Validity of Serology for American Trypanosomiasis with Eluates from Filter Paper

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This study evaluates whether blood collected on filter paper kept at 4 °C and tested at different intervals of time (1, 7, 15, 30 and 60 days after collection) would present similar results when compared to the serum samples and whether the type of filter paper influences the results. Eluates from filter paper samples were tested for Trypanosoma cruzi *antibodies using indirect immunofluorescence antibody test (IFAT), indirect haemagglutination (IHA) and enzyme-linked immunosorbent assay (ELISA) as reference, the antibody titer in sera. Analysis of data showed that results obtained with IFAT, IHA (cut off point = 1:40) and ELISA in sera had similar sensitivity and good concordance among reactions. The use of a multiple linear regression model indicated that titer fall in eluates occurs up to the 7th day after the collection, and it is more marked for samples with lower antibodies titers. However, no significant differences were observed by IFAT, IHA (cut off point = 1:20) and ELISA in the proportion of positive reactions between sera and eluates. The results also showed that Melitta, Klabin or Whatmen (reference) filter papers could be indicated for surveys, since they have shown similar capacity of maintenance of anti-*T. cruzi *immunoglobulins.*

Key words: serodiagnosis - anti-*Trypanosoma cruzi* antibodies - filter paper - serological epidemiology

In 1979, the National Health Foundation (FNS - MS) introduced a large scale campaign against triatomine bugs in an attempt to control Chagas' disease in Brazil (Ministério da Saúde 1978). The prophylactic measures have been assessed by periodic epidemiological surveys, which have also been used to establish which areas need to be included in the program (Camargo et al. 1984). Serological methods have been used routinely to assess the prevalence of the disease (Paul & White 1973, Zicker et al. 1990). The most common means of assessment are indirect immunofluorescence antibody test (IFAT), indirect haemagglutination (IHA) and enzyme-linked immunosorbent assay (ELISA) (Guimarães 1984).

Eluates from blood dried on filter paper are commonly used in serological tests. This procedure makes the collection, transport and storage of samples easier (Guimarães 1983). However, precise estimates of eluate dilution and denaturating of antibodies during filter paper storage in high environment temperatures and high relative humidity have revealed discrepancies between serum and eluate titers in paired sample studies (Marinkelle et al. 1978).

The purposes of this study are: (1) to determine if blood smears on filter paper, mailed long distances and tested at different intervals of time, provide similar results in comparison with serum samples; (2) to establish the discriminative titer between positive and negative reactions of eluates; (3) to observe if the type of filter paper used influences the results. The third of these aims was included in the study because of the possibility of filter papers of Brazilian manufacture be used in the next national serologic survey of Chagas' disease.

MATERIALS AND METHODS

Human sera - Sera from 131 individuals with chronic Chagas' disease, without specific treatment, were collected in Bambuí/Minas Gerais, Brazil. These sera were positive for anti-*T. cruzi* antibodies by IFAT and IHA. Specimens from 20 healthy individuals without history of Chagas' disease, were used as controls. Sera were processed within 24 hr and stored at -20°C. Matching filter paper blood specimens, obtained concurrently from each patient, were prepared. Blood was collected by venipuncture and several samples were immediately spread on three different absorbent papers: Melitta filter paper for coffee, Klabin 80 GRS, and Whatman No. 4. A vo-

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lume of blood sufficient to saturate an area of ± 100 cm^2 was used. The preparation was dried at room temperature, cut into discs of 6 mm diameter, placed in plastic bags, labelled and stored at 4°C to be tested 1, 7, 15, 30 and 60 days after collection (DAC). Ten percent of each sera and filter paper specimens were run in duplicate and stored under the same conditions. Both sets were tested together.

Elution - Ten paper discs were put into tubes containing 0.58, 0.68 or 0.7 ml of phosphate-buffered saline (PBS) at pH 7.2 (Alvarez et al. 1971) to elute the blood dried from the filter paper Melitta, Klabin and Whatman filter paper, respectively. The suspension was then shaken for 30 min at room temperature before the test to obtain a final dilution of 1:20.

Serological tests - Eluates were tested for *T. cruzi* antibodies using IFAT, IHA and ELISA taking the sera antibodies' titer on the 1st day after collection (DAC) as reference. All tests were conducted independently and double-blind. The positive reference serum used was obtained from a person with a clinical and parasitological diagnosis of Chagas' disease.

