Mass screening for *Trypanosoma cruzi* infections using the immunofluorescence, ELISA and haemagglutination tests on serum samples and on blood eluates from filter-paper

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Methods used to diagnose Trypanosoma cruzi *infection differ in their ability to discriminate between sera from infected and uninfected individuals. We compared the results of an immunofluorescence (IF) test, a haemagglutination (HA) test, and an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of* T. cruzi *infections in a large population-based survey in central Brazil using blood eluates from filter-paper and venous blood samples. The sensitivities of the tests on eluates, compared with results on serum samples, were low: ELISA (78.1%), IF (69.2%) and HA (64.6%). The level of agreement between the tests on eluates was very poor, with the best co-positivity for IF and ELISA. Both the positive and negative predictive values of the three tests on eluates were similar (around 96%) to those for sera. Higher co-positivity values were obtained for the three tests on sera. The implications of these results are discussed in relation to blood screening, routine medical practice, sero-epidemiological surveys, and the follow-up of patients admitted to therapeutic trials.*

Introduction

Various serological techniques have been used to diagnose the chronic phase of Chagas disease, and of these, the indirect immunofluorescence (IF), indirect haemagglutination (HA), and complement fixation tests are the most common. Although these methods are widely employed, the results obtained vary within and between laboratories (*1*). The enzyme-linked immunosorbent assay (ELISA) for antibodies to *Trypanosoma cruzi* is both sensitive and simple to perform but is not used routinely (*2–4*), while a competition antibody enzyme immunoassay based on monoclonal antibodies is being developed (*5*).

Attempts have been made in reference laboratories to standardize some of these methods, but there are still considerable variations in procedures and in the criteria used to evaluate the results. Tests have usually been compared using a panel of serum samples from patients with Chagas disease and from sero-negative controls (*1, 6*). Under these circumstances, the predictive value of a test may be misleading (*7*). The IF tests have been evaluated in non-selected population surveys, but neither HA nor ELISA techniques have been assessed in this way (*13*).

In the present article, we compare the results of IF, HA, and ELISA, using both blood eluates from filter-paper and sera from venous blood samples, to diagnose *T. cruzi* infections, in a large population-based study.

Methods

Blood collection

A cross-sectional survey of the prevalence of *T. cruzi* infections was carried out in 1988 among unskilled workers in the city of Goiânia, central Brazil. The survey was part of a study to assess the health impact of Chagas disease in urban areas (*8, 9*). Seven institutions that employed manual workers were selected and 6222 subjects were screened. A blood sample was collected from each worker by finger-prick onto filter-paper (Whatman No. 1), filling two circles of 1.6-cm diameter (*10*). After drying at room temperature, the filter-papers were stored at 4 °C. For serological testing, one circle was cut out and eluted with 0.25 ml of 0.01 mol/l phosphate-buffered saline

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Reprint No. **5097**

(PBS) for 24 hours to obtain a final dilution of 1:10. Eluates from the filter-paper were each tested for *T. cruzi* antibodies using IF, ELISA, and HA tests. All the tests were conducted independently in different laboratories in Goiânia.

A total of 782 (12.6%) of the workers were seropositive in at least one of the three tests on blood eluates. An attempt was made 2–4 months after the survey to collect a venous blood sample from all seropositives and an age-, sex-, and institution-matched sample of seronegatives. A total of 69 seropositives could not be included since they had changed their place of employment; however, the remaining 713 were invited for an interview and 640 (89.8%) were examined. Of the 5440 seronegatives, 727 were selected at random, after stratification by age, sex, and institution (to correspond to the distribution of the seropositive workers) and, of these, 78 could not be traced. Of the remaining 649 seronegatives, 539 (83.1%) were examined. A sample of venous blood (10 ml) was obtained for each of the 1179 workers examined and *T. cruzi* antibodies were assayed by IF, ELISA and HA tests in the same laboratories that had performed these tests on the filter-paper eluates.

