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ORIGINAL ARTICLE

Identification of immunodominant components of an isolate of *Trypanosoma cruzi* by immunoblot and its standardization for diagnostic purposes

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Abstract

Introduction: *Conventional serology is used in the detection of T. cruzi infection, with variable sensitivity and specificity, since a large amount of samples yield uncertain or discordant results; for diagnostic confirmation, the development of complementary tests, such as immunoblot, is required.* **Objective:** *To identify the most relevant immunogenic proteins in a T. cruzi isolate and to establish the positivity criterion for the immunoblot system.* **Material and methods:** *Antigenic extraction and characterization was performed and immunoblot was standardized. A panel of 35 T. cruzi-reactive and 30 -non-reactive (NR) sera was used. Three statistical methodologies were applied to determine positivity criteria.* **Results:** *Positive sera shared a pattern with molecular weights (MW) ranging from 10 to 250 kDa. Twelve components had a recognition rate higher than 50%, with those of 27, 32, 34 and 38 kDa showing rates close to 100%. Among the assessed positivity criteria, the 27 and 32 kDa components showed sensitivity and specificity of 100%.* **Discussion:** *Immunoblot testing is suitable for of T. cruzi infection confirmation and, therefore, it is proposed as a highly specific positivity criterion, especially in discordant cases.* (Gac Med Mex. 2015;151:6-13)

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Introduction

Given the public health importance that Chagas disease has in Mexico, Latin America and, currently, in non-endemic countries, serodiagnostic reliability is highly valuable. The World Health Organization (WHO) recommends the indirect enzyme-linked immunosorbent assay (ELISA) technique due to its high sensitivity, as well as indirect immunofluorescence (IIF) and indirect hemagglutination (IHA) for diagnostic confirmation. The WHO stablishes as criterion to confirm positivity demonstration of reactivity in two conventional serological test. Conventional serology has been widely used for screening, with varying sensitivity and specificity results; however, since a large amount of samples, especially from blood banks, yield uncertain or discordant results and remained unconfirmed, the WHO suggests promoting the development of complementary tests, such as immunoblot (western blot), for its confirmation[1-3].

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In Mexico, research using western blot with national isolated extracts and Mexican patients' serum have been reported[4,5]. Our research group characterized a semi-purified extract from an isolate originating from the state of Morelos (ITRI/MX1991/TQ) that showed sensitivity in the ELISA test and, in western blot, it revealed five *T. cruzi*-specific components of 74, 44, 31, 25 and 18 kDa, all of them without cross-immunity with *Leishmania mexicana*[6].

The aim of this work was to identify the main immunogenic proteins in a *T. cruzi* isolate from the state of Morelos and to establish the positivity criterion for the immunoblot system for diagnostic purposes.

Material and methods

Protein extraction

The aim extract was obtained from epimastigote phase of the *T. cruzi* strain ITRI/MX/1991/TQ, obtained from *Meccus pallidipennis* from Morelos, which was part of the strain collection at our laboratory. The parasitic expansion was performed in liver infusion tryptose (LIT) culture medium suplemented with 5% of fetal bovine serum. The harvest was carried out at the end of the exponential growth phase. The antigenic extract was obtained following, in general terms, the methodology described by Bucio et al. in 1999[6]. After the parasitic harvest, 5 washes were performed with 7.2 ph phosphate buffer solution (PBS) at 2,500 g/20 min/4 °C. The parasitic mass was then quantified and suspended in 50 nM of pH 7.4 Tris hydrochloric acid (HCL) solution in a 1:4 dilution and protease inhibitors (Complete mini Roche®) were added. The parasitic lysis was performed by sonication using a Vibra Cell VC 50 ultrasonic processor (Sonics); 60 s pulses were applied at 40 W in cold bath and the solution was centrifuged at 43,000 g/60 min/4 °C: Finally, the supernatant was separated and stored in 1.5 ml test tubes with screw caps at –40 °C until use. Proteins were quantified using the bicinchoninic acid method with a commercial reagent (Pierce® BCA PoteinAssay Kit, Thermo Scientific).

