

## *Trypanosoma cruzi* Recombinant Complement Regulatory Protein: a Novel Antigen for Use in an Enzyme-Linked Immunosorbent Assay for Diagnosis of Chagas' Disease

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Currently, diagnosis of Chagas' disease is based on serological methods, but due to the high occurrence of inconclusive results, more reliable methods are needed. The use of recombinant antigens for serodiagnosis of Chagas' disease is recommended in order to increase the sensitivity and specificity of the serological tests. The *Trypanosoma cruzi* complement regulatory protein (CRP) is a surface glycoprotein present on the trypomastigote forms of the parasite, and the recombinant CRP (rCRP) was cloned in a mammalian expression system and purified by affinity chromatography. The purified recombinant protein was used as an antigen in an enzyme-linked immunosorbent assay (rCRP ELISA) in order to verify its sensitivity and specificity compared with other established methods. In this evaluation, a panel of 184 serum samples distributed among chronic chagasic patients ( $n = 65$ ), blood bank donors ( $n = 100$ ), and patients infected with *Leishmania* spp. ( $n = 19$ ) was used. The sensitivity and specificity of the rCRP ELISA were 100% when compared to conventional serology and complement-mediated lysis tests from these groups. When hemoculture and PCR tests were evaluated for diagnosis of chronic chagasic patients, using the rCRP ELISA as a reference test, the positivities were found to be 64.62 and 81.54%, respectively, showing a higher degree of sensitivity of the test. The data demonstrate that rCRP ELISA was able to discriminate between chronic chagasic patients and nonchagasic individuals, such as blood donors and patients with leishmaniasis. Thus, the rCRP is an excellent antigen for use in Chagas' disease diagnosis, due to the absence of false-negative or false-positive results.

Chagas' disease, first described by Carlos Chagas in 1909, remains a public health concern even after several efforts to reduce the incidence of cases, which affect 16 to 18 million people in Latin America (52). The etiological agent, *Trypanosoma cruzi*, is normally transmitted by infected triatomid bugs; however, cases associated with blood transfusion, organ transplant, and congenital infection have been described even in countries to which the disease is not endemic (7, 18, 45, 47).

The diagnosis of Chagas' disease can be made by the detection of the parasite by indirect parasitological methods (xenodiagnosis and hemoculture) or, more frequently, by detection of immunoglobulin G (IgG) antibodies against *T. cruzi* in sera from chronically chagasic patients. The parasitological methods show high specificity but are limited in terms of sensitivity due to the low parasitemia in the chronic phase. By such methods parasites are detectable in only 25 to 75% of individuals known to be infected by *T. cruzi* (15, 44). The use of conventional serologic tests (enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence, and indirect hemagglutination) is still the main method for the Chagas'

disease diagnosis in the chronic phase. The epimastigote form of *T. cruzi* is generally used as a source of antigen because it is easily cultured in acellular medium and adequate amounts of antigen can be readily obtained (13). However, its antigenic fraction contains complex molecules, which favors the appearance of false-positive reactions and cross-reactivity with sera from patients bearing other parasites, especially *Leishmania* spp. and *Trypanosoma rangeli* (3, 16). In addition, this antigenic heterogeneity does not allow the differential diagnosis between the acute and chronic phases and also among the clinical manifestations (48). Indeed, discrepancies or inconclusive results, such as false-positive reactions caused by cross-reactivity with antibodies induced by other pathogens (mainly *Leishmania*) and false-negatives, may occur (35, 41, 42). For these reasons, the World Health Organization recommends using at least two of those serologic tests in parallel (52). The use of well-characterized antigens and their preparation under quality-control conditions have introduced a source variability in the final agent, and controversial results have been obtained with the reagents from whole or semipurified extracts of *T. cruzi* (12).

In recent years, development of recombinant DNA techniques permitted construction of different bacterial and eukaryotic gene expression systems which allow production of parasite antigens in large amounts and with a high level of purity. Several *T. cruzi* genes were isolated and cloned, and some recombinant antigens have been assayed and tested for diagnosis. The results, however, showed variable efficacy for

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the different tested antigens, and it was observed that some of them displayed better performance when used in combination than separately, producing results with sensitivities and specificities that reached up to 100% (1, 2, 17, 23, 24, 31, 32, 40, 49, 54).

More recently, use of the PCR was suggested as an alternative means of diagnosing Chagas' disease (6, 9, 51); however, variable levels of sensitivity in the detection of *T. cruzi* DNA have been reported by several laboratories. The initial data reported values from 96 to 100% compared with conventional serology (6, 51), but lower sensitivity levels were also observed (9, 22, 25) due to the extremely low parasitemia in the chronic phase of disease. The occurrence of these problems affecting sensitivity and specificity in the various tests cited above points to the necessity of developing more-reliable assays with well-defined antigen preparations for diagnosis (4).

The complement-mediated lysis (CoML) test detects the presence of protective antibodies during chronic infections with 100% sensitivity and specificity, but not in the uninfected, immunized host (26, 30). These antibodies, which induce lysis of *T. cruzi* in the presence of complement and are referred to as lytic antibodies, recognize antigens on the surfaces of living trypomastigotes and represent a class of antibodies distinct from those detected by conventional serologic tests (30). In addition, the CoML test may be the best indicator of an ongoing infection and an indication of treatment failure in chagasic patients (32). However, this method is not applicable for routine diagnosis in the clinical laboratory due to the manipulation of live infectious parasites, although the test is excellent for use as a reference (30). One of the antigenic targets of lytic antibodies was found to be a 160-kDa trypomastigote-specific surface glycoprotein (34, 36). This protein was later purified from the parasites and characterized as a complement regulatory protein (CRP) that functions to inhibit complement activation and lysis of the parasites (37). It was shown that antibodies to this protein could neutralize the complement inhibitory function, thus supporting complement-mediated lysis. Since the CRP is a target of complement lytic antibodies and the CoML test has been shown to be one of the best tests of ongoing infection, we sought to determine if a recombinant CRP-based ELISA could effectively replace the CoML and other diagnostic tests for detection of ongoing *T. cruzi* infection.

A full-length cDNA encoding the *T. cruzi* CRP was previously isolated (38) and stably transfected in mammalian cells, allowing its large-scale production and purification (M. Beucher, W. S. F. Meira, V. Zegarra, L. M. C. Galvão, E. Chiari, and K. A. Norris, submitted for publication). In this study, we report the standardization of an ELISA test using the recombinant CRP (rCRP) of *T. cruzi* and the evaluation of its efficacy in Chagas' disease diagnosis compared with well-established methods, such as hemoculture, PCR, complement-mediated lysis, and conventional serology.

#### MATERIALS AND METHODS

**Human sera.** A total of 184 sera were used in this study. Sixty-five serum samples from chronic chagasic patients as determined by positive results in conventional serological tests, such as indirect immunofluorescence, indirect hemagglutination, and enzyme-linked immunosorbent assay, were collected at the "Ambulatório de Doença de Chagas, Hospital das Clínicas, Universidade

Federal de Minas Gerais" (Belo Horizonte, Brazil). One hundred serum samples found to be negative by serological testing of blood donors from the blood bank of the "Clínica Romeu Ibrahim de Carvalho, Hospital Felício Rocho" (Belo Horizonte, Brazil) were used as negative controls. Serum samples of 10 patients with visceral leishmaniasis and 9 patients with cutaneous leishmaniasis were selected by positive direct parasitological examination to be tested in the rCRP-ELISA. This study was carried out with the consent of the participants and was approved by the Ethics Committee of the "Hospital das Clínicas/087/99, Universidade Federal de Minas Gerais, Belo Horizonte" (Minas Gerais, Brazil).

**Protein extraction and purification.** For *T. cruzi* rCRP extraction, we used the clone pcDNA3-CRP-his-stop transfected in CHO-K1 cells (Beucher et al., submitted). A total of  $3 \times 10^8$  transfected cell lines were centrifuged, the pellet was solubilized at  $10^7$  cells/ml with 2% Triton X-114 (Pierce Chemical Co., Rockford, Ill.) in Tris-buffered saline (50 mM Tris [pH 7.4], 150 mM NaCl), and the aqueous phase was collected after centrifugation and incubation at 37°C for 3 min. Protease inhibitors (leupeptin, aprotinin, and E-64; Sigma Chemical Co., St. Louis, Mo.) were added to buffers at a final concentration of 1 µg/ml each. Nickel-nitrilotriacetic acid affinity chromatography purification was performed using 4 ml of a ProBond Ni<sup>2+</sup> resin washed with 30 ml of ddH<sub>2</sub>O and with 50 ml of binding buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub> [pH 7.8], 500 mM NaCl). The total protein extracted from the cell lysate (~27 mg) was added to the resin in binding buffer and incubated for 1 h at room temperature with shaking. The resin was washed with 50 ml of washing buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub> [pH 6.0], 500 mM NaCl) containing 25 mM of imidazole, and the fractions were eluted with the same washing buffer but with a crescent concentration gradient of imidazole from 50 to 300 mM. The fractions were collected in 2-ml aliquots, and the purified recombinant protein was eluted at an imidazole concentration of 100 to 150 mM of in elution buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub> [pH 6.0], 500 mM NaCl). Protein fraction concentrations were determined using Bio-Rad (Hercules, Calif.) protein assay dye reagent concentrate. The *T. cruzi* rCRP was also purified from culture media after 3 ml of the culture medium was incubated with 200 µl of the ProBond Ni<sup>2+</sup> resin for 2 h at room temperature with shaking. This mixture was centrifuged, and the pellet was suspended in 200 µl of a 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer for protein elution. The supernatant was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Beucher et al., submitted).

**ELISA.** An ELISA for antibody detection in serum was standardized basically as described previously (50). The assay was performed using flat-bottom 96-well microtiter plates for enzyme immunoassay-radioimmunoassay (Hybond-polyethylene plates; Costar, Cambridge, Mass.) coated with 50 µl of solution containing 2.5 µg of purified *T. cruzi* rCRP/ml in carbonate buffer (0.05 M, pH 9.6), incubating for 24 h at 4°C. After incubation, the plates were washed two times with phosphate-buffered saline–0.05% Tween 20 and blocked for 2 h at 37°C with 200 µl of 5% nonfat milk (Nestlé Brasil Ltda., Araçatuba, São Paulo, Brasil) in phosphate-buffered saline (block solution) per well. Fifty microliters of sera diluted 1:200 in block solution was added per well. Plates were incubated for 16 h at 4°C and washed three times as previously mentioned. Then, 50 µl of peroxidase-conjugated rabbit anti-human IgG (DAKO Corp., Carpinteria, Calif.) diluted 1:7,500 in block solution was added, and the plates were incubated for 1 h at 37°C. After four washes as already described, the reaction was developed by addition of 100 µl of 3,3',5,5'-tetramethylbenzidine chromogen (DAKO Corp.) and incubation for 20 min at 37°C. The reaction was stopped with 100 µl of 1 M H<sub>2</sub>SO<sub>4</sub>, and the optical density at 450 nm (OD<sub>450</sub>) was determined in an ELISA reader (Benchmark Microplate Reader; Bio-Rad). The cutoff (CO) values were calculated for each plate as follows: CO =  $m + 2SD$ , in which  $m$  is the absorbance average of the negative controls ( $n = 8$ ) and SD is the standard deviation.

**Complement-mediated lysis.** Lytic antibodies were detected by the CoML test (21, 26, 29) as follows:  $6 \times 10^6$  to  $7 \times 10^6$  trypomastigotes per ml were incubated with human serum as the complement source (HuC) at 37°C for 30 min and counted to assure total resistance to complement lysis in the absence of immune serum. Fifty microliters of a suspension of such trypomastigotes and 50 µl of the test serum, diluted two- and fourfold, were mixed in 5-ml plastic tubes and incubated at 37°C for 30 min and then placed on ice. Fifty microliters of HuC were added to 50 µl of each sample, and the parasites were counted in a hemocytometer. The tubes were incubated at 37°C for 45 min and replaced on ice, and the parasites were recounted. The lysis percentage was calculated as  $100 - (\text{number of parasites in sample at 45 min}) \times 100 \div (\text{initial number of parasites in sample})$ .

**Hemoculture.** The technique of Chiari et al. (15) was used as follows: 30 ml of venous blood was collected into vacuum tubes containing sodium heparin and centrifuged for 10 min at  $300 \times g$ , at 4°C, to separate red blood cells from plasma. The plasma supernatant was centrifuged at  $900 \times g$  (4°C) for 20 min, and 3 ml

TABLE 1. Sensitivity, specificity, and positive percentage comparison between rCRP ELISA, conventional serology, CoML, hemoculture, and PCR tests with sera obtained from individuals infected with *T. cruzi* or *Leishmania* spp. and blood donors<sup>a</sup>

Sera group (n) <sup>b</sup>	Result (%)									
	rCRP ELISA		Conv. serology		CoML		Hemoculture		PCR	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
CCP (65)	100	0	100	0	98.46	1.54	64.62	33.38	81.54	18.46
NC (100)	0	100	0	100	5.0	95.0	ND	ND	ND	ND
Leish (19)	0	100	ND	ND	ND	ND	ND	ND	ND	ND

<sup>a</sup> CCP, chronic chagasic patients; NC, nonchagasic individuals (blood donors); Leish, patients infected with *Leishmania* spp.; ND, not done; Conv., conventional; Pos., positive; Neg., negative.

<sup>b</sup> n, no. of serum samples.

of liver infusion tryptose medium (LIT) (10) was added to the pellet. The packed red blood cells were washed by the same centrifugation and resuspended in 6 ml of LIT, homogenized, and distributed into six plastic tubes (Falcon) each containing 3 ml of LIT. All tubes were maintained at 27 to 28°C, mixed gently twice weekly, and examined monthly for up to 120 days. Ten microliters of each preparation was examined microscopically under 22-mm<sup>2</sup> coverslips at magnification  $\times 150$ .

**Preparation of DNA for PCR.** In order to obtain DNA for PCR, 10 ml of blood samples was collected simultaneously with hemoculture and added to 50-ml plastic tubes (Falcon) containing 10 ml of a mixture of 6 M guanidine-HCl and 0.2 M EDTA (Sigma), pH 8.0 (5). The samples were stored for 5 days at room temperature, boiled in water at 100°C for 15 min to shear the DNA molecules, and stored at 4°C until the time of use, when a 200- $\mu$ l aliquot was collected from each sample and DNA extraction was performed (8, 22).

**PCR conditions.** PCR amplification was performed in a total volume of 20  $\mu$ l containing 0.1% Triton X-100, 10 mM Tris-HCl (pH 9.0), 75 mM KCl, 5 mM  $\text{Cl}_2\text{Mg}_3$ , 0.2 mM (each) dATP, dTTP, dGTP, and dCTP (Sigma), 1  $\mu$ l of *Taq* DNA polymerase (Promega Corp.), 20 pmol of 121 (5'-AAATAATGTACGG (T/G)GAGATGCATGA-3') and 122 (5'-GGTTCGATTGGGGTGGTGTAA TATA-3') primers (Operon Technologies Inc.), and 2  $\mu$ l of DNA of each sample (20, 22). The reaction mixture was overlaid with 30  $\mu$ l of mineral oil (Sigma) to prevent evaporation and subjected to 35 cycles of amplification in an automatic thermocycler (MJ Research programmable thermal controller PTC-100) using plastic 0.5-ml microtubes. The temperature profile was as follows: 95°C for 1 min for denaturation (with a longer initial time of 5 min at 95°C), 65°C for 1 min for primer annealing, and 72°C for 1 min for extension, with a final incubation at 72°C for 10 min to extend the annealed primers. The PCR products were visualized by 6% polyacrylamide gel electrophoresis and silver stained (43). All DNA extraction steps and reaction mixtures used for PCR were monitored and compared with positive and negative controls. To avoid contamination, the reaction steps were performed in separate environments, using equipment and reagents destined exclusively to each stage. To test whether inhibition of the reaction was occurring, DNA from *T. cruzi* culture was obtained and used as a positive control. The sizes of the amplified bands were monitored using a 100-bp ladder molecular size marker (Promega).

