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Chagas' disease diagnosis: a multicentric evaluation of Chagas Stat-Pak, a rapid immunochromatographic assay with recombinant proteins of *Trypanosoma cruzi*

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Abstract

A rapid serologic test for diagnosis of *T. cruzi* infection (Chagas Stat Pak) was developed using recombinant proteins in an immunochromatographic assay. This cassette format test was evaluated first in blind with a panel of 393 coded serum samples. The Chagas Stat-Pak identified 197 infected (98.5% sensitivity) and 183 non-infected individuals (94.8% specificity). A second evaluation was performed with 352 sera from four Latin America countries tested independently in each country, showing a sensitivity of 100% and specificity of 98.6%. A third set of tests comparing sera with plasma and eluates from filter paper as well as serum preserved in 50% glycerol did show identical results as those obtained with serum. This rapid test (15 min) uses one device per sample, does not require refrigeration nor a laboratory structure or specialized skills to be performed, accepts different types of samples and may be stored for long periods of time for result checking and documentation. These attributes together with the high sensitivity and specificity demonstrated herein, make this test a suitable tool for field studies, small laboratories and emergencies at blood banks in the countryside of endemic areas.

1. Introduction

Chagas' disease, caused by the protozoan parasite *Trypanosoma cruzi*, is a debilitating illness affecting 16 to 18 million people in Latin America. Serologic diagnosis of *T. cruzi* infection is usually performed in endemic countries using two or three tests according to availability. Enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence (IIF) and indirect hemagglutination assay (IHA), also referred to as conventional tests, are often used. They usually employ crude antigenic *T. cruzi* preparations, are produced as kits, and are commercially available. Providing that good quality kits are selected and good laboratory practices followed, it is possible to obtain a sensitivity of 95 to 99% with the tests above mentioned. The sensitivity may be increased if a second test is included, as recommended by the World Health Organization (WHO, 2002). The specificity is lower with conventional tests (93-98%). Cross-reactivity to *T. cruzi* antigens has been detected in serum from patients with leishmaniasis (Chiller et al., 1990) or

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Trypanosoma rangeli infection (Guhl et al., 1987). In order to avoid false positives, the use of recombinant antigens and/or synthetic peptides has been proposed with success (Moncayo and Luquetti, 1990; Carvalho et al., 1993; Pastini et al., 1994; Peralta et al., 1994; Houghton et al., 1999 and 2000; Umezawa et al., 1999; Oelemann al., 1999; Saez-Alquezar et al., 2000; Rabelo et al., 1999; Franco da Silveira et al., 2001).

An ideal diagnostic test should be easy to perform, fast, involve a few steps, and have high sensitivity and specificity. Conventional tests are time consuming and include several steps, which increase the likelihood of errors by the operator (Luquetti and Rassi, 1999). ELISA is amenable for automation, but at an increased cost that cannot usually be afforded by most laboratories in underdeveloped countries, where *T. cruzi* infection is more prevalent. Many blood banks and routine diagnosis laboratories work with few samples per day, which makes automation impractical. Although IHA would be suitable in these conditions, it is not recommended as a single test, especially in blood banks, because its sensitivity is relatively low (96-98%) with the associated risk of liberating infected units of blood (WHO, 2002).

In a previous work (Umezawa et al., 1999), six *T. cruzi* recombinant antigens were evaluated by ELISA using sera from chagasic and non-chagasic individuals from nine countries of South and Central America. Based on the performance of each recombinant antigen (sensitivity and specificity values), three recombinant antigens were combined in a single ELISA resulting in a multi-antigen test that was very sensitive and specific for the diagnosis of Chagas' disease (Umezawa et al., 2003). Based on these results, a novel immunochromatography assay (Chagas Stat-Pak) for diagnosis of Chagas' disease was developed by combining relevant *T. cruzi* antigens. The objective of the present work was the evaluation of this multi-antigenic recombinant assay that accomplishes with some of the conditions of an ideal test: one step, results recorded by naked eye in few minutes, and high sensitivity and specificity. The diagnostic performance of the mixture of recombinant antigens was compared in blind with the conventional serology (ELISA, IIF and IHA). Furthermore, results obtained in field trials with sera from different countries are also reported.