Sodium dodecyl sulfate Polyacrylamide gel electrophoresis (SDS-PAGE)

The separation of the antigenic extract components by molecular weight was performed under reducing conditions by electrophoresis in discontinuous polyacrylamide gels with concentrating gel at 5% and separating gel at 12.5%. In general terms, the methodology described by Laemmli in 1970[7] was followed, in a Mini Protean III Cell equipment (Bio Rad®), at a constant potential difference of 150 V, with a 200/2.0 power source (Bio Rad®). The samples were prepared with a commercial buffer (Laemmli Sample Buffer, Bio Rad®) and β-mercaptoethanol and were subjected to boiling for 5 min. Commercial MW markers ranging from 10-250 kDa range (Bio-Rad) were employed. The gels were stained with Coomassie blue (Bio Rad®).

Human sera

To perform the initial titration, 3 control sera with previously-determined reactivities as being highly reactive (R++), low reactive (R+) and NR were used. Statistical assessment of the procedure was established with 35 *T. cruzi*-positive (+) sera and 30 negative (–) belonging to the serum bank of the laboratory and from different geeographic areas of Mexico, with reactivity initially determined with the ELISA, IHA and IIF techniques using "home-made" antigens and the commercial reagent CHAGATEK ELISA (Biomérieux, Argentina), which showed consistent reactivity when assessed in the National Institute of Parasitology Dr. Mario Fatala Chaben of Argentina.

Immunoblot (western blot)

Overall, the procedure described by Towbin et al.[8] was followed. After the electrophoretic run, transference to nitrocellulose membrane was performed with 0.45 µm pore using a Trans-Blot Semi-Dry Transfer cell equipment (Bio-Rad®) at a potential difference of 15 V/30 min. Antigen titration had been performed at the electrophoresis assay with the following amounts of protein per well: 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µg. The nitrocellulose membrane was blocked with pH 7.2 PBS/Tween 20 at 0.3% in semi-skeemed milk at 5% during 2 h at room temperature with light agitation. Three washes with PBS pH 7.2/Tween 20 at 0.3% were performed and the (+) control human serum was incubated at 1:100, 1:200, 1:300 and 1:500 dilutions and the (-) control human serum at 1:100 dilution with PBS/Tween 20 0.3%/milk 5%/4 ºC with light agitation overnight. Three washes were performed, followed by incubation with the peroxidase-conjugated anti-human IgG antibody (Invitrogen) in 1:200 dilution with PBS/Tween 20 0.3% for 2 h with light agitation at room temperature. Five washes were performed and the

Table 1. Basic contingency table and sensitivity and specificity determination

Immunoblot	Sera panels	
	Positive	**Negative**
Positive	TP	FP
Negative	FN	TN
	Sensitivity	**Specificity**
	$S = \frac{TP}{TP + FN}(100)$	$Sp = \frac{TN}{TN + FP}(100)$

TP: true positive; FP: false positive; FN: false negative; TN: true negative.

reaction was detected with a hydrogen peroxide and 3,3' diaminobenzidine (DAB) (Bio-Rad®) solution in pH 7.2 PBS, keeping it in the dark for 15 min. The reaction was stopped with distilled water. Then, the reactivity of the panels was analyzed with 20 µg of protein per well and sera diluted 1:100.

Statistical analysis

With the results of the western blot, the components detected per serum were quantified, separating positive and negative, and then the mean, mode and standard deviation (SD) values were calculated. Additionally, the MW of the components was determined and the percentage of the presence of each one in the immunoblot was calculated. Based on this, three positivity criteria were established: i) deterrmination of the amount of components, ii) presence of a specific immunogenic component and iii) presence of combinations of specific components[2,4,5,9]. The criteria were assessed for validity using contingency tables for the calculation of sensitivity, specificity and predictive values (Table 1). Finally, by means of receiver operating characteristic curves (ROC), the positivity criteria were determined, which provided more discriminating power.

Results

The parasitic and proteic yield was determined (Table 2).