**Slot blot hybridization.** All samples were submitted to the hybridization technique in a slot blot (Hoefer Scientific Instruments) with a specific probe that hybridized internally to the 330-bp fragment amplified by PCR. This probe consisted of the oligonucleotide 5'-TGGTTTGGGAGGGGCGTTCAAATT T-3' labeled with alkaline phosphatase (46) and was synthesized by Life Technologies (Rockville, Md.). This technique confirms the specificity of the amplified product and/or increases the sensitivity of the protocol. When PCR alone is employed, 10 fg of parasite DNA may be detected in polyacrylamide gel, while hybridization permits detection of as little as 0.1 fg (22).

**Data analysis.** The OD<sub>450</sub>s from different samples were plotted using computer graphics software (Prizm GraphPad, Version 2.01, San Diego, Calif.). The values of sensitivity and specificity were calculated according to the method of Camargo (11), and the degree of concordance between results was estimated by the Youden index (J) (53). The differences among positivity percentages were tested using the chi-square test with 1 degree of freedom by the statistics software package EPI-INFO/EPITABLE with the confidence interval at the level of 95%.

## RESULTS

**Development of the rCRP ELISA.** *T. cruzi* rCRP was produced in transfected cell lines and purified by affinity chroma-

tography on a nickel-nitrilotriacetic acid resin. A total of 783  $\mu$ g of purified recombinant antigen was obtained after the three immunoreactive eluted fractions were combined. The recombinant antigen was specifically recognized by a Western blotting assay against chicken anti-CRP IgY antibody (data not shown). After rCRP ELISA standardization, optimal results were obtained when the assay was performed under the following conditions: microtiter plates were coated with 50  $\mu$ l of the antigen at a concentration of 2.5  $\mu$ g/ml per well, the primary antibody was used in a dilution of 1:200, and the second antibody was diluted at 1:7,500.

**Sensitivity and specificity of rCRP ELISA.** The sensitivity and specificity of rCRP ELISA in relation to the conventional serology tests were calculated using serum samples from individuals in the chronic phase of Chagas' disease and from blood bank donors as positive and negative samples, respectively. The analysis of sensitivity, specificity, and concordance (Youden) showed a result of 100%. A negative percentage of 100% was also observed when sera from patients with visceral and cutaneous leishmaniasis were tested (Table 1). The rCRP ELISA results in all sera groups are also demonstrated in a scatter plot (Fig. 1). The cutoff value was calculated as described previously (50) and is indicated by the dashed line in the graphic. The comparison among the CoML and rCRP ELISA results demonstrated a sensitivity of 98.46%, a specificity of 95.0%, and concordance of 92% with a confidence interval from 0.85 to 0.98 (Table 1), confirming the high concordance between the methods. The difference of these two

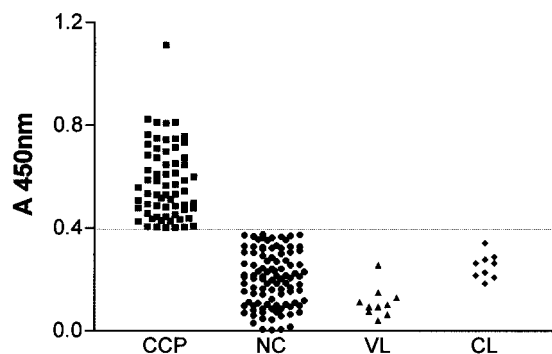


FIG. 1. Distribution of OD<sub>450</sub>s for different groups of individuals. CCP, chronic chagasic patients; NC, nonchagasic; VL, visceral leishmaniasis patients; CL, cutaneous leishmaniasis patients. The horizontal dashed line indicates the cutoff value calculated.

methods was not considered statistically significant in the sample tested according to the chi-square values calculated. These data showed clearly that the recombinant antigen was positive for all samples, without any false-positive or false-negative reactions. No cross-reaction was observed with leishmaniasis sera samples tested.

**Comparison between rCRP ELISA and other methods.** The hemoculture test and the PCR followed by hybridization were performed with the sera samples from chronic chagasic patients for comparison with the rCRP ELISA results. This analysis was done using the difference between positive percentage values obtained by rCRP ELISA (100%) and by hemoculture (64.62%) ( $\chi^2 = 27.94$ ;  $P = 0.00001$ ) (Table 1). The PCR-hybridization results also presented a significantly lower positivity of 81.54% ( $\chi^2 = 13.22$ ;  $P = 0.000277$ ) compared with the rCRP ELISA. The statistical analysis chi-square and  $P$  values demonstrated that the difference between the results of the different methods has high significance with a confidence interval of 95%. These data showed that the rCRP ELISA gives greater sensitivity than hemoculture and PCR in the diagnosis of Chagas' disease.

## DISCUSSION

Serological methods, based on the detection of antibodies against *T. cruzi*, are widely used for diagnosis of Chagas' disease. During the infection, the patients produce several antibodies against different *T. cruzi* antigens, and these antibodies show qualitative and quantitative differences. Among them, a small subset is considered specific for antigens from different developmental stages of the parasite. The majority of these antibodies usually show different degrees of nonspecific reaction with antigens from other closely related organisms, resulting in cross-reactions and consequently false-positive diagnosis (16, 33, 35). To overcome this problem, several antigens are being produced in different manners and evaluated for diagnosis of chagasic patients.

In the present report, we evaluated *T. cruzi* rCRP, a trypomastigote-specific antigen, in the development of a sensitive and specific ELISA system for the diagnosis of Chagas' disease. The method was standardized to establish the optimal antigen concentration and dilution of antibodies. The assay was performed with 184 individual sera, distributed between chronic chagasic patients ( $n = 65$ ), nonchagasic individuals ( $n = 100$ ), and patients with leishmaniasis ( $n = 19$ ). For the evaluation in the first two groups, we considered conventional serology as the reference test and compared these results with those obtained by the rCRP ELISA. This comparison showed a total concordance between the methods and allowed us to affirm that the new ELISA test shows sensitivity and specificity of 100%. When patients infected with *Leishmania* spp., considered negative samples, were tested by rCRP ELISA, no positive reaction was observed, confirming the high specificity of the method. The complement-mediated lysis was tested in the same group of patients and in the group of blood donors, resulting in a lower sensitivity and specificity than rCRP ELISA. This difference was not statistically significant for the number of samples analyzed. These data reinforce the strength of the rCRP ELISA as a Chagas' disease diagnostic tool.

The rCRP ELISA was considered a reference test to be

compared with hemoculture and PCR methods. The hemoculture, which is a highly specific test, was positive for 64.62% of the patients compared with ELISA results, confirming a low sensitivity of this method as reported previously. The positivity of 81.54% verified for the PCR also confirmed preceding data. Our results reinforce that both methods, although reliable, cannot be applied in clinical laboratory testing or in blood bank screenings due to the high level of variable results. The sensitivity of these methods seems to reflect the different genetic constitution of circulating *T. cruzi* strains, which vary according to geographic origin (19). The presence of the parasite in the blood at a given time depends on the parasite's life cycle as well as on the immunological equilibrium between the parasite and the host (14). The analysis of these results leads us to conclude that this recombinant antigen is a strong candidate for use in diagnosis of Chagas' disease.

In addition to the inadequacies of the present methods for diagnosing chronic Chagas' disease, there is also a lack of reliable or applicable means to verify cure of patients after drug treatment, particularly in the chronic phase of the disease. The effectiveness of treatment with nitroimidazole derivatives and other drugs has been difficult to assess, since conventional serologic tests may remain positive for several years in some patients despite repeated negative direct parasite detection tests, such as hemoculture or xenodiagnosis (15, 21, 26, 27, 28, 39). This lack of a means of detection of ongoing infection has hampered the development of drugs that may be effective in treating the chronic phase of the disease. The CoML test has been proposed by Kettli and Brener as a means to detect parasite clearance posttreatment (30). This method has opened new perspectives regarding the evaluation of cure in chagasic patients and shows clear discordance among conventional serology and lytic antibodies in both human patients and experimental animals, the former being classified as "dissociated" (35). It was previously shown that the *T. cruzi* CRP is a principal effector molecule restricting complement activation and lysis of the parasites and also elicits the production of lytic antibodies in humans (37). The results presented here suggest that the *T. cruzi* rCRP may be a candidate antigen for monitoring cure in chagasic patients after treatment.

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## Evaluation of a Recombinant *Trypanosoma cruzi* Mucin-Like Antigen for Serodiagnosis of Chagas' Disease<sup>▽</sup>

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Chagas' disease is caused by the protozoan parasite *Trypanosoma cruzi* and is one of the most important endemic problems in Latin America. Lately, it has also become a health concern in the United States and Europe. Currently, a diagnosis of Chagas' disease and the screening of blood supplies for antiparasite antibodies are achieved by conventional serological tests that show substantial variation in the reproducibility and reliability of their results. In addition, the specificity of these assays is curtailed by antigenic cross-reactivity with sera from patients affected by other endemic diseases, such as leishmaniasis. Here we used a highly sensitive chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) to evaluate a recombinant protein core of a mucin-like molecule (termed trypomastigote small surface antigen [TSSA]) for the detection of specific serum antibodies in a broad panel of human sera. The same samples were evaluated by CL-ELISA using as the antigen either a mixture of native *T. cruzi* trypomastigote mucins or an epimastigote extract and, for further comparison, by conventional serologic tests, such as an indirect hemagglutination assay and indirect immunofluorescence assay. TSSA showed ~87% sensitivity among the seropositive Chagasic panel, a value which was increased up to >98% when only parasitologically positive samples were considered. More importantly, TSSA showed a significant increase in specificity (97.4%) compared to those of currently used assays, which averaged 80 to 90%. Overall, our data demonstrate that recombinant TSSA may be a useful antigen for the immunodiagnosis of Chagas' disease.

American trypanosomiasis, or Chagas' disease, is a major health and economic problem in Latin America caused by the protozoan parasite *Trypanosoma cruzi*. It is estimated that 8 million people are currently infected and that 90 million individuals living in areas of endemicity are at risk of infection (27, 32). The parasite is transmitted to humans through the feces of infected, blood-sucking triatomine bugs, blood transfusion, organ transplantation, or congenital contamination or by the ingestion of tainted food and fluids (35). In recent years, several efforts have been successfully undertaken to control vectorial transmission in Latin American countries, with a concomitant decrease in the actual numbers of acute infections (28). However, since *T. cruzi* infection is asymptomatic in most cases, chronically infected individuals can serve as parasite reservoirs throughout their lifetimes. Thus, there is a consensus that congenital *T. cruzi* infection will be a pressing public

health problem for at least the next 20 years (30). In addition, the risk of acquiring Chagas' disease through infected blood transfusion is becoming a problem even in areas of nonendemicity, such as the United States and Europe, and some cases have already been reported (18, 23, 32). Owing to the risk of transmission by blood transfusion and organ transplantation, most blood donations in the United States have routinely been screened in recent years (1). Nevertheless, in many developed countries, the blood supply is not yet regularly tested for anti-*T. cruzi* antibodies (18, 32).

Due to the low parasite levels present in the chronic phase of the disease, its detection in blood samples by direct examination, hemoculture, or xenodiagnosis is difficult and time-consuming (19). Several PCR- and real-time-PCR-based procedures have been reported that, though highly specific and sensitive, might not be appropriate for routine use in blood supplies or health centers (16, 31). Detection of anti-*T. cruzi* antibodies is still the most effective method for demonstrating direct exposure to the parasite. At present, the most widely used serologic methods are indirect hemagglutination assays (IHAs), indirect immunofluorescence (IIF), and enzyme-linked immunosorbent assays (ELISAs) using total parasite homogenates or semipurified antigenic fractions from epimastigotes, the noninfective parasite form present in the digestive tract of the insect vector (19). However, these tests show vari-

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ations in the reproducibility and reliability of their results that can be attributed to the poor standardization of the reagents (12). The advent of recombinant DNA technology allowed the production and one-step purification of large amounts of highly pure *T. cruzi* immunodominant antigens, some of which were evaluated by way of multicenter trials (12, 19, 25). The use of linear and/or branched synthetic peptides spanning B-cell epitopes has also been successfully applied (20, 22, 34). Both recombinant antigens and synthetic peptides minimize the extent of specificity problems, one of the major drawbacks of immunodiagnosis of Chagas' disease (19). As previously shown, sera from individuals with leishmaniasis, mycoses, and/or certain autoimmune disorders cross-react with crude preparations of *T. cruzi* antigens (6, 33).

The mucin coat that covers the surfaces of bloodstream trypomastigotes (trypomastigote glycosylphosphatidylinositol [tGPI] mucins) is decorated with highly immunogenic  $\alpha$ -galactosyl ( $\alpha$ -Gal) epitopes (3, 4). Chagasic anti- $\alpha$ -Gal antibodies have a complement-independent lytic effect on bloodstream trypomastigote forms (26), and more importantly, their titer is considerably reduced in benznidazole-treated patients in the early stage of chronicity (5, 13), suggesting that they might also be useful for monitoring patients after drug treatment. The trypomastigote small surface antigen (TSSA) is a mucin-like glycoprotein displayed on the surface of infective trypomastigote forms (15) and potentially involved in host cell recognition (G. E. Cánepa and C. A. Buscaglia, unpublished data). Two main isoforms of TSSA were originally recognized in the two major lineages or subgroups into which the *T. cruzi* species has been divided (36): one isoform present in lineage I (currently known as *T. cruzi* discrete typing unit I [DTU TcI]) parasite stocks and one isoform present in TcIIe (now DTU TcVI) isolates. Sequence variations between the isoforms were shown to have a major impact on TSSA antigenicity, leading to negligible cross-reactivity between them (15). This property was proposed to have great epidemiological value, as it allowed for identification of the lineage of the infecting strain by simple serologic methods (9). Recent studies challenged this idea by showing that TSSA is significantly more diverse in amino acid structure than previously described (7), although the antigenic impact of these changes, if any, remains uncertain.

Here we report a thorough study of the sensitivity and specificity of TSSA which demonstrates that this recombinant antigen is a useful molecule for the immunodiagnosis of Chagas' disease. In order to improve the sensitivity of the assay, a high-throughput chemiluminescent ELISA (CL-ELISA) was employed (3).

## MATERIALS AND METHODS

**Study populations.** Human serum samples ( $n = 617$ ) were obtained from the Hospital das Clínicas da Universidade de São Paulo (HC-USP), the Laboratório de Investigação Médica-Parasitológica, Instituto de Medicina Tropical de São Paulo (IMT), Departamento de Moléstias Infecciosas, HC-USP, and the Fundação Hemocentro de Ribeirão Preto, São Paulo, Brazil. The use of these human serum samples was approved by the institutional review boards of the IMT and the Instituto de Ciências Biomédicas, USP (ICM-USP). Some of the samples were part of a serum panel stocked by the ICM-USP. These sera had been codified upon collection, and therefore no information (e.g., name, age, sex, etc.) regarding the patient was available. They were approved for use in the current study by the institutional review boards of both the ICM-USP and the IMT. Sera were collected from clotted blood obtained by venipuncture, diluted 1:2 with high-grade glycerol for conservation, and stored at  $-80^{\circ}\text{C}$  until use. Three panels

of human sera were used in this work. The first panel was composed of 237 samples collected from individuals that were grouped into seropositive Chagasic samples (those rendering positive results only for conventional serology tests [ $n = 185$ ]) or parasitologically positive Chagasic samples (those rendering positive results for both conventional serology tests and hemoculture [ $n = 52$ ]). The second panel was composed of 200 samples from healthy, noninfected individuals (NHS) rendering negative results for *T. cruzi* by three independent assays (ELISA using total parasite homogenate, IHA, and IIF) and was thus defined as the seronegative panel. The third panel, termed the specificity control panel, was composed of 180 samples collected from non-Chagasic individuals affected by unrelated diseases, as defined by the clinical and serologic diagnoses of their respective pathologies. Twenty-nine of these 180 samples were from patients infected with cutaneous leishmaniasis, 31 were from patients with visceral leishmaniasis, 4 were from histoplasmosis patients, 9 were from patients infected with *Mycobacterium leprae*, and 28 were from patients affected by different helminth and protozoan infections. Specifically, 4 of the last samples were from individuals infected with *Schistosoma* spp., 5 were from individuals infected with *Giardia lamblia*, 1 was from an individual infected with *Hymenolepis nana*, 4 were from individuals infected with *Tricuris trichiura*, 2 were from individuals infected with *Strongyloides stercoralis*, 2 were from individuals infected with *Ancylostoma* spp., 3 were from individuals infected with *Ascaris lumbricoides*, 1 was from an individual infected with *Enterobius vermicularis*, 3 were from individuals infected with *Toxocara canis*, and 3 were from individuals infected with *Cryptosporidium* spp. In addition, this panel contained 79 samples from individuals afflicted by autoimmune disorders: 53 with rheumatoid arthritis and 26 with systemic lupus erythematosus.

