and cardiac fibroblasts associated with vasospasm, vascular damage, cardiovascular remodeling and inflammation (Kedzierski and Yanagisawa, 2001). Mice infected with *T. cruzi* exhibit increased levels of ET-1 and endothelin converting enzyme (ECE), the enzyme responsible for the conversion of the precursor to ET-1, in plasma, in the vasculature and in *T. cruzi*-infected myocardial cells (Huang et al., 2000; Petkova et al., 2000). Interestingly, phosphoramidon, an inhibitor of ECE, ameliorated the pathology and reduced the extent of cardiac remodeling in these animals (Jelicks et al., 2002). Moreover, ET-1 KO mice showed certain protection against chronic Chagasic cardiomyopathy (Tanowitz et al., 2005).

Overall, and despite multiple and disparate attempts, none of the serological and/or biochemical markers that have been explored so far translated into a reliable, easy to assay and interpret marker to asses cardiac damage and/or disease prognosis (recently reviewed in (Pinazo et al., 2015)). The complex and likely multifactorial nature of Chagas disease pathogenesis together with intrinsic difficulties in establishing appropriate experimental/epidemiological models converge in turning this area as the most challenging in terms of *T. cruzi* biomarker discovery.

TYPING OF INFECTING PARASITE STRAIN(S)

Pioneer studies aimed at fingerprinting the infecting parasite strain(s) directly in clinical samples were based on biochemical markers (i.e. MLEE, Multi-Locus Enzyme Electrophoresis) (Tibayrenc and Ayala, 2015). Recent advances in typing schemes based on RFLP (Restriction Fragment Length Polymorphism), PCR-RFLP, RAPD (Random Amplification of Polymorphic DNA), DNA hybridization, karyotyping and, particularly, sequence-based markers either using a single locus or multiple loci have greatly improved parasite genotypic resolution *in vitro* (Table 1) (Tibayrenc and Ayala, 2015). However, *in vivo*, these genotyping methods are time-consuming and costly, and require parasite isolation and amplification or a high quantity of DNA, therefore necessitating invasive sampling with medical risks. Moreover, the fact that concurrent infections with multiple *T. cruzi* strains appears to be the norm rather than the anomaly (Perez, Lymbery and Thompson, 2014), and the discovery that the most prevalent *T. cruzi* genotype present in the bloodstream can differ from the strain(s) found sequestered within organs (Manoel-Caetano Fda et al., 2008; Vago et al., 2000) further complicate this task. In this context, serotyping methods emerge as an appealing alternative to overcome these limitations, as demonstrated in other human infectious diseases (Dunbar et al., 2015; Maksimov et al., 2012). Serotyping is based on the use of specific antigens with qualitative and/or quantitative differences among parasite strains/DTUs to detect strain-specific antibody signatures.

TSSA (Di Noia et al., 2002) is a parasite adhesin displaying significant sequence homology to *TcMUC*, a huge family of polymorphic genes that code for the polypeptide backbones of

the trypomastigote mucin molecules (Buscaglia et al., 2004; Campo et al., 2006), some of which are terminally decorated with α-galactosyl residues and constitute the F2/3 antigenic fraction. Interestingly, detailed genetic characterization of the *TSSA* locus disclosed minor sequence variations between TSSA variants expressed by different parasite DTUs (Bhattacharyya et al., 2010; Di Noia et al., 2002). Some of these variations were shown to have major impact on TSSA biological function and antigenicity, thereby leading to differential antibody profiles between variants (Balouz et al., 2015; Bhattacharyya et al., 2014; Canepa et al., 2012; De Marchi et al., 2011; Di Noia et al., 2002). So far, TSSA remains the only polymorphic antigen that has been successfully used for the development of DTU-specific serology methods for *T. cruzi* infections of humans (Table 1) (Bhattacharyya et al., 2014; Bisio et al., 2011; Burgos et al., 2010; Longhi et al., 2014; Risso et al., 2011). Despite this, the resolution and specificity of TSSA-based serotyping assays need to be improved. Discrimination between certain DTUs remains challenging as they possess identical or almost identical *TSSA* alleles or they are poorly immunogenic (Bhattacharyya et al., 2014; Canepa et al., 2012).