Serological tests

ELISA. The method described by Voller et al. was employed (*4*) but with antihuman IgG conjugated to horseradish peroxidase and *o*-phenylenediamine as substrate. Duplicate tests were carried out in disposable polystyrene microtitration plates with 96 flat-bottomed wells using a crude antigen for epimastigote culture forms of *T. cruzi* Y strain. The absorbances at $\lambda = 492$ nm were determined using a portable microplate spectrophotometer reader. Two duplicates of negative control samples and three positive controls (with low, medium, or high titres of antibodies) were assayed on all plates. The cut-off value for ELISA positivity was taken as the arithmetic mean of the absorbances of the two negative control samples and the lowest of the positive controls.

Immunofluorescence test. This was performed using the method described by Camargo (*11*). All samples were initially screened at a 1:40 dilution and those that were positive were retested in doubling dilutions up to 1:1280.

Haemagglutination test. This was carried out as described by Camargo et al. using a commercial kit (*12*). Eluates from filter-paper were tested at a 1:20 dilution and sera were screened initially at a 1:2 dilution; thereafter, positive samples were tested at up to a 1:64 dilution.

Definition of seropositivity

A pilot study was carried out using serum and filter-paper eluates to standardize the techniques and the antigen, and to define cut-off titres. A subject was classified initially as "eluate-positive" if any of the serological tests on blood collected on filter-paper was "positive", defined as follows: IF titre $>1:40$; ELISA absorbance $\geqslant 1.2$ times the cut-off value; and HA test positive at a dilution of 1:20 in eluate. For the IF and ELISA tests on sera, the criteria for positivity were the same as those for eluates, while for the HA test a titre of $\geqslant 1:16$ was taken to be positive. All other results were classified as negative.

Statistical analysis

For each of the tests, we estimated the sensitivity, specificity, and positive and negative predictive values of the antibody assay for eluates compared to those for sera. Of the 782 subjects who were eluate-positive, 640 (81.8%) had a venous blood sample assayed, and of 5440 who were eluate-negative, 539 (9.9%) had a venous blood sample assayed.

The sensitivity of a given test for eluates versus that of the same test for sera was expressed as the estimated number of subjects in the study population who were both eluate- and serum-positive divided by the number estimated to be serum-positive in the same test. The specificity was expressed as the number of subjects in the study population who were both eluate- and serum-negative divided by the number who were negative in the serum test.

The positive and negative predictive values of tests on eluates were also estimated relative to the results on sera. The positive predictive value of a test was expressed as the estimated number of subjects in the study population who were both eluate- and serum-positive in a given test divided by the number estimated to be eluate-positive in the same test. The negative predictive value of a test was expressed as the estimated number of subjects in the study population who were both eluate- and serum-negative in a given test divided by the number estimated to be eluate-negative in the same test. The sensitivity, specificity, and positive and negative predictive values for each test on eluates were also estimated, taking as true positives or as true negatives only those individuals whose sera were, respectively, positive or negative in all three tests. The expressions used in the calculations are shown in the Annex.

Results

Based on the results of tests performed on blood eluates, 782 (12.6%) of the 6222 workers had anti-*T. cruzi* antibodies in at least one test. The seroprev-

Table 1: **Comparison of the results of the IF, HA, and ELISA tests for antibodies against *Trypanosoma cruzi* in filter-paper blood eluates from the 6222 study subjects**

	IF/+		IF/−		
	HA/+	HA/−	HA/+	HA/−	Total
ELISA/+	314 (5.0)[a]	162 (2.6)	111 (1.8)	77 (1.2)	664 (10.7)
ELISA/−	43 (0.7)	53 (0.9)	22 (0.4)	5440 (87.4)	5558 (89.3)
Total	357 (5.7)	215 (3.5)	133 (2.2)	5517 (88.6)	6222 (100.0)

[a] Figures in parentheses are the percentage of the total number of samples.

