

## Serodiagnosis of Chagas' Disease by Enzyme-Linked Immunosorbent Assay Using Two Synthetic Peptides as Antigens

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**An enzyme-linked immunosorbent assay (ELISA) was developed for detecting antibodies against *Trypanosoma cruzi*. Two synthetic *T. cruzi* peptides, TcD and PEP2, were used. The specificity and sensitivity of the peptide ELISA were determined with 260 serum samples from individuals living in an area in which Chagas' disease is endemic. ELISAs were performed with the peptides singly or in combination. The evaluation of these tests showed that 168 (93.8%) of 179 serum samples from *T. cruzi*-infected patients were positive when TcD peptide was used as antigen; 164 (91.6%) samples were positive with PEP2, and 178 (99.4%) samples were positive when the two peptides were combined. Thus, the sensitivity of the ELISA using the two peptides exceeded 99%. The specificity was evaluated by using a panel of 118 serum samples that included samples from 81 individuals living in an area of endemicity with negative serology for Chagas' disease and from 37 patients from areas in which *T. cruzi* was not endemic but with other pathologies, such as leishmaniasis, tuberculosis, and leprosy. Only two false-positive serum samples were found in this group of individuals, giving a test specificity of more than 98%. Because these peptides can be synthesized and are very stable at room temperature, the use of such reagents can improve the standardization and reproducibility of ELISAs for the serodiagnosis of *T. cruzi* infection.**

Chagas' disease, caused by the parasite protozoan *Trypanosoma cruzi*, is a severe endemic infection affecting several million people in Central and South America. The most important route of transmission, in areas of endemicity, is still by contact of a human being with an infected triatomid bug. However, blood transfusion is the dominant means of transmission in areas in which the disease is not endemic (16, 17). In view of this, screening of blood is mandatory for donors from both areas of endemicity and areas in which the disease is not endemic but in which a high migration of individuals from areas of endemicity occurs. The most specific way to diagnose *T. cruzi* infections is by detection of the parasite in blood samples by direct examination, hemoculture, or xenodiagnosis. However, these methods have a low sensitivity (less than 50%) and are time-consuming (2). Detection of antibodies to *T. cruzi* antigens by immunological methods is still the most powerful method to indicate infection. Different serological techniques have been used since Machado and Guerreiro described in 1913 a complement fixation test for the serodiagnosis of Chagas' disease. More recently, automated tests have become important in the screening of large numbers of samples in a short period of time. The enzyme-linked immunosorbent assay (ELISA) is a useful method that can be automated and used in blood banks to screen for most of the infectious diseases transmissible by blood transfusion (such as AIDS, syphilis, hepatitis, and human T-cell leukemia virus). In most of the ELISAs developed for detecting *T. cruzi* infection, complex mixtures of antigens obtained by lysis of epimastigotes or trypomastigotes have been used (1, 10). Advances in molecular biology and recombinant gene expression have permitted the

identification and recombinant expression of important *T. cruzi* antigen genes (7, 8, 11, 14). Evaluation of some of these defined antigens has shown promising results for the diagnosis of *T. cruzi* infection (6, 8, 13, 15). With that approach, peptides have also been obtained and used as antigens in ELISAs (3, 19, 20). Among the different peptides described, two (TcD and peptide 2) were shown to have high sensitivity and specificity in detecting specific antibodies in sera from *T. cruzi*-infected individuals. In previous studies, the TcD peptide (3) showed a sensitivity of 95% for detecting antibodies in sera from patients from different areas of endemicity. Similarly, peptide 2 (19) was shown to be very effective in detecting antibodies in 93% of serum samples obtained from a Chilean population with Chagas' disease. In view of these data, we decided to evaluate these two peptides individually and in a combination ELISA, using sera from individuals living in an area of Brazil where Chagas' disease is endemic. Serum samples were from individuals infected with *T. cruzi*, as confirmed by clinical diagnosis, xenodiagnosis, and three conventional serological tests, or with other infectious agents known to cross-react serologically with *T. cruzi*.

