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The unmet medical need for *Trypanosoma cruzi*-infected patients: Monitoring the disease status

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A B S T R A C T

Patients infected by *Trypanosoma cruzi* are typically diagnosed by detecting specific antibodies in serological assays. Persistence of the parasitic infection increases the risk of morbidity and mortality. There are indications that anti-parasitic therapies help to reduce these risks when comparing treated and untreated populations. However, at present, treatment efficacy cannot be properly evaluated on an individual patient basis by available laboratory methods. To monitor parasite clearance, it is essential to change the paradigm of serological methods: analyzing the broad spectrum of antibody diversity is more informative about clinical status than conventional serology tests designed merely for global detection of antibodies.

1. Introduction

Chagas disease (CD), a zoonosis affecting humans in endemic areas, remains neglected after more than a century of its discovery by Carlos Chagas. CD is recognized by the World Health Organization as one of the most neglected tropical diseases. An estimated eight million individuals are affected in endemic areas and more than twenty million are at risk of exposure. The incidence of mortality in CD patients is over 10,000 deaths annually [1]. The disease is endemic in the majority of Latin American countries and, due to population migration, it has been reported in the United States, Canada, many European and some Western Pacific countries.

The vector of this parasitic disease belongs to the Triatomine family, is present in a large animal and sylvatic reservoir spanning from Argentina to the Northern part of Mexican border and is impossible to fully control. *Trypanosoma cruzi* (*T. cruzi*) is a flagellar protozoan that can lead to American trypanosomiasis also known as Chagas disease if the infection is not resolved spontaneously or by medication. The vector acquires *T. cruzi* during a blood meal, it then transmits the infective form of the parasite to humans during future blood meals. Beside vector transmission, CD may also be transmitted through blood transfusion, organ transplantation, congenitally or by the oral route through consumption of unsterilized insect-infested fruit by-products.

Despite extensive technological progress, current serodiagnostic methods have not greatly contributed to resolving this major health problem in Latin America. Chagas cardiomyopathy is the primary cause of morbidity and mortality of infected patients [2].

The focus of this review is serodiagnosis, with the spotlight on an unmet medical need: monitoring *T. cruzi* infection. We further emphasize that the humoral immune response can be more easily translated to simple routine assays, as compared to cellular assays, in low-resource countries. Although cellular immunity and genetic parameters are equally important, intensive research efforts have not been translated into concrete applications. Therefore, they are not considered in this review due to the complexity of implementation in ordinary laboratory settings. This review represents a synopsis of some immunological characteristics of *T. cruzi* and, an attempt to elucidate what is still hampering methodological developments to address the unmet medical need of disease monitoring. The authors express their personal thoughts and not necessarily those of their respective institutions.

After entering the human host, the parasite rapidly establishes an acute infection, occasionally identified by an apparent clinical symptom (chagoma) or other non-specific symptoms such as fever, headache, muscle pain, swelling and abdominal pain. A massive and specific immune-response takes place against the majority of immunodominant proteins that are expressed in the trypomastigote forms of the parasite. Due to multiple homologies in *T. cruzi* protein sequences with mammalian proteins, part of this immune response may involve an autoimmunity that plays a significant role in the pathogenesis of CD. The acute infection might resolve spontaneously and if treated in its very early stage the parasite may be prevented from invading various tissues to establish a chronic infection. Some studies have shown that early trypanocidal treatment can reduce the progression rate to cardiomyopathy [3]. This is particularly the case in infants who seem more responsive to trypanocidal medication [4]; it is expected that parasite elimination results in a reduction of disease progression rate but follow-

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up for many years is required to ascertain this hypothesis. However, the majority of infected individuals living in remote geographical areas, and those who do not have access to efficient healthcare systems, will progress to the chronic stage with clinically indeterminate or less perceptible symptoms. Ultimately, about 30% - 40% of patients will experience more severe clinical symptoms with either cardiac, gastrointestinal or neurological disorders. The remaining patients may stay in the indeterminate phase for the rest of their lives without showing any apparent life-threatening condition although still infected with *T. cruzi* [2,5].

