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Applicability of a novel immunoassay based on surface plasmon resonance for the diagnosis of Chagas disease

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ABSTRACT

Background: We defined the methodological criteria for the interpretation of the results provided by a novel immunoassay based on surface plasmon resonance (SPR) to detect antibodies anti-*Trypanosoma cruzi* in human sera (SPRCruzi). Then, we evaluated its applicability as a diagnostic tool for Chagas disease.
Methods: To define the cut-off point and serum dilution factor, 57 samples were analyzed at SPRCruzi and the obtained values of SPR angle displacement ($\Delta\theta_{SPR}$) were submitted to statistical analysis. Adopting the indicated criteria, its performance was evaluated into a wide panel of samples, being 99 Chagas disease patients, 30 non-infected subjects and 42 with other parasitic/infectious diseases. In parallel, these samples were also analyzed by ELISA.
Results: Our data demonstrated that 1:320 dilution and cut-off point at $\Delta\theta_{SPR} = 17.2$ m° provided the best results. Global performance analysis demonstrated satisfactory sensitivity (100%), specificity (97.2%), positive predictive value (98%), negative predictive value (100%) and global accuracy (99.6%). ELISA and SPRCruzi showed almost perfect agreement, mainly between chagasic and non-infected individuals. However, the new immunoassay was better in discriminate Chagas disease from other diseases.
Conclusion: This work demonstrated the applicability of SPRCruzi as a feasible, real time, label free, sensible and specific methodology for the diagnosis of Chagas disease.

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1. Introduction

Chagas disease (CD) is caused by the hemoflagellate protozoan *Trypanosoma cruzi*, included in order Kinetoplastida and family Trypanosomatidae [1]. It is endemic to a wide region of Latin America, where occurs in 21 countries and affects approximately 7 million people, being responsible for a high burden of morbidity and mortality, including sudden death events [2]. However, in the last decades, *T. cruzi* infections have been increasingly found in non-endemic areas, such as the USA, Canada, many European and some Western Pacific countries, mainly due to the increase of infected population movements [3].

It is well referenced that the conduits for the diagnosis of CD are related to the phases of the infection that a patient present. Thereby, in acute phase, period that comprehends 1 to 4 months following initial infection, laboratorial diagnosis is carried out by the detection of trypomastigote forms directly in the blood [4]. On the other hand, during the successive chronic phase, which is a lifelong phase, the number of parasites in the peripheral blood is low and subpatent. Consequently, due to the low sensitivity of the parasitological methods, at this phase the laboratorial diagnosis is usually performed by indirect serological methods for detection of IgG antibodies anti-*T. cruzi*, which are present in different concentration in approximately 98% of the infected patients [5]. For this purpose, the serological tests currently employed are indirect hemagglutination assay (IHA), indirect immunofluorescence (IIF) and enzyme-linked immunosorbent assay (ELISA), so that a conclusive result is achieved when at least two different methodologies are used [6]. In this sense, according to the Brazilian Consensus on Chagas Disease, the diagnosis during the chronic phase should be carried out using a test with high sensitivity, i.e. ELISA with total antigen or semi-purified fractions of the parasite, IIF or IHA, in combination with another test with high specificity, i.e. ELISA employing *T. cruzi*-specific recombinant antigens [7].

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Although these serological tests are widely used in routine, they present some methodological limitations, including frequent cross-reactivity with other infectious agents also observed in endemic areas, e.g. trypanosomatids as *Leishmania* spp., and problems with sensibility and specificity, which lead to inconclusive results usually observed in clinical practice and screening of blood donors [8]. In addition, they also require a long pre-analytical and analytical time to be performed, skilled labor, refrigeration for reagents and expensive supplies [9]. Therefore, the searches for alternatives serological tests which combine robustness, simplicity, portability with adequate specificity and sensitivity have been explored in order to overcome such problems [10].

Surface plasmon resonance (SPR) has been applied by several authors as an innovative method for the evaluation of interfacial events and, consequently, the development of immunoassays, since it combines great selectivity and sensitivity to recognize biomolecular interactions in label free and real time analysis [11,12]. Recently our team proposed a novel immunosensor based on SPR for the detection of antibodies anti-*T. cruzi* (IgG) in human serum [13]. On that occasion, we also optimized the operational parameters of the new immunoassay — termed SPRCruzi, which employs crude soluble antigens of *T. cruzi* covalently immobilized on a SPR sensor gold chip modified with an organic mixed self-assembled monolayer (SAM). The direct addition of a serum sample infected with *T. cruzi* generates a displacement of the SPR angle ($\Delta\theta_{SPR}$), which is derived from the occurrence of antigen-antibodies interactions on the sensor surface, and is proportional to the amount of antibodies anti-*T. cruzi* present in the sample. On the other hand, a null or smaller SPR angle variation is obtained if a non-infect sample is injected.

Although we previously demonstrated that SPRCruzi represents a promising diagnostic tool for CD diagnosis, once it allowed clear segregation of infected and non-infected groups in simple and fast analysis, still is necessary to establish and confirm the methodological criteria for interpretation of the immunoassay, and conduct a thorough and wide evaluation of its applicability.