### MATERIALS AND METHODS

**Study population.** Serum samples were collected from 260 individuals living in an area in which Chagas' disease is endemic, Virgem da Lapa City, located in the state of Minas Gerais, Brazil. All individuals were evaluated by an epidemiologic questionnaire, and physical examinations were conducted. Laboratory evaluations were done by electrocardiogram, chest radiograph, or radiologic imaging of the gastrointestinal tract. Three serological tests (indirect immunofluorescence assay [IFA], indirect hemagglutination [IHA], and ELISA) for detection of anti-*T. cruzi* antibodies were performed. The individuals were grouped into patients with or

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without Chagasic infection according to the clinical and serological findings. In order to test the specificity of the peptide assays, 37 serum samples were collected from patients with other infectious diseases known to cross-react serologically with crude *T. cruzi* antigen preparations. These included 11 samples from patients with visceral leishmaniasis (*Leishmania chagasi* infection), 12 samples from patients with cutaneous leishmaniasis (*L. brasiliensis* infection), 7 samples from patients with tuberculosis, and 7 samples from patients with leprosy.

**Synthetic peptides.** Two different peptides (TcD and PEP2), synthesized on the basis of repeat units from *T. cruzi* trypomastigote antigens, were used. They were selected on the basis of their previous performance in serological assays for the diagnosis of *T. cruzi* infection (3, 20). The TcD peptide contains 15 amino acids representing 1.5 of the 10-amino-acid repeat (AEPKSAEPKP) present in a high-molecular-weight trypomastigote antigen. This antigen appears to be specific to and conserved among *T. cruzi* isolates (3). PEP2 is a 22-amino-acid peptide sequence (GDKPSFPGOAAAGDKPSPFGQA) representing 1 of a 12-amino-acid repeat (PSPFGQAAAGDK) and 10 amino acids from this repeat of recombinant clone 2, which encodes a portion of a *T. cruzi* antigen also present in trypomastigotes (20). Peptides were synthesized on an Applied Biosystems instrument (model 430A) by using *N*-tert-butoxycarbonyl chemistry and were purified by reverse-phase high-performance liquid chromatography. Peptides were characterized by amino acid analysis with a Beckman 6300 system and by plasma desorption mass spectrometry on a Bio-Ion 20 spectrometer (3).

**Serological tests.** (i) **IFA.** The IFA technique described by Camargo (4) was followed. *T. cruzi* epimastigotes, Y strain, were used as antigen, and goat anti-human immunoglobulin G, fluorescein isothiocyanate conjugated (Cooper; Organon Teknika, Durham, N.C.), was used as a second antibody.

(ii) **IHA and conventional ELISA.** Commercial reagents were used for both tests. A Chagas HAI-Immunoserum kit (Immunoserum-Tecnologia Imunologica Industria e Comercio, São Paulo, Brazil) was used for the IHA, and an ELISA-hemobio Chagas (EMBRABIO-Empresa Brasileira de Biotecnologia, São Paulo, Brazil) was used for the ELISA test. All tests were performed according to the manufacturer's instructions.

(iii) **Peptide ELISA.** Plastic 96-well plates (Corning Easy Wash High Binding; Corning Laboratories, Corning, N.Y.) were coated with 50  $\mu$ l of TcD peptide (10  $\mu$ g/ml), PEP2 peptide (2.5  $\mu$ g/ml), or a mixture of the two peptides (TcD, 10  $\mu$ g/ml; and PEP2, 2.5  $\mu$ g/ml) per well diluted in 0.05 M carbonate buffer (pH 9.6). Plates were incubated for 1 h at 37°C and maintained at 4°C until use for up to 1 month. For use, sensitized wells were washed with 0.01 M phosphate-buffered saline (pH 7.2)–0.3% Tween 20 (PBS/T). Positive control, negative control, and unknown serum samples were diluted 1:20 in PBS/T containing 0.5% bovine serum albumin, and 50  $\mu$ l was added to each well. After 30 min of incubation at 37°C, wells were washed six times with PBS/T, 50  $\mu$ l of protein A-peroxidase (Zymed Laboratories, San Francisco, Calif.) diluted in PBS/T-bovine serum albumin was added, and the plates were incubated as described before. Wells were washed eight times with PBS/T, and 100  $\mu$ l of 2,2'-azino-di-3-ethylbenzthiazoline sulfonic acid (ABTS) substrate solution (50  $\mu$ l of 50 $\times$  ABTS, 50  $\mu$ l of 1.5% H<sub>2</sub>O<sub>2</sub>, 2.5 ml of 0.1 M citrate buffer [pH 4.1]; Zymed) was added. After 15 min at room temperature, the enzymatic reaction was stopped by adding 100  $\mu$ l of 10% sodium dodecyl sulfate. A<sub>405</sub> values were determined with an ELISA reader (Titertek Multiskan; Flow