**Parasites.** Cell-derived trypomastigotes from the Y strain (DTU TcII) were collected from the supernatant of *Mycoplasma*-free LLC-MK2 cells (American Type Culture Collection, Manassas, VA) grown in Dulbecco's modified Eagle medium (DMEM) containing 4.5% glucose, 10% fetal bovine serum (FBS), and antibiotics (8). After three washings in phosphate-buffered saline (PBS), parasites were lyophilized and stored at  $-70^{\circ}\text{C}$  until use. Epimastigotes from the same strain were cultured in liver infusion tryptose (LIT) medium containing 10% FBS and 5% glucose (10).

**Purification of parasite tGPI mucins and preparation of total epimastigote extracts.** tGPI mucins from  $10^{10}$  parasites were purified from delipidated butanol-water extracts by hydrophobic interaction chromatography as described previously (4). A total epimastigote extract (EpEx) was obtained as described previously (3).

**Expression and purification of recombinant GST-TSSA proteins.** Genes coding for the Sylvio X-10/1 TSSA (TSSA I; GenBank accession number ACY02865.1) and the CL Brener TSSA (TSSA VI, formerly TSSA II; GenBank accession number ACY54510) have been described (7, 15). To reduce the risk of false-positive sampling, the recombinant TSSA proteins used in this work consisted only of the predicted, full-length, mature products, i.e., without most of the endoplasmic reticulum and GPI anchor signals (Fig. 1A). Briefly, previously described glutathione *S*-transferase (GST)-TSSA clones were reamplified by PCR using the oligonucleotides EMT5/s and EMT5/a (15), digested with BamHI/EcoRI, and cloned into the pGEX-2T vector (GE). Supernatants of *Escherichia coli* cultures induced for 3 h at  $28^{\circ}\text{C}$  with 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside were purified by glutathione-Sepharose chromatography (15) and dialyzed against PBS. The purity of GST-TSSA samples was assessed with silver-stained SDS-PAGE gels (Fig. 1B). Some of the faint bands that can be seen in the gel correspond to multimeric forms and/or degradation products of TSSA molecules, as judged by anti-GST Western blot analysis (not shown).

**CL-ELISA.** Polystyrene ELISA microplates (FluoroNunc; Nunc, Roskilde, Denmark) were coated with the corresponding antigen (50  $\mu\text{l}$  containing 1.2 ng tGPI mucins, 2.2 ng EpEx, or 10 ng TSSA per well), diluted in 50 mM carbonate-bicarbonate buffer, pH 9.6 (CBB). After 18 h at  $4^{\circ}\text{C}$ , plates were blocked with CBB containing 0.1% bovine serum albumin (CBB-BSA) and washed 3 times with 300  $\mu\text{l}$  of PBS, pH 7.2, containing 0.05% Tween 20 (PBS-T). Sera were diluted in PBS-T as follows: (i) 1:2,000 for a chemiluminescent ELISA (CL-ELISA) with tGPI mucins or EpEx and (ii) 1:200 for a CL-ELISA with TSSA I and TSSA VI (15). In all cases, sera were incubated for 1 h at  $37^{\circ}\text{C}$ , and plates were then washed as before and developed by the addition of biotin-labeled anti-human IgG antibody (50  $\mu\text{l}$ , 1:1,000 dilution; Amersham, GE Healthcare, Piscataway, NJ), followed by streptavidin coupled to horseradish peroxidase (HRP) (50  $\mu\text{l}$ , 1:2,000 dilution; Amersham, GE Healthcare). Plates were washed as before, and 50  $\mu\text{l}$  of ECL reagent (Amersham, GE Healthcare) diluted 1:20 in CBB was added. Reading of the plates was carried out with a Fluoroskan Ascent FL apparatus (Thermo Labsystems, Helsinki, Finland), and values were expressed in relative luminescence units (RLU). The cutoff value for each antigen was calculated with the equation,  $\text{cutoff} = k(m_n - m_b)$ , where  $k$  is the



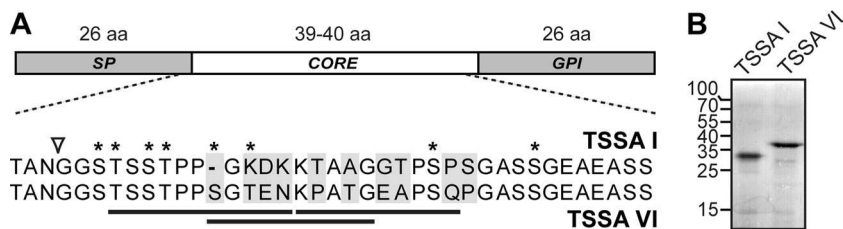


FIG. 1. Features of evaluated TSSA molecules. (A) Schematic illustration of TSSA products showing the predicted signal peptide (SP) and GPI-anchoring signals. The sequences of the TSSA I and TSSA VI regions expressed as GST fusion proteins are indicated. Variable positions between both proteins are shaded. The predicted SP cleavage site is indicated with an inverted triangle, and amino acids (aa) predicted to be *O*-glycosylated in the TSSA VI protein are indicated with asterisks. (B) The purity of the recombinant GST fusion proteins used for the CL-ELISAs was ascertained by silver staining of an SDS-PAGE gel containing 1 µg of the indicated protein. Molecular size markers (in kDa) are indicated.

number of standard deviations (SD) obtained for the negative-control sera separating the maximum RLU value for the negative-control sera and the minimum RLU value for the positive-control sera, and  $m_n$  and  $m_p$  are the mean values of RLU from the negative-control sera and the background (without the addition of serum), respectively. The  $k$  values were estimated as 3 for the tGPI mucins and 2 for EpEx and the GST-TSSA recombinant proteins. In order to compare the results of different CL-ELISAs using distinct antigens, serological titers were calculated by dividing the RLU values of individual serum samples by the cutoff value for each antigen.

**Conventional serologic tests (IHA, IIF, and ELISA).** Serum samples were tested by IHA and IIF as described previously (21), using commercial reagents (Cecon, São Paulo, Brazil). For the latter, fluorescein isothiocyanate (FITC)-coupled anti-human IgG antibodies were used (New England BioLabs, Beverly, MA). The cutoff value determined for both techniques was 1:40, and in both cases, a 1:20 titer was considered inconclusive. A commercial ELISA kit composed of a crude extract of epimastigote forms was purchased from Embrabio (São Paulo, Brazil) and used according to the manufacturer's guidelines. For ELISA and CL-ELISA, the corresponding cutoff value  $\pm 10\%$  was considered inconclusive.

## RESULTS

**Development of the *T. cruzi* mucin-based CL-ELISA.** The quality of the purified antigenic GST-TSSA I and GST-TSSA VI (formerly GST-TSSA II [15]) recombinant proteins was assessed with silver-stained SDS-PAGE gels (Fig. 1A). These proteins rendered a single band of the expected molecular mass (~35 kDa) (Fig. 1B), whereas purified tGPI mucins migrated as a broad smear ranging from 40 to 250 kDa, which is barely visible upon silver staining (4, 8; not shown). This heterogeneity is the result of the coexpression of multiple and heterogeneous apomucin polypeptides subjected to different extents of posttranslational modifications, mainly the addition of *O*-glycans (2, 8). The optimal concentration of protein per assay and the dilution of the serum samples were determined for each antigen by antigen-serum cross-titration, and they are defined as the antigen concentration and serum dilution that gave the highest ratio between a blindly selected pool of parasitologically positive sera ( $n = 10$ ) and a blindly selected pool of NHS from our seronegative panel ( $n = 10$ ). For tGPI mucins and EpEx, the optimal conditions were achieved at concentrations of 1.2 and 2.2 ng/well, respectively, and in testing the samples at a 1:2,000 dilution. In the case of the GST-TSSA VI molecule, optimal results were achieved by using 10 ng/well and diluting the serum samples 1:200. Accordingly, the same conditions were used for the evaluation of the GST-TSSA I recombinant protein. The cutoff and  $k$  values for each antigen were calculated as indicated in Materials and Methods by the

use of (i) 52 parasitologically positive sera and (ii) 100 NHS samples which were blindly selected from our seronegative panel.

**Sensitivity of the CL-ELISA using seropositive and parasitologically positive human sera.** A panel of 237 samples was analyzed by CL-ELISA using EpEx, purified tGPI mucins, and recombinant GST-TSSA VI and GST-TSSA I proteins as antigens. For comparison, all the samples were analyzed by three widely used serodiagnostic techniques: IIF, IHA, and ELISA. Two groups can be defined among the tested samples: those showing positive results in conventional serology tests and hemoculture (52 parasitologically positive samples) and those showing positive results only in conventional serology tests (185 seropositive samples), which were collected either in Brazil ( $n = 160$ ) or Chile ( $n = 25$ ). Box-and-whisker plots expressing the results obtained in each case are shown in Fig. 2, and

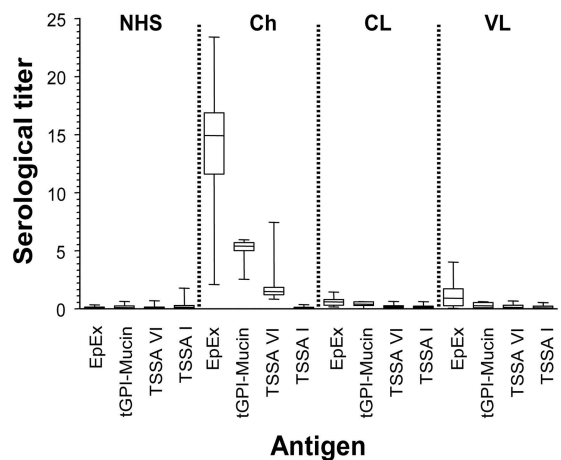


FIG. 2. Recognition of evaluated antigens by sera from *T. cruzi* and *Leishmania*-infected individuals. Reactivities of sera collected from individuals infected with *T. cruzi* (Chagasic [Ch]) ( $n = 237$ ) or *Leishmania* spp. ( $n = 60$ ) and from healthy individuals (NHS) ( $n = 200$ ) to the indicated *T. cruzi*-derived antigens were determined by CL-ELISA. Results are presented by box-and-whisker plots, indicating the median serum titer (horizontal line inside box), SD (box's upper and lower lines), and maximal and minimal serum titers (whiskers). The serological titer for each sample was determined as described in Materials and Methods. Sera from *Leishmania*-infected individuals were grouped according to the manifestation of the disease. VL, visceral leishmaniasis ( $n = 31$ ); CL, cutaneous leishmaniasis ( $n = 29$ ).

TABLE 1. Sensitivities and specificities of CL-ELISA with distinct *T. cruzi* antigens and of conventional serologic methods

Index	No. of samples	Result (%) using CL-ELISA with:				Result (%) using:		
		EpEx	tGPI mucin	TSSA VI	TSSA I <sup>d</sup>	IHA	IIF	ELISA
Sensitivity	237	99.6	100	86.9	0	97.5	98.7	100
Specificity <sup>a</sup>	200	100	100	99	NA	100	100	100
Specificity <sup>b</sup>	180	69.4	100	94.4	NA	77.8	58.3	57.8
Specificity <sup>c</sup>	380	85.5	100	97.4	NA	89.5	80.3	80

<sup>a</sup> Specificity determined with seronegative human sera.

<sup>b</sup> Specificity determined with specificity control human sera.

<sup>c</sup> Specificity determined with seronegative and specificity control human sera.

<sup>d</sup> NA, not applicable (did not render a positive value).

the raw data are summarized in Table 1. When tGPI mucins were used as antigens, 100% sensitivity was obtained for both groups of samples, in accordance with previous data (3). This value is even higher than that obtained by IHA or IIF and also higher than that obtained with EpEx, with which 1 out of 237 samples rendered negative results. On the other hand, the overall sensitivity of GST-TSSA VI is 86.9% and thus in the range of values for other recombinant parasite antigens proposed for Chagas' disease immunodiagnosis (12, 25). However, and according to previous results, sensitivities clearly differed between hemoculture-tested and -untested sera (15). The value for the first group was >98% (only 1 out of 52 samples produced inconclusive results), whereas a significant reduction to 83.7% was verified for the second group. No significant differences in the sensitivity indexes were observed when sera collected from Brazil and Chile were compared (83.7 and 84.0%, respectively), although the mean values recorded for the Brazilian samples were consistently higher (not shown). In line with previous results, none of the tested sera rendered positive values when evaluated by the control GST-TSSA I protein (Table 1), which could be attributed to (i) low levels of expression/antigenicity of the TSSA isoform present in DTU *TcI* parasite stocks and/or (ii) the predominance of non-DTU *TcI* infections in the domestic and peridomestic cycles of Chagas' disease in the Southern Cone countries of Latin America (11, 15).

**Specificity of the CL-ELISA.** The specificity of each antigen in our CL-ELISA was analyzed by using 380 serum samples collected from non-Chagasic individuals (Table 1). Among these samples, 200 were from healthy, noninfected blood donors (NHS; seronegative panel) and 180 from individuals affected by other infectious or autoimmune diseases which might elicit cross-reactive humoral responses toward *T. cruzi* constituents (specificity panel). No reactivity against tGPI mucins was observed among NHS samples, leading to a 100% specificity (3). The same results were obtained when these samples were evaluated either by conventional serology techniques (IIF, IHA, and a commercial ELISA kit) or by CL-ELISA using EpEx as the antigen. In the case of GST-TSSA VI, one inconclusive and one positive value were recorded. It is noteworthy that the latter sample also rendered positive results when tested with GST-TSSA I (not shown), suggesting that the reacting antibodies recognize a common determinant on both proteins, thus showing a specificity different from that of antibodies present in samples from patients with chronic Chagas' disease (15). Whether this recognition is directed toward the GST molecule or toward a TSSA epitope lying outside the

central and variable regions between both isoforms was not tested.

The most striking differences between conventional serologic assays and our proposed TSSA VI antigen were obtained when the specificity panel of serum samples was evaluated (Table 2). IIF, IHA, and ELISA techniques and the EpEx-based CL-ELISA displayed high levels of recognition of cross-reacting antibodies, particularly those from *Leishmania*-infected patients and individuals with autoimmune disorders (Fig. 1 and Table 2, respectively). In addition, EpEx-based and nonchemiluminescent ELISAs using a commercial kit yielded positive results for some samples from individuals infected with protozoan or multicellular parasites. In sharp contrast, the specificity recorded for tGPI mucins was 100%. GST-TSSA VI showed a slightly reduced specificity (94.4%) when evaluated with the specificity panel of serum samples, which is still significantly better than that reported for conventional serodiagnostic tests, such as IIF, IHA, and ELISA (ranging from 58 to 78%). Overall, these results highlight that the major advantage for the use of TSSA is at the specificity level.