Table 2: **Comparison of the results of the IF, HA, and ELISA tests for antibodies against *Trypanosoma cruzi* in blood eluates and in serum samples for "eluate-positive" and "eluate-negative" subjects[a]**

		Eluate/+ (n=640)		Eluate/− (n=539)	
		Serum/+	Serum/−	Serum/+	Serum/−
IF	Eluate/+	462 (72.2)[b]	26 (4.1)	—	—
	Eluate/−	131 (20.5)	21 (3.3)	9 (1.7)	530 (98.3)
HA	Eluate/+	386 (60.9)	18 (2.2)	—	—
	Eluate/−	162 (26.5)	74 (10.4)	6 (1.1)	533 (98.8)
ELISA	Eluate/+	525 (82.0)	14 (2.2)	—	—
	Eluate/−	89 (13.9)	12 (1.9)	7 (1.3)	532 (98.7)

[a] For each test method individually.
[b] Figures in parentheses are the percentage of the total number of eluate-positive and eluate-negative samples, respectively.

alences estimated by IF, HA, and ELISA were 9.2%, 7.9%, and 10.7%, respectively. The results of the three antibody tests on each blood eluate are summarized in Table 1. All three methods were positive for only 314 (40.2%) of the 782 samples that were positive in any one method. Of those that were positive in IF or HA tests, 50.6% were positive in both; for IF and ELISA the corresponding proportion was 62.6%; and for ELISA and HA, 58.3%. The following samples were positive in only one test: IF, 53 (6.8%); HA, 22 (2.8%); and ELISA, 77 (9.8%).

Table 2 shows the results obtained with each method on eluates and serum samples for the 1179 subjects examined. Results are shown separately for those classified as eluate-positive and eluate-negative since this facilitates calculation of the sensitivity, specificity, and predictive values of each test for eluates compared with those for sera (see Table 3). The ELISA had the highest sensitivity (78.1%), followed by IF (69.2%), and HA (64.6%); each of the tests had a specificity of around 99.5%. The positive predictive values for IF, HA, and ELISA were 94.7%, 95.5%, and 97.4%, respectively, while the corresponding negative predictive values were 95.5%, 95.5%, and 96.8%. It should be noted that these levels represent only the correspondence between tests on eluates and sera and not the absolute sensitivity or specificity.

Table 3: **Sensitivity, specificity, and positive and negative predictive values of the IF, HA and ELISA tests for *Trypanosoma cruzi* antibodies in filter-paper blood eluates relative to those for the same tests using venous blood samples**

Test	Sensitivity (%)	Specificity (%)	PPV[a] (%)	NPV[a] (%)
IF	69.2	99.4	94.7	95.5
HA	64.6	99.6	95.5	95.5
ELISA	78.1	99.7	97.4	96.8

[a] PPV = positive predictive value; NPV = negative predictive value.

Table 4 compares separately the results obtained with each method on sera for those classified as eluate-negative and eluate-positive. The results indicate that 16 (2.5%) of the 640 eluate-positive subjects were serum-negative in all three serum tests, while of

Table 4: **Comparison of IF, ELISA, and HA tests for antibodies against *Trypanosoma cruzi* in sera from subjects who were eluate-positive or eluate-negative**

	IF/+		IF/−		
	HA/+	HA/−	HA/+	HA/−	Total
Eluate-positive (n=640)					
ELISA/+	515 (80.5)[a]	70 (10.9)	25 (3.9)	4 (0.6)	614 (95.9)
ELISA/−	6 (0.9)	2 (0.3)	2 (0.3)	16 (2.5)	26 (4.1)
Total	521 (81.4)	72 (11.2)	27 (4.2)	20 (3.1)	640 (100.0)
Eluate-negative (n=539)					
ELISA/+	4 (0.7)	3 (0.6)	0 (0.0)	0 (0.0)	7 (1.3)
ELISA/−	1 (0.2)	1 (0.2)	1 (0.2)	529 (98.1)	532 (98.7)
Total	5 (0.9)	4 (0.7)	1 (0.2)	529 (98.1)	539 (100.0)

[a] Figures in parentheses are percentages.

