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Advances in Serological Diagnosis of Chagas' Disease by Using Recombinant Proteins

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1. Introduction

Chagas' disease is an infection caused by the parasite *Trypanosoma cruzi*, mainly occurring in American countries where the parasite vector bug, *Triatoma infestans*, is widespread. One hundred million individuals are currently under threaten of infection, as well as 16 million people are considered affected by the illness in Latin America alone.(Editorial, 2009) Considering indicators such as the disability-adjusted life years, DALY, and from a social point of view, Chagas' disease accounts for the third most important tropical illness of the World, following malaria and schistosomiasis.(Bitran *et al.*, 2009) Moreover, Chagas disease epidemiology nowadays impacts in non-endemic regions due to globalization, being the infection disseminated all over the world (Gascon *et al.* 2009). Certainly, the non-vectorial disease transmission (mother to child, transfusional and by organ transplantation) is the way the illness spreads in Europe, North America, Japan, and Australia, because many infected people have migrated from endemic regions to distant cities. (Bowling & Walter, 2009; Lescure *et al.*, 2009; Yadon & Schmunis, 2009)

The parasite distribution and living habits of rural Latin-American people have determined that the main transmission route is the vectorial one (via *Triatoma infestans*), leading to up 80% of human infection.(WHO, 2003) The main contagion way in urban areas arise from blood-transfusion, being responsible for 5-20% of the reported cases, while vertical transmission (mother-to-child) accounts for 2-10% of the infections.(Carlier & Torrico, 2003)

The infection transmission by oral route because of consumption of contaminated food and drinks is lower than that reported for the previously-mentioned routes.(Dias *et al.*, 2011) However, it has to be taken into account that the success of the non-oral infection prevention has increased the importance of the oral route of transmission.(Dias *et al.*, 2011; Nóbrega *et al.*, 2009) It is noteworthy that the acute outcome of the oral infection is particularly severe.(Bastos *et al.* 2010).

From the clinical point of view, the illness presents variable unspecific symptoms, depending on its stage: the acute one, shortly after primary infection, and the chronic stage, which may last many years if the individual is not treated. Human infection typically

appears with an incubation period of 4-10 days, which is generally asymptomatic. Afterwards, the infection may advance to the short acute phase, followed by the long-lasting chronic stage, which may occur in a symptomatic or asymptomatic way.

The acute stage of the infection usually lasts 2-4 months. When symptoms appear, these are often light and atypical. The infection proceeds generally unnoticed, and this prevents its diagnosis. Most of the patients recover within 3-5 months. However, global mortality during this phase reaches 5-10%, (WHO, 2002) and may be higher in children. (Pinto *et al.*, 2004) Deaths can be caused by complications related to myocarditis and/or meningoencephalitis, (Pittella, 2009) which has been proved by the presence of the amastigote form of parasites in cardiac, skeletal, glial and soft tissue cells. (da Silveira *et al.*, 2007)

The undetermined, chronic phase generally begins 8-10 weeks after the acute infection, and may last several years or even the whole life of the individual. The illness overtakes asymptotically, and generally, infected individuals keep their full working capacity without being aware of the infection. However, during this stage, patients display positive serology for specific IgG antibodies and low parasitemia. (Coura, 2007) Over 50% of the infected individuals show themselves healthy and do not develop serious outcomes. In the other cases, the infection is often detected once its sequels appear, after 10-20 years of the first parasite entry. The illness features that mainly develop are cardiac, digestive and/or neurological damages. Chagas' pathologic alterations can be summarized as chronic myocarditis, 27%, esophageal or colonic expansion, 5%, and abnormalities of central nervous system, ca. 3%. (Teixeira *et al.*, 2006) The most severe impact of the illness occurs during chronic phase, and after many years that the infection has been established, leading to high mortality rate among people who have developed cardiac pathology. (Rassi, Jr. *et al.*, 2007)

Undoubtedly, the most effective alternative to prevent Chagas' disease spreading is to control vectorial and transfusional ways of transmissions. Nevertheless, once the individual has been infected by *T. cruzi*, he/she may be treated. Two main aspects are covered by therapy, aiming to eliminate the parasite with trypanocidal medication, and/or medicating to relief symptoms of the several clinical outlines of the illness. Nowadays, two chemicals are used as trypanocidal agents namely, nifurtimox (Lampit®, Bayer; 5-nitrofuranyl-3-methyl-4-(5'-nitrofurfurylideneamino) tetrahydro-4H-1,4-thiazine-1,1-dioxide) and benznidazole (Rochagan® and Radanil®, Roche; N-benzyl-2-nitroimidazole acetamide). When infection is treated during the acute stage, it has been reported that parasitemia disappeared in 60% of the cases and serology turned into negative. Nevertheless, the outcome after therapy is finished shows variable effectiveness percentages, depending mainly on the age of the individual and the geographical region. (Perez-Molina *et al.*, 2009)

Currently, studies trying to expand the indicators on treatment effectiveness are in progress and will be accomplished by 2012. (Marin-Neto *et al.*, 2008) Though these studies have not yet finished, there is a consensus on treating Chagas' disease, taking into account the clear-cut results when using trypanocidal medication with children, during the indeterminate phase of the infection. (Lescure *et al.*, 2010; Perez-Molina *et al.*, 2009; Sosa-Estani & Segura, 2006) It therefore follows that it is highly advisable to count with reliable methods to early diagnose the disease, since this may accelerate the patient treatment, helping to reduce the serious consequences that the long term infection may cause.

1.1 *T. cruzi* infection diagnostic during the different stages of the illness

The infection diagnosis is not a simple task because the features of the illness development as well as the immunological response of the host must be taken into account when performing the laboratory diagnosis. Different diagnostic methods are used, depending on the illness stage and the particular clinical entity of the patient.

The alternative ways to get acute Chagas' infection are mainly vectorial and vertical transmission, and the reactivation of the chronic disease in immunosuppressed individuals. Therefore, diagnosis at this stage limits to: *i*) the uncommon cases of symptomatic patients, *ii*) newborns delivered from chagasic mothers, and *iii*) immunosuppressed patients that have been previously diagnosed as infected.

