Diagnosis of *Trypanosoma cruzi* infection status in saliva of infected subjects

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ABSTRACT

Chagas disease has the highest prevalence of any parasitic disease in the Americas, affecting 6 to 7 million people. Conventional diagnosis requires a well-equipped laboratory with experienced personnel. The development of new diagnostic tools that are easy to use and adapted to the reality of affected populations and health systems is still a significant challenge. The main objective of this study was to measure *T. cruzi* infection status using saliva samples of infected subjects. Blood and saliva samples from 20 *T.cruzi*-seropositive individuals and 10 controls were tested for *T. cruzi* infection using two different commercial serological tests. We have shown that detection of Chagas infection is possible using saliva samples, supporting the potential use of saliva to diagnose Chagas disease in humans. This method could provide a simple, low-cost but effective tool in the diagnosis of *T. cruzi* infection. The non-invasive nature of it makes it particularly well suited to endemic areas.

INTRODUCTION

Chagas disease (CD) is a major public health problem in Latin America, affecting approximately 6 to 7 million people. In addition, travel and immigration patterns have increased the relevance of *T. cruzi* infection outside of endemic areas[1,2,3]. *T. cruzi* can be transmitted to humans by reduviid insects that inhabit housing made of mud, thatch, and other natural materials in endemic areas[4], by blood transfusion, organ transplant from infected donors[5], congenital from mother to infant[6] and by ingestion of food or drink contaminated by infected triatomines[7].

CD still faces multiple challenges. There are no preventive vaccines for human or veterinary use and the two available treatments are not optimal as they are plagued by side effects and inconsistent efficiency[8,9,10,11]. Additionally, the diagnosis of this infection confronts several limitations. In the acute phase of the infection, diagnosis is based on the microscopic detection of trypomastigotes in blood. However, during the chronic phase of the infection parasite persistence is low and the detection of *T. cruzi* using this method is difficult and unreliable. Serology is the gold standard for *T. cruzi* infection diagnosis during the chronic phase of the disease. At least two different serological methods (preferably based on different antigens) are used to detect the presence of IgG antibodies against *T. cruzi* antigens. Currently, no single assay for chronic *T. cruzi* infection has shown enough sensitivity and specificity to be used alone for diagnosis[12].

CD particularly affects poor rural and peri-urban areas of Latin America, where health-care access is limited. Developing new diagnostic tools which are easy to use and adapted to the reality of affected populations and health systems, is still a substantial need[13]. The purpose of this study is to address this challenge by exploring the detection of *T. cruzi* infection status using saliva samples.

MATERIAL AND METHODS

Ethics statement

The study protocol was approved by the Ethics Committee of the Hospital Clinic of Barcelona and the Scientific Committee of the Barcelona Institute for Global Health (ISGlobal). Individual written informed consent was obtained from all study participants before the collection of the samples.

Design and setting

The study was designed as a pilot project to detect *T. cruzi* infection status using saliva of infected individuals. A total of 20 *T. cruzi*-seropositive individuals were enrolled in one group (GA), and 10 *T. cruzi*-seronegative individuals in another group (GB). GB was included as a negative control group. GA patients were originally from Latin America countries where CD is endemic. Among them, 10 received benznidazole treatment between 2005 and 2015 (all of them completed the treatment regime), and 10 of them did not receive treatment during the length of the study. GB subjects came from different areas of the world.

Selection of subjects

Inclusion criteria: adult subjects (over 18 and less than 50 years-old); weight over 40 kg, with serologic tests confirming or excluding *T. cruzi* infection. All the participants were recruited at the Center for International Health at the Hospital Clinic of Barcelona, Spain.

Participants

Subjects of both genders and aged between 18 and 50 years old were included in the study.

Sample collection and procedures

(a) Blood samples: 10 mL blood sample per individual was collected for diagnosis of *T. cruzi* infection in serum. Samples were centrifuged at 1600 g for 10 minutes at room temperature.

(b) Saliva samples: Subjects also provided 10 mL of unstimulated saliva samples. Saliva samples were centrifuged using ultrafiltration membranes (Amicon Ultra-15 devices) at 4000 g for 15 minutes at room

temperature.

ELISA Tests

300 microliters (µL) of concentrated saliva samples and 10 µL of blood serum were used to perform the serological test. Two different commercial serological tests were used: Bioelisa CHAGAS from Biokit (BioELISA Chagas; Biokit S.A., Lliçà d'Amunt, Barcelona, Spain), and DRG *Trypanosoma cruz*i IgG (DRG International Inc., Springfield, U.S.A).

Data analysis

Statistical analysis was performed by analysis of variance and unpaired t test using the GraphPad PRISM 5.0 software.