2. Materials and Methods

2.1. Chagas Stat-Pak test

Chagas Stat-Pak (Chembio Diagnostic Systems, Medford, NY, USA) is a rapid immunochromatographic screening test for detection of anti-*T. cruzi* antibodies in serum, plasma or whole blood. It employs a combination of a specific antibody-binding protein, which is conjugated on dye particles, and *T. cruzi* recombinant antigens which are bound to the membrane. As the test samples flow laterally through the membrane, the antibody-binding protein-dye conjugate binds to human immunoglobulins in the sample. If the sample contains anti-*T. cruzi* antibodies the complex binds to the antigens on the solid phase in the test window producing a pink/purple band. In the absence of specific antibodies there is no line in the positive reaction zone. The liquid continues to migrate along the membrane and produces a pink/purple band in the control zone confirming that the reagents are functioning properly (Figure 1). The test can be read as soon as the pink control line appears, which usually occurs within 5 to 15 min.

Five μL of serum are placed in the sample hole of the cassette and 240 μL of buffer, provided with the kit, are added. In approximately 5 min, the serum/buffer mixture migrates to the top of the device. The end of the reaction is indicated by the development of a colorful line on the top showing the positive control. Reading the sample region can be done immediately after the test is performed, recording a strong or weak line as positive and its absence as negative (Figure 1). Reading may be done also days after, since the result has been the same even in devices stored for more than three years at room temperature.

2.2. Conventional serologic tests

Serum samples from chagasic and non-chagasic individuals (healthy individuals and patients with leishmaniasis and other diseases) were selected by each participating center (Argentina, Bolivia, Brazil, Honduras and Venezuela) and their status determined with conventional serologic techniques (IIF, ELISA and IHA) for *T. cruzi* infection using crude epimastigote extracts as antigens. Since some reagents and methods used in the conventional serology could present variations, all sera employed in this work were sent just to the laboratory of one of us (AOL, Goiania, Brazil) for confirmation with IHA, IIF and ELISA. A commercial kit for IHA (Chagas HAI-Immunoserum-Tecnologia Imunológica Industria e Comércio, São Paulo, Brazil) was used. The results were expressed as the reciprocal of titer dilution and titers equal to 8 or above, after 2-β mercaptoethanol treatment, considered positive (Zicker et al., 1990). IIF was performed using formaldehyde-treated epimastigote forms of *T. cruzi* (Y strain) and a fluorescein-isothiocyanate-conjugated sheep anti-human IgG (Fluoline G, Biolab Diagnóstica SA, RJ, Brazil), and results were considered as positive above the 1/40 dilution (Zicker et al., 1990). ELISA was performed using a crude extract of Y strain epimastigotes and results were expressed as an index obtained dividing the optical density (OD) of the sample by the OD of the plate cut-off. An index equal or higher than 1.2 was considered positive (Zicker et al., 1990). Detection of anti-*T. cruzi* antibodies by the three serologic tests was the criterion used to characterize the infected individual. Patients with leishmaniasis or with autoimmune diseases, such as systemic lupus erythematosus, were chosen by epidemiologic, clinical and parasitological data. Muco-cuta-