539 eluate-negative subjects, 10 (1.8%) were serum-positive in at least one test.

For serum samples the agreement between tests was better than that for eluates. The IF, HA, and ELISA tests were all positive for 519 (81.9%) of the 634 serum samples that were positive in at least one of the tests. Individually, the IF test detected 602 (95%); the HA, 554 (87.4%); and the ELISA, 621 (97.9%) of the samples that were positive in at least one of the tests on sera.

Of those samples that were positive in IF or HA tests, 83.5% were positive in both; for those positive in IF or ELISA tests, 93.8% were positive in both; and for ELISA and HA tests the corresponding proportion was 86.2%. The following proportions of positive serum samples were positive in only one test; IF (0.5%), HA (0.5%), and ELISA (0.6%).

The sensitivity and positive predictive values of the tests on eluates, taking as true positives only those sera that were positive in all three tests, are shown in Table 5. The sensitivity increased to 72.4% for IF, 67.0% for HA, and 83.2% for ELISA, while positive predictive values decreased to 81.4% for IF, 90.8% for HA, and 84.6% for ELISA. A very small increase in specificity and a decrease in the negative predictive value occurred when true negatives were taken to be only those sera that were negative in all three tests.

Table 5: **Sensitivity, specificity, and positive and negative predictive values of the IF, HA and ELISA blood-eluate tests for *Trypanosoma cruzi* antibodies relative to those for sera that were positive or negative in all three tests**

Test	Sensitivity (%)	Specificity (%)	PPV[a] (%)	NPV[a] (%)
IF	72.4	99.7	81.4	95.0
HA	67.0	99.9	90.8	93.5
ELISA	83.2	99.8	84.6	96.5

[a] PPV = positive predictive value; NPV = negative predictive value.

Discussion

Serological tests are the most reliable and practical procedures for detecting *T. cruzi* infection. Parasitological methods such as xenodiagnosis can be helpful during the acute phase of infection, but during the chronic phase a positive result is obtained for only about 50% of patients. In endemic areas, serodiagnosis has been widely used to screen blood donors, in clinical practice and, less frequently, in epidemiological surveys (*13*).

Serological methods vary considerably in their ability to discriminate between sera from infected and uninfected subjects; this occurs when different tests are used in the same laboratory and also when the same test is used in different laboratories (*1, 6*). The agreement between the results obtained for different methods and laboratories has been assessed by comparing the results of tests on sera for individuals with clinical Chagas disease with those on sera from individuals from non-endemic countries (*14, 15*); however, the test performance in areas where Chagas disease is endemic may be different from that observed with a panel of serum samples. Moreover, the estimated sensitivity and specificity of a given test are measured relative to other methods and may differ from the true values.

With the emergence of acquired immunodeficiency syndrome (AIDS) in Brazil, all the screening procedures for blood donors are being revised (*16*).

Because of their low cost, HA and complement fixation tests are the most widely used techniques for the serodiagnosis of Chagas disease in public blood banks; the IF test has usually been reserved for use in instances when inconclusive results are obtained. However, IF tests can only be carried out if a fluorescence microscope and a trained technician are available. Only IF has been assessed previously on blood eluates in a population-based survey (*13*).

We used a restricted definition of seropositivity and only the first result for each assay with each sample was analysed. The performance of each test was determined after adjusting for the sampling fraction of seropositive and seronegative subjects in the population that exhibited the correct test performance in a field survey. Measured in this way, the performance of the tests was not satisfactory. The sensitivities for eluates compared with those for serum samples were not high, except for the ELISA (78.1%), but were 69.2% for the IF, and 64.6% for the HA tests. The higher sensitivity of the ELISA relative to that of the other tests has been reported previously (*2, 3, 6*). The level of agreement between the results of tests on eluates was very poor, with the highest co-positivity for IF and ELISA (62.6%). The three tests had a positive or negative predictive value of about 96% (Table 3).