RESULTS

We first detected *T. cruzi* infection status with the current serological tests using enzyme-linked immunosorbent assay (ELISA) from Biokit. As expected, seropositive patients showed higher levels of IgG anti-*T. cruzi* antibodies compared to *T. cruzi*-seronegative subjects (Figure 1).

Previous to running ELISA assays on the saliva samples of *T. cruzi* infected individuals, we tested whether a filtered and therefore a more concentrated saliva sample would exhibit a higher sensitivity than an unfiltered sample. Filtered saliva samples showed higher levels of response to *T. cruzi* antigen than their unfiltered counterparts, although these differences were not statistically significant (Figure 2A). Additionally, different volumes of filtered saliva samples were tested and the results showed a higher sensitivity when using 300 µl compared to other volumes (Figure 2B).

Then, we tested 20 *T. cruzi*-seropositive and 10 *T. cruzi*-seronegative serum and saliva samples using the same test. Results are summarized in figure 3. *T. cruzi*-seropositive subjects presented higher levels of IgG anti *T. cruzi* antibodies compared to *T. cruzi*-seronegative subjects, using serum or saliva samples (Figure 3A). ELISA results demonstrated consistency between serum and saliva samples. The salivary test presented a specificity

of 100%: all *T. cruzi*-seronegative individuals were negative using saliva as a sample. The sensitivity of the test was 70%: 6 of the 20 *T. cruzi*-seropositive individuals were negative using saliva as a source. 4 of these subjects were previously treated with benznidazole, and 2 of them were not previously treated. ELISA's cut-off value was computed by the usual cut-off formula of the form "mean + 3 standard deviation of negative controls".

We also compared levels of *T. cruzi* antibodies for both saliva and serum of previously treated and untreated seropositive subjects. Differences in levels of serum IgG anti *T. cruzi* antibodies were not found between treated and untreated individuals. Interestingly, we found differences in levels of *T. cruzi* antibodies between treated and untreated individuals using saliva (Figure 3B). However, these results were not statistically significant.

DISCUSSION

The most common method of diagnosing CD is detecting serum antibodies against parasite antigens mainly using ELISA tests. However, there are some limitations of using serum as a diagnostic sample. Blood collection is invasive, and it demands specially trained personnel.

Saliva has the potential to become a first line diagnostic sample of choice. The use of this fluid for diagnostic purposes is increasing in popularity: it is easy to collect, store and transport, it does not require highly trained personnel, and it is safer for medical staff to handle compared to other body fluids. In addition, it is noninvasive and the donation process is relatively stress free, minimizing donor discomfort. This can help improve access to diagnosis and treatment for people living in rural areas far from healthcare centers. These characteristics make salivary diagnosis especially valuable for vulnerable populations. In addition, analysis of saliva may provide a cost-effective approach for the screening of large populations[14,15]. Due to its many potential advantages, salivary diagnosis provides an attractive alternative to more invasive, time-consuming, complicated, and expensive diagnostic approaches.

Pinho et al. explored this area of research in 1999[16] for *T. cruzi* infection, showing that detection of *T. cruzi*

infection was possible using saliva samples. Our data confirms these findings, and thus supports saliva as a possible source of detection of CD.

Although our results have shown consistency between serum and saliva samples of *T. cruzi*-seropositive and *T. cruzi*-seronegative subjects, the low sensitivity of the ELISA test using saliva as a source of diagnostic could represent a challenge. It is common to find low concentrations of molecules in saliva compared to blood[15]. This weakness could be partially overcome using ultrafiltration membranes to concentrate saliva samples.

Our results have shown differences in the levels of IgG *T. cruzi* antibodies between previously treated subjects and untreated patients. Currently, the gold standard for evaluating treatment efficacy is the seroconversion of conventional serological tests, which may take years to decades to assess[17]. Although this is a preliminary finding, saliva could play an important role in evaluating treatment efficacy.

CONCLUSIONS

T. cruzi specific salivary IgG detection provides a simple, low-cost but effective tool in the diagnosis of the disease. The non-invasive nature of this method makes it particularly well suited to endemic areas. The salivary test presented a specificity of 100%. Unfortunately, the sensitivity of the test was lower: 30% of *T. cruzi*-seropositive individuals were negative using saliva as a diagnostic sample.

In the future, saliva could play a key role in diagnosis of *T. cruzi* infection and evaluation of treatment efficacy. Additional studies exploring other salivary molecules such as IgA anti *T. cruzi* levels could be helpful, due to the high concentration of this immunoglobulin in saliva. More research is needed with larger sample sizes to further investigate this method, which has the potential to revolutionize diagnosis and treatment of Chagas disease.