The IFAT reference reaction was carried out according to Camargo (1966), using formalde-hyde-fixed epimastigotes (Y strain) of *T. cruzi* as antigen. The conjugate was prepared from rabbit anti-human IgG serum marked with fluorescein isothiocyanate, F/P ratio = 9.

The IHA was performed according to Vitor and Chiari (1987). The hemagglutination reagent was human red blood cells (group O, Rh negative) tannized and adsorbed with *T. cruzi* epimastigotes (Y strain) extract obtained with NaOH 0.15 M (Hoshino-Shimizu et al. 1978) in glutaraldehyde solution of 15 µg/ml.

In both tests all samples were tested by doubling dilutions of 1:20 to 1:640, taking the cut off point at 1:40.

ELISA was performed according to Voller et al. (1975). Protein (10 µg/ml) of the crude antigen already mentioned for IHA was used for coating polystyrene microtitre ELISA plates (Hemobag Ltda). Comparison of serial dilutions using positive and negative sera showed that 1:80 sera dilution was optimal for screening Chagas' disease patients. The diluent used was PBS pH 7.2, containing 2% casein and 0.05% Tween 20. Peroxidase-labeled goat anti-human IgG (Sigma Co.) was used as conjugate and o-phenylenediamine was used as the substrate. The absorbance at 492 nm was determined by using an ELISA reader (BIO RAD model 2550). The cut off point value for ELISA positivity was taken as the optical density reading in 1:80 dilution corresponding to the mean plus 2 standard error obtained by testing 33 normal controls (negative for IFAT and IHA). A correction factor was calculated for each test run using positive and negative reference sera and was multiplied by the raw absorbance value for all the test samples of that specific run.

Statistical analysis - McNemar's χ^2 test and Student's t-test (Armitage & Berry 1987) was used for comparison of proportions and means in two related samples. Co-positivity (sensitivity) and co-negativity (especificity) indices were determined according to Buck and Gart (1965), and positive and negative predictive values were determined according to Vecchio (1966).

Since was accepted the premise that no serum significant decrease through the time (Guimarães et al. 1978) the antibody titer in sera were taken as reference for evaluate reproductibility of IFAT, IHA and ELISA in eluates. The model used to analysis was a multiple linear regression (Kleibaum & Kupper 1978) with dummy variables such as:

$Y = a + b_1D_1 + b_2D_2 + b_3D_3 + b_xX$

Y = eluate titer (mean)

X = serum initial titer (reference titer)

a = eluate titer when serum titer was zero

b_1, b_2, b_3 = regression coefficients

b_x = coefficient of regression for eluates in relation to the initial titer in serum

D_1, D_2, D_3 = dummy variables

The dummy variable values were: on the 7th DAC, $D_1 = 0$, $D_2 = 0$, $D_3 = 0$; on the 15th DAC, $D_1 = 1$, $D_2 = 0$, $D_3 = 0$; on the 30th DAC, $D_1 = 0$, $D_2 = 1$, $D_3 = 0$; on the 60th DAC, $D_1 = 0$, $D_2 = 0$, $D_3 = 1$.

For statistical purposes, eluate and serum titers (X) were transformed into $\log_{10}$ (X+1) or $\log_{10}$ [(1000) (X+1)] to absorbance (X).

RESULTS

IFAT (reference reaction), IHA (Table I) and ELISA (Table II) in sera were similarly sensitive

TABLE I

Crossed distribution of positive and negative reactions in sera, through IFAT (reference) and IHA (cut off points 1:40)

IFAT	IHA		
	Positive	Negative	Total
Positive	132	0	132
Negative	4	15	19
Total	136	15	151

$\chi^2_{1gl} = 2.25$ NS

proportion of positives IFAT = 0.87 co-positivy = 1.00

proportion of positives IHA = 0.90 co-negativy = 0.79

predictive positive = 0.97

predictive negative = 1.00

and good agreement was obtained between reactions, though IHA presented lower specificity.

High indices of co-positivity and co-negativity were observed with IFAT (Table III) and IHA (Table IV) for eluates from all filter papers tested with a cut off point of 1:40. However, the proportion of positive samples was significantly lower than that observed with sera. When a dilution of 1:20 was taken as the discriminating titer for eluates, no significant differences were observed between the proportion of positive reactions of sera and eluates for IFAT (Table V) and IHA (Table VI), although there was some decrease in the specificity of reactions.