Only when those serum samples that were positive in all three methods were taken as "positive" did the sensitivity of the IF, HA and ELISA tests on eluates increase to 72.4%, 67.0%, and 83.2%, respectively. This was expected, since those sera that were positive in all the tests were likely to include a higher proportion of true positives. However, this had the effect of decreasing the positive predictive values for all three tests, since a higher proportion of sera from eluate-positive subjects was not positive in all three tests. The overall performance of tests on sera and the level of agreement between the results were much better than that on eluates.

The tests were run in parallel (all three tests on eluates and on sera were performed simultaneously) and all samples that were positive in any one test were taken to be positive overall. This approach resulted in a screening procedure that had a relatively high sensitivity and low specificity. An alternative procedure is to test samples using one method and then to retest using a different method all those samples that were negative. The sensitivity obtained with such a "series" approach is lower than that with parallel testing, but the specificity is higher (*7*). The choice of an appropriate screening strategy and the selection of tests should be based on simplicity, cost, sensitivity, specificity and the experience of technicians.

The implications of our findings for the choice of screening procedures for the serodiagnosis of *T. cruzi* infection are not straightforward, different situations requiring different approaches.

Transfusion trasmission of trypanosomiasis has been the focus of much attention in Brazil since more new infections probably occur as a result of this route than through vector transmission. In blood banks the main objective of screening is to exclude potentially infected blood, and for this purpose a highly sensitive procedure is required whose specificity is of smaller consequence. A low cut-off point for positivity should be defined in order to exclude all those serum specimens that give borderline results and two or more screening tests could be used in parallel to increase the sensitivity.

For routine purposes, the sensitivity of a test is less important than its specificity; a false positive result may cause unnecessary distress, and even individuals who are truly seropositive can be offered little medical help if they have no clinical evidence of disease. In such situations, a single test for trypanosomiasis (IF or HA) might be sufficient. Diagnosis should be pursued using a combination of several methods if any clinical disorder is detected that could be caused by *T. cruzi* infection.

For the follow-up of subjects who are admitted to therapeutic trials, all available screening techniques should be employed, particularly if the criteria of cure rely on negative seroconversion. Isolation of parasites from peripheral blood by xenodiagnosis is not a sensitive technique during the chronic phase of infection.

For seroepidemiological surveys the ELISA test appears to be the best choice since it is relatively cheap and requires only small quantities of eluate, large number of samples can be processed daily, and the results can be read spectrophotometrically. The collection and storage of blood samples on filter-paper is still the best available procedure for mass screening and appears also to be reliable for ELISA tests, although rapid deterioration of IgG and IgM has been reported (*17*).

In a large screening survey for classifying blood samples for *T. cruzi* antibodies, we have shown reasonable agreement between the results of IF, ELISA, and HA tests. More consistent results were obtained with sera than with eluates. The choice of method, number of tests, and strategy for serological testing depend upon the purpose of the test; however, ELISA is probably a good method for field surveys.

Acknowledgements

This investigation was supported financially by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors are also grateful to Dr R.M. de Oliveira for

assistance with the fieldwork, Mr M.A. Chaves for help in analysing the data, and Miss D.H. de Rezende for secretarial assistance.

Résumé

Dépistage de masse des infections à *Trypanosoma cruzi* par immunofluorescence, titrage ELISA et hémagglutination sur des sérums et des éluats de sang recueilli sur papier-filtre

Les résultats de la recherche d'anticorps dirigés contre *Trypanosoma cruzi* par trois méthodes différentes—immunofluorescence indirecte (IF), titrage immuno-enzymatique (ELISA) et hémagglutination indirecte (HA)—ont été comparés à l'occasion d'une enquête effectuée sur une population urbaine du centre du Brésil où la maladie de Chagas est endémique. Pour cela, des prélèvements de sang ont été faits sur 6 222 travailleurs non qualifiés et recueillis sur papier-filtre. Des prélèvements de sang veineux ont également été pratiqués sur 640 sujets chez lesquels au moins une des épreuves mentionnées ci-dessus avait donné un résultat positif et sur un échantillon aléatoire de 539 sujets (stratifiés en fonction de l'âge, du sexe et de l'institution) chez lesquels les trois épreuves avaient été négatives. Les résultats obtenus avec les sérums ont ensuite été comparés aux résultats de l'examen des éluats correspondants.