Serotyping could be a rapid, sensitive, cost-effective and relatively non-invasive alternative to stringent *T. cruzi* genotyping in humans, and may also be used in animal reservoirs for epidemiological studies (Bhattacharyya et al., 2015; Cimino et al., 2011). Most importantly, development of novel serotyping tools will facilitate the unraveling of possible relationships between parasite genetic variability and clinical features, a major issue in Chagas disease applied research.

POINT-OF-CARE DIAGNOSIS

People living in Chagas disease endemic areas have restricted access to laboratory facilities and/or to appropriate health centers. This, together with the associated costs and expertise needed for conventional diagnostic methods point to a number of technological and economic barriers that further stress the need to deploy PoC tests for *T. cruzi* infection diagnosis. PoC devices, in addition, allow the patients to see the results for themselves, which contributes to a better working relationship between local communities and people carrying out the testing (e.g., during field surveys). Moreover, the need for follow-up visits to surveyed individuals and therefore the operational costs and risks of possible attrition bias are reduced. From an epidemiological point of view, a reliable PoC test would allow intervention strategies to be implemented *in situ*, such as for serologic surveillance, vaccine or clinical trials; as well as for rapid initiation of treatment of infected individuals during outbreaks of acute cases, such as those recently reported (Alarcon de Noya et al., 2010; Segovia et al., 2013).

Several rapid diagnostic and PoC tests to detect infection based on immunochromatography, particle agglutination, immunofiltration, immunodot, lateral-flow immunochromatographic assays (LFIA) or DNA detection are available for a variety of infectious diseases (Nair et al., 2016; Natoli et al., 2014; Teles and Fonseca, 2015). These may be either qualitative or semi-quantitative and are characterized by the delivery of quick results, in most cases without the need for electrical equipment. In the case of Chagas disease, several LFIAs based on antigenic fractions or recombinant antigens and a single PoC test aimed at the capture and

concentration of *T. cruzi* TESA antigens in urine samples (Chunap, see above) were developed (Table 1) (Castro-Sesquen et al., 2014; Houghton et al., 2009; Reithinger et al., 2010). Serological tests, however show substantial variations in their sensitivity in different geographical areas (Verani et al., 2009), and display sub-optimal performances (Sanchez-Camargo et al., 2014). Unfortunately, and despite being widely used in different parasitic diseases (Mondal et al., 2016; Sriworarat et al., 2015), PCR-based methods applicable in PoC settings such as those based on simple colorimetric loop-mediated isothermal amplification or Recombinase Polymerase Amplification (RPA) techniques are not yet available for *T. cruzi* detection.

As a first step towards the development of alternative PoC devices for diagnosis, a new diagnostic platform based on superparamagnetic microbeads coated with recombinant antigens and fluorescent (FMBIA) or electrochemical (EMBIA) detection was recently developed (Cortina et al., 2016). The EMBIA platform, including antigen-functionalized magnetic microbeads, disposable electrochemical cells-electrode cartridges and a portable potentiostat, was evaluated for serodiagnosis of human and cattle infectious diseases. In the particular case of Chagas disease, a more extensive validation was performed showing that the EMBIA platform displayed an excellent diagnostic performance almost indistinguishable from the well-established ELISA methods (Cortina et al., 2016).

DIAGNOSTIC APPLICATIONS FOR CHAGAS D ISEASE: THE ROAD AHEAD

As discussed throughout this chapter, parasite-specific immune signatures provided a prime source of biomarker candidates for development of Chagas disease diagnostic applications (see Table 1). However, they may now be re-explored using modern and powerful ‘omics’-based fingerprint approaches. Indeed, the availability of complete *T. cruzi* genomes from several strains together with a variety of recent genome mining exercises were performed to identify potential serodiagnostic reagents and vaccine candidates (Bhatia et al., 2004; Carmona et al., 2012; Cooley et al., 2008; Goto, Carter and Reed, 2008; Reis-Cunha et al., 2014). Importantly, most of the antigens that emerged from these *in silico*-guided screenings were not included in the ‘parental repertoire' (Table 1), and could thereby entail novel diagnostic capabilities, such as early assessment of drug efficacy (Alvarez et al., 2016; Laucella et al., 2009). Genome-wide approaches could be also used as a starting point to identify novel DNA-based DTU-resolution markers (Cosentino and Aguero, 2012) and/or novel serotyping reagents. As previously shown, genetic variation among *T. cruzi* DTUs translates into differential proteomes (Telleria et al., 2010) and, therefore also likely in distinct epitope collections ('epitomes') recognized during human infections. By carrying out genome-wide B-cell epitope prediction on proteins derived from allelic pairs of the hybrid *T. cruzi* CL Brener genome, the group of Dr. Bartholomeu in Brazil was able to identify three novel polymorphic epitopes potentially able to discriminate between parasite DTUs (Mendes et al., 2013). Unfortunately, this study and those mentioned above were somehow limited by the use of low-to-medium throughput antigen expression/synthesis approaches, and/or by the use of non-human sources of serum samples.