Diagnosis of patients in the chronic phase (indeterminate or symptomatic form) is currently based on serological assays that detect specific antibodies to *T. cruzi* antigens. There are several immunoassays marketed with various technological platforms that have acceptable performance. All existing immunoassay techniques have been designed and optimized for screening for anti-*T. cruzi* antibodies at a very high sensitivity and the best possible specificity. Obviously, the sensitivity and specificity balance depends, to a large extent, on the nature and quality of antigens and reagents used in the assays. Indeed, *T. cruzi*-strains are classified into seven different genomic variants DTU I - DTU VI and DTU bat. Multiple sources of antigen variation exist in wild type *T. cruzi* and are related to the different geographical strains [6]. In the early days of Chagas immunoassays, antigens were extracted from *in vitro* culture of parasites. Cultured parasites express different antigens compared to those expressed in natural infective cycles by amastigotes (intracellular replicating form) and trypomastigotes (circulating flagellar form). Various antigen compositions are responsible for a significant amount of false- and cross-reactivity, which can only be sorted out by a complex interpretation algorithm involving several technologies [7]. For instance, a large evaluation study by Saez-Alquézar et al. compared three different screening strategies applied in blood bank settings, namely: immunofluorescence, haemagglutination and ELISA to confirm or rule-out a positive serology result. More recently, the use of recombinant proteins and synthetic peptide antigens has significantly improved serodiagnostic performance [8]. Nevertheless, differences between immunoassay methods persist, and extensive comparison studies can classify the assays into the most sensitive or most specific for anti-*T. cruzi* antibody screening [9]. Unfortunately, research and development efforts practiced by the *in vitro* diagnostics industry are often guided by the largest available market size, in this case blood bank screening. Such organizations require highly sensitive immunoassays to pick up a tiny number of anti-*T. cruzi* antibodies, irrespective of their clinical relevance. Accordingly, all *in vitro* diagnostic manufacturers have boosted the sensitivity parameters to a level that causes poor and unrelated specificity when it comes to disease monitoring. This strategy unavoidably detects clinically irrelevant levels of anti-*T. cruzi* antibodies in blood samples of already “cured” individuals. Detecting residual traces of specific antibodies may be helpful for epidemiological studies and for an ultra-secure blood supply, but not for resolving clinical situations. The particular challenge for *T. cruzi* clinical diagnostics is monitoring the infection and identifying patients that have cleared the infection and those that still carry the pathogen. This goal is virtually impossible to accomplish with classical single immunoassays that use a mixture of antigens such as those assays designed for blood bank screenings.

Many research groups have shown interest in and continue to strive for the identification of biomarkers that can discriminate between patients with active infection (parasite persistence) and those that have resolved the infection (parasite clearance). The latter would have a better prognosis with regard to CD evolution [10]. Furthermore, stratification of patients that still carry the pathogen represents a better strategy to assess therapeutic efficacy of existing trypanocidal drugs or drugs in ongoing clinical trials.

A new generation of immunoassays is being developed to address the clinical need for *T. cruzi* and CD monitoring. These assays focus on candidate biomarkers with different properties to those employed for screening purposes.