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Figure 1: Serum IgG response to *T. cruzi* antigen in *T. cruzi*-seropositive and *T. cruzi*-seronegative subjects.

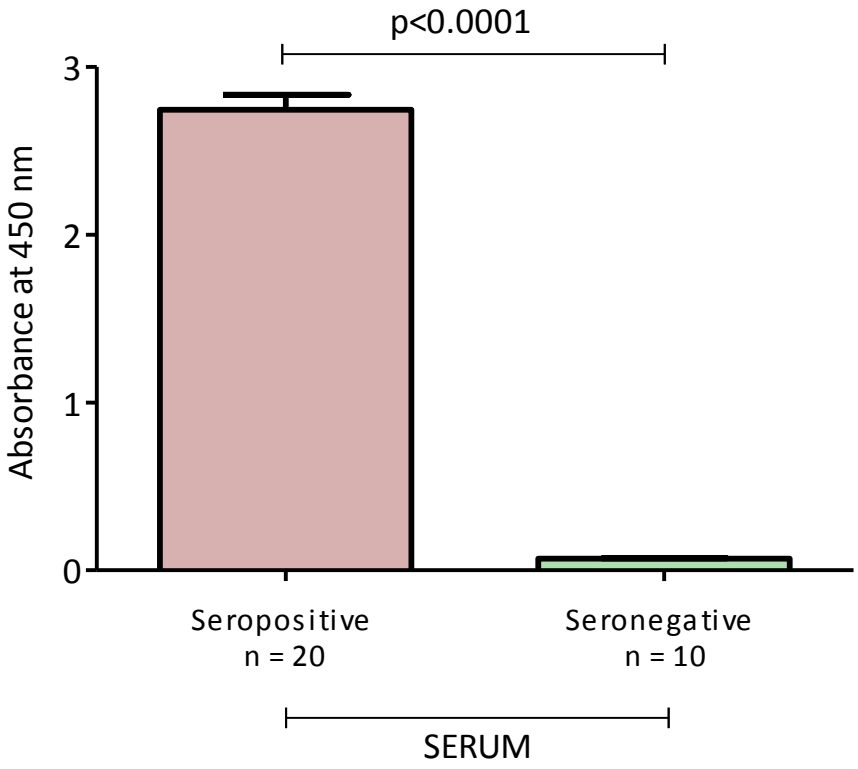

Figure 2A: Saliva IgG response to *T. cruzi* antigen using ultrafiltration membranes.

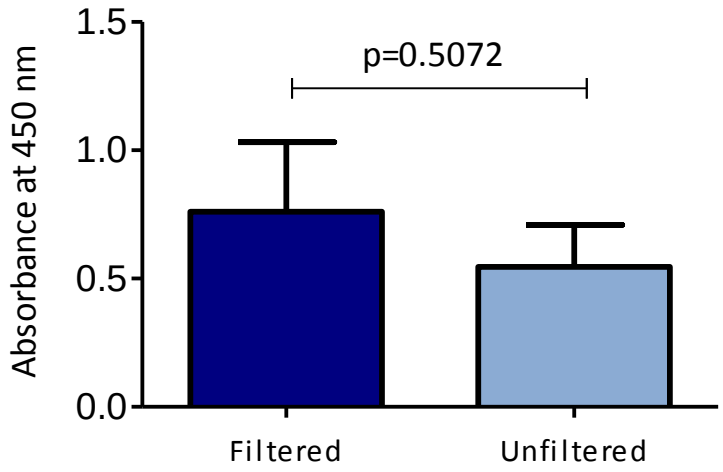

Figure 2B: Saliva IgG response to *T. cruzi* antigen in *T. cruzi*-seropositive and *T. cruzi*-seronegative subjects using different sample volumes

