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Short Report: Evaluation of In-House ELISA Using *Trypanosoma cruzi* Lysate and Recombinant Antigens for Diagnosis of Chagas Disease and Discrimination of Its Clinical Forms

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Abstract. The aim of this work was to investigate the potential usefulness of *Trypanosoma cruzi* lysate, recombinant protein JL7, and peptides P013, R13, JL18, JL19, and P0β as serological markers for human Chagas disease. We analyzed 228 sera from Brazilian Chagas disease patients classified into four clinical groups and 108 from non-chagasic patients. We defined the diagnostic sensitivity, specificity, and Kappa index measured by enzyme-linked immunosorbent assay (ELISA). As previously described, the highest values of diagnostic parameters were achieved for *T. cruzi* lysate and JL7; peptide P013 showed high specificity but low sensitivity. The other peptides resulted in lower sensitivity and specificity in our ELISA than *T. cruzi* lysate and JL7 protein. Antibodies against JL7 protein were mainly detected in sera from patients with severe chagasic cardiomyopathy, compared with those from the indeterminate form, whereas peptides failed to discriminate between the clinical forms of the disease.

Chagas disease, caused by the hemoflagellate *Trypanosoma cruzi*, is a widespread tropical disease affecting at least 8 million people primarily in Latin American countries.[1] The acute phase lasts 1 or 2 months and is usually symptomless,[2] however most of the infected individuals enter a life-long chronic phase with its asymptomatic and symptomatic forms. The asymptomatic, also called indeterminate form is characterized by the absence of clinical symptoms. However, ~40% of persons with chronic *T. cruzi* infection develop symptoms of visceral damage, which may include cardiac lesions, digestive alterations, or both clinical manifestations (cardiac plus digestive).[2]

In the chronic phase, the primary method for diagnosis is the search of antibodies by serological testing, whereas the secondary diagnostic techniques are parasitological tests.[1] So far, conventional serological tests include complement fixation,[3,4] indirect immunofluorescence assay (IFA),[5] indirect hemagglutination assay (IHA),[6] direct agglutination with 2-mercaptoethanol (DA-2ME),[7] and enzyme-linked immunosorbent assay (ELISA).[8–10] These tests usually use crude or semi-purified parasite preparations, often derived from a stage present only in the insect and in cultures at 26°C (epimastigote) but absent in the human host. Other assays incorporate more defined parasite components, like multiple fusion proteins containing epitopes from various *T. cruzi* antigens.[11–15] Moreover, a rapid immunochromatographic assay (Chagas Stat-Pak, Chembio Diagnostic Systems, Medford, NY) has also been developed by employing a mixture of *T. cruzi* recombinant antigens, presenting a different degree of performance.[16–18] However, in the absence of a true gold standard, it is still necessary to carry out at least two different serological tests to establish a reliable diagnosis of Chagas disease. Indeed, the World Health Organization (WHO) consensus guidelines recommend to perform a third assay or repeat sampling to confirm or exclude the diagnosis if two serological tests are in disagreement.[19]

In 1991, a Workshop organized by the Ibero-American Project of Biotechnology evaluated the diagnostic potential of phage expressed *T. cruzi* recombinant antigens, Antigen 2, Antigen 13, SAPA, H49, A13, JL5, JL7, JL8, JL9, and RAI by phage dot array immunoassays.[20] Results showed that the best recombinant antigen to be used in serodiagnosis of chronic *T. cruzi* infection was JL7 (also called H49 or Antigen 1), however a more efficient format than dot array was suggested.[20] In addition, the serodiagnostic efficiency of the ELISA for six recombinant antigens (H49, JL7, B13, JL8, A13, and 1F8) was carried out by using sera from different geographical areas of Latin America.[21] One of the goals of that work was to shed light on the usefulness of combined antigens to minimize the individual variation and promote a high sensitivity test for the routine diagnosis of Chagas disease.[21]

Until now, an effective assay for predicting the clinical evolution of a chronic infection is lacking.[15,22,23] Accordingly, in this study, we evaluated the diagnostic potential of different *T. cruzi* antigens, namely peptides P013, R13, JL18, JL19, and P0β by ELISA,[21] and investigated the potential use of

Table 1

Clinical features of the study population*

Serology	Disease form	No. of individuals	Age limits (years)	Mean age (years)	Sex (male/female)
Positive	IC	58	12–51	35.3	25/33
	DC	59	12–55	35.9	34/25
	MCC	57	24–55	39.2	29/28
	SCC	54	14–57	43.1	39/15
Negative	HI	59	19–55	39.6	35/24
	nCh	49	12–55	37.8	28/21
Total		336	12–57	38.5	190/146

*IC = indeterminate form of Chagas disease (normal electrocardiogram (ECG) and thoracic radiography with normal esophagus and colon x-rays); DC = digestive form of Chagas disease (megacolon and/or megaesphagus with normal ECG and thoracic radiography); MCC = mild chagasic cardiomyopathy (altered ECG, such as complete right bundle branch block, anterior left fascicular block, premature ventricular beating and alterations in ventricular repolarization with normal chest radiography and normal esophagus and colon x-rays); SCC = severe chagasic cardiomyopathy (altered ECG, high frequency of premature heart beating, ventricular auricular block, atrial fibrillation, clinical symptoms of cardiac alterations and cardiomegaly visualized by thoracic radiography); HI = healthy individual (normal ECG); nCh = no Chagas patients (other diseases).