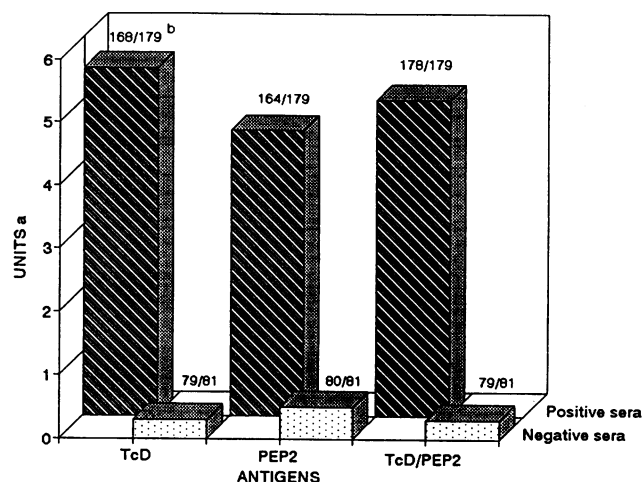


FIG. 1. Reactivity of serum samples from 260 individuals living in an area where Chagas' disease is endemic; samples were analyzed by ELISA, using TcD and PEP2 peptides as antigens. <sup>a</sup>Units = mean of sample absorbance/cutoff absorbance; <sup>b</sup>number of serum samples in ELISA peptide/number of serum samples in IHA-IFA-ELISA.

Laboratories, McLean, Va.). For each test, five negative control serum samples and two positive Chagas' patient serum samples were included. Test results were considered acceptable only when negative control sera had absorbances above 0.2 and positive control sera had absorbances between 0.6 and 0.8 (low positive), or between 1.2 and 1.4 (high positive). The cutoff was determined for each test by using the following calculation: mean of negative sera plus 3 standard deviations.

## RESULTS

Sera from 260 individuals living in an area of endemicity for Chagas' disease were assayed by IFA, IHA, and ELISA for the diagnosis of *T. cruzi* infection. One hundred seventy-nine positive serum samples and 81 negative serum samples in all three tests were chosen for further study, using the TcD and PEP2 ELISA (Fig. 1). From the data shown in Fig. 1, the TcD ELISA was found to be 93% sensitive and the PEP2 ELISA was 91% sensitive. As observed in Table 1, only one serum sample did not react with either peptide. In view of these results, an ELISA test using a mixture of both peptides was designed and evaluated. Using this assay, only one positive serum sample was missed, and the test was shown to have a sensitivity of greater than 99%. The specificity of the peptide test was evaluated with sera from individuals living in an area of endemicity for Chagas' disease who had negative *T. cruzi* serology, as well as with sera from patients with other pathologies. In these samples, 2 of 81 samples were positive, but no

TABLE 1. Reactivity of serum samples from 260 individuals living in an area where Chagas' disease is endemic analyzed by IFA, IHA, and ELISA versus ELISA with TcD and PEP2 peptides

IFA-IHA-ELISA result	No. of samples					
	ELISA TcD		ELISA PEP2		ELISA TcD/PEP2	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive (n = 179)	168	11	164	15	178	1
Negative (n = 81)	2	79	1	80	2	79

TABLE 2. Number of positive serum samples from a group of healthy individuals and patients with different infectious diseases tested by different serological assays used for detection of *T. cruzi* infection

Disease	No. of samples tested	No. of samples positive					
		ELISA				IHA IMUNO <sup>a</sup>	IFA
		EMBR <sup>b</sup>	TcD	PEP2	TcD/PEP2		
Cutaneous leishmaniasis	12	2	0	0	0	0	1
Visceral leishmaniasis	11	3	0	0	0	0	5
Leprosy	7	0	1	0	0	1	1
Tuberculosis	7	0	0	1	0	0	0
Healthy <sup>c</sup>	81	0	2	1	2	0	0
Total	118	5	3	2	2	1	7
Specificity (%)		95	97	98	99	99	94

<sup>a</sup> IMUNO, commercial indirect hemagglutination assay from IMMUNOSERUM.

<sup>b</sup> EMBR, commercial ELISA from EMBRABIO.

<sup>c</sup> Individuals from an area where Chagas' disease is endemic but with negative serology.

false-positive results were found among the 37 serum samples from individuals with other pathologies (Table 2). All cutaneous and visceral leishmaniasis serum samples were negative in the mixed-peptide assay, including those that were positive by IFA and ELISA. It should be pointed out that with the commercial IHA test, in which a *T. cruzi* crude extract is used as antigen, no false-positive results were found.