2. Direct biomarkers

In the very early days of Chagas diagnostics, xenodiagnosis was the method of choice for establishing a specific and direct diagnosis of *T. cruzi*-infected patients. Naïve vectors were nurtured on the skin of a patient suspected of infection and after several blood meals the insect was sacrificed for microscopic examination. Although xenodiagnosis is highly specific, this method lacked sensitivity and required skilled operators throughout the cumbersome, long and error-prone procedure. As more advanced molecular technologies appeared, xenodiagnosis methods were widely abandoned to the benefit of immunoassays. Direct parasite detection methods in the blood (such as microscopy or PCR) lack sensitivity due to the very low and fluctuating parasitemia during the chronic indeterminate phase. It is obvious that microscopy lacks sensitivity, given the limitations of observing blood smears in the case of low parasitemia. However, PCR techniques are reputed to be of high sensitivity and able to pick up a single parasite in 10 mL of blood; so the main cause of poor sensitivity is not the PCR technique itself, but rather wavering parasitic cycles in the blood stream. Nevertheless, treated patients that are persistently positive by PCR are clearly treatment failures. Indeed, sustained PCR positive results can indicate unsuccessful parasite elimination post-medication. Negative PCR results cannot confirm whether the parasite is eliminated since a negative result could be due to a “valley” in the parasitemia cycle. These limitations have guided research and development efforts towards indirect biomarkers for maintaining the same objective: monitoring the infection for improving medical care.

3. Indirect biomarkers

A large amount of research work has been dedicated to indirect non-specific biomarkers such as cytokines, transcriptomic and inflammatory biomarkers [18]. Attempts to associate such biomarkers to disease staging have not yet resulted in major successes. The number of patients that can be enrolled to be able to derive statistically sound conclusions is limited because of the complexity and high-costs of such studies. Consequently, validation studies to determine a robust set of biomarkers that can irrevocably indicate parasitological cure or persistence are still lacking.

It is more relevant to investigate indirect but specific biomarkers, such as anti-*T. cruzi* antibodies, because they have specific features that link their presence to the parasite. Such biomarkers are derived either from *T. cruzi*-specific antibodies or proteomic signature studies. There is a large consensus among the community of Chagas experts that converting serological status from seropositive to seronegative (seroreversion) provides solid evidence of parasite clearance [11,12]. Patients who, in the indeterminate stage, convert from positive to negative serology, most probably succeeding parasite clearance, are less susceptible to develop clinical Chagas disease. The pathogenic mechanisms underlying cardiac and/or gastro-intestinal clinical symptoms development are intimately associated with both the parasitic status and the patient's immune response. This can be easily understood since the immune system needs continuous antigenic stimulation to maintain the necessary oligoclonal activation of the lymphoid cellular repertoire to produce specific antibodies. Patient seroconversion from negative to positive status after parasite acquisition is a steady and cumulative buildup for implementing a robust immune response; the humoral component of this response cannot vanish as soon as the parasite has disappeared, whether spontaneously or subsequent to medication. “Rewinding” the immune response occurs once antigenic stimulation terminates (*e.g.* parasite clearance); polyclonal reactivity will then gradually decrease [10] and may ultimately reach full seronegative status decades later. This is a consequence of B-cell anergy and occurs at a different pace depending on the individual, and probably on the

infecting strain and the duration of infection since its onset.

One of the most remarkable facets of antibody-related detection approaches is, in addition to its intrinsic specificity, its power to unmask hidden parasites. While direct detection methods are limited to parasitemia, that is the actual presence of the parasite in the blood stream, immunological methods can indirectly reveal parasites hidden in the tissues. Indeed, the immune system acts as a potent and specific sensor throughout the body tissues and is able to reveal cryptic parasites by stimulating the secretion of specific antibodies. However, in the context of monitoring a therapy, the major drawback of antibody-related biomarkers resides in its relatively slow kinetics. The massive number of memory cells in the *T. cruzi*-specific B-cell repertoire continues to secrete antibodies years after the parasite has been eliminated from an individual. Another drawback is related to the auto-immune nature of certain antibody specificities; the inflammatory process and induced autoimmunity may partially withstand the stimulation of lymphocytes even after the parasite has been eliminated from an individual.

