COMPARISONS OF IMMUNOLOGICAL TESTS FOR SERODIAGNOSIS OF CHAGAS DISEASE IN BOLIVIAN PATIENTS

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Abstract. Enzyme linked immunosorbent assay (ELISA) and immunoelectrophoresis (IEP) were evaluated and compared to the classical immunofluorescence (IF) and complement fixation test (CFT) in the immunological diagnosis of Chagas' disease, using 407 sera from Bolivian patients. 72.7 to 79.5% of randomised sera, coming from patients living in endemic areas for Chagas' disease were considered as positive, according to the test limits, previously determined. The techniques could be classified according to their percentage detection as ELISA > IF > CFT > IEP.

The quantitative correlations between the tests were excellent ($p < 0.001$). 92.8% of the sera were positive or negative for the four tests, 6.1% for three tests and 1.1% for only two tests. The agreement between the tests ranged from 94.6 to 99.2%, co-positivity from 95.5 to 100% and co-negativity from 88.5 to 100%. IF gave the best results, and could be considered as the reference test since it was easy and rapid to perform. However to avoid errors or discrepancies between laboratories, two tests, such as IF and CFT, might be associated. ELISA can be used if higher sensitivity is required. IEP showed 1 to 14 precipitation bands in 96% of the sera from infected patients. The precipitation band 5, previously demonstrated as *Trypanosoma cruzi* specific, was present in 73% of these sera, indicating the interest to use immunoprecipitation test, if more specificity is required for the immunodiagnosis of Chagas' disease.

Key words: Chagas' disease; *Trypanosoma cruzi*; serodiagnosis; ELISA; immunoelectrophoresis; immunofluorescence; complement fixation test; comparisons of immunological tests; Bolivia.

Introduction

In the acute phase of Chagas' disease, blood trypomastigotes of *Trypanosoma cruzi* are easy to detect by direct microscopy. By contrast, in the chronic stage of the infection, parasitological investigations such as xenodiagnosis or blood culture only lead to 31 to 50% of diagnosis [1-4]. In chronic cases without blood forms, the diagnosis of *T. cruzi* infection must be based only on the presence of anti-*T. cruzi* circulating antibodies. This demonstrates the importance of the immunological diagnosis of Chagas' disease.

Various techniques have been applied: the complement fixation test (CFT), pioneered by Guerreiro & Machado [5] direct agglutination [6], hemagglutination (IHA) [7-10], immunoprecipitation [11, 12], immunofluorescence (IF) [13-17] and more

recently enzyme-linked immunosorbent assay (ELISA) [18-23] and thin layer immunoassay [24]. However few comparative studies between more than two tests have been performed. Camargo [25] evaluated CFT, IF, IHA and direct agglutination together recommending the association of two tests. Fuchs [26] compared CFT, IHA, IF and ELISA and showed IF and ELISA more related to clinical pictures of Chagas' disease.

In the present study, ELISA, which is largely used for sensitive diagnosis of other parasitosis [27, 28] and immunoelectrophoresis (IEP), recommended for specific diagnosis of helminthiasis [29], were evaluated and compared to the classical IF and CFT in the immunological diagnosis of Chagas' disease in Bolivian patients. Moreover, advantage was taken of the immunoelectrophoretic analysis of human antibodies to study the frequency of precipitating antibodies anti-antigen 5, previously demonstrated as specific of *T. cruzi* and without cross-reactions with other flagella [30, 31].

Material and methods

Antigenic extract of T. cruzi Epimastigotes of *T. cruzi* (Tehuantepec strain) were obtained from culture in cell-free GLSH monophasic medium at 28°C [32]. The parasites were collected by centrifugation at 2000 g, washed three times in Hank's balanced solution and divided into two samples. The first one was directly used in smears for immunofluorescence. The second was suspended in NaCl 1% o, frozen three times for cell disintegration using an hydraulic press at 18000 PSI (× press I.KB). It was then centrifuged at 26.000 g for 1 h. at 4°C. The supernatant was dialysed and lyophylized. Such crude *T. cruzi* antigenic extract was used for IEP, CFT and ELISA.

Human sera. For preliminary determination of the limits of each test, well referenced sera (group 1) were used: 76 came from *T. cruzi* infected Bolivian patients with positive xenodiagnosis; 68 were from control Bolivian patients living in highlands (altiplano) without Chagas' disease, never having travelled in known Bolivian endemic areas and for which parasitological and clinical investigations were negative; 10 were from European control patients living outside the Bolivian chagasic endemic areas and without parasitological or clinical Chagas' disease.

For the comparative study between the four serological tests, 263 randomised sera (group 2) came from patients living in various endemic areas of Bolivia.

Serological tests. IF was performed using *T. cruzi* epimastigotes fixed by 1% glutaraldehyde [16]. The FITC labelled anti-human immunoglobulins conjugate (Institut Pasteur Production-Paris) was used diluted 1/200.

