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A simple *Trypanosoma cruzi* enzyme-linked immunoassay for control of human infection in nonendemic areas

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Abstract

An enzyme linked immunosorbent assay (ELISA) was developed for detecting IgM and IgG antibodies against *Trypanosoma cruzi* in blood bank donors from endemic or nonendemic areas. A crude extract of trypomastigotes from cultures was used as antigen. A total of 494 serum samples from patients with acute, congenital, or chronic form of Chagas' disease, and from healthy French individuals were studied. The sensitivity of the ELISA was determined with 89 serum samples from chagasic patients and was evaluated to 98.8%. The specificity was determined with 405 serum samples from French blood transfusion centers donors and evaluated to 98.3%. Two hundred and eighty-five serum samples from blood donors from Argentina and Brazil were also tested. Furthermore, in order to assess the absence of cross-reactivity with other protozoan infections, we studied 86 serum samples including (i) 32 individuals with cutaneous leishmaniasis living in a *T. cruzi* endemic region of Bolivia, and (ii) 54 patients from nonendemic area for Chagas' disease, 19 of them with kala-azar and 35 others with malaria.

Keywords: *Trypanosoma cruzi*; Chagas' disease diagnosis; Sensitivity; Specificity; Blood bank screening; Transfusion

1. Introduction

Chagas' disease or American Trypanosomiasis caused by the protozoan *Trypanosoma cruzi* (*T. cruzi*) affects approximately 18 million people in Latin America and remains a significant public health problem in these areas. To-day many endemic countries have developed campaigns of eradication of Triatominae from the houses and, consequently, the frequency of natural or vectorial transmission has decreased [1]. In these countries, the acute cases of Chagas' disease have become rare although non-vectorial transmission still exists through blood transfusion or congenital transmission [2–5]. The control of blood bank donors is necessary because chronic infected individuals, that do not develop clinical signs of the disease, carry circulating parasites. The few parasites present in the blood are difficult to be observed microscopically but PCR techniques allowed the detection of parasite DNA in blood and tissue from chronic infected patients [6–8]. Neverthe-

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less, serological diagnosis is the most frequently used method for detection of *T. cruzi* infected blood in blood banks [9,10].

Due to increased migration of rural populations to the cities, transmission by blood transfusion is increasing in the main cities of endemic countries [2,3]. Moreover, the number of travellers and/or immigrants from endemic to nonendemic regions is growing, causing *T. cruzi* transmission in areas where Chagas' disease is not endemic and where *Trypanosoma cruzi* is not usually found [11,12]. Consequently, infected individuals may be diagnosed in nonendemic areas where the vector is absent [13–18]. Indeed, there is quite a need to develop simple, specific and sensitive methods to perform diagnosis of *T. cruzi* infection in nonendemic areas in order to eliminate *T. cruzi* positive blood samples from blood banks, to follow accidental *T. cruzi* infection (mostly applied to scientific workers in research centers), or cases of unknown aetiology, where *T. cruzi* infection has to be discarded.

To comply with these needs, we, together with South American laboratories, prepared an ELISA that uses as reactive reagent the crude extract of trypomastigotes from in vitro cultures. Trypomastigotes were chosen because they were representative of the circulating bloodforms of the parasite and because they gave a significantly less false positive and cross-reactive responses than the commonly used epimastigote antigen (Hontebeyrie and Brenière, unpublished results). The ELISA was tested on blood bank samples of French donors, and blood bank samples coming from endemic areas, as well as samples from patients at different stages of infection. Serum samples of chagasic patients were assessed for both parasite specific IgM and IgG antibodies. Cross-reactivity was tested against serum samples from people infected with *Leishmania* or *Plasmodium*. Our findings demonstrate that the ELISA based on the blood form of the parasite can detect specific anti-parasite antibodies, with a very low level of cross-reaction with other parasitic diseases.