Under the strict regulatory and technical requirements for safety in blood banks and routine screening, the vast majority of immunoassays available on the market have been essentially designed to detect tiny amounts of *T. cruzi*-specific antibodies. Therefore, currently existing conventional serological assays cannot address the question of infection monitoring by measuring antibodies. While negligible amounts of antibodies reveal a past-infection for blood screening purposes, they are insignificant for clinical practice. Nevertheless, low amounts of anti-*T. cruzi* antibodies during the indeterminate asymptomatic stage may point towards a favorable prognosis for the disease.

Countless efforts have been made to identify biomarkers that indicate therapeutic efficacy (lytic antibodies, anti-Tc24, anti-F29) [11], or more global host responses (T-cell responses, transcriptomics and differential gene expression) or even cytokine profiling in chronic Chagas patients [13]. However, none of these efforts have resulted in a significant breakthrough so far. Consequently, seroreversion, as measured by conventional serology tests, remains the standard for monitoring parasitological cure. Yet, this approach is not suitable for routine testing nor for clinical trials, since it requires multi-year follow-up. As a matter of fact, full seroreversion measured by conventional serology may take up to twenty years and differs between individuals and possibly regions [14,15]. Hence, there is a need for methods that can capture the trend to seroreversion as soon as possible after parasitic cure.

It has become clear that antibody patterns reflecting the diversity of immune responses can more easily identify patients that undergo major changes in their immune status [10]. Such changes can be induced by anti-parasitic medication or simply after spontaneous elimination of the parasite. To study antibody diversity, new multiparametric assays needed to be designed. This has been recently achieved where fifteen different antigens selected for their individual sensitivity and specificity balance were used in a single microplate [16]. Fig. 1 illustrates the changes that can be observed in the B-cell response in situations of parasite persistence (Fig. 1a) or parasite clearance (Fig. 1b). Both patient's samples are reported clearly seropositive in conventional screening assays. Only by looking at the antibody patterns in terms of diversity and signal intensity one can distinctly notify the two different clinical states. Pattern recognition can provide an impressive performance after an appropriate training of computerized image analysis using machine learning techniques. Major changes in the pattern may be recognized by naked-eyes of a well-trained human being. However, machines learning techniques enable a computerized analysis to recognize subtle changes in the patterns that are more complex to observe by naked-eyes of a laboratory operator. Deep learning consists of training a computer for recognizing a large number of images (over three thousands) obtained from well categorized samples (training dataset). The interpretation algorithm can be fine-tuned with an independent validation dataset of new images. This iterative process is then challenged and finalized when test images are perfectly recognized in the correct classification category.

Besides the antibody pattern markers, other biomarkers identified from comparative proteomic studies can provide indications about the presence of the parasite. In fact, the enzymatic machinery of the parasite is able to process certain endogenous proteins in the patients' blood stream. This is the case for two common proteins: fibronectin and A1-Apolipoprotein. These endogenous proteins are cleaved by the overload of parasitic enzymes to produce fragments of a defined size. Ndao et al. have identified such fragments in the blood of *T. cruzi*-infected patients. The fragments were identified by mass spectrometry studies and the relative ratios of cleaved fragment and native protein can be correlated to the presence of the parasite [19]. However, the mass spectrometry approach is not suitable for testing in the field. Novel immunoassays are under development to specifically measure Apo-A1 fragments without interference with the full-length Apo-A1 protein.

Notwithstanding the controversial findings of the BENEFIT trial [17], successful treatment of *T. cruzi*-infected patients in the chronic indeterminate phase, if it results in parasite elimination, significantly increases the chance of preventing evolution to Chagas cardiomyopathy. However, there are three major limitations to using current medication to treat *T. cruzi*-infected patients *i*) the relatively lengthy therapy protocols (60 days for benznidazole and 90 days for nifurtimox), *ii*) the severe side effects which often lead to a premature termination of treatment and, *iii*) lack of reliable tools to assess treatment efficacy after a reasonably short period of follow-up. New drugs and drug regimens are being developed to shorten treatment duration, and thus reduce related side effects. When it comes to drug development, it is of utmost importance to identify patients in need of medication and to be able to measure drug efficacy within a period of time that doesn't exceed the timeline of the clinical trial. Significant research efforts have been made to develop simple laboratory methods that help with patient stratification and follow-up. Many unspecific inflammatory markers have been investigated in isolation or in combination with genetic markers. These investigations have contributed to the understanding of inflammatory mechanisms of cardiomyopathy but have not yet resulted in a clear-cut methodology to monitor the disease. Therefore, seroreversion remains the most solid evidence of parasite clearance. Multiparametric assays, as opposed to conventional serology, can capture the trend to seroreversion sufficiently early and provide valuable information about the parasite clearance in progress.