In one of our first attempts to evaluate the TcD peptide, only 65% sensitivity and low absorbance values were found among positive sera. NUNC Immuno modules (Nunc Labs, Roskilde, Denmark) were used in this experiment (data not shown). In view of these results, a comparison of two different brands of plates, NUNC and Corning, was done. The latter had been used in the original TcD evaluation. Of five *T. cruzi*-positive serum samples, three samples were found to be negative when both peptides were used on NUNC plates, whereas all five samples were strongly positive on the Corning plates. These results probably reflect the differential ability of the plates in binding the synthetic peptides.

## DISCUSSION

Serological methods are widely accepted for the immunodiagnosis of Chagas' disease. Among them, IFA and IHA have been commercially available for a long time and consequently are the tests most often used in clinical laboratories and blood banks. Recently, enzyme immunoassays have been introduced and used for the screening of blood donors. However, complex mixtures of components extracted from parasites at different stages of the life cycle have been used as antigen (1, 10). Different *T. cruzi* strains and antigen extraction techniques have also been used, introducing a source of variability in the final reagent and giving controversial results.

To overcome these problems, we developed and evaluated an ELISA that uses a mixture of two *T. cruzi* trypomastigote-derived synthetic peptides as antigens. Initially, when TcD and PEP2 peptides were individually used as antigens in ELISAs, the sensitivities of the tests were 93 and 91%, respectively. These results are in agreement with previous data in which TcD showed 95% sensitivity (3) and PEP2 showed 92% sensitivity (20). It should be pointed out that both peptides were described as having high specificity and showed no cross-reactivity with leishmaniasis patient sera. These sera are considered one of the most commonly cross-reactive groups in the IFA (5, 9). We obtained similar results, although two serum samples from patients without Chagas' disease showed

positive results with TcD and one sample was positive with PEP2. Other peptides have been used as antigens for the diagnosis of *T. cruzi* infection, but their sensitivities in detecting specific antibodies were shown to be lower than those of the peptides evaluated in this study (19). Considering these results, we decided to combine the TcD and PEP2 peptides and use them as a single antigen in an ELISA. The use of a mixture of defined antigens in an ELISA for the serodiagnosis of Chagas' disease has been tested before with two recombinant antigens, CRA and FRA (13). In this case, the test showed 100% sensitivity and 100% specificity. However, a mixture of synthetic peptides was not tested, although reports have suggested that a combination of synthetic peptides would increase the sensitivity in detecting most of infected patients (19). With a combination of TcD and PEP2 peptides, we found high levels of sensitivity (99.7%) and specificity (99%). The only serum sample that showed a false-negative result was obtained from a patient who had received specific treatment for Chagas' disease. This serum sample also showed a weak positive reaction in the commercial ELISA test and IFA (1:40). It has been documented that after specific treatment antibody responses to *T. cruzi* antigens can decrease, and sometimes conventional serological tests become negative (12, 18).

Recombinant polypeptides are powerful reagents for serological diagnosis, mainly due to the larger spectrum of epitopes on their structure. However, synthetic peptides can be very useful because they can be readily produced in large amounts and standardized. Our results demonstrate the applicability of two well-defined synthetic peptides, particularly when used in combination, to the diagnosis of Chagas' disease. Their use in an ELISA would be important because of their stability, reproducibility, and reliability.

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## REFERENCES

1. Anthony, R. L., C. M. Johnson, and O. E. Sousa. 1979. Use of micro-ELISA for quantitating antibody to *Trypanosoma cruzi* and *Trypanosoma rangeli*. Am. J. Trop. Med. Hyg. 28:969-973.
2. Brofen, E., F. S. Assis Rocha, G. B. Machado, M. M. Perillo, A. I. Romanha, and E. Chiari. 1989. Isolation of *Trypanosoma cruzi* samples by xenodiagnosis and hemoculture from patients with chronic Chagas' disease. Mem. Inst. Oswaldo Cruz 84:237-240.