## DISCUSSION

Serologic methods are widely employed for the diagnosis of Chagas' disease, particularly in the indeterminate and chronic phases, when parasitemia is extremely low and very hard to detect. The World Health Organization has long emphasized the need to employ defined antigens as a way of improving the serodiagnosis of Chagas' disease. In order to be useful, these antigens must meet several criteria: (i) they should be present in *T. cruzi* isolates from different areas of endemicity, (ii) they should be absent from other infectious disease agents, (iii) they should be highly immunogenic in populations with different genetic backgrounds, and (iv) they should be stable and easily amenable to quality control tests to guarantee reproducibility (19). Considering these guidelines, we herein evaluated the recombinant protein core of the TSSA antigen, derived from a mucin molecule expressed by the bloodstream forms of *T. cruzi*, in the development of a sensitive and specific CL-ELISA system for the diagnosis of Chagas' disease. The choice was based on previous results in which the TSSA isoform from DTU *TcVI* parasite stocks displayed a high reactivity with human infection sera from Southern Cone countries (15). Although the spectra of circulating parasite stocks, and therefore of their expressed TSSA isoforms, seem to vary in different areas of endemicity, a high frequency of TSSA VI recognition, as assessed by Western blotting, was also recently observed in

TABLE 2. Specificities of CL-ELISA with distinct *T. cruzi* antigens and of conventional serologic methods to heterologous human sera

Disease or infection (n) <sup>a</sup>	Test result <sup>b</sup>	No. of samples with each result with CL-ELISA and:			No. of samples with each result with:		
		EpEx	tGPI mucin	TSSA VI	IHA	IIF	ELISA
Leishmaniasis (60)	Positive	36	0	0	35	46	40
	Inconclusive	6	0	1	3	5	0
	Negative	18	60	59	22	9	20
	Specificity	30	100	98.3	36.7	15	33.3
Histoplasmosis (4)	Positive	0	0	0	0	0	0
	Inconclusive	0	0	0	0	0	0
	Negative	4	4	4	4	4	4
	Specificity	100	100	100	100	100	100
<i>Mycobacterium leprae</i> (9)	Positive	0	0	0	0	3	0
	Inconclusive	0	0	0	0	2	0
	Negative	9	9	9	9	4	9
	Specificity	100	100	100	100	44.5	100
Helminth and protozoan infections (28)	Positive	2	0	0	0	0	5
	Inconclusive	2	0	1	0	0	2
	Negative	24	28	27	28	28	21
	Specificity	85.7	100	96.4	100	100	75
Rheumatoid arthritis (53)	Positive	4	0	3	0	4	20
	Inconclusive	1	0	4	0	5	0
	Negative	48	53	46	53	44	33
	Specificity	90.6	100	86.6	100	83.1	62.3
Systemic lupus erythematosus (26)	Positive	4	0	1	2	4	9
	Inconclusive	0	0	0	0	6	0
	Negative	22	26	25	24	16	17
	Specificity	84.6	100	96.2	92.3	61.5	65.4

<sup>a</sup> n, number of samples from affected individuals.<sup>b</sup> Specificity is expressed as a percentage.

patients with chronic Chagas' disease from Colombia, Venezuela, and Mexico (29).

By using a much larger and more comprehensive serum panel, we estimated the overall sensitivity of GST-TSSA VI as 86.9% (Table 1). Even though the sensitivity of this antigen is in the range of other recombinant proteins proposed for Chagas' disease immunodiagnosis (12, 25), its sensitivity among parasitologically positive samples was >98%. More importantly, TSSA VI showed minimal cross-reactivity with blood samples from *Leishmania*-infected individuals, a common source of false-positive results in conventional serodiagnosis tests for Chagas' disease (Tables 1 and 2). In accordance, TSSA seems to be restricted to *T. cruzi*, since no homologous gene has been detected in the genome databases of other trypanosomatids (reference 17 and our unpublished results).

For comparison purposes, the same samples were evaluated in parallel using tGPI mucins, a putative gold standard for the diagnosis and follow-up of the treatment of Chagas' disease (3, 5,

13), which resulted in 100% sensitivity and 100% specificity with the extensive panel of serum samples tested here. The basis for this sensitivity can be attributed in part to the abundance of tGPI mucins on the surface of a parasite (26) and to the high antigenicity of their nonreducing, terminal  $\alpha$ -Gal epitopes (4). Accordingly, treatment of tGPI mucins with  $\alpha$ -galactosidase abolished most (80 to 100%) of the reactivity of individual human Chagasic sera against these GPI-anchored glycoproteins (I. C. Almeida, unpublished data). In spite of its optimal specificity and sensitivity, some drawbacks could be envisaged for the routine implementation of tGPI mucins in serodiagnosis. First, the material is purified from cultured infective forms of the parasite, and this process is expensive and time-consuming. In addition, purification is a multistep process, requiring lyophilization of the parasites, extraction in organic solvents, and hydrophobic-interaction chromatography. These disadvantages are eased by the fact that very small amounts of tGPI mucins are required (~1 ng/well). Therefore, a single-batch preparation from 10<sup>9</sup> trypomastigotes should

render enough material to perform 20,000 assays. Different protocols to speed up and simplify the tGPI mucin purification processes should be evaluated.

It becomes clear that in order to improve the sensitivity of TSSA VI, it should be supplemented with other antigens, either by including it in existing serologic diagnostic kits or by developing a new mixture of antigens that might well include a synthetic terminal  $\alpha$ -Gal-containing epitope(s) recognized by Chagasic anti-Gal antibodies in tGPI mucins. In this regard, it is worth noting that chemical synthesis of complex oligosaccharides attached to *T. cruzi* mucins has been recently achieved (14, 24). We speculate that the presence of both peptide and glycan epitopes in a single serodiagnostic reagent may render optimal results in terms of sensitivity and specificity. In summary, our results show that TSSA VI is a better alternative to the epimastigote extracts currently employed in *T. cruzi* serodiagnosis. It could be combined with other recombinant antigens to improve the sensitivities of current kits or used alone or in combination with other antigens as a confirmatory diagnostic test, as recommended by the World Health Organization (19). The CL-ELISA developed here also has a highly increased specificity, in particular with respect to *Leishmania* infections, compared to other, more cumbersome diagnostic techniques for *T. cruzi* detection, such as IHA and IIF.

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# Multiepitope Synthetic Peptide and Recombinant Protein for the Detection of Antibodies to *Trypanosoma cruzi* in Patients with Treated or Untreated Chagas' Disease

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A tetrapeptide and a recombinant protein, each representing 4 immunodominant epitopes of *Trypanosoma cruzi*, were tested by use of ELISA for the detection of serum antibodies. Sera from individuals with Chagas' disease, including persons untreated and successfully or unsuccessfully treated, were tested. These assays detected antibody in 100% of the parasitemias. The antibody reactivity decreased based on the success of treatment. Higher sensitivity was observed for tetrapeptide/recombinant protein assays than for lysate-based ELISA, and specificity was improved, particularly with *Leishmania* sera. The results indicate that multiepitope antigens provide a more sensitive and specific alternative to lysate for detection of anti-*T. cruzi* antibodies, as required for developing blood screening assays.

Chagas' disease is caused by the parasite *Trypanosoma cruzi* and is routinely diagnosed by using serologic methods, with crude antigens or fixed whole organisms in many cases [1–6]. In fact, in most cases, multiple assays—for example, immunohemagglutination, complement fixation, and ELISA with parasite lysates—are performed to establish a consensus-positive result [7, 8]. In some cases, xenodiagnosis is performed, whereby uninfected reduviid insects are allowed to feed on the blood of a patient, after which the insects are killed and are analyzed for the presence of parasites [9]. Hemoculture is also used to detect parasites [10]. In addition, electrocardiograms or radiographs are frequently obtained, depending on the stage of the disease [11]. Therefore, there is a need for improved test methods for *T. cruzi* antibody detection to minimize the amount of repeated testing required.

Several recombinant antigens have been identified that have serologic utility [12–15]. However, the most effective antigens have been those with immunodominant, repeating B cell epitopes [16–19]. In many cases, synthetic peptides have been made containing these repeating epitopes [16–19]. In particular, we initially synthesized a tripeptide combining the 3 epitopes PEP-2, TcD, and TcE [21–23]. A consensus-positive but serum-negative result for this peptide was used to identify, by serologic expression cloning, a fourth epitope, TcLo1.2 [23]. This was then incorporated into a branched tetrapeptide and subse-

quently into a linear recombinant protein (TcF) [23]. These were evaluated to determine their utility in detecting antibodies to *T. cruzi* in several groups of individuals [21–25]. These groups included (A) controls from an endemic area; (B) patients with untreated disease in whom parasites were demonstrable; (C) hemoculture-positive patients with treated but not cured Chagas' disease; (D) hemoculture-negative patients with treated but not cured Chagas' disease; and (E) patients with Chagas' disease that was considered to be cured [24, 25]. In addition, these same patient groups were evaluated by use of an ELISA with crude *T. cruzi* lysate and a Gull Chagas' ELISA to determine whether the peptide/recombinant protein would enable the development of a more effective test for serodiagnosis of Chagas' disease. Further studies were also performed on sera from individuals who were confirmed parasite positive by xenodiagnosis, as well as on sequential serum samples from an individual who was accidentally infected with *T. cruzi* and subsequently treated [26].

## Materials and Methods

**Recombinants and synthetic peptides.** There are several core antigens used in development of the multiepitope peptide and recombinant protein. The TcD peptide contains a 15-amino acid sequence containing one and a half units of a 10-amino acid repeat required for optimal immunoreactivity with *T. cruzi* antibodies in serum [19, 20]. The TcE peptide comprises a 7-amino acid repeat sequence, as described elsewhere [18, 21–23], and contains 3 repeats with each of the degeneracies for full activity. The TcE peptide was identified from expression cloning by use of a DNA library from amastigotes screened with TcD-negative sera [19, 23]. PEP-2 is a 22-amino acid peptide containing a base 12-amino acid repeat [16] and 10 additional amino acids from the same repeat, and it has been shown to complement the serologic reactivity of TcD [20].

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The TcLo1.2 sequence was obtained by expression cloning using a single consensus-positive serum that was negative for reactivity with PEP-2, TcD, and TcE and also contained a 15-amino acid repeat with conserved substitutions of a single amino acid [21–23].

The branched tetrapeptide 2/D/E/Lo1.2 was synthesized from a lysine core residue by use of orthogonal protection, as described elsewhere [23]. By using this approach, 2 independent amino acid sequences can be built from a “core” lysine residue. This approach was used to synthesize a peptide with PEP-2, TcD, and TcE epitopes on the alpha amino group and TcLo1.2 on the epsilon amino group [23]. Purification of this peptide was performed by reverse-phase high-performance liquid chromatography methods, as described elsewhere [23].

The recombinant TcF was a linear protein, 2/D/E/Lo1.2, with a hexahistidine tag at the amino terminus that was expressed in *Escherichia coli* and was purified by metal-ion affinity chromatography, as described elsewhere [23]. *T. cruzi* lysate was prepared as described elsewhere [19, 20].

**Study population.** Serum samples were obtained from individuals with different disease categories to evaluate the use of the tetrapeptide/recombinant protein in ELISA. These included sera from individuals given a diagnosis of Chagas’ disease, obtained both before and during treatment, as well as sera from individuals with xenodiagnosis-confirmed parasitemias. Forty-nine sera from individuals shown by xenodiagnosis to be infected with *T. cruzi* were obtained from Dr. Alejandro O. Luquetti, Federal University of Goias, Goiana, Brazil, and 7 plasma samples from parasite-positive individuals were obtained from Dr. Roberto Badaro, Federal University of Bahia, Salvador, Brazil. The sera from patients with treated and untreated Chagas’ disease were obtained from Dr. Antoniana Krettli, Federal University of Minas Gerais, Belo Horizonte, Brazil [24, 25]. The samples were grouped as follows: (A) 10 control samples from an endemic area, (B) 20 samples from individuals with untreated disease who were shown to be parasite positive, (C) 20 samples from patients who were treated but not cured and who remained hemoculture positive, (D) 11 samples from patients who were treated but not cured and who became hemoculture negative, and (E) 20 samples from patients who were successfully treated and considered to be cured. Group D was shown to be positive for complement-mediated lysis, whereas group E was negative [25]. Individuals were treated with either benzimidazole or nifurtimox [25].

A further 80 samples from individuals with treated and untreated *Leishmania* infections were also evaluated to assess specificity in a group known to have cross-reactivity with *T. cruzi* lysate. Sequential serum samples from a patient infected with *T. cruzi* (laboratory accident) and subsequently treated were obtained from Frank Steurer (CDC, Atlanta) [26]. Nonendemic control samples were obtained from Boston Biomedica (West Bridgewater, MA).

**ELISA.** Indirect fluorescent antibody test titers were determined according to published methods [6]. ELISA for *T. cruzi* lysate, tetrapeptide, or recombinant TcF was performed as follows: 96-well microtiter plates (Corning Easiwash; Corning Costar, Cambridge, MA) were coated overnight at 4°C with *T. cruzi* lysate (100 ng/well) and the branched tetrapeptide 2/D/E/Lo1.2 or TcF (200 ng/well). Plates were then aspirated and blocked for 2 h at room temperature with PBS containing 1% (w/v) bovine serum albumin (BSA). This was followed by washing in PBS containing 0.1%

Tween 20 (PBST). Serum (1 : 50) dilution in PBS containing 0.1% BSA was added to wells and incubated for 30 min at room temperature. This was followed by washing 6 times with PBST and then incubating with protein A-HRP conjugate (1 : 20,000 dilution; Sigma, St. Louis) for 30 min. The plates were then washed 6 times in PBST and incubated with tetramethylbenzidine (TMB) substrate (Kirkegaard and Perry, Gaithersburg, MD) for 15 min. The reaction was stopped by the addition of 1 N sulfuric acid, and the plates were read at 450 nm by use of an ELISA plate reader (EL311; Biotek Instruments, Hyland Park, VA).

The positive cutoff for assays was determined from the mean of the negative population plus 3 SDs of the mean. Samples within 10% of the cutoff were considered equivocal. Serial dilutions of Chagas’ serum samples were tested in ELISA with *T. cruzi* lysate, and titers at cutoff were determined. The lysate-based Chagas’ ELISA (Gull Laboratories, Salt Lake City) was performed according to the manufacturer’s specifications. Cutoff and the determination of positive, equivocal, and negative status were achieved by reference to the standards and controls supplied with the test. Signal/cutoff is the ratio of the sample optical density to that of the cutoff. Xenodiagnosis and hemoculture were performed according to procedures described elsewhere [9, 10].

## Results

**Peptide/recombinant ELISA with culture-confirmed or xenodiagnosis-positive sera.** Fifty-four samples from individuals shown to be parasite positive (culture confirmed, xenodiagnosis positive) were tested with ELISA using either *T. cruzi* lysate, tetrapeptide (2/D/E/Lo1.2), or recombinant TcF as the solid phase, and results were compared with those from the Gull Chagas’ ELISA. The distribution of TcF reactivity for these samples, relative to the negative population (nonendemic US blood donors), is shown in the histogram in figure 1. A similar distribution was seen for the tetrapeptide (data not shown). In all cases, the reactivity was considerably greater than the cutoff level, with 49 of 54 samples having ELISA optical density values >1 and indicative of a sensitivity of 100% on parasite-confirmed samples. All of these samples were also fully reactive in the *T. cruzi* lysate and tetrapeptide ELISA, as well as in the Gull Chagas’ ELISA. Table 1 presents a summary of data for the parasite-positive samples. These data indicate that the peptide/recombinant assays perform equally as well as the Gull Chagas’ ELISA or the crude lysate ELISA. As shown in figure 1, the specificity of the TcF assay was >99%. A specificity >99% was also observed for the branched tetrapeptide 2/D/E/Lo1.2.