2. Materials and methods

2.1. Serum samples

Serum samples were collected from 494 individuals: 405 French blood transfusion center donors used as controls, 89 clinically and biologically well-characterized chagasic patients from Argentina or Bolivia. Those sera, except control sera, were previously assessed for *T. cruzi* infection by indirect hemagglutination and/or immunofluorescence. In addition, serum samples of 285 individuals presenting themselves to blood bank at Hospital Eva Peron, Buenos Aires, Argentina ($n = 185$) or at the Hemocentro, Brasilia, Brazil ($n = 100$) were analyzed.

A total of 86 serum samples from patients with non-related infections were studied to test the cross-reactivity: 51 with leishmaniasis (19 with well-characterized kala-azar from Mediterranean regions and 32 with cutaneous leishmaniasis from Bolivia) and 35 with clinical malaria from Africa or Asia.

2.2. T. cruzi antigen

Antigen was prepared from trypomastigotes of the Brener CL strain of *T. cruzi* grown on monolayers of Vero cells in RPMI 1640 supplemented with 5% calf serum, 2 mM L-glutamine, 50 IU penicillin-50 μg streptomycin mixture (Flow laboratories, France), 5% calf serum (Eurobio, Paris, France) at 33°C. The trypomastigotes were collected from the supernatant after five to seven day cultures, washed three times with serum-free RPMI 1640 and adjusted to

Table 1
Sensitivity (i.e., true positive ratio), specificity, and 1−specificity (i.e., false positive ratio) of specific *T. cruzi* IgG ELISA determined around the threshold positive value obtained from 405 control serum samples from nonendemic area

Threshold (O.D.)	Sensitivity	se (%)	Specificity	Sp (%)	1-Sp
0.240	89:89	100	329:405	81.2	0.187
0.262	88:89	98.8	364:405	89.9	0.101
0.302	88:89	98.8	398:405	98.3	0.017
0.333	88:89	98.8	405:405	100	0.000

10^8/ml in RPMI 1640 before freezing at −80°C. They were disrupted by three cycles of freeze-thawing, centrifuged at 10000×*g* during 20 min and the supernatant was kept at −20°C. The protein content was measured by the Bradford method (Bio-Rad S.A., Ivry/Seine, France).

2.3. ELISA: IgM and IgG detection of anti-T. cruzi antibodies

Microtest 96-well immunoplates (Nunc, Roskilde, Denmark) were coated with 50 µl of *T. cruzi* antigen (2.5 µg total protein/ml) in 0.15 M phosphate-buffered saline (PBS), pH 7.2, and incubated overnight at 4°C. All plates were blocked with PBS added with 0.1% Tween 20 (Sigma, St. Louis, MO, USA) and 2.5% non-fat dried milk (PBS-TM) for 1 h at room temperature. The plates were then washed extensively with PBS-Tween 20. Serum samples were diluted 1:200 in PBS-TM and 50-µl samples were added to each well. All samples were assayed in duplicate and one negative and one positive controls were added to each microplate. Plates were incubated 1 h at 37°C and washed 5 times with PBS-Tween 20. Fifty µl of a 1:1000 dilution of goat $F(ab)'_2$ peroxidase-conjugated anti-human IgM (Caltag, San Francisco, CA, USA) or 1:4000 dilution of goat $F(ab)'_2$ alkaline-phosphatase-conjugated anti-human IgG (Fc specific), (Caltag, San Francisco, CA, USA) were added to each well and the plates were incubated for 1 h at 37°C and then washed 5 times with PBS-Tween 20. Peroxidase was revealed with 50 µl of a 1:1 mixture of 2.2′-azino-di-3-ethylbenzothiazoline sulfonate and hydrogen peroxide (ABTS Peroxidase Substrate System, Kirkegaard Perry Laboratories Inc., Gaithersburg, MD, USA) added to each well. The reaction was stopped by addition of 50 µl of 10% sodium azide solution. Alkaline phosphatase was revealed by addition 50 µl of a solution of 1 mg/ml p-nitrophenyl phosphate (Sigma, St. Louis, MO, USA) diluted in 0.1 M Tris-HCl buffer, pH 8.2, added with 1.5 M NaCl, followed by an incubation for 30 min at 37°C. The reaction was stopped by addition 50 µl of 1 M NaOH.