CFT was carried out according to Kent and Fife [33].

ELISA was performed according to Carlier [27]. Preliminary assays showed the optimal *T. cruzi* antigenic extract coating concentration to be 10 µg/ml. The sera were diluted 1/100. The peroxydase-labelled anti-human immunoglobulins (Institut Pasteur Production, Paris) were used at 500 ng/ml with H_2O_2 and orthodianizine as substrate. Extinction values were measured at 405 nm.

IEP was carried out according to Biguet [29] in 1% agarose using sera three times concentrated by lyophylisation. The precipitation band 5 was identified by its particular intensity and position on immunoelectrophoresis [34].

The same *T. cruzi* antigenic extract was used for these three last techniques though each test was performed independently and the results compared *a posteriori*.

Results

Determination of the specific limit of the tests. The results of the group 1 sera are expressed in *table 1*. The detection limits of 1/40 for IF and 1/2 for CFT were selected. The limit extinction value of ELISA was determined as $m + 2\sigma$ of the control Bolivian group: $0.07 + 2 \times 0.05 = 0.17$.

The IEP was considered positive with the presence of, at least, one well-defined precipitation band.

Table 1. Frequency of the different results obtained for group 1 of selected sera.

Tests	*Results*	*Control Europeans*	*Control Bolivians*	T-cruzi *infected Bolivians (positive xenodiagnosis)*
	≤10	10	64	0
IF	20	0	4	0
titre	40	0	0	7
	80	0	0	10
	≥160	0	0	59
	<2	10	67	0
CFT	2	0	1	5
titre	4	0	0	16
	8	0	0	24
	≥16	0	0	31
	0	10	68	3
IEP	1-2	0	0	2
(precipi-	3-4	0	0	22
tation	5-6	0	0	21
bands)	>6	0	0	26
ELISA	Ext.values	0.07 ± 0.03	0.07 ± 0.5	0.39 ± 0.07

Results obtained in the randomised sera group. The frequency of the different titres, number of precipitation bands and extinction values obtained for the group 2 sera are expressed in *table 2*. IEP showed 1 to 14 precipitation bands with a mean of 6 ± 3. 72.7 to 79.5% of these sera were considered as positive and the techniques can be classified according to their percentage detection as ELISA > IF > CFT > IEP.

Correlation studies between IF, CFT, and ELISA. The quantitative correlation values between the logarithmic transformations of the titres of IF and CFT and the arithmetic extinction values of ELISA, of the group 2 sera were obtained by analysis of the regression curves and are expressed in *table 3*.

Agreement between the tests. Among the 263 randomised sera, 244 (92.8%) were positive or negative for the four tests, 16 (6.1%) for three tests and 3 (1.1%) for only two tests (doubtful results).

Consequently, only 260 sera could be classified as positive or negative according to any three of the four tests. The agreement, co-positivity and co-negativity of each test, alone or associated, with such a classification are expressed in *table 4*. The general agreement ranged from 94.6 to 99.2% as IF > ELISA, CFT > IEP, co-positivity from 95.5 to 100% as IF, ELISA > CFT > IEP, and co-negativity from 88.5 to 100% as IEP > IF CFT > ELISA. However, no significant differences could be noted between these results.

Frequencies of the precipitation band 5 in IEP. The frequencies of the band 5, and of the total number of precipitation bands observed in IEP are expressed in *table 5*. There is no difference between the results of the group 1 of sera with positive xenodiagnosis (72.6%) and the group 2 (73.3%).

Table 2. Frequency of different results obtained for group 2 of randomised sera.

IF			*CFT*			*IEP*			*ELISA*		
titre	*n*	%	*titre*	*n*	%	*bands*	*nb*	%	*O.D.*	*n*	%
<40	60	22.8	<2	62	23.5	0	72	27.3	<0.17	54	20.5
40	29		2	28		1-2	12		0.17-0.27	17	
80	59		4	38		3-4	51		0.28-0.37	54	
160	65	77.2	8	62	76.5	5-6	62	72.7	0.38-0.47	111	79.5
320	34		16	59		7-8	40		0.48-0.57	27	
≥640	16		≥32	14		≥9	26		0.58-0.67	0	

Table 3. Results of the quantitative correlation studies between IF, CFT and ELISA (r = correlation coefficient; t = value of student's t; p = probability).

Correlations	*r*	*t*	*P*
CFT/ELISA	0.32	4.76	0.001
IF/ELISA	0.43	9.36	0.001
IF/CFT	0.23	3.41	0.001

Table 4. Agreement, co-positivity and co-negativity between the tests alone or associated with the 260 sera classified as positive or negative according to any 3 of the 4 tests.