Electrophoretic pattern

The electrophoretic pattern of the extract showed 33 components; those with the highest relevance were those of 38, 48, 50, 89 and 96 kDa (Fig. 1)

Immunoblot with human sera

Reactivity of *T. cruzi*-positive sera, shows a rich pattern of rich in components with a wide range of MWs (Fig. 2), the banding pattern of which is resumed in table 3. The negative sera immunoblot is shown in figure 3.

When these reactivities are analyzed, *T. cruzi*-positive sera are found to have a very diverse reactivity pattern, which encompasses a wide range of MWs, ranging from 10 kDA to over 250 kDa, and recognize 13.7 components on average; however, a shared banding pattern is observed in all positive sera (Table 4).

Statistical analysis

The first positivity criterion, with regard to the number of recognized components, was assessed by means of true and false positive and negative analysis (Table 5). Based on this criterion, sensitivity and specificity patterns were calculated (Fig. 4), and elevated specificity was observed to occur from 10 components on, in detriment of sensitivity.

For the assessment of the second criterion by means of the presence of a relevant immunogenic component in positive sera, the 250, 140, 75, 64, 38, 34, 32, 27, 22, 21 and 18 kDa components showed a recognition rate higher than 50% (Table 3). When positivity was determined in the immunoblot by the presence of these specific components, sensitivity and specificity values

Table 2. Parasitic and proteic yield

Culture (ml)	Parasitic mass (g)	Antigen (ml)	Concentration (mg/ml)	Proteic yield (mg)
2,000	3.76	18.8	11.5	216.2

Figure 1. *T. cruzi ITRI/MX/1991/TQ isolate proteic extract components. separated by SDS-PAGE (staining with Coomassie blue).*

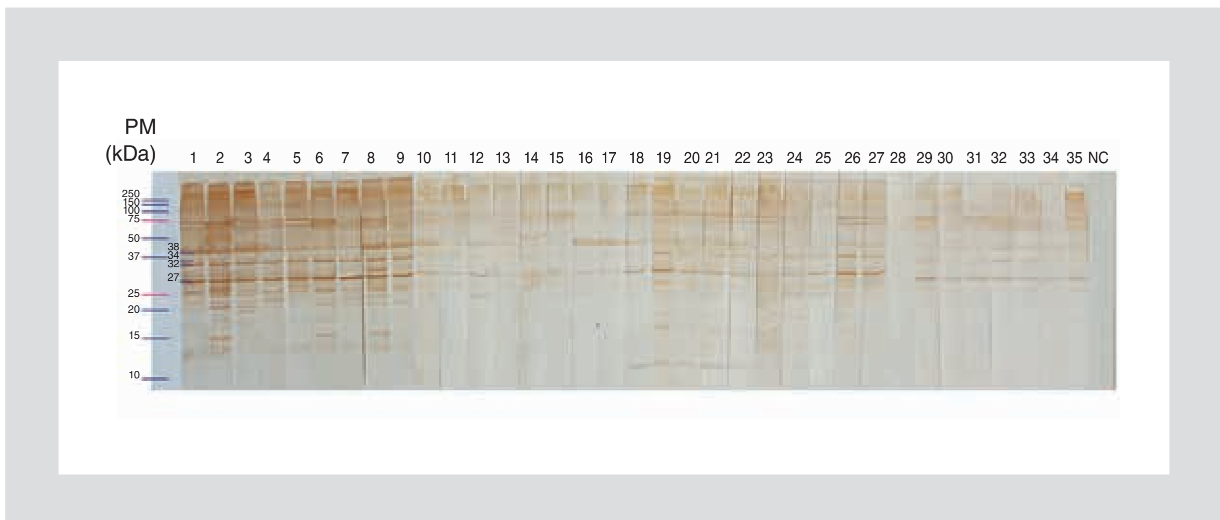

Figure 2. *Western-blot of 35 T. cruzi-reactive sera. NC: negative control.*

Figure 3. *Western blot of 30 NR to T. cruzi sera. PC: positive control.*

higher than 70% were obtained, and a maximum certainty point was attained with the 32 kDa component (Table 6).

Finally, in order to determine the cutoff point that allows for the highest sensitivity and specificity to be obtained, combinations of the components with the highest percentages of recognition were considered as a criterion, an analysis that determines that, for the employed sample, higher sensitivity and specificity percentages are obtained when the 27 and 32 kDa components are present together (Table 7).