Optical density was measured at 492 nm for peroxidase activity and at 414 nm for alkaline phosphatase activity in an automated ELISA reader (Titertek Multiskan MCC/340 – Lab Systems, France).

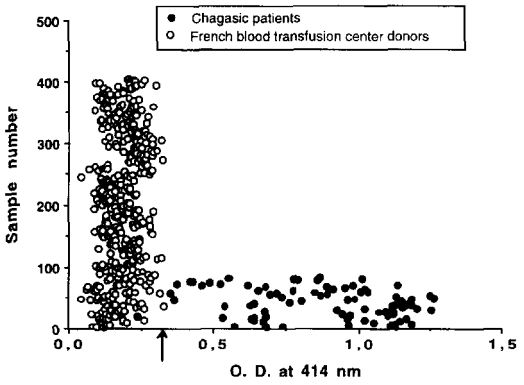

Fig. 1. Distribution of O.D. values obtained from IgG *T. cruzi* ELISA with control serum samples from French blood transfusion center donors ($n=405$) (○) and with serum samples from chagasic chronic patients ($n=89$) (●). The cut off value is equivalent to 0.302 (↑).

Sera were considered as positive when the mean of duplicate optical density values reached a value equivalent to or more than the mean value obtained from the 405 controls ±2 SD.

3. Results

Serum samples from French blood transfusion center donors and from patients with Chagas' disease were analyzed by ELISA using a crude extract of *T. cruzi* trypomastigotes derived from in vitro cell cultures. The cut off values for IgM and IgG *T. cruzi* antibodies were determined comparing the values obtained from a population of 405 French blood transfusion center donors with the ELISA measurements of 89 chagasic patients. In the present study, the cut off evaluated at 0.302 was equivalent to the mean optical density value of the 405 uninfected individuals ±2 SD ($0.186 \pm 2 \times 0.058$). The cut off value for IgM antibodies determined under the same conditions was equivalent to 0.153 (not shown).

The distribution of optical density of negative and positive samples for IgG antibodies is shown in Fig. 1. This allows the frequency of false negatives in the chagasic population and of false positives in the control population to be determined. At this cut off value, the percentage of false negatives and false positives were 1.1% (1 of 89) and of 1.7% (7 of 405), respectively.

Table 2
Sensitivity (i.e., true positive ratio), specificity, and 1-specificity (i.e., false positive ratio) of specific *T. cruzi* IgG ELISA determined around the threshold positive value obtained from 52 control serum samples from Brazil

Threshold (O.D.)	Sensitivity	se (%)	Specificity	Sp (%)	1-Sp
0.240	89:89	100	36:52	69.2	0.308
0.302	88:89	98.8	45:52	86.5	0.135
0.333	88:89	98.8	50:52	96.1	0.039
0.353	88:89	98.8	52:52	100	0.000

The sensitivity and specificity of the ELISA was determined by ROC (Receiver Operating Characteristic) curves [19]. The sensitivity represents the ratio of patients that have the infection (true positives) to the total patients (true positives+false negatives). The specificity represents the ratio of control individuals (true negatives) to the total negatives (true negatives+false positives). In Table 1, the values of true positives and false positives (1−specificity) determined at four different thresholds in the IgG antibody response are shown. For the cut off of 0.302, the sensitivity was evaluated as 98.8% and the specificity, considering only the serum samples from nonendemic region, was evaluated as 98.3%. The predictive value of a positive result representing the probability that the subject was infected when the test was positive, was estimated as 92.6% (88 positive samples/88 positive +7 false-positive samples). The predictive value of a negative result representing the probability that the subject was noninfected when the test was negative, was estimated as 0.25% (1 false-negative sample/1 false-negative +398 negative samples). The percentage of correctly classified samples was determined to be 98.3% (88 positive samples from chagasic patients +398 negative samples from control individuals/494 total samples).