Tests	*agreement*		*co-positivity*		*co-negativity*	
	n	%	*n*	%	*n*	%
IF	258	99.2	199	100.0	59	96.7
ELISA	257	98.8	199	100.0	58	95.1
CFT	257	98.8	198	99.5	59	96.7
IEP	252	96.9	191	96.0	61	100.0
IF/ELISA	255	98.0	199	100.0	56	91.8
IF/CFT	255	98.0	198	99.5	57	93.4
CFT/ELISA	234	97.6	198	99.5	56	91.8
IF/IEP	250	96.1	191	96.0	59	96.7
IEP/ELISA	249	95.7	191	96.0	58	95.1
IEP/CFT	249	95.7	190	95.5	59	96.7
ELISA/IF	252	96.9	198	99.5	54	88.5
ELISA/IF/IEP	247	95.0	191	96.0	56	91.8
CFT/IEP/IF	247	95.0	190	95.5	57	93.4
ELISA/CFT/IEP	246	94.6	190	95.5	56	91.8

Table 5. Frequencies of the precipitation band 5 in IEP in group 1 (positive xenodiagnosis) and group 2 (randomised) sera.

Total	*positive xenodiagnosis sera*			*group 2 sera*		
bands	*n*	*band 5*	%	*n*	*band 5*	%
0	3	0	0.0	72	0	0.0
1-2	2	1	50	12	6	50.0
3-4	22	12	54.5	51	33	64.7
5-6	21	17	80.9	62	44	70.9
>6	28	23	82.1	66	57	86.3
	73	53	72.6	191	140	73.3

Discussion

The high frequency of positive results with the studied sera coming from patients living in endemic areas underlines the frequency of *T. cruzi* infection in Bolivia, where 35% of the total population is considered as infected [34].

The comparative study shows a good agreement between CFT, IF, ELISA and IEP, since no significant differences could be observed between the results. The co-positivity can be considered as a parameter of the relative sensitivity of the tests and the co-negativity as a relative specificity parameter. The results obtained are in accordance with those of Camargo [25] who obtained relative sensitivity of 99.9 for IF and 99.2 for CFT and Voller [18] who obtained 98% agreement for IF and ELISA. Fuchs [26] obtained sensitivity of 98.5% with ELISA, 95.1% with IF and 73.1% with CFT. Spencer [21] noted 87.4% agreement between ELISA, IF and CFT. Our slightly higher results than in these two last studies could be explained by the use of the same batch of *T. cruzi* antigenic extract, prepared from fresh *T. cruzi* epimastigotes and lyophilized to avoid conservation problems.

IF gave the best results, and can be considered as the reference test, since it is easier and more rapid to perform than CFT, which confirms many previous works [13, 16, 17, 25, 26].

IEP was able to detect 96% of the sera with positive xenodiagnosis (group 1) or serology (group 2), with the highest relative specificity. The *T. cruzi* specific band 5 could be identified in 73% of the two groups of sera having precipitating antibodies, bringing the certitude of the *T. cruzi* infection. Such results are in accordance with a preliminary work of Afchain [35] performed with few sera. They indicate the high immunogenecity in man of the antigen 5 and the interest to use such immunoprecipitation test, cheap and simple to perform, for the immunodiagnosis of Chagas' disease.

ELISA appears with a high sensitivity but a lower specificity than in the other tests. This could be due to the use of a crude *T. cruzi* antigenic extract [28].

The use of purified specific antigens as the 90 Kd molecular weight glycoprotein [23] or anti-antigen 5 monoclonal antibody in competition EIA [36] allows considerable improvement in specificity. However, at the present time, such reagents are not available for routine study and only crude antigenic extract can be used.

Such comparisons also clearly show that the use of two or three associated tests do not improve the relative sensitivity or specificity of the serodiagnosis. One single, well-chosen, test can be sufficient. However to avoid discrepancies or errors between laboratories [37] it is preferable to associate two techniques.

In terms of equipments and cost, IF and ELISA are more expensive than CFT and IEP. Indeed IF needs a fluorescent microscope and fluorescein conjugate, while ELISA needs a spectrophotometer and enzyme conjugate (it is also possible to use ELISA as a semiquantitative test, using serum dilution and visual determination of a titer, avoiding the use of a spectrophotometer). CFT and IEP need only disposable material (plates, slides). The antigen consumption is higher in IEP and CFT than in IF, using smears of epimastigote forms and ELISA using only very low amount of antigenic extract. The required technical skill is quite the same for all the tests. IEP, CFT and ELISA are more time consuming than IF. Such considerations on easy handiness, rapidity, slow antigen consumption, sensitivity and specificity lead to the conclusion that IF is the best test, beside the need of fluorescence microscope which can be used in many other applications in a routine laboratory. According to the possibilities of the laboratory, IF and CFT could be recommended or IF and ELISA, if more sensitivity is necessary or IF and IEP, if more specificity is required.

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