**Patients with untreated and treated Chagas’ disease.** The second set of samples tested in the tetrapeptide and TcF ELISAs consisted of 5 groups: (A) 10 endemic controls, (B) 20 samples from individuals with untreated Chagas’ disease, (C) 20 samples from patients who were unsuccessfully treated and were hemoculture positive, (D) 11 samples from patients who were unsuccessfully treated and were hemoculture negative, and (E) 20 samples from patients who were considered to be cured. All these samples were also tested with the crude lysate assay and

with the Gull Chagas' ELISA to determine how the tetrapeptide/recombinant assays compared. Figure 2 shows the distribution of ELISA reactivities expressed as signal/cutoff ratio for the different assays with the 5 different groups of samples (groups A–E). Higher signal/cutoff ratios were seen in the tetrapeptide and TcF ELISAs than in the Gull Chagas' or *T. cruzi* lysate ELISAs. A summary of the sensitivities of the different peptide/recombinant ELISAs versus the Gull Chagas' and crude lysate ELISAs is presented in table 1.

Whereas all assays were similar in sensitivity (100%) in samples from untreated individuals and in confirmed parasite-positive samples, there were differences in the other patient groups. In particular, differences were seen between the samples from the treated group that became hemoculture negative and the samples from the group that was considered to be cured. These are groups where low antibody titers would be expected, and assay differences reflect the different abilities of the tests to detect low-titer sera. Both the tetrapeptide and recombinant ELISAs were more sensitive in these groups. The mean ELISA signal/cutoff ratios for the tetrapeptide, TcF, and *T. cruzi* lysate assays in each group of samples (A–E) are shown as horizontal bars in figure 2. In all cases, the mean values, which are in-

**Table 1.** Reactivity of different ELISAs with sera of treated and untreated patients with Chagas' disease.

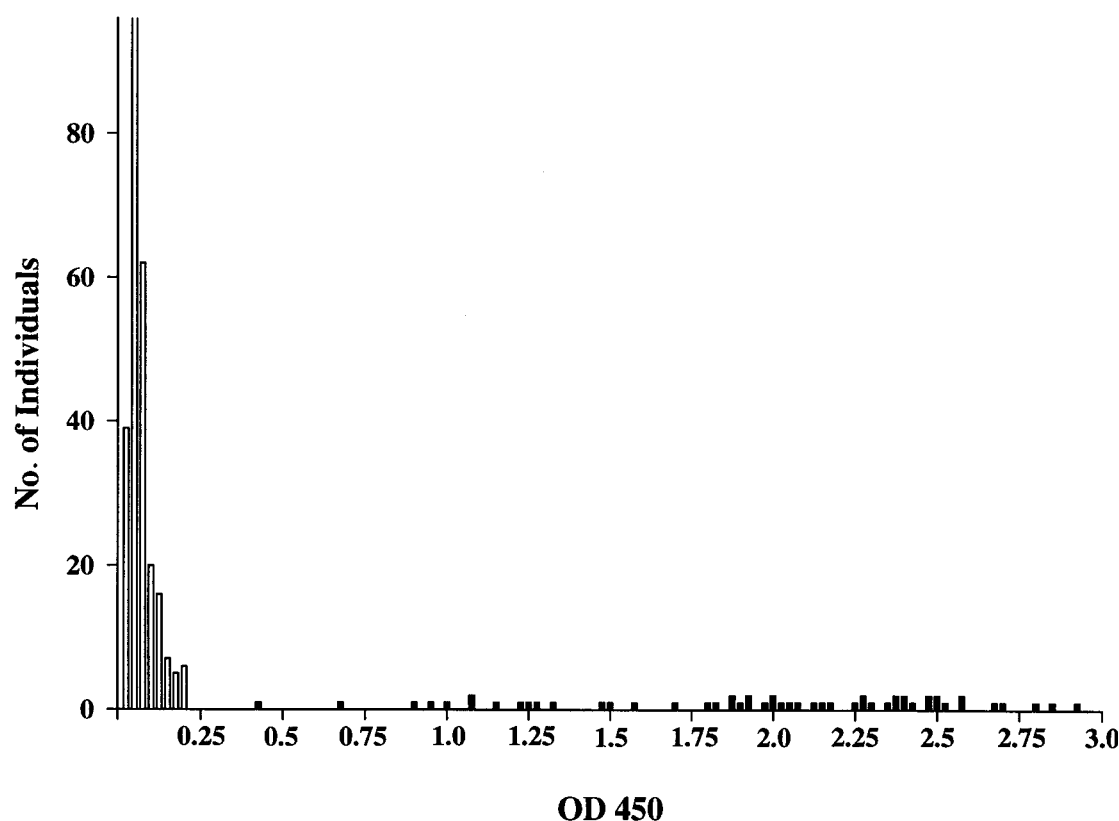
Patient group	No. of ELISA-positive patients			
	Lysate	2/D/E/ Lo1.2	TcF	Gull Chagas'
Untreated	20/20	20/20	20/20	20/20
Treated, not cured, hemoculture positive	20/20	20/20	20/20	20/20
Treated, not cured, hemoculture negative	10/11	10/11	10/11 +1 Eq	10/11
Considered to be cured	17/20	19/20	19/20	15/20 +1 Eq
Endemic normals	0/10	0/10	0/10	0/10
<i>Leishmania</i>	14/80 +5 Eq	6/80 +2 Eq	3/80 +4 Eq	14/80 +5 Eq
Parasite positive	56/56	56/56	54/54 <sup>a</sup>	54/54 <sup>a</sup>

NOTE. Summary of ELISA reactivity of *Trypanosoma cruzi* lysate, tetrapeptide (2/D/E/Lo1.2), recombinant TcF, and Gull Chagas' ELISAs with samples from individuals treated and untreated for Chagas' disease, xenodiagnosis positive, and *Leishmania* positive and from endemic normals. Eq, within equivocal zone of the assay (within 10% of cutoff).

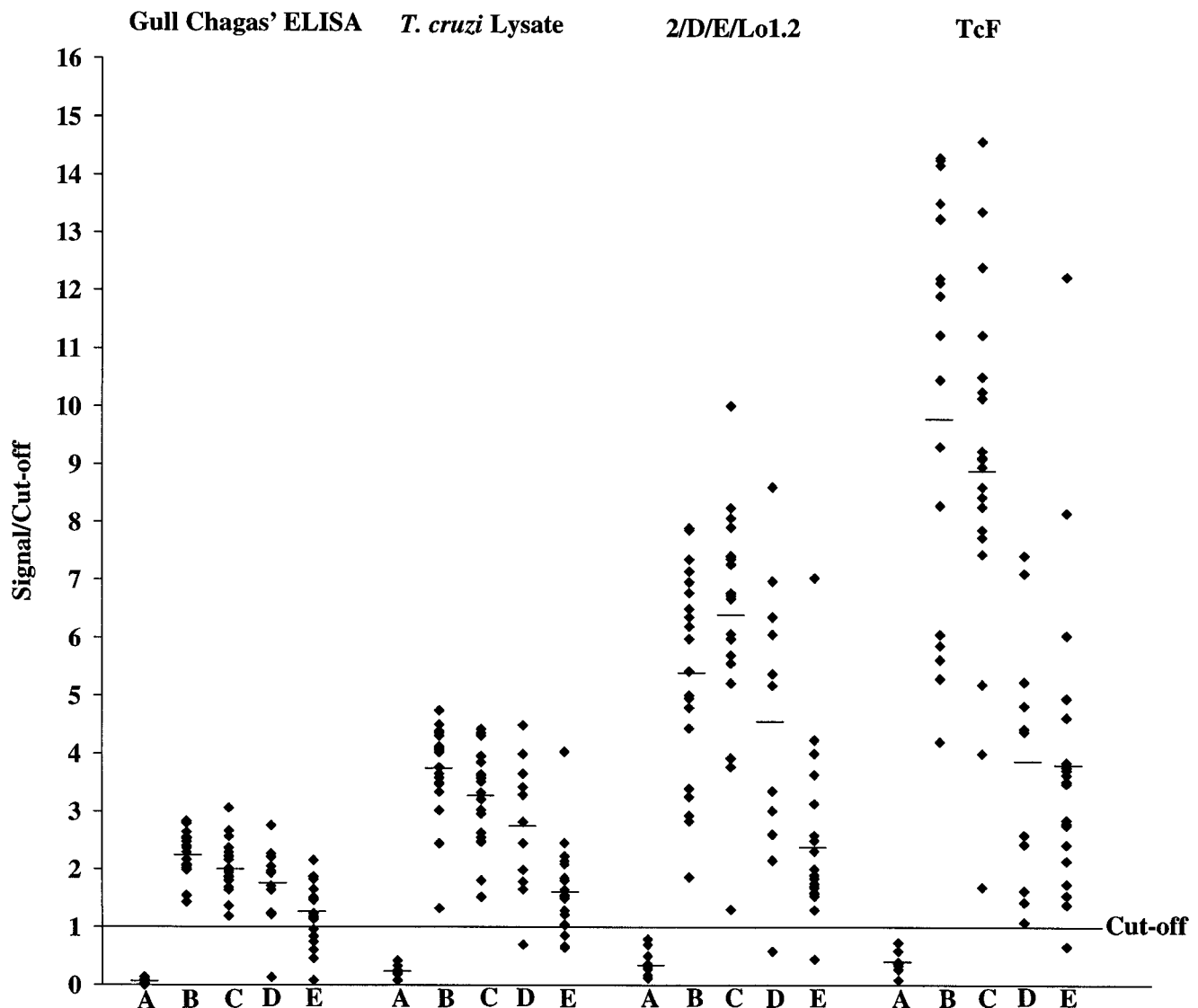
<sup>a</sup> Insufficient sample for 2 xenodiagnosis samples.

dicative of changes in antibody titer, decline in accord with the extent of success of the treatment protocol.

The reduction in parasite burden, as indicated by the hemo-



**Figure 1.** Population histogram showing ELISA reactivity with recombinant TcF of samples ( $n = 54$ ) from individuals who were positive for the *Trypanosoma cruzi* parasite and of control sera ( $n = 251$ ). OD, optical density.



**Figure 2.** Antibodies to *Trypanosoma cruzi* in patients with treated and untreated Chagas' disease as detected in ELISAs with solid phases of Gull Chagas' test, *T. cruzi* crude lysate, tetrapeptide 2/D/E/Lo1.2, and recombinant TcF. The 5 groups of samples tested include, from left to right for each assay, (A) 10 control samples from an endemic area; (B) 20 samples, from individuals with untreated disease, shown to be parasite positive; (C) 20 samples from patients who were treated but not cured and who remained hemoculture positive; (D) 11 samples from patients who were not cured and who became hemoculture negative; and (E) 20 samples from patients who were successfully treated and considered to be cured. Activities are expressed as signal/cutoff ratios for the individual assays. Horizontal lines in each data set indicate the mean signal/cutoff for each group.

culture status of the patient or the patient's being cured, led to significant decreases in signal but still remained greater than the parasite burden observed in uninfected individuals from an endemic area. This indicated the persistence of low antibody titers even in individuals considered cured. For individuals with leishmaniasis, the values were higher in the lysate ELISA than in the tetrapeptide or recombinant assays and reflect the lower specificity when lysate is used as antigen. As indicated in table

1, both the tetrapeptide and recombinant assays detected fewer *Leishmania* sera than the lysate-based assays. Actual end-point antibody titers, which were determined for the lysate-based ELISA by use of serial dilutions, were shown to decrease from  $1325 \pm 773$  in the untreated group to  $109 \pm 128$  in the group considered cured.

**Seroconversion studies.** Serial samples from an individual accidentally infected with *T. cruzi* and subsequently treated with



nifurtimox [26] were tested with the recombinant TcF in Western blot analysis. The data (not shown) indicate that the antibody to *T. cruzi* increased immediately after infection and began to decline after treatment, with residual antibody still detectable years after the infection. The level of antibody in this patient, however, was considerably lower than that typically seen in highly infected patients. The reactivity of the serial samples in lysate ELISA closely correlated with that seen in the tetrapeptide and TcF assays.

## Discussion

A synthetic branched tetrapeptide (2/D/E/Lo1.2) and a recombinant (TcF) containing 4 immunodominant repeat epitopes were compared in ELISAs with *T. cruzi* lysate-based assays for the detection of antibodies present in individuals infected with the agent of Chagas' disease. The study group included samples from untreated, parasite-positive individuals as well as from individuals successfully or unsuccessfully treated. Control samples from endemic and nonendemic areas (United States) and *Leishmania* infection sera were studied. The peptide/recombinant assays were highly sensitive in detecting anti-*T. cruzi* antibodies in individuals who were parasite infected and also showed responses to changes in antibody titer that were similar to those seen in lysate-based ELISA as a result of successful treatment. In all cases, the antibody titer in tetrapeptide-, recombinant-, and lysate-based ELISA was seen to decrease based on the expected parasite burden in each of the groups, based on success of treatment.

In comparison with the Gull Chagas' ELISA, both the tetrapeptide and recombinant ELISAs exhibited a higher clinical sensitivity. These data also show an improved specificity in *Leishmania* samples versus lysate-based assays. This indicates that the tetrapeptide 2/D/E/Lo1.2 and the recombinant TcF provide an alternative to lysate in the development of immunoassays for the detection of anti-*T. cruzi* antibodies. In particular, both ELISA with the tetrapeptide 2/D/E/Lo1.2 and ELISA with recombinant TcF showed high signal/cutoff ratios, compared with lysate-based assays with a high degree of sensitivity and specificity. When used in Western blot, recombinant TcF was effective in detecting antibodies immediately after infection, as well as after treatment in the one individual tested. Previous studies [22, 23] have also shown that a strong correlation exists between the tetrapeptide/TcF ELISA and the radioimmunoprecipitation assay frequently used as a confirmatory assay in the United States [27–29]. The ability of the TcLo1.2 epitope to increase reactivity of low-positive sera [22, 23] indicates the need for either the tetrapeptide 2/D/E/Lo1.2 or the recombinant TcF in the development of an assay suitable for use in the blood bank [21–23]. The data presented indicate that use of a multiepitope peptide, in particular the recombinant TcF, can provide for the development of reproducible, easily

manufactured reagents that can provide more sensitive and specific alternatives to *T. cruzi* lysate in immunoassay development.

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# Chagas disease: recombinant *Trypanosoma cruzi* antigens for serological diagnosis

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**Diagnosis of individuals infected by *Trypanosoma cruzi* is performed mainly by serological tests using crude antigens, which might crossreact with other infections. In the past ten years, many recombinant *T. cruzi* proteins and synthetic peptides have been described, and some are already on the market. Managers of laboratories and blood banks need to make decisions on a cost-benefit basis whether to include these new-generation tests. Here, we indicate antigens that are likely to prove most useful.**

Chagas disease affects around 12 million people in the Americas and, owing to migration, infected individuals can be found in nearly every country of the world. Although control of vector transmission has been achieved in at least three countries after the successful Southern Cone Initiative<sup>1</sup>, and blood supplies are screened in this area, new cases are still increasing in countries without proper control. Furthermore, infected individuals need to be diagnosed and eventually treated<sup>2</sup> and candidates for blood donation should be adequately screened.

Aetiological diagnosis of American trypanosomiasis, which is required in various circumstances (Table 1), is based on the presence of antibodies against the protozoan parasite *Trypanosoma cruzi* in the serum of infected individuals. These antibodies are usually detected by an array of serological tests, from the outdated complement-fixation reaction to enzymatic immunoassays such as the enzyme-linked immunosorbent assay (ELISA). Of these conventional tests, the most widely used are indirect haemagglutination (IHA), indirect immunofluorescence (IIF) and ELISA, because of their simplicity, low costs and good performance in terms of both specificity and sensitivity. All are based on whole or semipurified antigenic fractions from *T. cruzi* epimastigotes (the noninfective form of the parasite). The WHO<sup>3</sup> recommends using at least two tests in parallel; if both tests are performed by trained technicians with good-quality kits, it is possible to define the status of more than 98% of sera. Nevertheless, in busy, routine diagnostic laboratories, hospitals and blood banks, use of the commercially available diagnostic kits might yield figures well below 98%<sup>4</sup>.