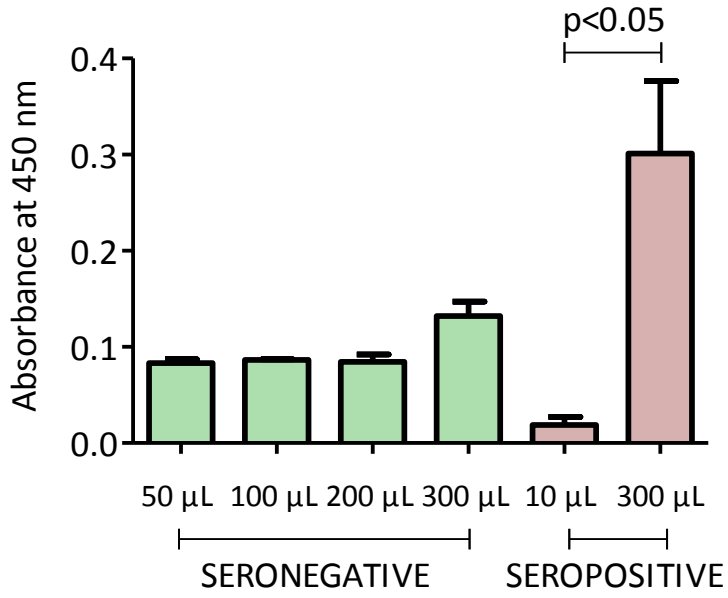

Figure 3A: Serum and saliva IgG response to *T. cruzi* antigen in *T. cruzi*-seropositive and *T. cruzi*-seronegative subjects.

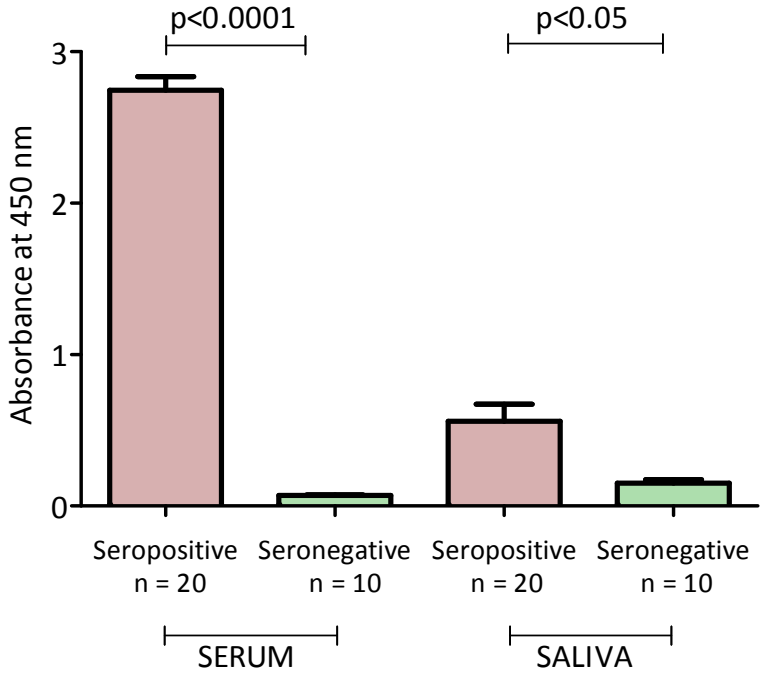

Figure 3B: Serum and saliva IgG response to *T. cruzi* antigen in *T. cruzi*-seropositive subjects previously treated with benznidazole and *T. cruzi*-seropositive subjects untreated.

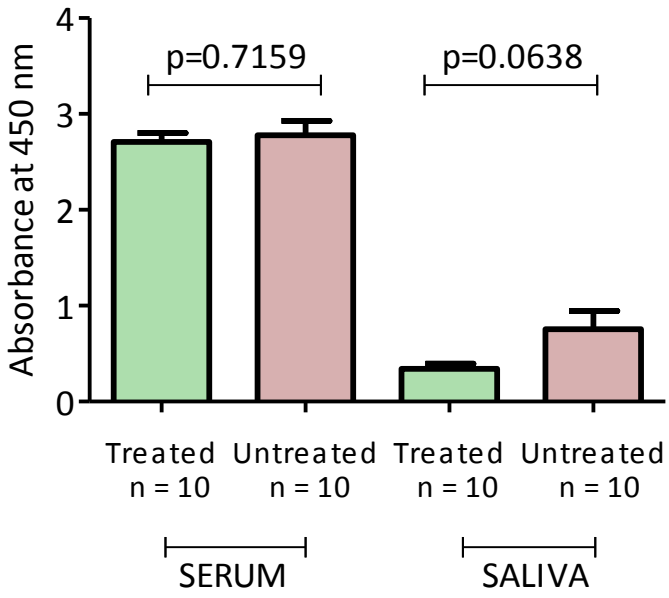

Table 1: Age and gender of study participants.

GA	Age	Gender
Untreated patients	38	Female
	31	Female
	44	Male
	44	Female
	42	Female
	39	Female
	44	Male
	42	Female
	34	Male
	33	Female
Treated patients	54	Female
	46	Female
	43	Female
	50	Female
	33	Female
	49	Female
	40	Female
	48	Female
	50	Female
	41	Female

GB	Age	Gender
European controls	39	Female
	32	Male
	31	Male
	35	Female
	26	Female
Endemic countries controls	28	Female
	43	Male
	30	Female
	39	Male
	28	Female