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TABLE 2
Sensitivity, specificity, and Kappa index of each antigen determined by ELISA*

Antigens	Median values (AU)		Sp (%)	Se (%)	Kappa index
	ChD (*N* = 228)	nChD (*N* = 108)			
Trypanosoma cruzi	4.31 (2.61–6.05)	0.39 (0.09–1.34)	89.8	99.6	0.91
JL7	4.97 (1.11–9.99)	0.55 (0.12–0.89)	100	95.2	0.93
P013	2.46 (0.35–13.64)	0.20 (0.00–0.60)	97.2	82.5	0.74
R13	1.38 (0.21–10.29)	0.21 (0.01–1.64)	85.1	61.4	0.41
JL18	0.83 (0.22–2.66)	0.52 (0.11–1.59)	78.7	37.3	0.14
JL19	0.87 (0.20–2.44)	0.58 (0.05–1.67)	75.0	40.4	0.13
P0β	0.44 (0.00–7.04)	0.23 (0.00–1.98)	86.1	28.5	0.12

*ELISA plates were coated with 50 ng protein/well of *T. cruzi* epimastigote lysate, 2 μg/well of recombinant protein JL7 or 2 μM of peptides coupled to bovine serum albumin (BSA) in 50 μL buffer carbonate. Serum samples were diluted 1/400. All samples were tested in duplicate, and sera from six healthy individuals were loaded on the same plate to determine cut-off value, as the optical density (OD) mean value plus three standard deviations. Antibody level, in arbitrary units (AU), was calculated as ratio (OD value of each serum samples/cut-off value) and, the results were expressed as median values (5th–95th percentiles).
ChD = Chagas disease; nChD = no Chagas disease; N = number of individuals; Sp = specificity; Se = sensitivity.

T. cruzi lysate, JL7 protein, and these peptides as markers for prognosis of the disease. Peptide R13 (EEEDDDMGFGLFD) was derived from the 13 C-terminal amino acids of TcP2β, whereas P013 (EDDDDDFGMGALF) and P0β (AESEE) were derived from the C-terminal region of TcP0 protein.[24] Peptide JL18 (AYRKALPQEEEEDVGPRH) and the contiguous JL19 (VDPDFCRSTTQDAYRPVDP) were derived from *T. cruzi* recombinant protein JL9.[24]

Test sera were obtained from a careful selection of patients from the Laboratory of Chagas Disease, Hospital das Clinicas, Federal University of Goias-Goiania, Brazil. The existence of *T. cruzi* infection was assessed serologically by IHA, DA-2ME, IFA, and ELISA using *T. cruzi* Y strain (DTU Tc II) epimastigote form as antigen. Random codes were given to all samples and aliquots were sent to the Laboratorio de Biología Molecular de la Enfermedad de Chagas, INGEBI–CONICET, Buenos Aires, Argentina, for conformational testing. Moreover, 20% of sera were selected at random and recoded to use them as internal controls in different tests. At the end of the study, all codes (from Brazil) and test results (from Argentina) were sent to the manager of the Task Force on Chagas disease, Special Program for Research and Training in Tropical diseases (TDR-WHO), where codes and results were matched.

According to a conventional serological test (ELISA, IHA, IFA, DA-2ME) results, a total of 228 sera from patients with Chagas disease and 108 from patients without chagasic infection were used (Table 1). Coded sera from Chagas disease patients were classified into four groups according to clinical, electrocardiographic and radiological results, namely indeterminate form of Chagas disease (IC), digestive form of Chagas disease (DC), mild chagasic cardiomyopathy (MCC), and severe chagasic cardiomyopathy (SCC) (Table 1).