Variation in the reproducibility and reliability of these tests has been reported and explained by poor standardization of the reagents<sup>5</sup>. Crossreactivity

occurs with antibodies elicited by other pathogens (mainly *Leishmania*). In order to solve these problems, several purified antigens have been described, tested and used in research with good results, but they have not been included in kits for technical and economic reasons. Large-scale production and purification of parasite antigens by classical biochemistry is a very difficult and time-consuming task, and only very small amounts of antigenic components are obtained. Some of these problems have been overcome with the development of recombinant DNA technology, which has led to the construction of various bacterial and eukaryotic gene expression systems that allow the production of parasite antigens in large quantities, with a high degree of purity and standardized quality.

## Isolation of *T. cruzi* recombinant antigens

*Trypanosoma cruzi* antigen genes have been cloned by screening genomic or cDNA expression libraries with sera from chagasic patients or *T. cruzi*-infected animals<sup>6</sup>. Libraries were constructed in phage vector ( $\lambda$ gt11 or  $\lambda$ ZAP) using randomly generated fragments of genomic DNA or cDNA molecules transcribed from mRNAs of epimastigotes or trypomastigotes<sup>6,7</sup>. *Trypanosoma cruzi* recombinant antigens relevant for serodiagnosis have been isolated by several laboratories (Table 2). Although many antigens show sequences that are either identical or very similar to each other, they have been given different names (e.g. CRA, Ag30, JL8 and TCR27). For this reason, identical or similar genes are grouped together in Table 2. Several of the genes have tandemly repeated sequences and so the predicted lengths of the amino acid repeat units are also given.

Comparisons of genes cloned from different *T. cruzi* strains and isolates showed that the sequence of the repeat units is almost identical, indicating that the repetitive domains of these antigens are highly conserved. For instance, antigens FRA, Ag1, JL7 and H49 are built up of repeats of 68 amino acids that are very conserved between strains and isolates of *T. cruzi*<sup>8</sup>. The high frequency with which antigens bearing repetitive domains are isolated could be explained by a high concentration of specific antibodies against repeats in sera from infected individuals and/or by the fact that such antibodies

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**Table 1. Serological tests for American trypanosomiasis**

Use	Testing laboratory	Requirements	Observations/pitfalls
Confirmation of etiology in a patient	Diagnostic laboratory	High specificity	Mislabelling of sample may give a wrong result. A false positive result may have serious consequences as rejection for a job and psychological fear of a severe disease
Exclusion of blood from a donor	Blood bank	High sensitivity	A false negative may transmit the infection through blood to the recipient
Epidemiological work (certify area free of infection)	Public health service network	High sensitivity	Crossreaction with leishmaniasis may give false positives
Follow up after etiological treatment	Research laboratory (comparison with antibody concentration before treatment)	High specificity and sensitivity	Need long period of observation. Stored sample of serum for comparison of titres
Check up in immunosuppressed/ AIDS/transplant	Diagnostic laboratory	High specificity	Possibility of reactivation of the infection
Suspected acute phase without detectable parasites	Diagnostic laboratory (assay with IgM conjugates searching for specific IgM)	First priority to search for parasites. If negative results, proceed with IgM search	If positive, should be specifically treated (Ref. 2)
Suspected congenital infection: (1) immediately after delivery, proceed as acute phase. (2) otherwise, recall infant after six months of age	Diagnostic laboratory	Mother's serology should be positive. Search for specific IgG after six months of age	If specific IgG is present after six months of age, the infant should be treated with trypanocidal drugs (Ref. 2)

bind with high affinity. However, this strategy also allowed the identification of nonrepetitive *T. cruzi* antigens such as the ribosomal P protein JL5, the 24 kDa flagellar Ca<sup>2+</sup>-binding proteins (FCaBP, 1F8, Tc-24, Tc-28), A13, Tc40, heat-shock proteins, flagellum-associated membrane proteins (FL-160, CEA, CRP), and ubiquitin.

#### Evaluation of the diagnostic potential of *T. cruzi* recombinant antigens

Three multicentre studies have been performed using 17 recombinant antigens<sup>21–23</sup>. The first study<sup>21</sup>, undertaken by the WHO, used sera from 50 chagasic and non-chagasic individuals from endemic areas of Central Brazil. Ten out of 17 antigens tested had a kappa index (KI)  $\geq 0.80$  and their specificities and sensitivities were 0.86–1.00 and 0.95–1.00, respectively. (The KI measures the agreement of results obtained by a given antigen with reference samples<sup>21</sup>; that is, it can compare results obtained in different laboratories with the same samples. Identical results to the reference are given a KI of 1.0 and a KI  $>0.80$  indicates excellent agreement.) The CRA antigen was found to be the best diagnostic antigen (KI = 1.00), followed by antigens B13 (KI = 0.96) and H49 (KI = 0.92). It is noteworthy that antigens carrying common amino acid repeats presented different KI values. For instance, CRA, Ag30 and JL8 (Table 2) share identical or similar 14-amino acid repeats but had KI values of 1.00, 0.84 and 0.80, respectively<sup>21</sup>. These differences could reflect different protocols used to isolate them and the type of the immunoassay used (radioimmunoassay, ELISA, phage dot blot immunoassay).

In a second study<sup>22</sup>, co-ordinated by the Project of Biotechnology of the Science and Technology for

Development organization (CYTED), ten recombinant antigens (Ag2, Ag13, SAPA, H49, A13, JL5, JL7, JL8, JL9 and RAI) were examined in a reference laboratory using the phage dot blot immunoassay. Sera from 215 individuals were included in this study: 148 chagasic patients from different endemic areas for American trypanosomiasis in Argentina, Brazil and Venezuela, and 67 non-chagasic subjects. Antigens JL7, H49, Ag2 and A13 were the best diagnostic reagents in this study, with KI values of 0.82–0.93. However, none of these antigens could detect specific antibodies in sera from four chronic chagasic patients out of 148 (2.7%), indicating that single antigens would not be suitable for serological diagnosis.

The third study<sup>23</sup> examined the diagnostic efficiency of six antigens (H49, JL7, JL8, A13, B13 and 1F8) using ELISA on a panel of 541 serum samples (from 304 infected and 237 non-infected individuals) from nine countries in South and Central America (Argentina, Brazil, Bolivia, Chile, Colombia, Venezuela, El Salvador, Guatemala and Honduras). Four antigens (1F8, H49, JL7 and B13) showed high sensitivity (93.4–99.0%). Many individuals living in endemic areas produced specific antibodies against repetitive amino acid antigens (H49, JL7 and B13). Interestingly, the sensitivity (99%) and specificity (99.6%) of the 1F8 antigen were comparable to those of the repetitive antigens, indicating that chronic chagasic patients also display antibodies against non-repetitive antigens. Serum samples from infected individuals reacted with at least one recombinant antigen, suggesting that a mixture of recombinant antigens could detect anti-*T. cruzi* antibodies in all serum samples used in this study. The positivity of a hypothetical antigenic mixture comprising the

**Table 2. *Trypanosoma cruzi* recombinant proteins or synthetic peptides with potential clinical and epidemiological use**

Antigen <sup>a</sup>	Repeat length (amino acids)	Native protein <sup>b</sup> (kDa)	Remarks	Diagnosis/use	Refs
CRA	14	225	Cytoplasmic antigen	Chronic infections	6
Ag30		180–225			6
JL8		>170			6
TCR27		150–200			6
FRA	68	>300	Cytoskeleton-associated protein	Chronic infections	6
Ag1		205			6
JL7		>170			6
H49		>300			8
B13	12	116–140	Trypomastigote surface protein	Chronic infections	9
Ag2		85			6
TCR39		82			6
PEP-2					10
Ag36	38	85	Microtubule-associated protein	Chronic and acute infections	6
JL9		110			6
MAP					6
SAPA	12	105–205	Trans-sialidases (TS family)	Acute and congenital infections	6
TCNA					6
TS					6
Ag13	5	85		Chronic and acute infections	6
TcD		260			11
B12	20	200–230		Chronic infections	9
TcE	7	35	Ribosomal protein	Chronic infections	12
JL5	None	38	Ribosomal P protein	Cardiac clinical forms	6
A13	None	230		Chronic and acute infections	6
FCaBP	None	24	Flagellar Ca <sup>2+</sup> binding protein	Chronic infections monitoring of cure	6
1F8		24			6
Tc-24		24			13
Tc-28		28			14
Tc-40	None	38–100		Chronic infections	15
cy-hsp70	None	70	Heat shock proteins	Chronic infections monitoring of cure	6,16
mt-hsp70		70			16
grp-hsp78		78			16
FL-160	None	160	Flagellum-associated surface protein (TS-like family)	Chronic infections monitoring of cure	17
CEA					18
CRP					19
SA85-1.1	None	85	Trypomastigote surface protein (TS-like family)	Chronic infections	17
Ubiquitin	None			Chronic infections	20

Abbreviations: CEA, chronic exoantigen of 160 kDa; CRA, cytoplasmic repetitive antigen; CRP, complement regulatory protein of 160 kDa; cy-hsp70, cytoplasmic heat shock protein of 70 kDa; FCaBP, flagellar Ca<sup>2+</sup>-binding protein; FL-160, flagellar surface protein of 160 kDa; FRA, flagellar repetitive antigen; grp-hsp78, endoplasmic reticulum heat shock protein of 78 kDa; MAP, microtubule associated protein; mt-hsp70, mitochondrion heat shock protein of 70 kDa; SAPA, shed acute-phase antigen; SA85-1.1, surface protein of 85 kDa; TCNA, *Trypanosoma cruzi* neuraminidase; TS, trans-sialidase.

<sup>a</sup>Several different names were given to identical or similar peptides, and they are grouped together.

<sup>b</sup>Sizes of some native proteins may differ among different *T. cruzi* strains or isolates.

peptides H49 or JL7, B13 and 1F8 was calculated to be 100%. Furthermore, results indicated that one of the major advantages of recombinant ELISA for the serodiagnosis of Chagas disease was the lack of cross-reaction with other parasitic diseases, such as leishmaniasis.

#### Evaluation of mixtures of recombinant antigens or synthetic peptides

##### Recombinant antigen mixtures

Serodiagnostic tests with recombinant antigens were improved using a mixture of the antigens CRA and FRA in an ELISA<sup>24–26</sup> (Table 3). The performance of

the CRA+FRA mixture was compared with four commercial ELISA kits, IHA and IIF tests using 524 well-defined chagasic and non-chagasic human serum samples from endemic areas of Brazil and from blood donors of the State Blood Bank of São Paulo, as well as 60 serum samples that had given discrepant results with conventional serology<sup>26</sup>. The CRA+FRA mixture showed 98.3% sensitivity and 100% specificity, and no crossreactivity was observed in the recombinant ELISA with 58 sera that were positive for other diseases. The use of the CRA+FRA recombinant ELISA might lead to a reduction of more than 50% in the number of discordant sera<sup>26</sup>.

**Table 3. Diagnostic performance of serodiagnostic assay using different combinations of *T. cruzi* recombinant proteins or synthetic peptides**

Antigens <sup>a</sup>	Assay	Type of antigen	Sensitivity (%)	Specificity (%)	Refs
CRA+FRA mixture	ELISA	Fusion protein ( $\beta$ -galactosidase)	100.0 98.3	100.0 100.0	24,25 26
Ag1+Ag2+Ag30+SAPA mixture	Enzyme immunoassay (immunodot) <sup>b</sup>	Fusion protein (glutathione-S-transferase)	99.6	99.1	27
FCaBP+hsp70 mixture	ELISA	Fusion protein (His <sub>6</sub> -tagged peptide)	97.0	92.3	16
(CRA+FRA+Tc-24+SAPA+MAP+TcD+Ag39) <sup>c</sup>	Line immunoassay <sup>d</sup>	Recombinant proteins and synthetic peptides	100.0	99.3	28
			99.4	98.1	29
TcD+PEP-2 mixture	ELISA	Synthetic peptide	99.7	99.0	34
TcD+Ag2+TcE mixture	Particle gel immunoassay <sup>e</sup>	Synthetic peptide	96.8	94.6	35
TcD+TcE+PEP-2 multi-epitope	ELISA	Linear synthetic peptide	99.6	99.3	12
			100.0	100.0	36
TcD+TcE+PEP-2+TcLo1.2 multi-epitope	ELISA	Branched synthetic tetrapeptide	100.0	ND	12
			100.0	93.3	37
TcD+TcE+PEP-2+TcLo1.2 multi-epitope	ELISA	Linear synthetic peptide	100.0	ND	12
TcD+TcE+PEP-2+TcLo1.2 multi-epitope	ELISA	Linear fusion protein (His <sub>6</sub> -tagged peptide)	100.0	ND	12
			100.0	96.6	37

<sup>a</sup>Abbreviations: CRA, cytoplasmic repetitive antigen; FCaBP flagellar Ca<sup>2+</sup>-binding protein; FRA, flagellar repetitive antigen; hsp70, heat shock protein of 70 kDa; SAPA, shed acute-phase antigen; ND, not determined.  
<sup>b</sup>Enzyme immunoassay (EIA), Dia Kit<sup>TM</sup> Bio-Chagas assay®, Gador S.A., Argentina.  
<sup>c</sup>Recombinant proteins or synthetic peptides are fixed individually in a single strip.  
<sup>d</sup>Line immuno assay (LIA) Chagas antibody, Innogenetics, Belgium.  
<sup>e</sup>Particle gel immunoassay (PaGIA), DiaMed AG, Switzerland.

A new immunodot assay (Dia Kit Bio-Chagas assay®, Gador, Buenos Aires, Argentina) has been developed that uses a mixture of five recombinant antigens (Ag1, Ag2, Ag13, Ag30 and SAPA) coated in a single line onto a reinforced nitrocellulose membrane, together with a second human immunoglobulin G (IgG) control line to monitor the conjugate- and colour-development steps<sup>27</sup>. This study used 995 chagasic and non-chagasic serum samples from Argentina, Brazil and Chile. The test displayed a sensitivity of 99.6% and a specificity of 99.1% (Table 3) but four false positive reactions were observed among 16 sera from patients with visceral leishmaniasis. The combination of recombinant antigens hsp78 (grp78) and FCaBP in ELISA (Table 3) has also been tested with a panel of 176 serum samples from Brazilian chagasic and non-infected individuals<sup>16</sup>. The mixture improved sensitivity in comparison with hsp78 and FCaBP alone (from 90% to 97%), but it also increased the cross-reactivity with serum from patients with cutaneous leishmaniasis (from 3% to 8%).

The loss of specificity found with different antigenic mixtures could be due to the packing of antigen molecules in a limited physical space, which might interfere with the binding of the antibodies. To overcome this problem, a line immunoassay (INNO-LIA® Chagas Ab, Innogenetics, Ghent, Belgium) combining recombinant antigens and synthetic peptides was developed and evaluated with a panel of 1062 serum samples from patients and healthy individuals from four Brazilian regions endemic for American trypanosomiasis<sup>28</sup>. Seven

recombinant antigens (CRA, FRA, Ag39, TcD, Tc24, SAPA and MAP) were coated as discrete and independent lines onto a nylon membrane with plastic backing, and the assay gave 100% sensitivity and 99.3% specificity (Table 3). No crossreactivity was found with a set of 40 sera from patients with leishmaniasis. A second study using 1604 serum samples from the State Blood Bank of São Paulo, Brazil, suggested that the INNO-LIA Chagas could be used as a confirmatory test in serological diagnosis<sup>29</sup>.