La séroprévalence globale de l'infection à *T. cruzi* a été trouvée égale à 12,6%. Les épreuves ELISA, IF et HA ont donné respectivementde 10,7%, 9,2% et 7,9% de résultats positifs. Sur les 782 cas déclarés positifs dans au moins une des épreuves pratiquées sur les éluats, seulement 314 (40,2%) ont été positifs dans les trois épreuves.

On a calculé la sensibilité, la spécificité et la valeur prédictive (positive et négative) des épreuves pratiquées sur les éluats par rapport aux épreuves sur sérum. La sensibilité a été maximale pour l'ELISA (78,1%), suivie par l'IF (69,2%) et l'HA (64,6%), tandis que la spécificité des trois épreuves était d'environ 99,5%. La valeur prédictive positive a été de 97,4% pour l'ELISA, 95,5% pour l'HA et 94,7% pour l'IF. Enfin, la valeur prédictive négative des trois méthodes a été voisine de 96%.

Les performances des trois méthodes ont également été estimées en considérant comme "vrais positifs" les sérums qui avaient été déclarés positifs par les trois méthodes et comme "vrais négatifs" ceux pour lesquels les trois méthodes avaient donné un résultat négatif. Dans ces conditions, la sensibilité a atteint 83,2%, 72,4% et 67,0% respectivement pour l'ELISA, l'IF et l'HA, tandis que la valeur predictive positive était réduite à 84,6% pour l'ELISA, 81,4% pour l'IF et 90,8% pour l'HA. Quant à la spécificité et à la valeur prédictive négative, elles n'ont pratiquement pas changé. En général, la concordance entre les trois méthodes a été meilleure pour les sérums que pour les éluats. L'article se termine par une analyse des leçons que l'on peut tirer de ces résultats pour le diagnostic sérologique de la maladie de Chagas.

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Annex

Procedure used to estimate the sensitivity, specificity, and positive and negative predictive value of antibody tests based on filter-paper blood eluates compared with tests based on venous blood samples

	"Eluate-positive" ($N_1 = 782$)[a]	"Eluate-negative" ($N_2 = 5440$)[b]
No. of venous blood samples assayed	$n_1 = 640$	$n_2 = 539$
Tests that used a specific essay:		
	Serum +ve −ve	Serum +ve −ve
Eluate +ve	*a* *b*	— —
−ve	*c* *d*	*g* *h*
	(a+b+c+d=640)	*(g+h=539)*

[a] No. of eluate samples classified as antibody positive in one or more of the tests.
[b] No. of eluate samples classified as antibody negative in all three of the tests.

nESP = estimated number of subjects who were eluate- and serum-positive for a particular test in the population = $N_1 a/n_1$

nSP = estimated number of subjects who were serum-positive for a particular test in the population = $N_1(a+c)/n_1 + N_2 g/n_2$

nESN = estimated number of subjects who were eluate- and serum-negative for a particular test in the population = $N_1 d/n_1 + N_2 h/n_2$

nSN = estimated number of subjects who were serum-negative for a particular test in the population = $N_1(b+d)/n_1 + N_2 h/n_2$

Estimated sensitivity of the particular test = *nESP/nSP*

Estimated specificity of the particular test = *nESN/nSN*

Estimated positive predictive value of the particular test = $a/(a+b)$

Estimated negative predictive value of the particular test = $nESN/[N_2 + N_1(c+d)/n_1]$