Discussion

In the SDS-PAGE protein analysis (Fig. 2), 33 protein bands ranging from 10 to over 250 kDa were observed, with those of 38, 48, 50, 89 and 96 kDa standing out, although low MW bands were also observed. This pattern is consistent with those observed in previous works[6,10,11].

When the *T. cruzi*-positive sera panel reactivity was assessed by immunoblot, reactivity was found with 37 components, out of which 12 showed a rate of recognition higher than 50%. In this analysis, the presence of 3 components with recognition rates of 100% and one with 97%, with weights of 27, 32, 34 and 38 kDa, respectively, stands out. Some of the encountered components are consistent with immunogenic proteins referred in literature, such as the 25, 27, 34, 38, 40, 68, 75 and 84 kDa antigens[4,6,10-14].

With regard to previous studies performed using western blot for diagnostic purposes, only some components have been observed to appear in common in different research; these differences in antigenic profiles are probably due not only to the *T. cruzi* isolate used, but also to other factors associated with the extraction and standardization methodologies of the used procedures and techniques[2].

With the *T. cruzi* isolate and the methodology employed in the present research, a large amount of immunodominant components were obtained, out of which 12 were recognized by more than 50% of positive sera, as opposed to other works where only 5-8 immunodominant bands were recognized[2,4,10].

In order to determine the positivity criterion, statistical analysis of the results was carried out using three criteria: presence of components, presence of a specific immunogenic component and presence of a combination of specific components.

With the first criterion, 100% specificity was obtained with the presence of 10 or more components; however, sensitivity decreased as the number components increased, as shown by the ROC curve (Fig. 4). The amount of components has been previously reported as a positivity criterion, even if the presence of a single component is considered as being reactive[5]. In this study, the number of components in this case was established not to be able to be regarded as sufficient criterion to determine seropositivity, since sensitivity was too low.

Table 3. Components that showed recognition higher than 50%

Component (kDa)	% of sera that recognize the component (n = 35)
> 250	82.4*
250	71.4*
166	2.9
140	65.7*
128	34.3
100	2.9
95	5.7
90	2.9
75	80*
68	28.6
65	2.9
64	74.3*
56	2.9
52	11.4
50	5.7
46	5.7
42	22.9
38	94.3*
34	94.3*
32	97.1*
30	5.7
29	11.4
28	45.7
27	100*
23	8.6
22	62.9*
21	74.3*
20	11.43
19	48.6
18	51.4*
17	8.6
16	40
15	2.9
14	37.1
13.5	20
13	17.1
12	22.9

*Components with > 50% recognition.

Table 4. Basic statistics

	Positive sera n = 35	Negative sera n = 30
Mean	13.7 ± 4.7	4.5 ± 2.1
Mode	13	3

With regard to the criterion of presence of one specific component, the analysis of the 32 and 38 kDa components, even when they showed sensitivity and specificity patterns ranging from 93 to 97% in an independent manner, could also not be useful to determine seropositivity.

The last analyzed criterion was the existence of combinations of components with sensitivity and specificity higher than 90% and that, when combined, showed sensitivity and specificity of 100%. In this work, the 27 and 32 kDa polypeptides met this criterion. There are studies that propose the presence of one out of a group of 12 specific components (25, 28, 30, 32, 40, 42, 65, 70, 83, 90, 100 and > 100 kDa)[4], 3 out of a group of 7 specific bands (14, 19, 27, 30, 34, 37 and 75 kDa)[9] or at least 5 out of a group of 10 (25, 30, 33, 37, 39, 45, 52, 70, 75 and 95 kDa)[2] as positivity criteria.

Based on the analysis of the results of this work, the demonstration of reactivity to the 27 and 32 kDa components is proposed to be established as a highly specific positivity criterion for the diagnosis of *T. cruzi* infection. On the other hand, the immunoblot, when it has been standardized to be employed with human sera, is a useful tool for diagnostic confirmation. However, it is well known that these procedures are highly expensive, in addition to being very laborius and requiring specialized personnel and equipments, making its use difficult for massive purposes. For this reason, these antigens are proposed as candidates to be used in other diagnostic systems of the Dot-ELISA or immunochromatographic-type for quick diagnostic strips.