Recent advances in computerized photolithography and photochemistry, however have allowed the development of a novel peptide-chip technology where up to 1 million

individual peptides are synthesized *in situ* on a glass slide (Buus et al., 2012). This *in situ* synthesis makes the production of the chip highly cost-effective and allows, for the first time, to interrogate complete proteomes. Recent work in our laboratories demonstrated both the high technical reproducibility as well as epitope mapping consistency of this platform when compared with earlier technologies (Balouz et al., 2015; Carmona et al., 2015). Most importantly, by screening the complete length of 457 parasite proteins (~7% of the *T. cruzi* deduced proteome) we were able to identify 2,031 Chagas disease-specific peptides and 97 novel parasite antigens (Carmona et al., 2015). Together with above mentioned studies, and besides emphasizing the huge potential of genomic/proteomic methods as major driving forces in antigen discovery, these findings indicate that a vast majority of the *T. cruzi* antigenic repertoire remains uncharacterized. We aim now at interrogating the entire *T. cruzi* deduced proteome, including inter-strain polymorphisms revealed by previously developed genetic diversity maps (Ackermann et al., 2012; Panunzi and Aguero, 2014). The use of samples collected from particular patients populations such as infected with different parasite DTUs or showing distinct Chagas disease-associated pathologies followed by a final integration of the emerging data will put us in position to discriminate between the common linear B-cell 'epitome' of *T. cruzi* and sets of epitopes showing differential recognition among distinct Chagas disease populations.

Considering the huge functional and diagnostic significance of carbohydrates on *T. cruzi* biology (de Lederkremer and Agusti, 2009), it could be hypothesized that similar high-throughput approaches carried out on glycan and/or lectin microarrays (Fernandez-Tejada, Canada and Jimenez-Barbero, 2015) will have a major impact on Chagas disease biomarker discovery. These methodologies have been successfully explored in different parasitic infections (Anish et al., 2013; Aranzamendi et al., 2011; Martin et al., 2013).

From a wider perspective, high-throughput strategies may be also pursued to develop novel biomarkers based on the detection of *T. cruzi*-derived molecules (Castro-Sesquen et al., 2014; de Titto and Araujo, 1988) and/or parasite-induced modifications o n host molecules and/or cells (Li et al., 2016; Mucci et al., 2002; Muia et al., 2010; Risso et al., 2007; Trocoli Torrecilhas et al., 2009). Theoretically, these strategies would have the additional advantage to discriminate between active from past infections and/or to assist in monitoring disease progression, as validated in other human diseases (Karsdal et al., 2010; Leeansyah et al., 2013; Tritten et al., 2014). In this context, it is important to keep in mind i) the extent of variability due to host genetic background on setting these putative biochemical markers and ii) that *T. cruzi*-derived molecules, and particularly during the chronic phase of Chagas disease, are present in biological fluids at extremely low concentrations, and likely aggregated in membrane-coated vesicles (Bayer-Santos et al., 2013; Fernandez-Calero et al., 2015; Lantos et al., 2016; Trocoli Torrecilhas et al., 2009).