In the above mentioned cases, parasites are usually, easily found in blood, therefore being their direct microscopic observation the chosen method for a safe diagnosis. (WHO, 2002; Rosenblatt, 2009) In contrast, indirect serological techniques present low sensitivity in recently infected individuals because humoral immune response is delayed. (Zuniga *et al.*, 2000) Moreover, even specific IgM immunoglobulin has a window period that requires up to several weeks to be produced at detectable concentrations. Other pitfalls such as the lack of IgM anti-*T. cruzi* commercial kits prevents from choosing serology as the infection diagnostic method. This methodology is only used when the parasite is not found in fresh blood smears, by Strout and/or microhematocrit methods. (Luquetti & Schmunis, 2010)

During the chronic phase, the *T. cruzi* infection diagnosis is required, *i*) as pregnancy or occupational routine control analysis -in countries where according legislation is in force-, *ii*) when in presence of a Chagas' compatible cardiopathy, *iii*) during transfusion and transplantation screening, and *iv*) to achieve a reliable illness prognosis of patients who were already diagnosed. One particular case among the latter ones is monitoring the antiparasitic treatment effectiveness.

Conversely to what happens in the acute phase, during the chronic infection, a significant humoral response is found in immunocompetent individuals, along with low parasite concentration in blood. Under these circumstances, indirect techniques are highly sensitive, while the direct ones fail. Hence, Chagas' disease serologic diagnoses intend to verify the occurrence of several specific antibodies against *T. cruzi*. However, conventional serology presents different sensitivity and selectivity, depending on the immunological technique used to perform the determination, and mainly in the nature of the antigen used to capture the specific antibodies. (Belluzo *et al.*, 2011)

In this Chapter, we will describe the most recent contributions of our and other groups to improve the analytical tools available to properly and safely diagnose Chagas' disease using recombinant proteins, in each one of the clinical entities mentioned above.

2. Conventional diagnosis of *T. cruzi* infection

Direct parasite detection in whole blood is the simplest, regular procedure used to diagnose acute infection whereas, indirect serological tests are the chosen ones to diagnose the undetermined, chronic state. (Rosenblatt, 2009; WHO, 2002)

2.1 Parasitological and serological diagnosis of acute entities

The direct microscopic observation of parasites from patients peripheral blood is the elected methodology to confirm acute infection. The Strout concentration method is the routinely

performed parasitologic analysis in adults since more than 50 years,(Strout, 1962) and it has been reported about 95% sensitivity.(Freilij & Storino, 1994)

The other commonly used concentration method is the pediatric, more recent version, named the direct micromethod or microhematocrit, which requires a lower blood volume than Strout method, and is mainly used to diagnose congenital Chagas' disease and acute infection in children.(Freilij *et al.*, 1983;Freilij & Altcheh, 1995) However, newborn babies usually present low parasitemia, therefore making difficult to perform a proper conventional parasitologic analysis. It is then recommended to perform serologic tests to diagnose the congenital infection. The evaluation of specific anti-*T. cruzi* IgA and IgM is not recommended due to the high rate of false-negative results in neonates.(Moya *et al.*, 2005) Considering that maternal specific anti-*T. cruzi* IgG antibodies are commonly present in newborn circulating blood, even up to the ninth month, it is not advisable to perform serologic IgG determinations as routine, in newborns younger than 9 months old. In this line, if the micromethod is negative or if it has not been performed during the first months of life of the newborn, then congenital infection should be serologically diagnosed using peripheral blood not before the child is 9 months old, once maternal antibodies have disappeared.(Gomes *et al.*, 2009) Following, when specific IgG presence is negative after the ninth month of life, then vertical transmission is ruled out. Alternatively, during the first months of life of babies, it is possible to forego results using other non-standard, more expensive techniques such as the polymerase chain reaction, PCR.(Diez *et al.*, 2008) This technology is particularly preferred when the health center counts with the supplies to carry out the methodology.

Indirect parasitological methods are also used, mainly when the parasite is not easily found in samples. These methods are the hemoculture and xenodiagnosis, and consist of enriching the parasites present in the patient's blood sample, through allowing their replication.(Chiari *et al.*, 1989) Both of these latter techniques are also used when diagnosing chronic infection. These methods demand long periods of time to arise to the results (weeks or months), together with other drawbacks. For example, xenodiagnostic method has the disadvantage of producing rather variable sensitivity results, 20-50%, alongside the requirement of a suitable building infrastructure and trained personnel to deal with insect breeding. Thus, this method is not commonly performed in basic health centers.(Luquetti & Schmunis, 2010)

When searching for reappearance of acute infection in immunosuppressed individuals under risk, negative serological results are not always associated with absence of the infection. This is a consequence of the immunological status of the patient that shows difficulties to produce detectable amounts of specific IgG. As mentioned previously, in the particular, difficult cases, expensive PCR techniques are the recommended diagnostic method.

2.2 Serological diagnosis of chronic entities

The widely used serological assays to diagnose *T. cruzi* infection in present clinical practice are indirect haemagglutination (IHA), indirect immunofluorescence (IIF), and enzyme-linked immunosorbent assay (ELISA).(WHO, 2002;Yadon & Schmunis, 2009) The analyst's choice of the particular technique depends on sanitary-authority recommendations, market impositions, and the lab-technician preference. This latter one is generally related to the

methodology simplicity, and the personal confidence he/she has in a particular technique after having performed it for a long while.

IHA is an inexpensive technique, which is easy to be performed and interpreted, and it has been used for more than 50 years, therefore being appropriately settled among lab technicians. Similarly, IIF was developed in the sixties and presents equivalent features to IHA though, more skillful technicians are required to perform the analysis and produce accurate readings, as well as it needs a fluorescence microscope. IIF is a very sensitive, specific and cheap alternative for those who have the equipment and the trained personnel. However, regular health centers do not count with both of them.

ELISA is a more recent technique which was firstly described during 1975 to diagnose Chagas' disease,(Voller *et al.*, 1975) and its usage was settled just at the ends of the eighties. This technique has the advantage of being widely extended as a diagnostic tool of many infections. Therefore, most of regular laboratories have the required equipment and trained personnel to appropriately perform the analysis. Contrarily to IHA and IIF, ELISA may be performed with automatic equipment at large health institutions. Moreover, even though ELISA is more expensive than the other two techniques, its notable performance in terms of sensitivity and specificity, has made of this the preferred methodology to diagnose *T. cruzi* infection.(Saez-Alquezar *et al.*, 1997)

Recently, one very fast technique with a different format has been developed namely, lateral chromatography.(Ponce *et al.*, 2005;Barfield *et al.*, 2011) This methodology uses small volume samples such as one serum drop, and allows acquiring results in 15 min, therefore being useful to perform the test in the field, without the need of refrigerator to preserve the reagents. Several multicenter studies have demonstrated that a commercial lateral chromatography kit show more than 92% sensitivity, whereas specificity is ca. 96%.(Ponce *et al.*, 2005;Brutus *et al.*, 2008;Roddy *et al.*, 2008;Sosa-Estani *et al.*, 2008)

The fundamental problems of *T. cruzi* infection serological diagnostic methods are the lack of reproducibility that sometimes occur, deficient immunological reaction specificity, what produces false-positive results, and the occasional insufficient sensitivity translated into false-negative outcomes.