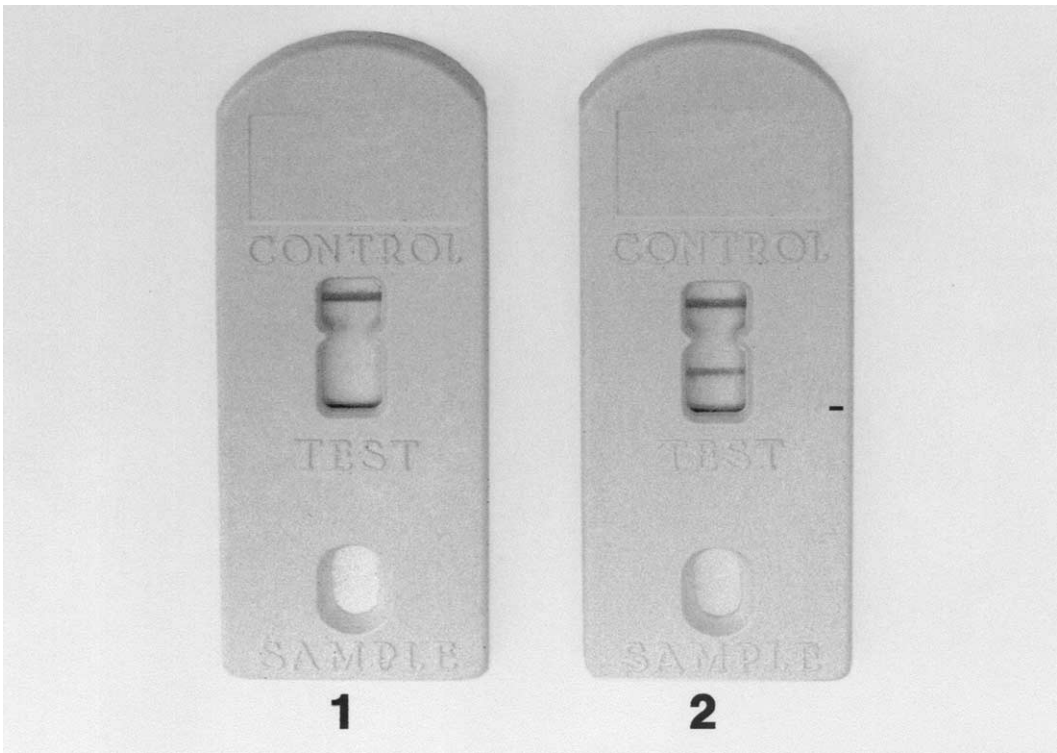

Fig. 1. Typical diagnosis test with Chagas Stat-Pak. 1) Serum sample from a healthy individual. Only one pink band appears in the control window, and no visible band in the test window. 2) Serum sample from a chronic chagasic patient. In addition to the control band, a clearly visible pink band also appears in the test window.

neous leishmaniasis was certified by skin biopsies. All visceral leishmaniasis patients had amastigote forms of *Leishmania* sp in bone marrow aspirates. Four cases of suspected simultaneous infection with *T. cruzi* could not be assessed because patients did not return after treatment for kala azar.

2.3. Serum selection

A panel of 393 sera from patients from endemic areas of Central Brazil, mainly Goiás, Minas Gerais and Bahia states was selected at one of the laboratories involved (AOL, Goiania, Brazil) and sent to another participating laboratory (ESU, São Paulo, Brazil) where the samples were tested with the Chembio Chagas Stat-Pak device in blind. Samples were sent at 4°C in 50% glycerol to preserve the original reactivity of each serum. Out of the 393 sera, 200 samples were from well-defined chagasic patients (age limits 8 to 69 years old, mean 45.3 years) presenting with cardiopathy and/or megaesophagus and/or megacolon. These sera presented three positive tests of conventional serology (IIF, IHA, ELISA). An additional 150 samples were selected from healthy individuals, matched by sex and age, and with three negative tests for *T. cruzi* infection. Another group comprising 43 serum samples were from non-*T. cruzi* infected individuals, with other diseases: 9 with kala azar, 10 with muco-cutaneous leishmaniasis, 11 with hepatitis B diagnosed by positive HbsAg, 3 with active HIV-infection and 10 with autoimmune diseases (systemic lupus erythematosus, esclerodermia, with or without rheumatoid factor). Three conventional tests were performed also in this group of sera, with variable results: most of sera from kala azar and some from muco-cutaneous leishmaniasis had positive IIF test titers from 1/40 to 1/320.