Nevertheless, modern ‘-omic’ technologies are offering alternative strategies for such a difficult system biology exploration (Cantacessi et al., 2015; Hockl et al., 2016; Preidis and Hotez, 2015). In particular, mass spectrometry (MS) methods provide a robust, versatile and sensitive analytical technology allowing high-throughput detection with mass accuracy, precise quantitation and verification of protein variants, splice isoforms, metabolites and disease-specific post-translational modifications (Crutchfield et al., 2016). Indeed, using

MS-based approaches, global changes in metabolic profiles in Chagasic patients showing acute myocarditis (Girones et al., 2014) and serological biomarkers showing differences between Chagasic and healthy subjects (Santamaria et al., 2014) were recently identified. In the latter case, biomarker peaks with the best discriminatory power were further characterized by a range of proteomic and immunological techniques, indicating that specific fragments derived from proteolysis of apolipoprotein A-I and one fragment of fibronectin are specifically upregulated in Chagasic patients (Santamaria et al., 2014). Interestingly, these biomarkers returned to normal values more rapidly than whole parasite-based serological tests in patients treated with nifurtimox, thus supporting their potential use for evaluation of therapeutic efficacy (Table 1) (Santamaria et al., 2014).

In addition to the obvious impact in the diagnostic application field, it is expected that the utilization of these and other high-throughput, '-omic' techniques will provide a vast amount of putative biomarkers to be explored in other Chagas disease research areas such as molecular epidemiology and prioritization of targets for vaccine development. Most importantly, together with appropriate animal models and robust bioinformatics, molecular and cellular tools (Aguero et al., 2008; Crowther et al., 2010; Katsuno et al., 2015; Lewis et al., 2015; Magarinos et al., 2012; Moraes et al., 2014), these biomarkers will be instrumental to screen, prioritize and evaluate safety and efficacy of novel drugs/treatments.

CONCLUDING REMARKS

Over 100 years after its discovery, and despite its huge medical, economic and social burden, Chagas disease remains a major threat in several countries of Latin America and an emergent global health problem. Great efforts have been made and are still being made in Latin America and other developed countries to halt *T. cruzi* transmission. However, one of the key issues concerning Chagas disease control remains that of diagnosis. Without accessible and effective diagnostics tools and methods, infected individuals cannot be timely identified and hence treated. Even if treated, the success of treatment cannot be efficiently assessed.

As discussed here, current diagnostic methods are highly accurate in detecting most of *T. cruzi* infections in humans. However, there are still some clinical and/or epidemiological situations in which their performance is severely impaired. In addition, the complex epidemiological features of Chagas disease and the remarkable phenotypic diversity displayed by distinct *T. cruzi* strains, which may be associated to certain aspects of disease progression/outcome, also stress the necessity of developing new methods able to be deployed in PoC settings and capable of typing the infecting parasite(s) directly in clinical samples. The implementation of modern ‘-omic’, high-throughput and aggressive strategies constitutes an appealing alternative to move on towards filling current diagnostic gaps. Emergent diagnostic tests integrating these novel and tailored tools will provide a significant impact on the effectiveness of current intervention schemes and, most importantly, will improve the clinical management of Chagasic patients by providing the intervening physician with an accurate and integrated diagnosis.