The ELISA tests carried out with eluates did not show significant numbers of false positive or false negative reactions (Table VII). The results show that ELISA is a very sensitive and specific assay, able to detect low levels of anti-*T. cruzi*

TABLE II

Crossed distribution of positive and negative reactions in sera, through IFAT (cut off point 1:40 - reference) and ELISA (cut off point 0.260 nm)

IFAT	ELISA		
	Positive	Negative	Total
Positive	130	2	132
Negative	0	19	19
Total	130	21	151

$\chi^2_{1gl} = 0.5$ NS
proportion of positives IFAT=0.87 co-positivity= 0.99
proportion of positives ELISA=0.86 co-negativity=1.00
predictive positive=1.00
predictive negative=0.91

TABLE III

Validate indices of the IFAT for *Trypanosoma cruzi* antibodies in filter paper blood eluates, in days 1, 15 and 60 after collection, relative to sera IFAT (cut off points 1:40)

	Days after collection								
	1			15			60		
	M[a]	K[a]	W[a]	M[a]	K[a]	W[a]	M[a]	K[a]	W[a]
co-positivity	0.89	0.94	0.87	0.93	0.92	0.92	0.92	0.93	0.91
co-negativity	1.00	0.95	1.00	1.00	1.00	1.00	1.00	1.00	1.00
predictive positive	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00
predictive negative	0.58	0.69	0.53	0.68	0.63	0.63	0.63	0.68	0.61
p (sera)	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87
p (eluate)	0.78	0.83	0.75	0.82	0.80	0.80	0.80	0.82	0.80

M: Melitta; K: Klabin; W: Whatman; p: ratio of positive reactions
[a]: ratio of positive reaction with significative difference ($p<0.05$)

TABLE IV

Validate indices of the IHA for *Trypanosoma cruzi* antibodies in filter paper blood eluates, in days 1, 15 and 60 after collection, relative to sera IHA (cut off points 1:40)

	Days after collection								
	1			15			60		
	M	K	W	M[a]	K[a]	W[a]	M[a]	K[a]	W[a]
co-positivity	0.96	0.96	0.95	0.94	0.91	0.93	0.92	0.90	0.93
co-negativity	0.87	0.93	0.73	1.00	1.00	1.00	1.00	1.00	1.00
predictive positive	0.98	0.99	0.97	1.00	1.00	1.00	1.00	1.00	1.00
predictive negative	0.68	0.70	0.61	0.65	0.56	0.60	0.58	0.52	0.60
p (sera)	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90
p (eluate)	0.87	0.87	0.88	0.85	0.82	0.83	0.83	0.81	0.83

M: Melitta; K: Klabin; W: Whatman; p: ratio of positive reactions
[a]: ratio of positive reaction with significative difference ($p<0.05$)

TABLE V

Validate indices of the IFAT for *Trypanosoma cruzi* antibodies in filter paper blood eluates (cut off point 1:20), in days 1, 15 and 60 after collection, relative to sera IFAT (cut off points 1:40)

	Days after collection								
	1			15			60		
	M[a]	K	W	M	K[a]	W	M[a]	K	W
co-positivity	0.95	0.99	0.96	0.98	0.95	0.96	0.95	0.97	0.96
co-negativity	1.00	0.79	0.89	1.00	1.00	1.00	1.00	1.00	1.00
predictive positive	1.00	0.97	0.98	1.00	1.00	1.00	1.00	1.00	1.00
predictive negative	0.76	0.94	0.77	0.86	0.73	0.79	0.76	0.83	0.79
p (sera)	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87	0.87
p (eluate)	0.83	0.89	0.85	0.85	0.83	0.84	0.83	0.85	0.84

M: Melitta; K: Klabin; W: Whatman; p: ratio of positive reactions
[a]: ratio of positive reaction with significative difference ($p<0.05$)

TABLE VI

Validate indices of the IHA for *Trypanosoma cruzi* antibodies in filter paper blood eluates (cut off point 1:20), in days 1, 15 and 60 after collection, relative to sera IHA (cut off points 1:40)