3. Burns, J. M., Jr., W. G. Shreffler, D. E. Rosman, P. R. Sleath, C. J. March, and S. G. Reed. 1992. Identification and synthesis of a major conserved antigenic epitope of *Trypanosoma cruzi*. Proc. Natl. Acad. Sci. USA **89**:1239-1243.
4. Camargo, M. E. 1966. Fluorescent antibody test for the serodiagnosis. Technical modification employing preserved culture forms of *Trypanosoma cruzi* in slide test. Rev. Inst. Med. Trop. São Paulo **8**:227-234.
5. Camargo, M. E., and C. Rebonato. 1969. Cross-reactivity in fluorescence test for trypanosoma and leishmania antibodies—a simple inhibition procedure to ensure specific results. Am. J. Trop. Med. Hyg. **18**:500-505.
6. Cetron, M. S., R. Hoff, S. Kanh, H. Eisen, and W. C. Van Voorhis. 1992. Evaluation of recombinant trypomastigote surface antigens of *Trypanosoma cruzi* in screening sera from a population in rural Northeastern Brazil endemic for Chagas' disease. Acta Trop. **50**:259-266.
7. Cotrim, P. C., G. S. Paranhos, R. A. Mortara, J. Wanderley, A. Rassi, M. E. Camargo, and J. Franco da Silveira. 1990. Expression in *Escherichia coli* of a dominant immunogen of *Trypanosoma cruzi* recognized by human chagasic sera. J. Clin. Microbiol. **28**:519-524.
8. Gruber, A., and B. Zingales. 1993. *Trypanosoma cruzi*: characterization of two recombinant antigens with potential application in the diagnosis of Chagas' disease. Exp. Parasitol. **76**:1-12.
9. Guimarães, M. C. S., B. J. Celeste, E. A. Castilho, J. R. Mineo, and J. M. P. Diniz. 1981. Immunoenzymatic assay (ELISA) in mucocutaneous leishmaniasis, Kala-azar and Chagas' disease: an epimastigote *Trypanosoma cruzi* antigen able to distinguish between anti-trypanosoma and anti-leishmania antibodies. Am. J. Trop. Med. Hyg. **30**:942-947.
10. Gusmão, R. A., J. M. Rezende, A. Rassi, A. A. Gam, and F. A. Neva. 1982. Antibody levels to *Trypanosoma cruzi* in infected patients with and without evidence of chronic Chagas' disease. Am. J. Trop. Hyg. **31**:452-458.
11. Ibanez, C. F., J. L. Afranchino, R. A. Macina, M. B. Reyes, S. Leguizamón, M. E. Camargo, L. Aslund, U. Pettersson, and A. C. C. Frasch. 1988. Multiple *Trypanosoma cruzi* antigens containing tandemly repeated aminoacid sequence motifs. Mol. Biochem. Parasitol. **30**:27-34.
12. Krettli, A. U., J. R. Cançado, and Z. Brener. 1982. Effect of specific chemotherapy on the level of lytic antibodies in Chagas' disease. Trans. R. Soc. Trop. Med. Hyg. **76**:334-340.
13. Krieger, M. A., E. Almeida, W. Oeleman, J. J. Lafaille, J. Borges-Pereira, H. Krieger, M. R. Carvalho, and S. Goldenberg. 1992. Use of recombinant antigens for the accurate immunodiagnosis of Chagas' disease. Am. J. Trop. Med. Hyg. **46**:427-434.
14. Lafaille, J. J., J. Linss, M. A. Krieger, T. Souto-Padron, W. De Souza, and S. Goldenberg. 1990. Structure and expression of two *Trypanosoma cruzi* genes encoding antigenic proteins bearing repetitive epitopes. Mol. Biochem. Parasitol. **35**:127-136.
15. Lorca, M., A. Gonzales, C. Veloso, V. Reyes, and U. Vergara. 1992. Immunodetection of antibodies in sera from symptomatic and asymptomatic Chilean Chagas' disease patients with *Trypanosoma cruzi* recombinant antigens. Am. J. Med. Hyg. **46**:44-49.
16. Moncayo, A. 1992. Chagas' disease: epidemiology and prospects for interruption of transmission in the Americas. World Health Stat. Qu. **45**:276-279.
17. Schmuñis, G. A. 1991. *Trypanosoma cruzi*, the etiologic agent of Chagas' disease: status in the blood supply in endemic and nonendemic countries. Transfusion **31**:547-557.
18. Schmuñis, G. A., A. Szarfman, L. Coarasa, C. Guilleron, and J. M. Peralta. 1980. Anti-*Trypanosoma cruzi* agglutinins in acute human Chagas' disease. Am. J. Trop. Med. Hyg. **29**:170-178.
19. Vergara, U., M. Lorca, C. Veloso, A. Gonzalez, A. Engstrom, L. Aslund, U. Pettersson, and A. Frasch. 1991. Assay for detection of *Trypanosoma cruzi* antibodies in human sera based on reaction with synthetic peptides. J. Clin. Microbiol. **29**:2034-2037.
20. Vergara, U., C. Veloso, A. Gonzalez, and M. Lorca. 1992. Evaluation of an enzyme-linked immunosorbent assay for the diagnosis of Chagas' disease using synthetic peptides. Am. J. Trop. Med. Hyg. **46**:39-43.