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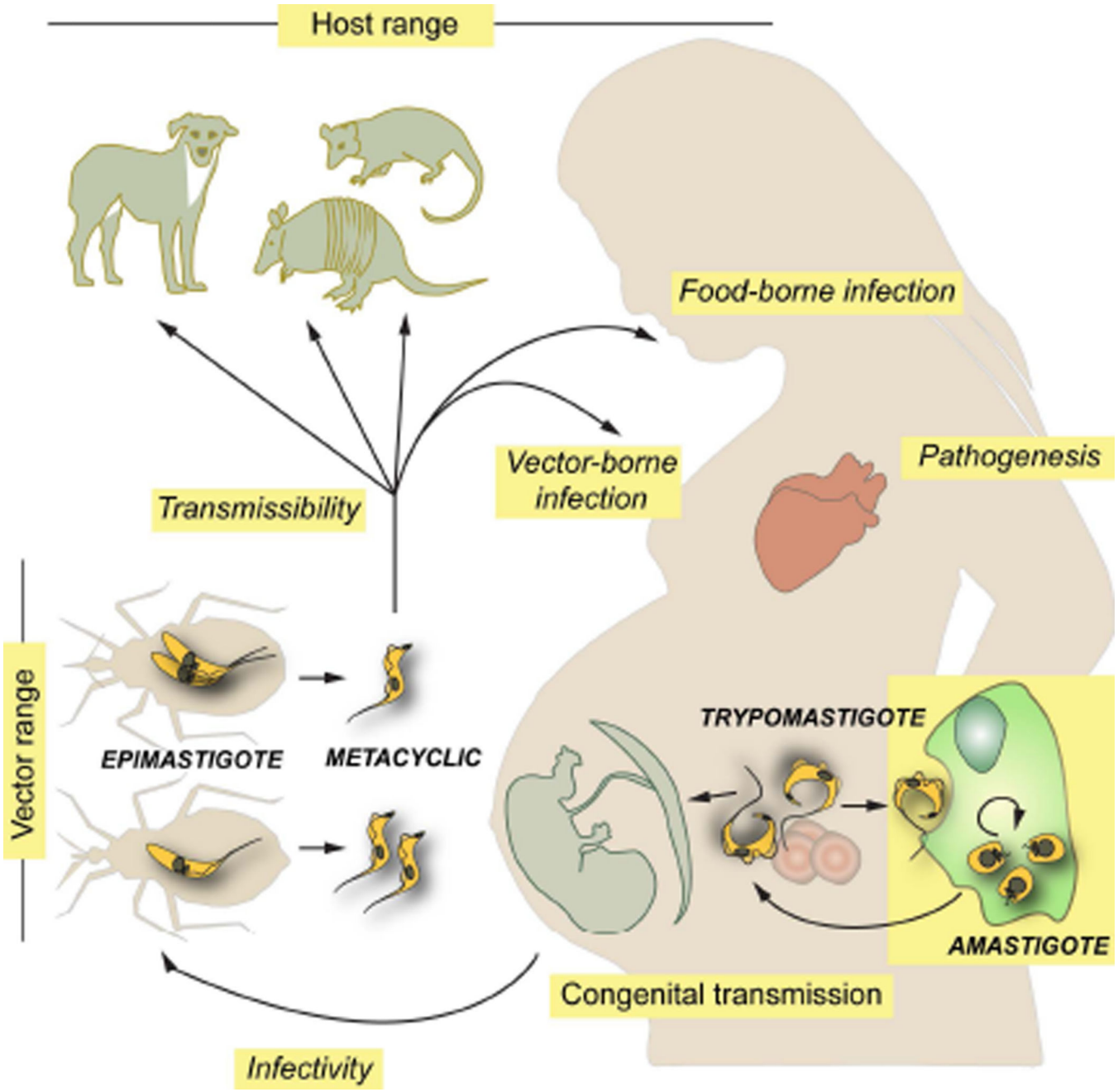

FIGURE 1.

Schematic diagram showing the *T. cruzi* life cycle and different biological features that contribute to ensure its transmission and the establishment of multiple interactions with insect vectors and infected humans. Those features for which there is direct or indirect experimental evidence suggesting inter-strain variability are denoted in italics.

Table 1

Overview of performance and features of available diagnostic applications for Chagas disease.