Chagasic infection serology tests may produce cross-reactions with antibodies produced during the course of other illnesses. In this line, unspecific reactivity has been described for infections caused by *T. cruzi* phylogenetically related microorganisms, such as *T. rangeli* and *Leishmania sp.*(Soto *et al.*, 1996;Araujo, 1986;Saez-Alquezar *et al.*, 2000) Moreover, other false-positive results due to cross-reactions have been described when testing samples from patients with autoimmune diseases,(Reiche *et al.*, 1996) or from individuals suffering from other acute infections or pregnant women who display an important, polyclonal unspecific response.(Konishi, 1993)

The clinical practice often finds an important number of inconsistent results regarding reproducibility and confidence when diagnosing chagasic infection. The lack of reproducibility and confident results has also been reported in a multicenter study.(Saez-Alquezar *et al.*, 1997) In this work, it was proved the deficiency of reagents standardization, what produced incongruent results when testing the same serum panel. Along the same direction, since the early nineties, several works accounted for the huge losses caused by disposal of whole blood reservoirs typified as undetermined for *T. cruzi* infection.(Carvalho *et al.*, 1993;Salles *et al.*, 1996;Saez-Alquezar *et al.*, 2000) Taking into account tests discrepancies, one of WHO

recommendations states that *T. cruzi* infection must be diagnosed when the sample produces positive results by two different serological methods, whereas the undetermined condition is established for samples rendering dissimilar outcomes.

Traditionally, whole parasites, or extracts from laboratory strains of *T. cruzi* epimastigotes cultures, have been the source of antigens used for the serological infection diagnosis. However, this yields to complex protein mixtures of unknown composition, which display severe difficulties to be standardized, and additionally lead to false-positive results.

The diagnostic problems arising from serology deficient specificity, as well as the deprived reagents standardization, can be resolved through the use of defined antigens, such as the proteins obtained by molecular biology technology procedures. (Saez-Alquezar *et al.*, 2000; Umezawa *et al.*, 2003; Umezawa *et al.*, 2004; Aguirre *et al.*, 2006)

The following sections will be focused in this issue and the most important contributions that several research groups have recently made.

3. Use and prospects of recombinant DNA technology

Since the emergence of recombinant DNA technology, many protein molecules have been designed and prepared to eventually be assessed for serological diagnosis. The proteins obtained through this technology may be used as antigens to capture antibodies, to evaluate exclusively defined molecules, avoiding potential interferences from other components that usually occur when the antigens have been obtained by purifying native source proteins. (da Silveira *et al.*, 2001) It follows that the usage of recombinant proteins as antigens to detect or quantify specific antibodies markers of a disease permits enhancing the specificity of the immunological reaction involved, therefore leading to more accurate diagnosis. (Aguirre *et al.*, 2006; Camussone *et al.*, 2009)

In this methodology the proteins are usually prepared by heterologous expression, mainly in *Escherichia coli* cells. (da Silveira *et al.*, 2001) Sequences of *T. cruzi*-protein codifying-DNA are inserted in a bacterial plasmid, which is transformed in competent bacteria. The proteins encoded by the plasmid are expressed in the bacterial culture, and are afterward purified into a highly pure product. The advantage of the proteins thus obtained is that they are an entirely characterized antigen, which may be evaluated individually for antibody determination in different clinical conditions. The prepared antigens can therefore be characterized considering the clinical information they provide, and may then be used to prepare specific diagnostic reagents. These proteins count with one desired feature of diagnostic reagents, as it is that their production and evaluation can be highly standardized. From another point of view, recombinant antigens do not require manipulation of the infective agent as do the antigens obtained by purification procedures from rough cultures. This has been a significant progress when considering the characteristics of viral infective agents, for which reagents production has substantially switched to that derived from recombinant DNA technology. Not less important is the major saving financial benefit of these reagents. Indeed, once bacteria are transformed into competent, protein producing strain, they can be used to prepare substantial amounts of antigen with low cost of production.

Using this technology, many gene expression clones have been create, a fact that has made available the obtainment of massive amounts of highly pure, standardized *T. cruzi* proteins. (da Silveira *et al.*, 2001)

During the latest three decades, many parasite antigens have been cloned and characterized. The cloned antigens correspond to different parasite stages namely, the trypomastigote sanguineous, the amastigote intracellular and the epimastigote, which is the form found inside the vector bowel and that can be cultured. Several of these antigens were obtained by immunological tracing through expression of cDNA libraries from chagasic patient sera, as well as from immunized animals.(Lafaille *et al.*, 1989;Affranchino *et al.*, 1989;Levin *et al.*, 1989;Cotrim *et al.*, 1990;Gruber & Zingales, 1993) The antigen codifying genes have been identified from cDNA present in the libraries accomplished from epimastigote or trypomastigote forms.(Affranchino *et al.*, 1989;Levin *et al.*, 1989;Gruber & Zingales, 1993;Godsel *et al.*, 1995) Lately, Da Rocha *et al.* have proposed using amastigote proteins since this is the intracellular parasite form, being these antigens more significant for serodiagnosis.(DaRocha *et al.*, 2002)

The usage of DNA technology brought into light the existence of many parasite antigens with repetitive sequences, a fact that had been previously described when cloning proteins of other parasites.(Hoft *et al.*, 1989) Generally, these are the most immunogenic antigens, and are the mainly selected when performing immunological tracing in cDNA libraries cloned in phages. Therefore, it was initially stated that these were the most valuable antigens for diagnosis.(Frasch & Reyes, 1990) However, it was afterward proved that some non-repetitive antigens have equivalent diagnostic value than repetitive ones. Certainly, a multicenter study evaluated in parallel 4 repetitive recombinants antigens (H49, JL7, B13, JL8) together with 2 non-repetitive ones (A13 y 1F8).(Umezawa *et al.*, 1999) The results demonstrated that both type of antigens were similarly useful for *T. cruzi* infection diagnosis, and the authors suggested that if they were to be used together in a mixture, they could supplemented each other enhancing the sensitivity of the assay. This was afterwards proved by the same group, see Tables 1 A,B and C.(Umezawa *et al.*, 2003)