The remaining coded sera from individuals without Chagas disease were divided into two groups (Table 1), healthy individuals with normal ECG and sero-negative for *T. cruzi*, however born in endemic regions, and patients with other diseases, 5 with VL (Kala-azar), 4 with muco-cutaneous leishmaniasis, 19 with autoimmune diseases (12 with Systemic Lupus Erythematosus), 16 with cardiomyopathies of non-chagasic ethiology, and 5 with another disease such as juvenile diabetes, schistosomiasis, idiopathic megaesophagus, and South American Blastomycosis. Age limits in this group were from 12 up to 55 years of age (Table 1). All sera have had no antibodies (Ab) against *T. cruzi*, whereas in 1 out of 5 patients with visceral leishmaniasis (VL), the IFA test was positive. Furthermore, an additional six patients with VL (1–10 years of age) were tested and two of them also resulted seropositive for IFA test, pointing to the high degree of cross-reactivity between Chagas disease and VL.

We first analyzed the Ab levels against *T. cruzi* lysate, recombinant JL7 protein, and peptides P013, R13, JL18, JL19, and P0β by ELISA. As shown in Table 2, the Ab levels against *T. cruzi* lysate, JL7 protein, and peptides P013, R13, and P0β were higher in sera from Chagas disease patients compared with those from non-chagasic individuals. As previously reported,[12,20] *T. cruzi* lysate, and JL7 protein showed high sensitivity (Se) and specificity (Sp), providing the best Kappa indexes of 0.91 and 0.93, respectively, compared with the other tested antigens.

The peptides P013 and R13 presented Kappa values of 0.74 and 0.41, respectively (Table 2). For peptide P013, Sp and Se achieved a value of 97.2% and 82.5%, respectively; peptide R13 presented a Sp of 85.1% and a Se value of 61.4%. The remaining peptides resulted in Kappa indexes

TABLE 3
Prevalence of reactive serum samples from patients of non-chagasic ethiology and healthy individuals*

Antigens	nChD (*N* = 108)	HI (*N* = 59)	VL (*N* = 5)	ML (*N* = 4)	AD (*N* = 19)	Other diseases (*N* = 21)
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Trypanosoma cruzi	11 (10.2)	5 (8.5)	1 (20)	2 (50)	1 (5.3)	2 (9.5)
JL7	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
P013	2 (1.9)	0 (0)	0 (0)	0 (0)	2 (10.5)	0 (0)
R13	16 (14)	10 (16.9)	0 (0)	2 (50)	3 (15.8)	1 (4.8)
JL18	23 (20.2)	16 (27.1)	0 (0)	1 (25)	0 (0)	6 (28.6)
JL19	27 (23.7)	14 (23.7)	0 (0)	1 (25)	3 (15.8)	9 (42.9)
P0β	13 (12.1)	3 (5.1)	0 (0)	3 (75)	1 (5.3)	6 (28.6)

*nChD = no Chagas disease; HI = healthy individual; VL = visceral leishmaniasis (Kala-azar); ML = muco-cutaneous leishmaniasis; AD = autoimmune disease; Other Diseases = no chagasic cardiomyopathy, juvenile diabetes, schistosomiasis, idiopathic megaesophagus, and South American Blastomycosis; N = number of individuals.

TABLE 4
Prevalence of reactive serum samples from patients with different clinical forms of Chagas disease*

Antigens	ChD ($N = 228$) N (%)	IC ($N = 58$) N (%)	DC ($N = 59$) N (%)	MCC ($N = 57$) N (%)	SCC ($N = 54$) N (%)
Trypanosoma cruzi	227 (99.6)	57 (98.2)	59 (100)	57 (100)	54 (100)
JL7	217 (95.2)	54 (93.1)	56 (94.9)	54 (94.7)	53 (98.1)
P013	188 (82.5)	49 (84.5)	48 (81.3)	45 (78.9)	46 (85.2)
R13	140 (61.4)	35 (60.3)	36 (61.0)	35 (61.4)	34 (63.0)
JL18	85 (37.3)	19 (32.8)	14 (23.7)	26 (45.6)	26 (48.1)
JL19	92 (40.4)	22 (37.9)	16 (27.1)	30 (52.6)	24 (44.4)
P0β	65 (28.5)	16 (27.6)	18 (30.5)	9 (15.8)	22 (40.7)

ChD = Chagas disease; IC = indeterminate form of Chagas disease; DC = digestive form of Chagas disease; MCC = mild chagasic cardiomyopathy; SCC = severe chagasic cardiomyopathy; N = number of individuals.

lower than 0.15 (Table 2) caused by their low Se (< 40%), although their Sp were all higher than 75%. Interestingly, peptide P013 rendered only two false positive results and none of them were sera from healthy individuals (Table 3).