			Diagnostic Performance[1]							
	Technique/Tool		Chronic cases	Acute cases	Congenital cases	Drug efficacy	Disease prognosis	Parasite typing	PoC setting[2]	Remarks
Parasitological Methods	Directbloodexamination tests						N.A.	N.A.		Microscopy-based methods, gold standard for diagnosis of acute and congenital cases
	Hemoculture					N.A.	N.A.	N.A.		Improved sensitivity due to parasite amplification, time-consuming, expensive
	Xenodiagnosis				N.A.	N.A.	N.A.	N.A.		Improved sensitivity due to parasite amplification, time-consuming, expensive, not well tolerated
Serological Methods	Whole Parasite-based	IHA[3]					N.A.	N.A.		Moderate specificity, trained personnel required
		IFA[3]					N.A.	N.A.		Moderate specificity, trained personnel and infrastructure required
		ELISA[3]					N.A.	N.A.		Method of choice for diagnosis of most clinical situations, cost-effective, easy to perform
		CoML	N.A.	N.A.	N.A.		N.A.	N.A.		Measures antibodies that are cleared in parasitological cured hosts early after drug treatment
	Parasite fraction	TESA[4]					N.A.	N.A.		Secreted/Excreted Antigens from trypomastigotes
		F2/3		?			?	N.A.		Highly *O*- glycosylated trypomastigote mucins, expensive, low purification yields
	Recombinant Antigens	Parental Repertoire[3,5]					N.A.	N.A.		High specificity but impaired sensitivity as compared to whole parasite-based methods.
		SAPA					?	?		Repetitive, surface antigen associated to enzymatically active *trans*-sialidases
		Ag13/TcD				?	?	?		Repetitive, surface antigen associated to enzymatically inactive *trans*-sialidases
		F29/FCaBP/Tc-24/Tc-28/1F8		?	?		?	?		Flagellar Calcium Binding Protein
		Ag1/JL7/FRA/H49						N.A.		Repetitive, internal antigen
		JL5		?	?	?		N.A.		Ribosomal antigen that elicits 'auto-antibodies' able to cross-recognize endogenous receptors
		TSSA		?						Mucin-like, surface antigen showing polymorphisms among strains
Molecular Methods	PCR, qPCR						N.A.			High specificity, trained personnel required
	Genotypification Markers[6]		N.A.	N.A.	N.A.	N.A.	N.A.			High specificity, trained personnel required, manual technique, expensive
	Biochemical Markers[7]		N.A.	?	?			?		Low specificity and sensitivity, in most cases only 'indicative/predictive' of cardiomyopathy

[1] Performance is arbitrarily indicated as appropriate (green boxes), non-appropriate (vermillion boxes) or intermediate/needs to be improved (yellow boxes) according to available data. N.A. and question marks (?) stand for non-applicable or not enough experimental data available to assess the performance, respectively.

[2] Adaptability to be deployed in PoC settings, which depends on specific features of the tool/technique and which is arbitrarily indicated as above.

[3] Commercially available from different vendors.

[4] TESA assays include diverse techniques that measure anti-TESA antibodies in serum samples (i.e. TESA-ELISA, TESA-dot blot, TESA-blot) and techniques that capture TESA antigens directly in urine samples (i.e. Chunap assays)

[5] The term 'parental' repertoire refers to *T. cruzi* antigens identified in large-scale initiatives carried out in the 80's, and includes: Ag1/JL7/FRA/H49; Ag2/B13/TCR39/PEP-2; Ag10; Ag13/TcD; Ag19; Ag26; Ag30/CRA/JL8/TCR27; Ag36/JL9/MAP; Ag54; JL1; JL5; JL9; SAPA; B12; TcE; A13; F29/FCaBP/Tc-24/Tc-28/1F8; Tc-40; HSP70; HSP78 and FL-160, according to current nomenclature. These antigens were assayed for conventional diagnosis in different combinations using a variety of technological platforms (ELISA, LFIA, Western blot, dot blot), some of which are commercially available from different vendors. The individual performance of some of these antigens showing particular features is indicated below.

[6] Include RFLP, PCR-RFLP, RAPD, Southern blot and other DNA hybridization methods, karyotyping methods and sequence-based methods either using a single locus or MLST.

[7] Include TNF-α, ACE2, BNP, ANP, ET-1 and other biochemical markers indicated in text.

All of abbreviations are defined in the text. Briefly: PoC, Point-of-Care; HIA, Hemagglutination Inhibition Assay; IFA, Immunofluorescence Assay; ELISA, Enzyme Linked Immunosorbent Assay; F2/3: Purified highly *O*-glycosylated and antigenic trypomastigote mucins; CoML, Complement-Mediated Lysis; TESA, Trypomastigote Excreted-Secreted Antigens; LFIA, Lateral-Flow Immunochromatographic Assays; SAPA, Shed Acute-Phase Antigen; TSSA, Trypomastigote Small Surface Antigen; PCR, Polymerase Chain Reaction; PCR-RFLP, PCR-based Restriction Fragment Length Polymorphism assay; MLST, Multi-Locus Sequence Typing assay; RAPD, Random Amplification of Polymorphic DNA; TNF-α, Tumor Necrosis Factor α; ACE2, Angiotensin-Converting Enzyme 2; BNP, Brain Natriuretic Peptide; ANP, Atrial Natriuretic Peptide; ET-1, Endothelin-1.