#### Synthetic peptide mixtures

Synthetic peptides derived from the amino acid sequences of repeated domains of *T. cruzi* antigens (Ag1, Ag2, Ag13, Ag30, Ag36 and SAPA) were used to develop ELISA and immunoradiometric assays<sup>30–32</sup>. Peptides derived from Ag2 and Ag36 reacted with 93% and 65%, respectively, of 60 serum samples from Chilean chronic chagasic patients<sup>32</sup>. By contrast, peptides based on the amino acid repeats of antigens Ag1, Ag13 and Ag30 gave poor results (44–65% sensitivity)<sup>30,32</sup> when compared with those obtained with the fusion proteins (>90% for all antigens). These results suggested that some synthetic peptides cannot mimic the immunodominant epitopes of native antigens. The correct identification of epitopes present in the repetitive domains of *T. cruzi* antigens will help the design of synthetic peptides. The synthetic peptide Ag30 (Refs 30,32) carries only one copy of the main immunodominant B-cell epitope<sup>33</sup>. This could explain why this peptide was only recognized by 60% of chagasic patients, whereas the recombinant protein

carrying many repeats reacts with 99% of sera<sup>9,11,21–25,28,29</sup>.

The synthetic peptides PEP-2 and TcD have been tested individually and combined in an ELISA format (Table 3) with 378 serum samples from chagasic and non-chagasic individuals (260 living in an endemic area for Chagas disease in Brazil and 118 healthy individuals and patients with different infectious diseases)<sup>34</sup>. Individually, TcD and PEP-2 peptides gave sensitivities of 93% and 91%, respectively. Combination of the TcD and PEP-2 in a single ELISA significantly improved the sensitivity (99.7%) and specificity (99%) of the test. However, in a separate study<sup>30</sup>, the peptide TcD (Table 2) showed a specificity of only 44%, suggesting that the result depends on the format of the test used.

The synthetic peptides Ag2, TcD and TcE were used to develop a particle gel immunoassay (ID-PaGIA®, Diamed, Cressier sur Morat, Switzerland)<sup>35</sup>. When coloured gel particles sensitized with the three peptides are mixed with the specific serum, they agglutinate and can be visualized after centrifugation. The ability of ID-PaGIA to discriminate between negative and positive sera was tested using 111 negative and 119 positive sera collected in four different Brazilian institutions. The sensitivity and specificity of this assay were 96.8% and 94.6%, respectively<sup>35</sup> (Table 3). The assay has the advantages of simplicity of operation and a rapid reaction time (20 min).

#### Multiepitope antigens

A multiepitope synthetic peptide or recombinant protein carrying *T. cruzi* repeating B-cell epitopes has recently been constructed and evaluated with sera from infected and non-infected individuals from Brazilian regions endemic for Chagas disease and from Ecuador<sup>12,36,37</sup> (Table 3). Consensus positive sera for *T. cruzi* infection from the Centers for Disease Control and Prevention (Atlanta, GA, USA) were also used<sup>12,37</sup>. Initially, the repeat units of antigens TcD, TcE and PEP-2 were combined into a single linear multiepitope synthetic peptide (Table 3). An ELISA based on this multiepitope peptide gave a sensitivity of 99.6% and a specificity of 99.3%.

A fourth repeating epitope, TcLo1.2, was isolated by expression cloning, using a serum sample from a chagasic patient who was negative for reactivity with the linear tripeptide TcD–TcE–PEP-2. TcLo1.2 was further combined into a branched tetrapeptide with TcD–TcE–PEP-2 on one arm and TcLo1.2 in the other. A linear multiepitope recombinant protein (TcLo1.2–TcD–TcE–PEP-2) was also expressed in *Escherichia coli*. An ELISA based on these constructs displayed 100% sensitivity (Table 3). However, the reactivities of the linear tripeptide TcD–TcE–PEP-2 or the branched tetrapeptide were greater than that seen for the individual mix of these three peptides<sup>11,36,37</sup>, suggesting that the

multiepitope constructs can minimize the problems of competition between different peptides for the solid phase.

Apart from the kits described above, other commercial serodiagnostic kits using recombinant or synthetic *T. cruzi* peptides are currently used: an ELISA based on the FRA+CRA mixture (EIE-Recombinante-Chagas®, BioManguinhos, Rio de Janeiro, Brazil); a rapid qualitative immunochromatographic test based on a combination of recombinant antigens (Chagas Stat-Pak®, Chembio Diagnostic Systems, New York, NY, USA); and a qualitative, membrane-based, immunoassay manufactured using *T. cruzi* and *Leishmania* recombinant antigens (Qualicode Chagas/Leishmania® kit, Immunetics, Cambridge, MA, USA). Some of these kits are still under evaluation.

#### Diagnosis of the acute phase using recombinant antigens

The serological profile in the first month of the acute phase of Chagas disease corresponds to a classical primary immune response<sup>38</sup>. Specific immunoglobulin M (IgM) appears early in the acute phase of *T. cruzi* infection and can be used in the diagnosis of congenital transmission<sup>38</sup>. The recombinant antigen SAPA reacted with IgM and IgG antibodies of sera from acute and chronic chagasic patients<sup>39</sup>. Anti-SAPA antibodies were detected in 90% of serum samples from acute chagasic patients, and in 10–48.7% of chronic patients<sup>22,30–32,39–42</sup>. SAPA reacted with foetal IgM and IgG antibodies present in the cord blood from *T. cruzi*-infected newborns, and it has been suggested that detection of IgM and IgG with SAPA could be used to distinguish congenitally infected infants from uninfected infants<sup>39</sup>.

#### Conclusions and perspectives

The inclusion of recombinant antigens and synthetic peptides for the serological diagnosis of *T. cruzi* infection has been a clear advance in terms of specificity increase. After the first multicentric studies with these new tools, it became clear that single antigens lack the required sensitivity when compared with conventional tests. There have been several successful attempts to increase sensitivity by the use of cocktails of recombinant antigens (both in mixtures and in different spots), mixtures of synthetic peptides or multiepitope antigens. All the products described, commercially available or not, have a higher specificity than conventional tests, and a number of them require fewer steps and hence are faster. The inclusion of one of these tools is recommended if performed in parallel with one of the conventional tests, mainly IIF or ELISA, which will give the desired specificity (given by a recombinant) and the required sensitivity (given by the crude antigenic preparations).

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## Serodiagnosis of Chronic Chagas Infection by Using EIE-Recombinant-Chagas-Biomanguinhos Kit

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*A kit based on an enzyme immunoassay, EIE-Recombinant-Chagas-Biomanguinhos, developed by the Oswaldo Cruz Foundation, was evaluated for the serodiagnosis of chronic Chagas disease. Evaluation was performed with 368 serum samples collected from individuals living in an endemic area for Chagas disease: 131 patients in the chronic phase with confirmed clinical, epidemiological, and serological diagnosis (indirect immunofluorescence, indirect hemagglutination or enzyme-linked immunosorbent assay) and 237 nonchagasic seronegative individuals were considered negative control. The EIE-Recombinant-Chagas-Biomanguinhos kit showed high sensitivity, 100% (CI 95%: 96.4-100%) and high specificity, 100% (CI 95%: 98-100%). The data obtained were in full agreement with clinical and conventional serology data. In addition, no cross-reaction was observed with sera from patients with cutaneous (n=14) and visceral (n=3) leishmaniasis. However, when these sera were tested by conventional serological assays for Chagas disease, cross-reactions were detected in 14.3% and 33.3% of the patients with cutaneous and visceral leishmaniasis, respectively. No cross-reactions were observed when sera from nonchagasic seronegative patients bearing other infectious disease (syphilis, n=8; HTLV, n=8; HCV, n=7 and HBV, n=12) were tested. In addition, sera of patients with inconclusive results for Chagas disease by conventional serology showed results in agreement with clinical evaluation, when tested by the kit. These results are relevant and indicate that the referred kit provides a safe immunodiagnosis of Chagas disease and could be used in blood bank screening.*

Key words: serodiagnosis - recombinant antigens - *Trypanosoma cruzi* - Chagas disease

Chagas disease is still a major health problem in Latin America where 16-18 million individuals are infected with the causative agent *Trypanosoma cruzi* and at least 90 million people are estimated to be at risk of infection (WHO 1996). Under natural conditions, infected reduviid bugs transmit the *T. cruzi* to humans when broken skin or mucous membranes contact metacyclics trypomastigotes from insect excreta. However, *T. cruzi* may bypass the vector bugs and be transmitted to man by a number of alternative mechanisms: blood trans-

fusion, congenital transmission, accidental laboratory contamination, organ transplantation from infected donors and transmission by oral route (Umezawa et al. 1996, Gomes 1997). Blood transfusion is the second most common means of infection and the human migration from endemic areas to urban centers is proving a rising risk of transfusional Chagas disease in all Latin America and in non endemic countries (Schmunis 1991).

Diagnosis of chronic Chagas disease is based on the detection of parasite by indirect parasitological methods (xenodiagnosis and hemoculture) or more usually on the detection of IgG antibodies against *T. cruzi* in the sera of patients by immunological methods (complement fixation-CF, indirect immunofluorescence-IIF, direct agglutination-DA, indirect hemagglutination-IHA and enzyme-linked immunosorbent assay-ELISA). The methods based on detection of the parasite, although highly specific, are of limited sensitivity, because parasites are

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detected in only 20-50% of individuals known to be infected, resulting in many false negative results (Gomes 1997). On the other hand, the methods based on the detection of an immune response to the parasite in the mammalian host lack specificity since they use crude or partially purified parasite extracts. Cross-reaction to *T. cruzi* has been observed with related protozoan diseases, particularly leishmaniasis. The problems with conventional assays (CF, IHA, DA, IIF and ELISA) may be overcome by using recombinant polypeptides containing specific *T. cruzi* epitopes that elicit an immune response in the majority of chagasic patients.

Several *T. cruzi* genes have been cloned and some of recombinant antigens have been assayed for their use in diagnosis (Affranchino et al. 1989, Levin et al. 1989, Almeida et al. 1990, Paranhos et al. 1990, Cotrin et al. 1990, Zingales et al. 1990, Goldenberg et al. 1991, Umezawa et al. 1999). Two recombinant antigens, CRA and FRA, expressed in the bacterium *Escherichia coli* were analyzed by Krieger et al. (1992) in a diagnostic test for Chagas disease. The data indicated that recombinant antigens displayed better results when used in combination than separately. These authors developed a direct ELISA which involves the use of peroxidase-labeled antigens to detect the immune-complexes. The results indicate that the recombinant (CRA+FRA) ELISA was better than the conventional ELISA in the diagnosis of Chagas disease, providing 100% specificity and sensitivity in all sera tested (Almeida et al. 1990, Krieger et al. 1992). These antigens were characterized and shown to display a repetitive epitope structure (Lafaille et al. 1989, Krieger et al. 1990). FRA (flagellar repetitive antigen) is located in the flagellum of the parasite and displays a 68-amino acid repeat, while CRA (cytoplasmic repetitive antigen) is distributed throughout the cytoplasm and has a 14-amino acid repeat (Lafaille et al. 1989).

Recently, a kit for diagnosis of chronic Chagas disease, using CRA+FRA antigens was developed by Oswaldo Cruz Foundation (Fiocruz), Rio de Janeiro, Brazil. The kit is based on enzyme immunoassay, the direct ELISA.

In the present work we report the evaluation of the EIE-Recombinant-Chagas-Biomanguinhos kit for the diagnosis of *T. cruzi* infection using characterized serum samples from individuals living in Chagas disease endemic areas and from individuals with other infectious diseases.

## MATERIALS AND METHODS

**Human sera** - Serum samples were collected from 368 patients between the ages of 5 and 76 years old from Hospital Universitário Oswaldo Cruz (Recife) living in Chagas disease endemic areas in the State of Pernambuco, Brazil: 131 pa-

tients in the chronic phase of the Chagas disease with confirmed clinical, epidemiological, and serological diagnosis and 237 nonchagasic individuals with negative serology were considered negative controls. Serum samples were previously classified as negative when two serological tests (ELISA, IIF or HAI) gave nonreactive results against *T. cruzi* antigens and as positive when two tests were reactive. Serum samples of 14 patients with cutaneous leishmaniasis (CL) and 3 with visceral leishmaniasis (VL) were also tested. Diagnosis of CL was based upon the collective analysis of a set of elements: presence of typical lesions, compatible epidemiological history and direct parasite detection. VL was diagnosed by clinical, epidemiological history, positive serological assay (IIF) and by detection of parasites in bone marrow aspirate. These sera were also tested by conventional serological assay to Chagas disease (ELISA and HAI) to evaluate cross-reaction.

Sera from patients with other infectious disease (8 with syphilis, 8 with HTLV, 7 with HCV, and 12 with HBV) as well as sera of patients with inconclusive results for Chagas disease (Table II) from blood center Fundação Hemope/Hemocentro, Pernambuco, Brazil were included in this study. All sera were analyzed by EIE-Recombinant-Chagas-Biomanguinhos kit. Blood samples from the individuals were taken by venopuncture and the sera obtained were stored at -20°C until use.

**Enzyme immunoassay** - The enzyme immunoassay, with the EIE-Recombinant-Chagas-Biomanguinhos, was performed according to manufacturer. Briefly, microplates sensitized with the recombinant antigens were incubated with undiluted patient sera (50 µl) at 37°C for 30 min. After washes to remove the unbound antibodies, the plates were incubated for 30 min at 37°C with 50 µl of peroxidase conjugated antigens. After repeated cycles of washes the immune complexes were revealed by the addition of hydrogen peroxide and 3, 3', 5, 5'-tetramethylbenzidine. The reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub>, and the optical density (OD) at 450 nm was determined in a ELISA reader (Bio-Rad 3550). The cutoff (CO) values as well as the gray zone were calculated for each plate according to manufacturer. Sera with OD values equal or greater than CO value were considered reactive, and consequently considered positive for antibodies to *T. cruzi*. Sera with OD values below CO were considered non-reactive and negative for antibodies to *T. cruzi*.

**Data analysis** - The figure of samples recorded as OD<sub>450nm</sub> was distributed by using computer graphics software. The values of sensitivity and specificity were calculated according to Camargo (1992). The confidence interval (CI) was calculated at the level of 95%.

## RESULTS

ELISA results are shown in the Figure. The CO and the gray zone values for each plate are shown in Table I. The high sensitivity of 100% (CI 95%: 96.4-100%) and specificity of 100% (CI 95%: 98-100%) show the excellent performance of the EIE-Recombinant-Chagas-Biomanguinhos kit. All 131 cases of confirmed Chagas disease, which had been diagnosed by clinical and conventional serology were positive and all 237 sera from nonchagasic individuals were negative.

Analysis of the sera negative for Chagas disease but positive for CL and VL shows that the responses do not give rise to false positive results (Figure). When these sera were tested by conventional serological assays for Chagas disease, cross-reactions were observed in 14.3% (2/14) of the patients with CL and 33.3% (1/3) patients with VL. No cross-reactions were observed when sera from patients with syphilis, HTLV, HBV and HCV were tested by the recombinant kit. The sera with inconclusive results for Chagas disease by using conventional serological assays were in agreement with the clinical evaluation (two chagasic patients and four nonchagasic individuals) when tested by recombinant kit (Table II).

TABLE I

Cutoff and gray zone values obtained for each plate in present report

Plates	Cutoff values (OD)	Gray zone values (OD)
1	0.233	0.233-0.279
2	0.244	0.244-0.293
3	0.198	0.198-0.237
4	0.192	0.192-0.231
5	0.178	0.178-0.213
6	0.183	0.183-0.220

TABLE II

Comparative evaluation of the sera with the inconclusive results by conventional serological tests and EIE-Recombinant-Chagas-Biomanguinhos kit

Sera	Conventional serological tests		EIE-Recombinant-kit
	ELISA OD/CO	IIF	OD/CO
1 <sup>a</sup>	0.289/0.180	NR	0.087/0.198
2 <sup>a</sup>	0.245/0.180	NR	0.087/0.198
3 <sup>a</sup>	0.286/0.180	NR	0.088/0.198
4 <sup>a</sup>	0.254/0.180	NR	0.096/0.198
6 <sup>b</sup>	1.188/0.180	NR	1.242/0.198
7 <sup>b</sup>	0.215/0.180	R	1.150/0.198

a: nonchagasic sera; b: chagasic sera; NR: non reactive sera; R: reactive sera; OD: optical densities; CO: cutoff.