When the presence of Abs against these antigens was analyzed in sera of the patients with different clinical forms of chronic Chagas disease, we observed that the positivity of *T. cruzi* lysate, JL7, and peptides P013 and R13 were similar for all of them (Table 4). On the other hand, a higher prevalence of sera reactivity against peptides JL18 and JL19 was found in patients with severe or mild cardiomyopathy. Twenty-two out of 54 sera from patients with SCC (40.7%) recognized P0β peptide. Sera from patients with digestive disease showed a higher percentage of positivity with *T. cruzi* lysate, JL7, and peptides P013 and R13 (Table 4). We further considered whether Ab levels against the different antigens might allow us to differentiate among the clinical forms of Chagas disease. By using a non-parametric analysis for more than two groups (Kruskal-Wallis test), we observed a significant difference between the Ab levels elicited against JL7 protein ($P < 0.025$) and peptide JL18 ($P < 0.028$) by patients belonging to four clinical forms (Figure 1). No differences were observed with the other antigens (Figure 1 and data not shown). To determine which clinical form differed from each other, a Tukey test to unequal samples was carried out, comparing the mean value of Ab levels elicited against JL7 protein by patients belonging to one clinical form to the mean value obtained in the other clinical situations. Interestingly, anti-JL7 Ab concentration (by optical density [OD]) was higher in sera from patients with SCC, compared with those from IC ($P < 0.019$) (Figure 1). No analysis was performed with data from peptide JL18 because it showed poor Se and Ab levels barely above the cut-off value.

Our current results, together with previous reports,[20,21] extend the repertoire of parasite proteins that can be used to perform an appropriate serological test framed in WHO requirements for *T. cruzi* infection diagnosis. Indeed, a WHO-TDR Committee declared in 2007 that searching of biomarkers to predict progression of Chagas disease is a priority research area in this field. Ongoing studies have focused on endothelin 1, tumor necrosis factor-α, B-type natriuretic peptide, angiotensin-converting enzyme, and also autoantibodies as candidates for disease prognosis.[23,25–27] Among the last ones, cross-reactive Abs against β1-adrenergic and M2 muscarinic receptors have been associated with different arrhythmogenic anomalies, which may contribute to the distinct cardiac alterations observed in patients with SCC.[28] Although receptor Abs are the result of molecular mimicry with parasite ribosomal P proteins,[29,30] R13, P013, and P0β peptides were not good candidates as serological markers of Chagas disease in this study. Only the concentration of Abs against JL7 protein showed a significant increase ($P < 0.019$)

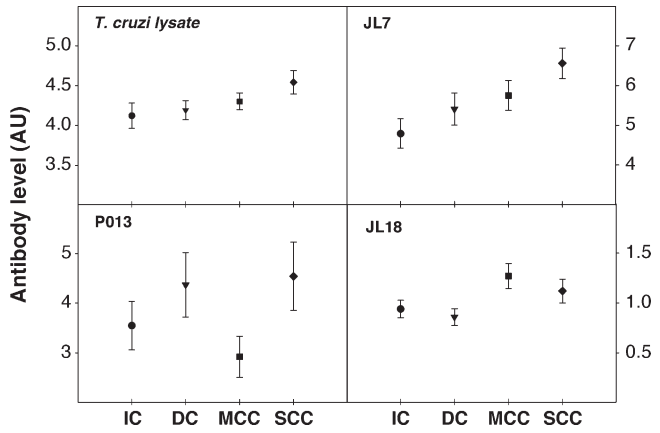

FIGURE 1. Performance of antigens for prognosis of Chagas disease. Enzyme-linked immunosorbent assay (ELISA) plates were coated with 50 ng protein/well of *Trypanosoma cruzi* epimastigote lysate, 2 μg/well of recombinant protein JL7 or 2 μM of peptides coupled to bovine serum albumin (BSA) in 50 μL buffer carbonate. Serum samples were diluted 1/400 and tested in duplicate. Sera from six healthy individuals were loaded on each plate to determine cut-off value, as the optical density (OD) mean value plus three standard deviations. Results were expressed as ratio calculated as (OD value of each serum samples/cut-off value) ±SE. IC = indeterminate form of Chagas disease; DC = digestive form of Chagas disease; MCC = mild chagasic cardiomyopathy; SCC = severe chagasic cardiomyopathy.

in sera from patients with SCC compared with those with the asymptomatic form, suggesting that this antigen may be useful for differential prognosis, i.e., a high concentration of Abs against JL7 may be a marker for progression of disease to a severe cardiopathy. The finding of a patient in the asymptomatic form with high levels of Abs against JL7 might indicate in this particular individual a future progression to a severe form of cardiopathy. Moreover, it is noteworthy that JL7 protein was not recognized by sera from patients with cardiomyopathies of non-chagasic ethiology, not even by those from patients with visceral and muco-cutaneous leishmaniasis, which confirms that this antigen is also an excellent diagnostic reagent for Chagas disease. This study was carried out with sera collected from patients resident in geographical regions in which strains belonging to TcII prevail. Thus, this antigen needs to be validated in cohorts of patients from different geographical origin in which different parasite lineages prevail to assess its potential for prognosis of Chagas disease severity in all endemic regions.

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