Once the complete genome sequence of *Trypanosoma cruzi* was annotated, (El Sayed *et al.*, 2005) alternative antigenic candidates have been searched in the parasite genome. The studies have been supported by bioinformatic prediction of putative proteins and antigenicity predictors.(Goto *et al.*, 2008;Cooley *et al.*, 2008;Hernandez *et al.*, 2010) Using these tools, it has been possible to choose antigens which display the lowest homology level with proteins of organisms related to *T. cruzi*.(Hernandez *et al.*, 2010) Moreover, the bioinformatic analysis has allowed describing for the first time a specific antigen to type discrete typing units (DTUs). (Di Noia *et al.*, 2002)

The results published by many different laboratories point towards considering recombinant proteins as the chosen molecules to be used in immunoassays to diagnose *T. cruzi* infection. Moreover, the lack of specificity leading to false-positive results can be overcome by deleting sequence regions encoding for proteins which cross-react when analyzing negative sera,(Aguirre *et al.*, 2006), or using recombinant proteins that are specific for anti-*T. cruzi* antibodies, yet keeping a high sensitivity.(Belluzo *et al.*, 2011;Camussone *et al.*, 2009) Indeed, the largest studies on the diagnosis reveal the convenience of using these antigens, regarding not only specificity but also the possibility of standardizing both, the methodology and the protein production.(Umezawa *et al.*, 1999;Saez-Alquezar *et al.*, 2000;Umezawa *et al.*, 2003)

The following table display the key recombinant antigens discussed in the present chapter, which were evaluated by several authors for *T. cruzi* infection diagnosis. Notice,

that many of these antigens particularly named by one author have amino acid sequences, which may be very similar to those obtained by other authors who have named them differently (e.g. FRA, Ag1, JL7, H49). Identical or highly similar antigens were grouped in the same row.

Antigen (grouped by high identity)	Characteristics	Diagnostic use	Described by
CRA Ag30 JL8 TCR27 RP4	Cytoplasmic antigen	Chronic infection	Lafaille <i>et al.</i> , 1989 Ibáñez <i>et al.</i> , 1988 Levin <i>et al.</i> , 1989 Hoft <i>et al.</i> , 1989 Camussone <i>et al.</i> , 2009
FRA Ag1 JL7 H49 RP1	Cytoskeleton associated protein	Chronic infection	Lafaille <i>et al.</i> , 1989 Ibáñez <i>et al.</i> , 1988 Levin <i>et al.</i> , 1989 Cotrim <i>et al.</i> , 1995 Camussone <i>et al.</i> , 2009
B13 Ag2 TCR39 PEP-2 RP5	Trypomastigote surface protein	Chronic infection	Gruber <i>et al.</i> , 1993 Ibáñez <i>et al.</i> , 1988 Hoft <i>et al.</i> , 1989 Peralta <i>et al.</i> , 1994 Camussone <i>et al.</i> , 2009
Ag36 JL9 MAP-like RP3	Microtubule associated protein	Chronic and acute infection. Antibodies against this protein render cross- reactions with mammal cell cytoskeleton.	Ibáñez <i>et al.</i> , 1988 Levin <i>et al.</i> , 1989 Kerner <i>et al.</i> , 1991 Camussone <i>et al.</i> , 2009

Table 1A. Relevant repetitive recombinant antigens proposed for diagnostic uses.

Abbreviations used: CRA, cytoplasmic repetitive antigen; FRA, flagellar repetitive antigen; MAP, microtubule associated protein. RP1, RP3, RP4 and RP5, repetitive peptide 1, 3, 4 and 5, respectively.

Antigen name	Characteristics	Diagnostic use	Described by
SAPA RP2	Trans-sialidase family	Acute and congenital infections. Chronic infection in leishmaniasis endemic regions	Frasch & Reyes, 1990 Russomando <i>et al.</i> , 2010 Breniere <i>et al.</i> , 1997 Gil <i>et al.</i> , 2011 Camussone <i>et al.</i> , 2009
TcLo1.2	Trans-sialidase family	Chronic infection	Houghton <i>et al.</i> , 1999
TcD	Trans-sialidase family	Chronic and acute infection	Burns, Jr. <i>et al.</i> , 1992
Trans-sialidase catalytic region	Trans-sialidase family	Confirmation of chronic infection	Buchovsky <i>et al.</i> , 2001
FL-160 CEA CRP160	Complement regulatory protein from TS-like family	Chronic infection and cure monitoring Chronic infection and cure monitoring Cure monitoring	Cetron <i>et al.</i> , 1992 Jazin <i>et al.</i> , 1995 Meira <i>et al.</i> , 2004
TSSAI	Trypomastigote muscin of TS-like family	<i>T. cruzi</i> typing (named lineage Tc I, in the previous nomenclature)	Di Noia <i>et al.</i> , 2002
TSSAII	Trypomastigote muscin of TS-like family	<i>T. cruzi</i> typing (named lineage Tc II, in the previous nomenclature) DTUII, V and VI in the current nomenclature Confirmatory diagnostic in Chagas and leishmaniasis co-endemic regions	Di Noia <i>et al.</i> , 2002 Bhattacharyya <i>et al.</i> , 2010 Cimino <i>et al.</i> , 2011

Table 1B. Relevant recombinant antigens which belong to trans-sialidase (TS) and TS-like family, proposed for diagnostic uses. Abbreviations used: CEA, chronic exoantigen (160 KDa); CRP, complement regulatory protein; FL-160, surface flagellar protein (160 KDa); RP2, repetitive peptide 2; SAPA, shed-acute phase antigen.