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Table 5. Sensitivity and specificity based on the number of reactive components

Number of components	TP	FN	FP	TN	Sensitivity	Specificity
1	35	0	30	0	100	0
2	35	0	28	2	100	6.7
3	35	0	26	4	100	13.3
4	35	0	18	12	100	40
5	33	2	14	16	94.3	53.3
6	33	2	9	21	94.3	70
7	32	3	5	25	91.4	83.3
8	32	3	3	27	91.4	90
9	31	4	1	29	88.6	96.7
10	31	4	0	30	88.6*	100*
11	27	8	0	30	77.1	100
12	25	10	0	30	71.4	100
13	20	15	0	30	57.1	100
14	15	20	0	30	42.9	100
15	14	21	0	30	40	100
16	11	24	0	35	31.4	100
17	9	26	0	35	25.7	100
18	8	27	0	35	22.9	100
19	6	29	0	35	17.1	100
20	6	29	0	35	17.1	100
21	3	32	0	35	8.6	100
22	2	33	0	35	5.7	100
23	1	34	0	35	2.9	100

TP: true positive; FN: false negative; FP: false positive; TN: true negative.
*Highest values.

Table 6. Sensitivity and specificity based on reactivty to a specific component

Component (kDa)	TP	FN	FP	TN	Sensitivity	Specificity
> 250	29	6	2	28	82.9	93.3
250	25	10	5	25	71.4	83.3
140	23	12	3	27	65.7	90
75	28	7	9	21	80	70
64	26	9	2	28	74.3	93.3
38	33	2	2	28	94.3	93.3
34	33	2	8	22	94.3	73.3
32	34	1	2	28	97.1*	93.3*
27	35	0	6	24	100	80
22	22	13	6	24	62.9	80
21	26	9	1	29	74.3	96.7
18	18	17	1	29	51.4	96.7

TP: true positive; FN: false negative; FP: false positive; TN: true negative.
*Highest values.

Table 7. Sensitivity and specificity based on the reactivity of combined specific components

Components (kDa)	TP	FN	FP	TN	Sensitivity	Specificity
27 and 32	35	0	0	30	100*	100*
27 and 34	33	2	2	28	94.3	93.3
27 and 38	33	2	1	29	94.3	96.7
32 and 34	33	2	0	30	94.3	100
32 and 38	33	2	0	30	94.3	100
34 and 38	31	4	1	29	88.6	96.7
27, 32, 34	33	2	0	30	94.3*	100*
27, 32, 38	33	2	0	30	94.3*	100
32, 34, 38	31	4	0	30	88.6	100
27, 32, 34, 38	31	4	0	30	88.6	100

TP: true positive; FN: false negative; FP: false positive; TN: true negative.
*Highest values.

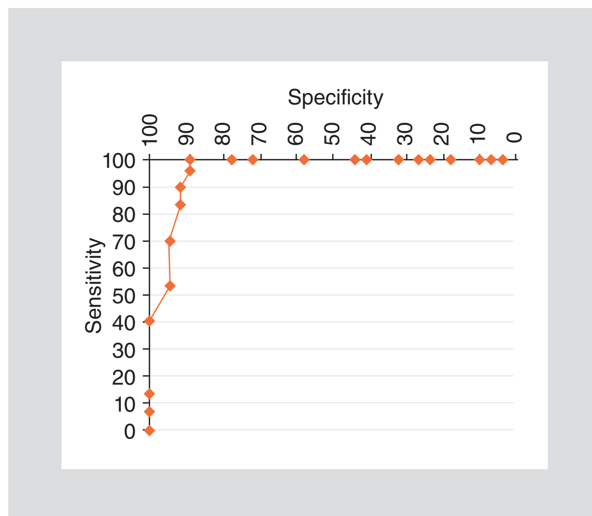

Figure 4. *Characteristics of the ROC curve with* T. cruzi*-reactive and NR sera at different number of components.*

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