	Days after collection								
	1			15			60		
	M	K[a]	W	M	K	W	M[a]	K[a]	W[a]
co-positivity	0.98	0.99	0.96	0.95	0.96	0.96	0.95	0.95	0.95
co-negativity	0.53	0.27	0.53	0.80	0.93	0.67	1.00	1.00	1.00
predictive positive	0.95	0.92	0.95	0.98	0.99	0.96	1.00	1.00	1.00
predictive negative	0.73	0.80	0.62	0.63	0.70	0.63	0.68	0.68	0.68
p (sera)	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90
p (eluate)	0.93	0.97	0.91	0.87	0.87	0.89	0.85	0.85	0.85

M: Melitta; K: Klabin; W: Whatman; p: ratio of positive reactions
[a]: ratio of positive reaction with significative difference ($p<0.05$)

TABLE VII

Validate indices of the ELISA for *Trypanosoma cruzi* antibodies in filter paper blood eluates, in days 1, 15 and 60 after collection, relative to sera ELISA (cut off point 0.260 nm in 1:80 dilution)

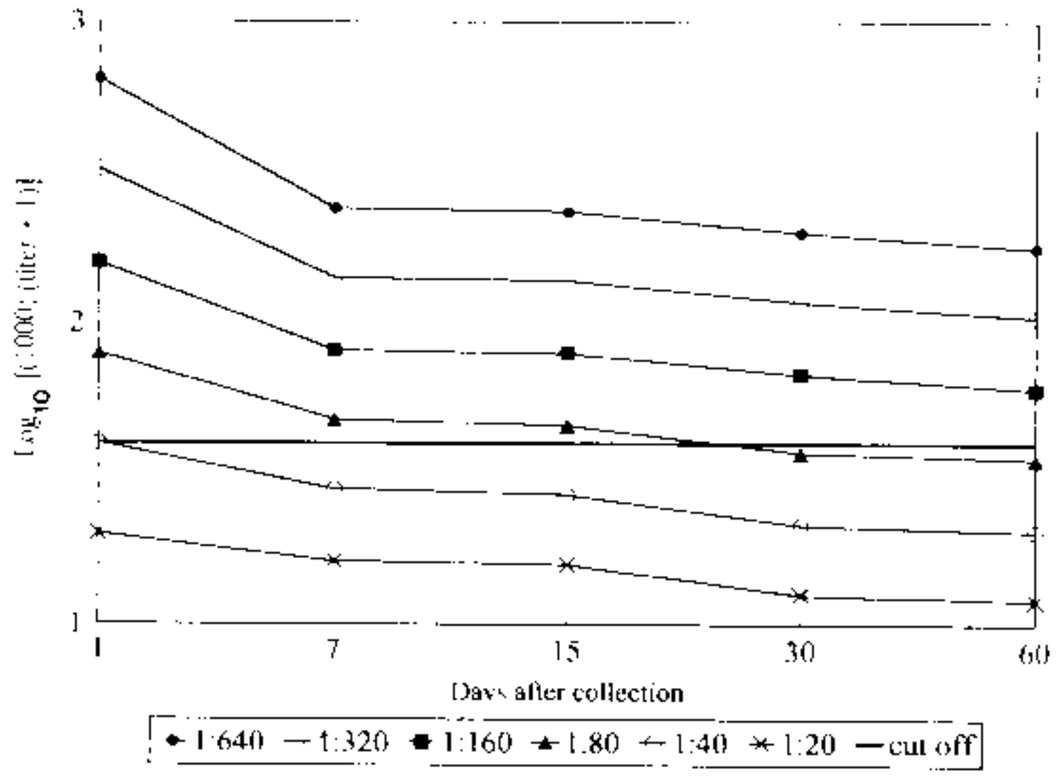
	Days after collection								
	1			15			60		
	M	K	W	M	K	W	M	K	W
co-positivity	0.99	0.99	0.99	0.99	0.99	0.99	1.00	0.99	0.99
co-negativity	0.94	0.94	0.94	0.94	0.94	0.82	0.88	0.88	0.79
predictive positive	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.97
predictive negative	0.94	0.94	0.94	0.94	0.89	0.94	1.00	0.94	0.94
p (sera)	0.89	0.89	0.89	0.89	0.89	0.89	0.89	0.89	0.89
p (eluate)	0.89	0.89	0.89	0.89	0.88	0.89	0.90	0.89	0.89

M: Melitta; K: Klabin; W: Whatman; p: ratio of positive reactions

immunoglobulins present in the filter papers and to discriminate negative and positive samples.