Antigen name	Characteristics	Diagnostic use	Described by
R13	Last 13 amino acids from ribosomal protein.	Specific of cardiac disease	Aznar <i>et al.</i> , 1995
P2 β	Full length ribosomal P2 β protein	Stages of cardiac disease Stages of cardiac disease All stages	Diez <i>et al.</i> , 2006 Fabbro <i>et al.</i> , 2011 Breniere <i>et al.</i> , 2002
TcE	Ribosomal protein	Chronic infection	Houghton <i>et al.</i> , 1999
FcaBP 1F8 Tc-24 F29 Tc-29 Calflagin	Flagellar calcium binding protein	Chronic and acute infection Chronic infection Cure monitoring patients Cure monitoring patients Chronic infection Chronic infection	Engman <i>et al.</i> , 1989 Gonzalez <i>et al.</i> , 1985 Krautz <i>et al.</i> , 1995 Fabbro <i>et al.</i> , 2007 Abate <i>et al.</i> , 1993 Marcipar <i>et al.</i> , 2005
cy-hsp70 mt-hsp70 grp-hsp78	Heat Shock Proteins	Chronic infection and cure monitoring	Krautz <i>et al.</i> , 1998
TcAg29	Alginate regulatory protein	Chronic infection	DaRocha <i>et al.</i> , 2002
TcAg48	RNA binding protein	Chronic infection	DaRocha <i>et al.</i> , 2002
Tc1	Repetitive proteins obtained by bioinformatic analysis of the genome	Chronic infection	Goto <i>et al.</i> , 2008
Tc3	Idem	Chronic infection	Goto <i>et al.</i> , 2008
Tc4	Idem	Chronic infection	Goto <i>et al.</i> , 2008
Tc9	Idem	Chronic infection	Goto <i>et al.</i> , 2008
Tc10	Idem	Chronic infection	Goto <i>et al.</i> , 2008
Tc12	Idem	Chronic infection	Goto <i>et al.</i> , 2008
Tc15	Idem	Chronic infection	Goto <i>et al.</i> , 2008

Table 1C. Other relevant recombinant antigens proposed for diagnostic uses. Abbreviations used: cy-hsp70, cytoplasmic thermal-shock protein; FcaBP, flagellar calcium-binding protein; grp.hsp 78, endoplasmic reticle thermal-shock protein (78 KDa); mt-hsp 70, thermal-shock mitochondrial protein (70 KDA).

4. Recombinant proteins use: Mixtures vs. fusion proteins

The first works dealing with a single recombinant protein for diagnostic purposes reported lack of sensitivity when using only one of those antigens.(Levin *et al.*, 1991;Moncayo & Luquetti, 1990;Peralta *et al.*, 1994) Consequently, most of these proteins have been evaluated not only alone and independently from others, but also together as part of mixtures or as fusion proteins, carrying several recombinant epitopes.(Umezawa *et al.*, 1999;Umezawa *et al.*, 2004;Camussone *et al.*, 2009;Foti *et al.*, 2009) Accordingly, a multicenter study evaluating 6 recombinant proteins separately with a serum panel composed by sera from patients of several countries, described that using the set of results of the 6 proteins together had yield a sensitivity and specificity compatible with the reference assays.(Umezawa *et al.*, 1999) Later, the same group evaluated the mixture of the 6 proteins, supporting the use of the mixture to reach the same sensitivity and specificity.(Umezawa *et al.*, 2003) Soon after, the reactivity of individual antigens vs. antigen mixtures was systematically assessed by ELISA.(Umezawa *et al.*, 2004) This study confirmed that the results obtained with recombinant protein mixtures led to higher media values of optical densities, ODs, than the results produced when using the individual recombinant proteins. Moreover, sera rendering low ODs when examined with individual recombinant proteins produced higher ODs outcomes when using the protein mixtures. Along with this, several commercial ELISA kits with recombinant protein mixtures display equivalent or even higher sensitivities and specificities than those produced by kits with total parasite homogenate.(Gadelha *et al.*, 2003;Pirard *et al.*, 2005;Remesar *et al.*, 2009;Caballero *et al.*, 2007) These works have studied kits using Ag1, Ag2, Ag30, Ag13 together with Ag36 recombinant antigens (Chagatest Rec from Wiener lab, Argentina), and FRA and CRA recombinant antigens (Biomanguinhos, Friocruz, Brazil). However, another study reported that Chagatest Rec v3.0 (Wiener) displayed a rather low 95% sensitivity.(Ramirez *et al.*, 2009)

One of the strategies proposed to enhance reagents production standardization is to obtain multiepitope molecules, designed as a unique construction by fusing several relevant diagnostic antigens.(Houghton *et al.*, 1999;Aguirre *et al.*, 2006;Camussone *et al.*, 2009) It has recently been proved that when using these constructions, the ODs of sera with low reactivity increases, as well as it had been reported for mixtures.(Camussone *et al.*, 2009) Moreover, by this approach the attachment of the antigen turned out to be homogenous and reproducible when using different surfaces such as ELISA plaques, latex particles or bioelectrodes.(Camussone *et al.*, 2009;Gonzalez *et al.*, 2010;Belluzo *et al.*, 2011) It has been proposed that when there is only one molecule exposed to the surface, competition for the active sites is prevented, therefore resulting in a uniform attachment. Furthermore, sensitivity may be increased because a higher number of freely accessible epitopes are available to capture the antibodies present in samples, as depicted in Fig. 1.(Camussone *et al.*, 2009)

A few articles report on the use of this strategy to produce commercial ELISA kits which have demonstrated to be highly satisfying. One of these works, analyzes the performance of the TcF antigen, previously described by Houghton et al in 1999, with which the Biolab Merieux reagent was prepared.(Ferreira *et al.*, 2001) In this case, the recombinant protein used bears the PEP2, TcD, TcE and TcLo1.2 peptides. Recently, Abbot Laboratories have presented a new kit which uses a 4-antigen multiepitope protein containing TcF, FP3 -built up with TcR27 and FcaBP-, FP6 -with TcR39 and FRA- and FP10 -with SAPA and MAP-.(Praast *et al.*, 2011) According to the authors, this kit performed even better than the Biolab Merieux one.