4. Conclusion

Despite impressive efforts in basic research to help with CD management, there has been little translation into products available in the clinic. The complexity of the parasite *in vivo* life cycle and its pathogenic relationship to severe clinical symptoms in CD patients, combined with a lack of methods to reliably assess treatment efficacy, makes disease control extremely challenging. Because *T. cruzi*-infected patients experience different outcomes, a novel strategy is required for patient stratification and disease monitoring. This strategy should tackle two issues *i)* parasite elimination and, *ii)* clinical evolution to symptomatic CD; these two sides of the disease are consecutively related. Thus, novel serological methods based on pattern recognition could pave the way for a better assessment of trypanocidal drugs and/or spontaneous parasite clearance. Indeed, a favorable decrease in the serological pattern acts as a surrogate marker for treatment efficacy and could also serve as guidance for adequate medical decisions. Furthermore, when it comes to vaccination research programs, the change in serology paradigm, from a simple “Yes/No” answer to a pattern recognition, will be instrumental to distinguishing adaptive immunity from vaccine-induced immunity. The pattern recognition approach represents a major step towards personalized medicine, which is precisely what all Chagas patients need.

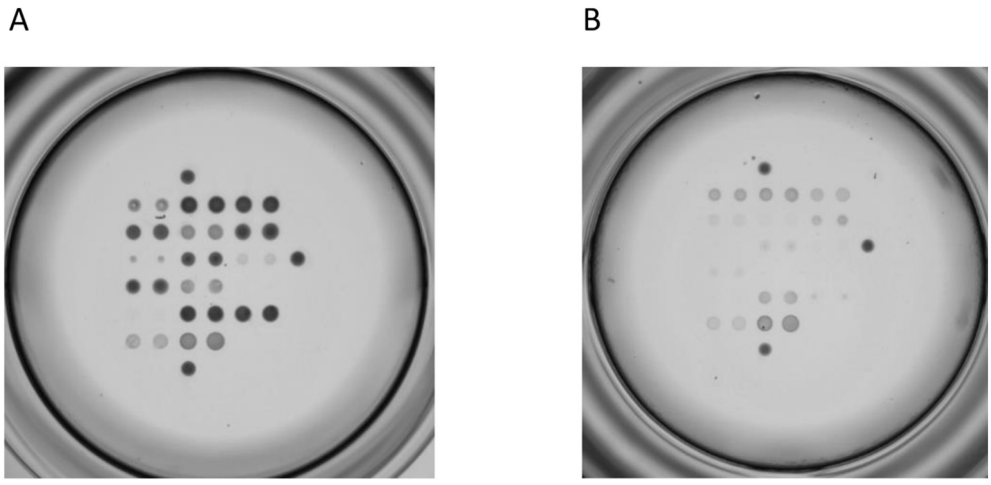

Fig. 1. Real images of serological patterns obtained with seropositive samples representative of parasite persistence (A) and parasite clearance (B) conditions. Both samples are seropositive in conventional screening assays. Technical details are described in Zrein et al. [16].

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Maan Zrein acts as Chief Scientific Officer employed by Infynity Biomarkers; this employment doesn't interfere with the ethical quality of the manuscript nor with any present or future interests.

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