On the other hand, each one of the participating laboratories selected their own samples: Honduras (CP and EP), 204 samples (157 chagasic and 47 non-chagasic individuals); Venezuela (NA), 45 samples (40 chagasic and 5 non-chagasic individuals); Bolivia (SR), 21 samples (10 chagasic and 11 non-chagasic individuals); Argentina (AS and MJL), 82 samples (72 chagasic and 10 non-chagasic individuals). All serum samples of chagasic patients ($n = 279$) and non-chagasic individuals ($n = 73$) were re-tested with three conventional serologic tests (IHA, IIF, ELISA) at the laboratory of one of us (AOL, Goiania, Brazil).

2.4. Detection of antibodies in different biologic specimens

The performance of the kit with samples diluted (50%, v/v) in buffered glycerol was compared with that in non-diluted sera in one of the participating laboratories (CP and

EP, Tegucigalpa, Honduras). Serum samples were collected from 29 blood donors (25 positives and 4 negatives for *T. cruzi* infection in the conventional serology), 14 chronic chagasic patients with positive serology for Chagas' disease (2 asymptomatics and 12 with cardiopathy), and 6 patients with leishmaniasis (3 muco-cutaneous and 3 visceral leishmaniasis cases) with negative serology for Chagas disease. Five μL of serum without dilution or 10 μL of serum diluted in glycerol were applied in the Chagas Stat-Pak device. The effect of the use of plasma (5 μL) from blood samples collected in sodium citrate or EDTA and stored at 4°C was tested in 8 blood donors (3 positive for Chagas' disease and 5 normal individuals).

The possibility of employing filter paper as support of whole blood was tested. Dried blood samples from 24 blood donors (13 positives and 11 negatives for Chagas' disease in the conventional serology) were eluted and tested. Elution from the filter paper (circles of 6 mm of diameter) with 50 μL of Stat-Pak diluent or 0.9% NaCl was performed, and 20 μL were used in the test. Finally, 10 μL of whole blood instead of serum, was tested directly in the cassette and compared with results obtained from serum of ten blood donors.

3. Results

The evaluation of the Chagas Stat-Pak test was performed in three steps. In the first one, a blind trial with a high number of serum samples was done by selecting sera from one laboratory and performing the test in blind in a different laboratory. Secondly, an open trial was carried out in different countries, where the sera were selected and the test performed. Finally, the performance of the test with samples different from sera (plasma, etc) was evaluated.

3.1. Blind trial with Chagas Stat-Pak test

Each serum received in blind from the panel of 393 was tested by one of us (ESU, São Paulo, Brazil) using the Chagas Stat-Pak device. Reading on each device was carried out by three different observers as positive or negative and recorded accordingly. Once codes were broken, those sera showing discordant results ($n = 13$) when comparing conventional serology and Chagas Stat-Pak were tested again and results scored again by three independent observers.

Table 1 shows the results of the evaluation of the Stat-Pak test with consensus positive and negative sera from chagasic patients ($n = 200$) and healthy individuals ($n = 150$), respectively. Three out of 200 positive samples were negative with the Chagas Stat-Pak, giving a sensitivity of 98.5%. The three false-negative samples were from adults with positive conventional serology and clinical symptoms for the digestive form of Chagas' disease. Six out of 150 healthy individuals samples gave a false-positive reaction with the Chagas Stat-Pak test. Results using sera from patients with other diseases that can potentially crossreact with *T. cruzi* antigens are also shown in Table 1. Four samples, two from patients with kala azar and two patients with hepatitis B, generated positive readings with the Chagas Stat-Pak test. It is interesting to note that 10 out of 19 samples (53%) from patients with leishmaniasis were positive in the three conventional serologic tests for Chagas' disease. There was no false positive result in the group of sera from patients with other diseases. These results show that the specificity of the Chagas Stat-Pak test was 94.8% (10 false positives out of 193 samples).