2. Population, materials and methods

2.1. Study population

Two groups of human serum samples were applied to achieve the proposed investigations of the present study. The first group (Group 1) was used during the standardization of the methodological criteria of SPRCruzi. It included 57 sera, being 20 of these from non-infected individuals (NI) and 37 from CD patients (CH). The latter were divided into three groups according to the reactivity index (RI) defined by ELISA: high ($n = 12$) ($2.9 \leq RI \leq 3.5$), medium ($n = 13$) ($2.0 \leq RI \leq 2.2$) and low reactivity ($n = 12$) ($1.0 \leq RI \leq 1.3$). The RI was calculated by dividing the absorbance value of reading from each sample assayed by the cut-off value adopted in the analysis.

The second population group (Group 2) was used for the evaluation of the applicability of SPRCruzi in the diagnosis of CD. It included 171 human sera samples, being 99 from CD patients (CH), 30 negative control sera from non-infected individuals (NI) and 42 from individuals not infected with *T. cruzi* but infected with other relevant parasitic/infectious diseases usually find in endemic areas for CD (other diseases), which were subdivided as patients with Visceral Leishmaniasis (VL) ($n = 7$), Cutaneous Leishmaniasis (CL) ($n = 6$), Acute Toxoplasmosis (ATX) ($n = 6$), Chronic Toxoplasmosis (CTX) ($n = 6$), as well as human T lymphotropic virus (HTLV) ($n = 8$), hepatitis B virus (HBV) ($n = 4$) and human immunodeficiency virus (HIV) ($n = 5$) infections.

All the serum samples CH and NI referred above were collected from individuals from the municipality of Berilo, located in the Jequitinhonha Valley, northeast of Minas Gerais State, Brazil, an important endemic region for CD. The diagnosis of all samples was previously confirmed by three serological tests, including ELISA, IIF and IHA. The collection of these samples from endemic area was approved by the research ethics committee of the René Rachou Research Center, under the process number 007/2002. The human sera with HBV, HTLV, HIV, ATX and CTX were kindly provided by the Ezequiel Dias Foundation, Belo Horizonte, Minas Gerais. Samples from CL and VL patients were provided by the biorepository of the Laboratory of Biomarkers for Diagnosis and Monitoring, René Rachou Research Center, Oswaldo Cruz Foundation, FIOCRUZ, Minas Gerais, Brazil. The diagnosis of other diseases samples was confirmed according to the recommended for each disease.

2.2. Preparation of crude soluble antigens

The crude soluble antigens used in SPRCruzi and ELISA were obtained by alkaline extraction from epimastigotes of *T. cruzi* Y-strain harvested in axenic culture in liver infusion tryptose (LIT) medium and maintained at 28 °C. Briefly, the parasites were submitted to lysis with NaOH 0.15 mol/l overnight in an ice bath under agitation. The addition of HCl 0.15 mol/l was carried out aiming the neutralization of the material. Then, the suspension was centrifuged at 15,000 rpm for 30 min at 4 °C and the supernatant was submitted to protein determination according to Lowry et al. [14]. The concentration was adjusted to 1000 $\mu g\ ml^{-1}$ and antigen aliquots were frozen at −80 °C until analysis. Posteriorly, dilutions of antigen for the use in the methodologies were carried out with HBS-EP pH 7.4 buffer solution.

2.3. SPR immunoassay (SPRCruzi)

The immunoassay based on SPR for the detection of anti-*T. cruzi* antibodies in tested serum samples was performed as optimized and described by Luz et al. [13]. In order to obtain the parameter $\Delta\theta_{SPR}$ from the injection of samples, and consequently detect the occurrence of interactions between immobilized antigen and antibodies present in serum, an SPR analyzer from Autolab Springle (Eco Chemie B.V., Netherlands) was used.

2.4. Analysis for standardization of methodological criteria of SPRCruzi

Aiming to ascertain the best serum dilution and cut-off point to be adopted for interpretation the values of $\Delta\theta_{SPR}$ provided by SPRCruzi, and thus identify the CD patients with assurance, the samples of Group 1 were diluted with HBS-EP buffer at pH 7.4 into three potential serial dilutions: 1:160, 1:320 and 1:640. The samples were first analyzed as a pool of sera in triplicate and then individually. Other dilution factors were not chosen because they did not provide a good separation between CH and NI individuals, as previously verified [13].

The results of SPR analysis were expressed as $\Delta\theta_{SPR,}$ and the obtained data were analyzed by receiver operating characteristic (ROC) curve analysis, a statistical tool that indicates the more appropriate cut-off point to discriminate negative from positive results. Moreover, the area under the curve (AUC) also provided the global accuracy of the test, which can be classified as low (0.51–0.61), moderate (0.62–0.81), elevated (0.82–0.99) and outstanding (1.0) [15]. The ROC curve analysis was performed using MedCalc 14.8.1.

2.5. Evaluation of the applicability of SPRCruzi

For the evaluation of the applicability of the immunoassay, the samples of Group 2 were analyzed individually with the most adequate serum dilution and cut-off previously defined. From the obtained data, the performance indexes were also calculated to determine how much SPRCruzi is possible of application for the diagnosis of CD in endemic areas.

2.6. Comparison of SPRCruzi and ELISA

In parallel, the detection of antibodies in sera from Group 2 was also evaluated by ELISA performed according to Voller et al. [16] using the

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