Table 2 depicts the different values of true positives and false positives (1−specificity) determined at four different thresholds for the IgG antibody response, in an endemic region. Fifty-two serum samples from the Hemocentro of Brasilia (Brazil) were selected as negative controls (serology-negative by indirect hemagglutination and indirect immunofluorescence). The sensitivity was evaluated to 98.8% at the different thresholds, i.e., 0.302, 0.333, 0.353. The different values of the specificity were 86.5%, 96.1% and 100% for the O.D. values of 0.302, 0.330 and 0.353, respectively. The predictive values of positive and negative results for IgG determination in the endemic region are shown in Table 3.

IgM anti-*T. cruzi* responses were evaluated with 7 samples of well-characterized acute chagasic patients. All these samples were positive compared to the cut off value of 0.153. In this case, the specificity was equivalent to 95.5% with the 405 samples of French blood transfusion center donors and the false positives were evaluated to 4.6%. In contrast, serum samples from congenital cases ($n = 6$) were negative for IgM anti-parasite responses. Fig. 2 shows the distribution of O.D. values for acute and congenital cases of Chagas' disease.

To evaluate the specificity of this *T. cruzi* ELISA towards other protozoan infections, 35 malaria patients from Africa or Asia were analyzed. All of them were negative for detection of *T. cruzi* IgM and IgG antibodies (estimated specificity = 100%). When patients with leishmaniasis were assessed for *T. cruzi* IgM and IgG antibodies, 50 out of 51 patients (estimated specificity = 98%) presented a negative reactivity. Consequently, the estimated specificity was equivalent to 96.8% for the 32 leishmania samples from Bolivia and was equivalent to 100% for the 19 leishmania patients outside the endemic regions for Chagas' disease, i.e., kala-azar.

Because the risk of *T. cruzi* transmission by blood

Table 3
Predictive values of positive and negative results and percentage of well-classified individuals in endemic region

Threshold (O.D.)	Predictive value (%)		Well-classified (%)
	Positive	Negative	
0.302	92.6	2.2	94.3
0.333	97.8	1.96	97.9
0.353	100	1.92	99.3

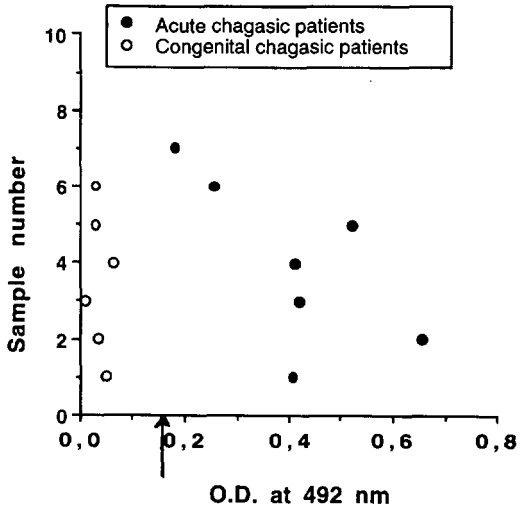

Fig. 2. Distribution of O.D. values obtained from IgM *T. cruzi* ELISA with serum samples of acute ($n=6$) (●) or congenital ($n=7$) (○) cases of Chagas' disease. The cut off value is equivalent to 0.153 (↑).

transfusion remains a public health problem in endemic regions, we analyzed 285 serum samples of blood donors of: (i) the Hospital Eva Peron, located in the suburbs of Buenos Aires, where the majority of blood donors was represented by migrants from endemic areas of North Argentina, and (ii) the Hemocentro from Brasilia, collecting blood samples from hyperendemic regions of Brazil (Bahia, Goias, Minas Gerais, Pernambuco, Paraiba), for the presence of IgM and IgG *T. cruzi* antibodies. Among them, 67 out of 285 serum samples (23.5%) and 184 out of 285 (64.5%) were positive for IgM and IgG *T. cruzi* antibodies, respectively. The very high level of positivity among these donors may be due to the selection of them in blood centers where the majority of people is coming from *T. cruzi* hyperendemic regions.