Table 1
Reactivity of Chagas Stat-Pak test with sera from chagasic patients, healthy individuals and patients with other diseases

Status of Infection	Number of Individuals	Positive cases with	
		Stat-Pak[a]	CS[b]
Chagasic patients	200	197	200
Healthy individuals	150	6	0
Patients with other diseases:			
Visceral leishmaniasis	9	2	9
Muco-cutaneous leishmaniasis	10	0	1
AIDS	3	0	0
Hepatitis B	11	2	0
Autoimmune Diseases	10	0	0
Total other diseases	43	4	10

[a] Stat-Pak Sensitivity = 98.5% and Specificity = 94.8%.
[b] CS, Conventional Serology determined by IIF, IHA, and ELISA.

3.2. Chagas Stat-Pak test with serum samples from patients from different geographical regions

The diagnostic efficiency of the Chagas Stat-Pak test was evaluated with 352 serum samples from four different countries. The tests were performed by each laboratory involved and the results are shown in Table 2. In three countries all the results obtained with the rapid test matched with those obtained with conventional serology. In Argentina one false positive out of 10 samples was found even after repetition of this sample. The sensitivity and specificity of Chagas Stat-Pak test was 100% and 98.6%, respectively (Table 2).

3.3. Chagas Stat-Pak test in different conditions and biologic samples

The possibility to employ preserved sera was confirmed by one of the participating laboratories (Honduras) using serum samples diluted in glycerol (50%, v/v) upon comparison with non-diluted sera (Table 3). There was no interference of the diluent in the performance of the test: anti-*T. cruzi* antibodies were detected in both non-glycerinated and glycerinated serum samples. Full agreement with conventional serology for Chagas' disease was obtained. Reliable results were also obtained using plasma (5 μL) from blood

Table 2
Diagnostic performance of Chagas Stat-Pak test with serum samples from different Latin America countries

Country	Number of Individuals	Chagasic		Non-chagasic	
		Positive/total		Positive/total	
		Stat-Pak[a]	CS[c]	Stat-Pak[b]	CS[c]
Honduras	204	157/157	157/157	0/47	0/47
Venezuela	45	40/40	40/40	0/5	0/5
Bolivia	21	10/10	10/10	0/11	0/11
Argentina	82	72/72	72/72	1/10	0/10
Total	352	279	279	1/73	0/73

[a] Stat-Pak Sensitivity = 100% and [b] Specificity = 98.6%.
[c] CS, Conventional Serology determined by IIF, IHA and ELISA.

samples collected in sodium citrate or EDTA and stored at 4°C. When results from eluates of filter paper as support of whole blood were compared with those obtained with serum from the same individuals, identical output was recorded. Results obtained with serum and whole blood agreed with conventional serology for Chagas' disease. Studies performed with plasma, diluted-glycerol samples, whole blood and eluates obtained from filter paper showed similar results, indicating that this assay may be employed with any of these supports or methods of preservation.

4. Discussion

In a blinded study, Chagas Stat-Pak test displayed 98.5% sensitivity with sera from endemic regions for Chagas' disease in Central Brazil (Table 1). Excluding serum samples from patients with other diseases ($n = 43$), sera were classified as either consensus positive ($n = 200$) or negative ($n = 150$) (Table 1) by matched results in three conventional serologic tests (IIF, IHA and ELISA). The percentage of agreement with positive and negative consensus sera was 98.5% and 96.0%, respectively. The specificity of the Chagas Stat-Pak test was also challenged with a collection of 19 sera from patients with leishmaniasis whose sera cross-reacted in the conventional serology for Chagas' disease (10 false positives out of 19 samples). Two of these sera (10.5%) reacted in the Chagas Stat-Pak, indicating a fairly optimized specificity with respect to cross-reactive antibodies specific for *Leishmania.*