No significant difference was observed comparing sera and eluates run in duplicate (10% of samples), in ELISA, IFAT and IHA.

At least, the reproductibility of the three tests was evaluated, by the double-blind and matched form, with eluate samples of blood dried on different filter papers preserved at 4°C for up to 60th DAC. For IFAT (Whatman filter paper) the fall in the titers occurs up to the 7th DAC, and it is more marked for samples with lower antibodies titers. After the fall of titers, they became stable until the 60th DAC (Fig.). Similar results were observed for the other tests and different filter papers. No changes from positive to negative results were observed for ELISA.



Reproductibility of the IFAT using samples eluated from blood dried on filter paper Whatman No. 4

DISCUSSION

It was possible to shown, with the use of serum, that IFAT, IHA and ELISA had similar capacities to detect anti-*T. cruzi* antibodies. The co-positivity high rates of ELISA (0.99) and IHA (1.00), in relation to that of IFAT, show equal sensitivity for the three reactions. These results are in accordance with Zicker et al. (1990), who compared the results of IFAT, IHA and ELISA for the diagnosis of *T. cruzi* infections. The co-negativity rate, higher in ELISA (1.00) than in IHA (0.79), can be explained by the low specificity of IHA. This discrepancy (four false positives by IHA, negative by IFAT) can be due to inespecific IgM, but this reactivity was not evaluate by the incubation of sera with 2 mercapto-ethanol. The disparity (two false negatives by ELISA, positive by IFAT) can be explained by inespecificity in one of both negatives in ELISA. This serum presented previously negative results during three years. In this work presented low titer (1:40) in sera (IFAT and IHA) and some eluates tested by IHA, being probably true negative. The other case was due to an experimental error since only for ELISA the serum was negative. However, these differences were not significant.

At the cut off point 1:40, we observed that the proportion of positive results of eluates was significantly lower than that of sera when tested in IFAT and IHA. Zicker et al. (1990) also found that the level of agreement between the tests on eluates was very poor. Our results, however, show a low negative predictive value.

The differences in the titer frequency distribution were more remarkable in respect to the low positive titers (1:40 and 1:80).

Kagan (1972) and Lobel et al. (1976) in malaria and Chiari et al. (1987) in goat toxoplasmosis, obtained similar results when comparing the sensitivity of serum and blood collected on filter paper for the screening of antibodies.

The discriminating titer of 1:40 between positive and negative sera adopted for IFAT and IHA in Chagas' disease has been preconized as a cut off point that permits better rates of sensitivity and specificity (Guimarães 1984, Vitor & Chiari 1987). Nevertheless, cut off point of 1:20 is justified when eluates are tested in face of the absence of significant differences, which was observed between the proportions of positive results in eluates and sera, tested by IFAT and IHA. This strategy allows an increase in the sensitivity of reactions without reducing the specificity of IFAT in eluates. Although the employment of the cut off point of 1:20 had increased the reaction sensitivity in IHA, it caused a considerable decrease in the specificity.

A possible decrease of specificity by the employment of a cut off point of 1:20 in eluates in one of these reactions could be resolved by using two-stage surveys. The first stage would constitute a trial of positive cases and the second would confirm the diagnosis and doubtful samples. The realization of diagnosis in the second stage, with titration, would permit discrimination of samples with titers above 1:40 from those with titers equal to or below 1:20. This would enable distinction between real positive and doubtful or negative cases. To define doubtful cases, it would be necessary to employ a technique that should show a sensitivity and specificity for eluates over 90%, in association with traditional methods. The results suggest that the most appropriate technique should be ELISA. In spite of a decrease of the antibodies levels having being observed along time, with ELISA this decrease did not exercise any influence upon the sensitivity and specificity of the reaction, unlikely what was observed regarding IFAT and IHA.

The present results show that the three tested filter papers (Melitta, Klabin and Whatman) have similar capacities to maintain anti-*T. cruzi* immunoglobulins. The following recommendations

are made: (a) in regional laboratories, eluates should be tested by IFAT with a cut off point of 1:20; (b) aliquots of blood dried on filter paper samples, with positive results, should be sent to a reference laboratory where the eluates could be assessed by IFAT, IHA and ELISA; (c) for samples giving consistently doubtful results, serum samples should be obtained to be tested in a reference laboratory.

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