4. Discussion

The *Trypanosoma cruzi* IgM and IgG ELISAs were developed in order (i) to screen blood bank donors, (ii) to perform diagnosis of Chagas' disease. This ELISA, for a cut off representing the mean value of 405 samples ±2 SD was highly sensitive for specific IgG detection. In the present study, the sensitivity was estimated to be 98.8% both with serum samples of individuals from endemic or nonendemic regions accompanied with a low frequency of false negatives (1 out of 89) (Tables 1 and 2). In nonendemic region, the specificity was estimated to be 98.3% with a low frequency of false positive (0.017) and without cross-reactivity with sera from patients with kala-azar (n=19) or malaria (n=35). In endemic region, where the prevalence of the infection was high, it was convenient to increase the cut off value in order to get a higher specificity. For example, the specificity reached 100% at the cut off of 0.353, with a similar sensitivity of 98.8% and absence of false positive among the sera of nonchagasic Brazilian individuals (characterized by two different tests negative for *T. cruzi* infection). At this cut off value, the percentage of well-classified patients was estimated to 99.3%. Recent data provided by multicentre studies with recombinant polypeptides have shown that the majority of parasite recombinant antigens gave a 100% specificity, like in our present work [20,21]. Nevertheless, the sensitivity of that type of assay was found to be lower than 98%. Table 4.

Transmission of Chagas' disease in regions that are not endemic for Chagas' disease has been recently reported, suggesting that control of blood bank donors is necessary in countries where immigrants coming from areas endemic for Chagas' disease are increasing. In fact, the majority of seropositive chagasic patients lacked clinical signs of disease

Table 4
Lack of cross-reactivity of trypomastigote *T. cruzi* ELISA with other protozoan parasitosis

	Positive samples	Negative samples	Specificity (%)
Patients with malaria ($n=35$)	0	35	100
Patients with leishmaniasis			
Kala-azar ($n=19$)	0	19	100
Muco-cutaneous from Bolivia[a] ($n=32$)	1	31	96.8

[a]These patients were coming from areas in Bolivia endemic both for leishmaniasis and for Chagas infection.

and had not been submitted to active search for the infection. The number of chronic patients with positive serology for Chagas' disease remains high in endemic areas where WHO estimated some 18 million people to be infected (WHO Special Program for Research and Training in Tropical Diseases, TDR News, November 1996). This is a strong argument for testing blood bank donors in Latin America, and for considering a possible risk of blood transmission in countries where infected people are migrating. The risk of infection appeared higher in immunosuppressed patients submitted to organ transplantation [11,12,18].

Detection of anti-*T. cruzi* IgM provides a test to discriminate in newborns and young children between congenital (IgM negative) and acute (IgM positive) cases of infection. High levels (mean ratio = 3.14 ± 1.2, range 1.41 to 5.05) of antiparasite IgM were detected in seven acute cases, while sera from congenital cases were negative for IgM and only positive for IgG, until 6 months after birth. Preliminary studies in a Bolivian endemic area, where vectorial transmission is still occurring, indicate that the anti-*T. cruzi* IgM detection by ELISA was suitable for screening a population of recently infected young children, when compared to PCR and direct parasite detection [22].

The results herein clearly indicate that the *T. cruzi* trypomastigote extract can be used as an excellent reagent for detecting the human infection, in regions where Chagas' disease is not prevalent. It seems to perform better than the epimastigote extracts giving lower background and showing almost no cross-reactivity with other protozoan infections. Regarding the complexity of infection in endemic countries, it can be hypothesized that a combination of this type of reagent with recently characterized recombinant peptides may lead to the development of a new generation of reagents for Chagas' disease, combining the outstanding performance of the trypomastigote extract, with the information derived from the reaction of a serum sample with each of the parasite recombinants. Interestingly, recent studies with recombinant polypeptides have shown the interest of such reagents to analyse the status of the disease and to perform epidemiological studies. Thus, recombinant polypeptides were shown to detect anti-SAPA (Shed Acute Phase Antigen) responses in sera from acute or congenital cases [23,24] and anti-JL5 or R-13 responses in sera from patients with severe Chagas heart disease or congenital cases [25,26], and to be useful for demonstrating lack of anti-p24 responses as a criterion to follow the efficacy of chemotherapy [27].

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