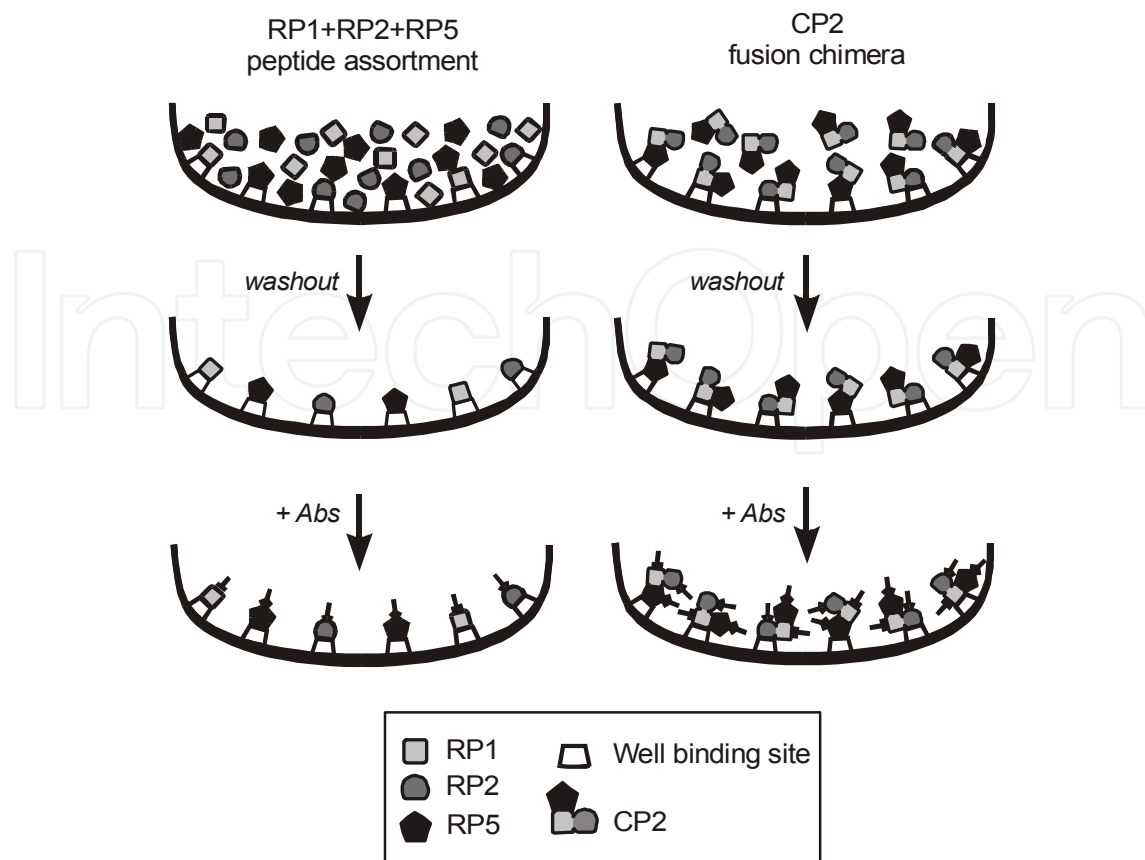


Fig. 1. Illustration of the ELISA plaque sensitizing process and the exposure to the sample: left-hand side, when using a protein mixture of three recombinant peptides RP1+RP2+RP5; right-hand side, when using a multi-epitope chimeric protein bearing the same peptides fused in a single protein, CP2. RP: recombinant peptide, CP: chimeric protein obtained by fusion of peptides RP1, RP2 and RP5 together in only one molecule. Reproduction from Camussone et al 2009.

5. Recombinant antigens use for the diagnosis of the different clinical entities

5.1 Acute phase diagnosis

As previously mentioned, during acute infection, serological methods are poorly sensitive. In the case of immunosuppressed individuals, as they do not develop an appropriate humoral immunological response, the direct method or molecular techniques are advisable. Serology is pertinent for in the neonatal congenital diagnostic when microhematocrit repeatedly shows negative results, or when diagnosis has not been made during the first month of life of the newborn. In these cases, it is necessary to serologically monitor congenital infection between 6 to 9 months, no matter if conventional reagents or recombinant ones are used.

Nevertheless, the search for antibodies which are usually triggered during acute phase could enhance results. In the early nineties a shed acute phase antigen (SAPA) was proposed to discriminate between acute and chronic infection. (Reyes *et al.*, 1990) This antigen was described when a panel of recombinant proteins obtained from a cDNA library was used to analyze the reactivity of IgG antibodies occurring in sera of chronic chagasic

mothers and their newborns. The recognized IgGs against different *T. cruzi* antigens produced the same signal in sera from newborns and their respective mothers but SAPA antigen was recognized most frequently by antibodies from the infected newborns than it was by antibodies occurring in their mothers serum. Accordingly, the authors proposed it to be used to detect specific anti-*T. cruzi* IgG antibodies in neonates. Other works report that antibodies anti-SAPA allows the discrimination between acute and chronic *T. cruzi* infection because they were not present in the later stage of the infection.(Lorca *et al.*, 1993) Nevertheless, later works described SAPA as reactive when assessed with sera from chronic infected individuals.(Breniere *et al.*, 1997; Camussone *et al.*, 2009) This apparent contradiction could be explained considering the significant differential reactivity of anti-SAPA antibodies generated during the different stages of the infection. Indeed, anti-SAPA antibodies are detected in almost all infected individuals but its reactivity is higher in the acute infection. It has recently been performed a study by following up of 2283 chagasic mothers, from which 209 transmitted the infection to the newborns.(Russomando *et al.*, 2010) This work provides evidence on SAPA utility to serologically diagnose congenital infection before the third month of life, thus turning the protein into a promising inexpensive reagent to reduce the required time to detect the neonatal infection, and proceed to its early treatment.

Although different reactivity patterns have been described in Western Blot assays which use native *T. cruzi* excretion antigens, to discriminate between acute and chronic infection (Umezawa *et al.*, 1996) no other useful recombinant antigen different from SAPA has been described to diagnose the acute phase.

5.2 Chronic infection diagnosis

It has been already mentioned above that, when *T. cruzi* homogenate is used to perform ELISA tests, the assay sensitivity is high leading to a quite reliable result, therefore some authors have suggested that a single assay could be enough to test sera in blood banks. (Sosa Stani *et al.*, 2008; Otani *et al.*, 2009)

Several multicenter studies carried out on samples from blood-banks, report that ELISA tests which use parasite homogenates perform similarly than those which used recombinant proteins.(Remesar *et al.*, 2009; Otani *et al.*, 2009) However, cross-reactivity of antibodies towards antigens from *T. cruzi* and *Leishmania sp* has been frequently informed, and can be explained considering the phylogenic proximity between both parasite species.(Chiller *et al.*, 1990; Vexenat *et al.*, 1996; Chiaramonte *et al.*, 1996; Desquesnes *et al.*, 2007; Aguirre *et al.*, 2006) When sera from patients infected with *Leishmania ssp* parasites are included in the evaluations, specificity of recombinant proteins are higher (Umezawa *et al.*, 1999; Ferreira *et al.*, 2001; Aguirre *et al.*, 2006; Caballero *et al.*, 2007; Camussone *et al.*, 2009)