It has been suggested that variability among *T. cruzi* isolates in conjunction with immunogenetic features of the human host could influence the performance of serologic assays. *T. cruzi* is not a homogeneous population of parasites; but consists of a pool of sub-populations that present a high heterogeneity in biologic parameters and genetic characteristics (Souto et al., 1996; Briones et al., 1999; Machado and Ayala, 2001). Furthermore, in a recent consensus meeting, the *T. cruzi* species were divided into two major phylogenetic lineages: *T. cruzi* I and *T. cruzi* II

Table 3
Detection of anti-*T. cruzi* antibodies by Chagas Stat-Pak in different biological specimens

Samples	Clinical Status	CS[a]		Stat-Pak	
		P[b]	N[c]	P[b]	N[c]
Glycerol diluted sera (v/v)[d]	Blood donors (n = 29)	25	4	25	4
	Chronic chagasic patients (n = 14)	14	0	14	0
	Patients with leishmaniasis (n = 6)	0	6	0	6
Whole blood[e]	Blood donors (n = 10)	6	4	6	4
Dried blood on filter paper[f]	Chronic chagasic patients (n = 13)	13	0	13	0
	Healthy individuals (n = 10)	0	10	0	10
Plasma[g]	Blood donors (n = 8)	3	5	3	5

[a] CS, Conventional Serology determined by IIF, IHA and ELISA.
[b] P, positive results with CS or Chagas Stat-Pak
[c] N, negative results with CS or Chagas Stat-Pak
[d] Serum diluted in glycerol (final concentration 50%) and stored at 4°C. Ten μL were applied on Chagas Stat-Pak apparatus.
[e] Ten μL of fresh blood samples were applied on Stat-Pak apparatus.
[f] Dried blood samples were eluted from the filter paper (circles of 6 mm diameter) with 50 μL of Chagas Stat-Pak diluent and 20 μL were applied on the cassette.
[g] Blood samples were collected in sodium citrate or EDTA and stored at 4°C. Five μL of plasma were applied on Chagas Stat-Pak cassette.

(Satellite Meeting, 1999). For this reason the Chagas Stat-Pak was also evaluated with sera from different regions of Latin America. Sensitivity and specificity of Stat-Pak were high in Central America, Venezuela and Bolivia, considered to be endemic areas with relatively high prevalence of *T. cruzi* infection. Although a relatively small number of samples has been analyzed, and most parasites isolated previously from human chronic phase infected individuals were *T. cruzi* II (Luquetti et al., 1986; Souto et al., 1996), the Chagas Stat-Pak gave comparable results in the different geographical regions studied.

Serologic methods are widely accepted for the immunodiagnosis of *T. cruzi* infection. Most tests that are commercially available today employ crude or semi-purified parasite fractions as antigens. Among them, IIF, IHA and ELISA have been commercially available for a long time and consequently are the most often used in clinical laboratories and blood banks. In developing countries of Latin America many blood banks, routine diagnostic laboratories and peripheral hospitals work with few samples per day, and often they can not afford the equipments and technicians normally employed in the high-throughput diagnosis systems.

The new generation of serodiagnostic tests for Chagas' disease employing *T. cruzi* recombinant antigens and synthetic peptides must require adaptation to these local facilities. Therefore, a rapid immunochromatographic assay (Chagas Stat-Pak) was developed employing a mixture of *T. cruzi* recombinant antigens (Umezawa et al., 1999 and 2003). This test allows the reaction to be viewed by naked eye against a background that is almost white. Other proven advantages of this test are the extremely easy manipulation, the short time for execution and reading and the lack of any apparatus for incubation, centrifugation or mechanical reading, which made it ideal for field studies. Due to the easy manipulation it does not require high skills in order to be performed and may be stored in the field, at room temperature. Another advantage, important for epidemiologic serologic surveys, is the preservation of the reaction on the cassette, without need of refrigeration and the possibility to carry out the test in the field and the readings be checked afterwards at the centers, by learned staff.

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