It has been recently reported that the antigen TSSA2 displays 87.8% sensitivity and 100% specificity to discriminate between chagasic and leishmaniasic patients.(Cimino *et al.*, 2011) TSSA2 is the only reported recombinant antigen, which has displayed specificity to type *T. cruzi* genotypes DTUII, DTUV or DTUVI by specific antibodies from infected patients. (di Noia *et al.*, 2002; Bhattacharyya *et al.*, 2010) As these DTUs are those predominant in South America, the authors proposed using this antigen in confirmatory *T. cruzi* infection diagnostic tests, in regions which are co-endemic for both infections. It was also described that SAPA antigen could be specific and sensitive enough to be used when trying to distinguish between chronic *T. cruzi* and leishmaniasic infections, in regions where both illnesses are co-endemic.(Gil *et al.*, 2011)

It should be considered that it is difficult to discard *T. cruzi* infection in patients suffering from leishmaniasis, because these are co-endemic diseases. That is why antigens cross-reactivity is normally assayed testing sera from patients who live in Chagas' disease non-endemic regions, because this allows ruling out *T. cruzi* infection from an epidemiological point of view. (Hernandez *et al.*, 2010; Caballero *et al.*, 2007; Aguirre *et al.*, 2006) It therefore follows the need to discuss and define new criteria to study the performance of immunochemical tests at those regions. In this regard, the enhanced sensitivity displayed by PCR techniques should allow overcoming the mentioned drawback.

The lack of cross-reactions of *T. cruzi* recombinant proteins towards samples from *T. rangeli* infected individuals was also described. (Caballero *et al.*, 2007) However, these are only preliminary results, and large evaluations have not been still performed since *T. cruzi* and *T. rangeli* are co-endemic and mixed infections are difficult to exclude with conventional or epidemiologic analysis. The first studies reporting discrimination of both infections using molecular approaches have been published in the last year and will allow to compose a serum panel with samples from patients suffering from either only one or both infections. (Botero *et al.*, 2010)

In spite of the advantages yielded when using recombinant antigens in ELISA, it has also been described that sensitivity varies according to the antigen used to sensitize plaques and the geographical region. Thus, sensitivity obtained when performing tests in Colombia using recombinant antigens related to the predominant strain in South America is different from that obtained when assays are carried out in regions at the South of the continent. (Ramirez *et al.*, 2009) The same holds true when examining samples from Panamá patients, where *T. cruzi* strains are very similar to those from Colombia. (Caballero *et al.*, 2007) These works point out that serum level of antibodies in Panamanian patients were significantly lower than those from Brazilian individuals, from where the recombinant proteins were obtained using parasite genotypes isolated in Brazil. These data are in agreement with the serological differential reactivity produced by experimental infections in a mouse model, when strains representatives of different DTUs are inoculated. (dos Santos *et al.*, 2009).

5.3 Laboratory treatment monitoring

The evaluation of treatment effectiveness is normally carried out through serological analysis. Direct parasitological techniques miss reliability because of the extremely low parasitemia, which after treatment diminishes more, even when total parasite elimination could have not been reached.

Conventional serology turns into negative for more than 80% of the patients treated during the acute phase, once passed about 2 years after treatment. However, this percentage drops to less than 10% for patients who have been treated during the chronic phase, this taking several years. (Cancado, 1999)

The patient status is classified, according to the laboratory tests results. Thus, patients are considered cured when parasitological tests and conventional serology are negative. When parasitological tests are negative and 2 of 3 conventional serological tests are positive, the patient is classified as dissociated. Patients are considered not to be cured when the 3 tests are positive.

The serological test that demonstrated to be especially useful to monitor treatment effectiveness is the assessment of lytic antibodies. This test showed 100% correlation with parasitological cure, when lytic antibodies were evaluated in sera from patients who were

confirmed to be cured.(Krettli *et al.*, 1979) The drawback of this test is the need to count with *in vivo* trypomastigotes culture, thus not being available in clinical diagnostic laboratories. Consequently, several recombinant antigens to evaluate patient's treatment response have been proposed and assessed by ELISA as follows.

The target antigen of lytic antibodies was identified as a 160 KDa molecule, a complement regulatory protein, usually named CRP.(Krettli, 2009) The assessment of antibodies against this protein displayed 100% correlation with that of lytic antibodies, when using both the native protein and the recombinant one.(Meira *et al.*, 2004)

Cruzipain and Tc24 are other recombinant proteins which were also evaluated to monitor patient's treatment, and displayed 70% and 80% correlation with the lytic antibodies method, respectively.(Gazzinelli *et al.*, 1993;Krautz *et al.*, 1995) F29, which is Tc24 homologous, was used to follow up the treatment in children younger than 12 years old, after 48 months of initiated the medication.(Sosa *et al.*, 1998) This work reports that 67% of sera from treated children showed lack of anti-F29 antibodies whereas 100% of untreated children showed positive results for the specific antibodies. Therefore, the authors proposed to confirm the lack of anti-F29 antibodies as a serological marker of children cure. More recently, F29 was again evaluated as antigen of treatment monitoring in adults.(Fabbro *et al.*, 2007) Results showed lack of the specific antibodies in 82.4% of sera from treated patients who still showed positive conventional serology. The same group has recently evaluated the levels of specific antibodies against the ribosomal protein TcP2 β , as a cure marker. (Fabbro *et al.*, 2011) Their results showed a significant decrease of specific anti-TcP2 β in sera from treated patients, although no negative results occurred, which is a similar behavior than that displayed when performing conventional serology. Therefore, anti-TcP2 β does not resemble to be an apparently good candidate to be used as an early marker of the infection cure.

Another interesting study is the one where CRA and FRA antigens were evaluated, and displayed a 67% correlation with the reference technique. This makes CRA and FRA as quite promising antigens to be used for cure monitoring. The results are interesting considering that the Bio-manguinhos, Fiocruz commercial kit, commercialized in Brazil is manufactured with a single mixture of these two recombinant proteins.

5.4 Chronic infection monitoring

In the context of Chagas disease autoimmune hypothesis, during the nineties, it was proposed that different antigens contributed to the generation of autoantibodies, which could be used as illness evolution markers.(Leon & Engman, 2001) Among these antigens we should mention cruzipain, (Giordanengo *et al.*, 2000;Goin *et al.*, 1999;Duschak *et al.*, 2001) sulfo-cerebrosides (Avila *et al.*, 1993) and the ribosomal protein TcP2 β .(Levitus *et al.*, 1991) Precursor works had described that this ribosomal antigen that shares the C terminal region with its homologous from humans, generated autoimmune antibodies, whose concentration was increased in patients who had developed chagasic cardiopathy.(Levin *et al.*, 1991;Aznar *et al.*, 1995). Our group evaluated the concentration of antibodies against cruzipain, sulfo-cerebrosides and ribosomal TcP2 β in three different groups of patients: those classified as asymptomatic, those who only displayed electrocardiographic alterations and those who had evident cardiopathy.(Diez *et al.*, 2006) In our experience, only those samples from patients with evident cardiopathy had increased specific anti-TcP2 β concentration. However, these results have not yet been confirmed analyzing a larger number of patients. In another study, anti-TcP2 β concentration was higher in sera from patients at indeterminate

stage than in sera from symptomatic individuals.(Breniere *et al.*, 2002) In a longitudinal evaluation of asymptomatic and cardiac groups of patients, we described that only the individuals who evolved to a more severe clinical status increased specific anti-TcP2 β concentration in late stages of the infection.(Fabbro *et al.*, 2011) However, the transversal comparison of the sera from patients with and without cardiopathy revealed that anti-TcP2 β concentration between both groups was not significantly different. The discrepant results mentioned above show that it is not still clear if anti-TcP2 β can be used as a serological marker of myocardic damage.

Also, muscarinic acetylcholine receptor subtype II, in this case a host antigen, has shown to be quite auspicious to monitor the chronic infection.(Goin *et al.*, 1999) Nevertheless, recent studies suggest that it is not apparent that this protein is useful to discriminate between different stages of the illness.(Tovar *et al.*, 2009;Talvani *et al.*, 2006)

The difficulties to establish clear illness evolution markers lead to the present state, where we do not count yet with useful tools to evaluate Chagas' disease prognosis.

6. Future prospects

The number of recombinant proteins assessed and proposed as candidates to be used as tools for *T. cruzi* infection diagnosis is quite high. However, there is no serum panel to be used as international reference. Therefore, the reports on results produced by using these proteins can hardly be compared. Usually, serum panels, previously typified with other validated methods are used to appraise new serology reagents. These already standardized serological reagents have their own sensitivity and specificity, which may lead to a bias when typifying the panel.

Currently, several diagnostic tests have been proposed as the reference one, such as immunofluorescence or different versions of Western blot.(Otani *et al.*, 2009;Caballero *et al.*, 2007) However, no consensus exists among researchers and regulatory agencies on which tests are preferable.

Another inconvenience is that there is evidence on the absence of humoral response in some patients of endemic regions, whose infection was proved by parasitological techniques or by tests evaluating the cellular immune response.(Salomone *et al.*, 2003;Olivera *et al.*, 2010) These reports alert us on a potential overestimation of the sensitivity and specificity underestimation of the immunochemical assays when they are assessed with serum panels typified by conventional serology.

Conventional serology is still a crucial tool to diagnose the different entities studied during the chronic phase of the infection. The main multicenter studies carried out in regions where leishmaniasis is not endemic have shown that ELISAs using both parasite extractive antigens, as well as the recombinant ones, display optimal sensitivity and specificity. Although both kind of antigens perform similarly in those leishmaniasis-free places, when determinations are carried out where Chagas' disease and leishmaniasis are co-endemic, ELISAs using recombinant proteins have demonstrated to be the most useful.

In the same line, presently, several authors who have evaluated ELISA commercial kits with plaques sensitized with parasite homogenate or with recombinant antigens, have shown that it is not mandatory to carry out 2 different tests. Certainly, when performing only one ELISA, it is feasible to diagnose the infection, if the result is positive. This is acknowledged because results obtained with both kinds of ELISA correlate appropriately. (Remesar *et al.*, 2009;Otani *et al.*, 2009) However, considering the poor sensitivity of IHA, this latter one would not be recommended as a second test. (Remesar *et al.*, 2009)

Even though some recombinant proteins have been used to monitor Chagas' disease treatment, it could not be still demonstrated that these proteins give diagnostic information to evaluate cardiopathy diagnosis and prognosis. During recent years, the description of the whole genome of *T. cruzi* has prompt to systematically analyze new antigens, some of which have been described as putative antigens, but has not yet confirmed. This is being evaluated nowadays by different research groups which, it is expected will suggest new interesting markers that are useful for cure monitoring and cardiopathy prognosis.

Lately, several research works on infection diagnostic tools have reported on the development of latex particle agglutination and amperometric biosensors to diagnose *T. cruzi* infection.(Gonzalez *et al.*, 2010;Belluzo *et al.*, 2011;Ribone *et al.*, 2006) Latex particle vs. conventional agglutination has the advantage of allowing particle sensitization with recombinant proteins, what leads to a more reproducible, standardized reagents production.(Gonzalez *et al.*, 2010) Biosensors technology admits reutilization of the device, potentially yielding to automation, thus facilitating laboratory operation. Moreover, the simplicity of the equipment required, permits the analysis to be performed in the field, which is an important attribute because infected people generally live in the countryside and do not attend health centers.(Belluzo *et al.*, 2011) The electrochemical biosensor technology developed follows the same ELISA format, exchanging the colorimetric signal readout by the amperometric one.(Belluzo *et al.*, 2011) Although no commercial device is yet available, the results of our studies are quite promising. This methodology could allow reducing costs and time of analysis in the near future, keeping the same or even higher standards of sensitivity and specificity than ELISA.(Belluzo *et al.*, 2011)

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Tropical Medicine has emerged and remained as an important discipline for the study of diseases endemic in the tropic, particularly those of infectious etiology. Emergence and reemergence of many tropical pathologies have recently aroused the interest of many fields of the study of tropical medicine, even including new infectious agents. Then evidence-based information in the field and regular updates are necessary. Current Topics in Tropical Medicine presents an updated information on multiple diseases and conditions of interest in the field. It includes pathologies caused by bacteria, viruses and parasites, protozoans and helminths, as well as tropical non-infectious conditions. Many of them are considering not only epidemiological aspects, but also diagnostic, therapeutic, preventive, social, genetic, bioinformatic and molecular ones. With participation of authors from various countries, many from proper endemic areas, this book has a wide geographical perspective. Finally, all of these characteristics, make an excellent update on many aspects of tropical medicine in the world.

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