## DISCUSSION

The serological conventional assays (CF, IIF, IHA and ELISA) for diagnosis of Chagas disease are used for individual diagnosis and for screening of donated blood, as well as in epidemiological studies. However, a persistent problem with these conventional assays has been the occurrence of false-positive results. Due to this problem, the World Health Organization recommends that serum specimens should be tested in two conventional assays before being accepted as positive (Gomes 1997). This approach carries with it an enormous logistic and economic burden, especially for a blood bank. Even if some blood banks use three different serological tests to reach a diagnosis, the amount of blood discarded that could be transfused, if there was a safe test, is significant. As an example, in the major Brazilian blood center (Fundação Pró-Sangue Hemocentro de São Paulo) some 10,000 blood units are discarded per year due their reactivity for Chagas disease in conventional serologic tests, at a cost of approximately US\$ 60.00 per bag (Carvalho et al. 1993). In addition, the diagnostic tests that give some false-positive results should be avoided because such tests can create social problems for false-positive chagasic patients. They can also produce erroneous data in epidemiological studies (Krieger et al. 1992).

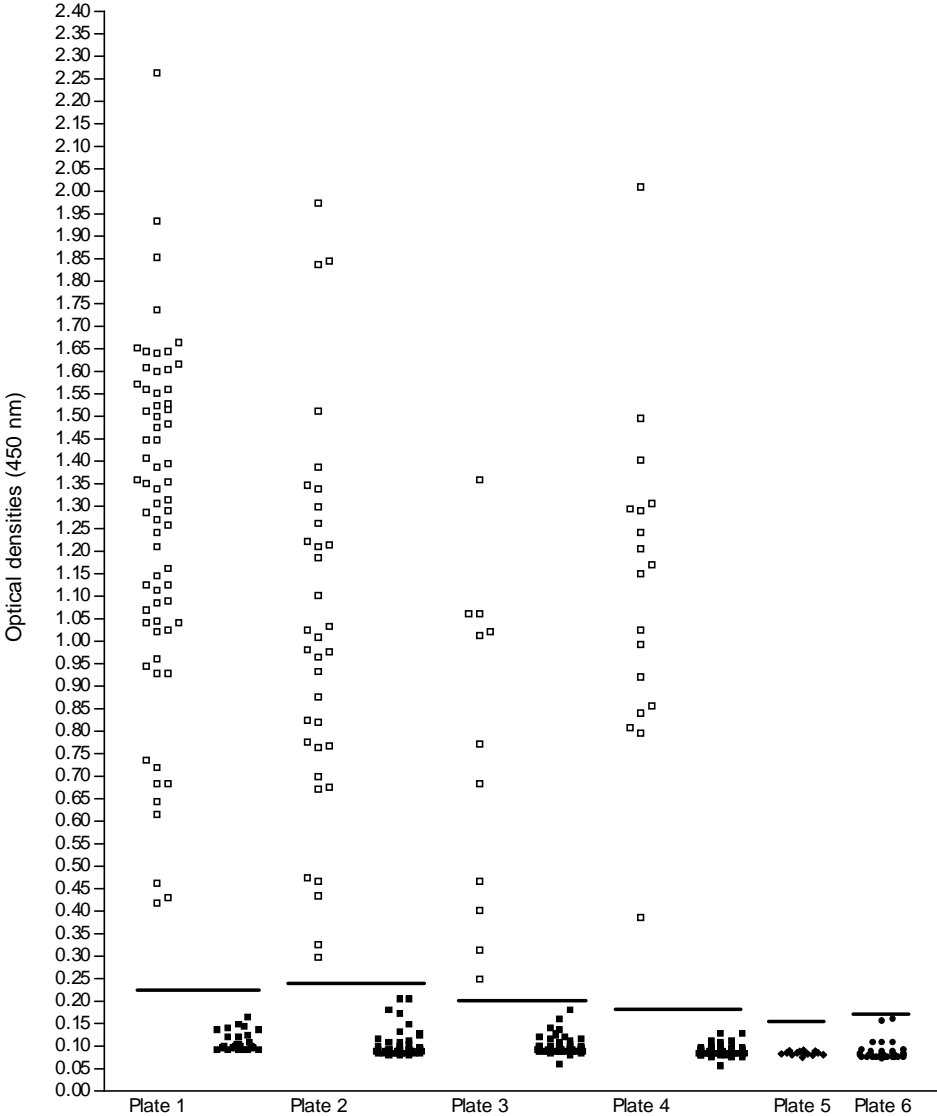
In order to overcome these problems, a new diagnostic kit EIE-Recombinant-Chagas-Biomanguinhos was developed by Fiocruz to detect antibody to *T. cruzi* in sera and plasma. The test is a direct ELISA that use the CRA+FRA recombinant antigens.

In the present report we have evaluated the EIE-Recombinant-Chagas-Biomanguinhos kit using serum samples from individuals living in Chagas disease endemic areas of State of Pernambuco, Brazil. The test showed to be highly sensitive and specific, detecting 100% of the chagasic (131/131) and nonchagasic individuals from endemic areas (237/237), respectively. Sensitivity and specificity were 100% (CI 95%: 96.4-100%) and 100% (CI 95%: 98-100%), respectively. In addition, no cross-reaction was observed with sera from patients with CL and VL. Even if low reactivity sera were not included in this study, we may predict a high sensitivity according to the results obtained with these confirmed chagasic patients. Several conventional serological tests as well as ELISAs commercially available in Brazil showed cross-reactivity to sera from patients with other diseases. These false-positive results are frequent in the case of patients with leishmaniasis (Carvalho et al. 1993). The absence of cross-reactions in EIE-Recombi-

nant-Chagas-Biomanguinhos shows its high specificity for Chagas disease.

According to Camargo (1992), a problem that occurs during screening of chagasic sera in blood banks is the variable percentage of samples showing a reactivity in the gray zone around the cutoff values. In order to verify this problem we tested by recombinant kit, sera from blood center Fundação Hemope/Hemocentro, Pernambuco, Brazil with inconclusive results for Chagas disease obtained by conventional serological assays. Reactivity in the gray zone was not observed and the results were in full agreement with clinical evalu-

ation. The kit EIE-Recombinant-Chagas-Biomanguinhos for the diagnosis of chronic Chagas disease has several advantages over other available methods: (i) the use of specific *T. cruzi* recombinant antigens avoids false-positive reaction; (ii) the direct ELISA increases the sensitivity of the method allowing the evaluation of low titer sera, and corroborates its specificity; (iii) the use of undiluted serum samples reduces the possibility of error due to manipulation; (iv) the procedure is quick (taking 2 h to perform), easily performed and reliable. Taken as a whole, these facts indicate that the EIE-Recombinant-Chagas-Biomanguinhos is



Distribution of optical densities values from chagasic and nonchagasic individuals using the EIE-Recombinant-Chagas-Biomanguinhos kit. The horizontal line inside the drops for each plate represents the cutoff values. Chagasic individuals (□); nonchagasic individuals (■); individuals with visceral and cutaneous leishmaniasis (◆); syphilis, HTLV, HCV and HBV infections (●)

suitable for the diagnosis of Chagas disease and could be used in blood bank screening. We are currently evaluating this kit for screening donated blood of the Fundação Hemope.

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## Highly Effective Serodiagnosis for Chagas' Disease<sup>▽</sup>

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Many proteins of *Trypanosoma cruzi*, the causative agent of Chagas' disease, contain characteristic arrays of highly repetitive immunogenic amino acid motifs. Diagnostic tests using these motifs in monomeric or dimeric form have proven to provide markedly improved specificity compared to conventional tests based on crude parasite extracts. However, in many cases the available tests still suffer from limited sensitivity. In this study we produced stable synthetic genes with maximal codon variability for the four diagnostic antigens, B13, CRA, TcD, and TcE, each containing between three and nine identical amino acid repeats. These genes were combined by linker sequences encoding short proline-rich peptides, giving rise to a 24-kDa fusion protein which was used as a novel diagnostic antigen in an enzyme-linked immunosorbent assay setup. Validation of the assay with a large number of well-characterized patient sera from Bolivia and Brazil revealed excellent diagnostic performance. The high sensitivity of the new test may allow future studies to use blood collected by finger prick and dried on filter paper, thus dramatically reducing the costs and effort for the detection of *T. cruzi* infection.

Chagas' disease is called a silent disease because most people do not realize they are infected with the parasite *Trypanosoma cruzi* until late in life, if ever. However, 20 to 30% of those infected will suffer from irreversible damage to the heart, esophagus, or colon, decades after acquisition of the parasite (5, 40). Heart failure is a common cause of sudden death early in life (26). In spite of intense attempts to eradicate the insect vector in past years, the disease still affects over 8 million persons in Latin America, and 75 million people are at risk of infection (40). In some areas of Bolivia, such as Tarija or in peripheral urban districts of Cochabamba, the infection rate among children was found to be as high as 28% (28), and the disease may account for 13% of all deaths in this nation (27).

This unbearable situation could be changed by diagnostic screening of the population at risk at regular intervals followed by therapy of positive cases. Unfortunately, the available drugs, nifurtimox and benznidazole, are only effective during the early stage of the infection. When used for the treatment of the later stages of the disease, parasite eradication is markedly less effective and the drugs frequently induce severe side effects (1). Treatment of adults, therefore, has to be considered with caution. However, treatment of all infected children and young adults up to 15 to 16 years of age appears to be a reasonable policy. This strategy combined with rigorous vector control could significantly reduce the infection rate of the whole population in the long term.

Different serologic assays are available for testing clinical and donor specimens for *T. cruzi* infection. The most widely

used procedures are an enzyme-linked immunosorbent assay (ELISA) and indirect hemagglutination (IHA). Most assays use crude lysates of the parasite as antigen, but more recent tests are based on recombinant proteins (3, 4, 7, 8, 14, 19, 22, 25, 29–31, 35–37). Most use a collection of short recombinant peptides as antigens. These peptides correspond to repetitive amino acid sequences that occur in high copy numbers in different parasite proteins. The sera of infected individuals frequently contain high titers of antibodies against these repetitive motifs. (9, 16, 35). Recently developed diagnostic tests contain combinations of monomers or dimers of these repeats. Even though tests based on recombinant antigens are generally highly specific, many yield only suboptimal sensitivity rates (12, 21, 32).

In this report, we describe the production of several such repetitive structures in higher oligomeric form and their performance in immunoassays. Oligomeric antigens had high reactivities with patient sera, especially when presented as a fusion of several different oligomers. A fusion of the antigens B13, CRA, TcD, and TcE, called TcBCDE, was found to be highly specific for *T. cruzi*, whereas some other known antigens led to cross-reactions with sera from leishmaniasis patients.

### MATERIALS AND METHODS

**Serum samples.** Serum samples from patients with Chagas' disease were collected in Bolivia from patients attending the health service center SEDES (Servicio Departamental de Salud) in Cochabamba, the Laboratory of Parasitology at INLASA, La Paz, as well as Percy Boland Maternal Hospital, Santa Cruz. In Brazil samples were collected at the Hospital das Clinicas, Universidade Federal de Goiás, Goiania. Finally, samples from Latin American immigrants in Spain at different health centers were delivered to the Laboratory of Parasitology at the Universidad de Barcelona for research purposes. All sera included in this study were classified as positive or negative by two or three different tests as specified below for the individual trial results. Sera from patients with visceral or cutaneous leishmaniasis were either collected from patients at the Universitat de

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TABLE 1. Synthetic tandem repeat antigens of *T. cruzi*

Antigen	Amino acid sequence	No. of repeats in genome	Similarity with <i>Leishmania</i> spp.
MAP	PRHVDPDHFRSTTQDAYRPVDPSPAYKRALPLEEEEDVG PRHVDPDHFRSTTQDAYRPVDPSPAYKRALPLEEEEDVG PRHVDPDHFRSTTQDAYRPVDPSPAYKRALPQEEEDVG	135	40% ( <i>L. infantum</i> ), 52% ( <i>L. braziliensis</i> )
JL8	AAEATKVAEAEKQR AAEATKAVEAEKQR AAEATKVAEAEKQK	140	Very weak similarity
CRA	KVAEAEKQKAAEAT KVAEAEKQKAAEAT KVAEAEKQKAAEAT	130	No similarity
B13	PFGQAAAGDKPS PFGQAAAGDKPS PFGQAAAGDKPK	103	No similarity
FRA	AFLDQKPEGVPLRELPLDDSDSFVAMEQERRQLEKDPRRNARE IAAL EESMNARAQELAREKKLADR AFLDQKPEGVPLRELPLDDSDSFVAME QERRQLEKDPRRNAKEIAALEESMNARAQELAREKKLADR AFLDQK	14	70% ( <i>L. infantum</i> ), 63% ( <i>L. braziliensis</i> )
FRA2 <sup>a</sup>	MEQERRQLEKDPRRNAREIAALEESMNARAQELAREKKLADRAF PDSPNS MEQERRQLEKDPRRNAREIAALEESMNARAQELAREKKLADRAFNSPD MEQERRQLEKDPRRNAREIAALEESMNARAQELAREKKLADRAF	14	55% (with both <i>L. infantum</i> and <i>L. braziliensis</i> )
TcD	PKPAE PKPAE PKPAE PKPAE PKPAE PKPAE PKPAE PKPAE	430	No similarity
TcE	PAKAAA PPAKAAA PPAKAAA PPAKAAA PPAKAAA PPAKAAA	70	No similarity
SAPA	PVDSSAHGTPST PVDSSAHGTPST PVDSSAHGTPST PVDSSAHGTPST PADSSAHSTPST	>130	No similarity
TcMyo	LAQREADNEKLAED LAQREADNEKLAEE LAQREADNEKLTED LAQREADNEKLAED	>170	No similarity

<sup>a</sup> Underlined residues indicate introduced proline-rich spacer sequences.

Barcelona, Barcelona, Spain, or were donated by Abdullatif Ali, University of Dhamar, Dhamar, Yemen. Leishmaniasis had been confirmed by identification of the parasites in smears of skin lesions by microscopy and/or PCR analysis (C. Riera, unpublished results). Uninfected human serum samples were obtained from blood donors at the Academic Hospital at the University of Giessen, Giessen, Germany. Sera from Mongolian patients with syphilis and brucellosis were donated by Zandraa Jamba, Health Science University of Mongolia, Ulaanbaatar, Mongolia.

**Synthetic gene construction.** Oligonucleotides designed by using DNA-Works 3.1 software (17) were assembled to complete DNA fragments by a PCR method essentially as described previously (39). By this way, genes encoding up to nine identical amino acid repeats were constructed for the antigens MAP (20), JL8 (24), CRA (22), B13 (14), TcD (3), TcE (19), SAPA (34), and TcMyo. The two versions of the FRA antigen were bought as synthetic DNA from a commercial supplier (ATG:biosynthetics GmbH, Merchausen, Germany). For easy purification of the resulting translation products by metal affinity chromatography, the open reading frames, on average 400 to 500 bp in length, were fused with a hexahistidine affinity tag contained in the expression vector pQE-30 (Qiagen, Hilden, Germany) via a BamHI site at the 5' end and a HindIII site at the 3' end. The primary structures of the antigens resulting from the different synthetic genes are listed in Table 1.

In a second step, the recombinant genes were fused as follows. The 5' end of CRA was ligated to a BamHI site near the 3' end of B13. The TcD and TcE antigens were fused with HindIII-BamHI adapter oligonucleotides (5'-AGCTG CCGAGCCTGAGCA and 5'-GATCTGCTCAGGCTCGGC) that encode a peptide with two proline residues as a hinge region to facilitate folding of the individual antigens in an optimal conformation. Finally, the two resulting dimeric sequences were connected to a single DNA construct by using another HindIII-BamHI adapter (5'-AGCTCTCTCCGCTGCCGA and 5'-GATCTCGGCAGC GGAGAG). The amino acid sequence of the biggest fusion, B13-CRA-TcD-TcE (TcBCDE), including the vector-derived histidine tag, is MRGSHHHHHH GS PSPFGQAAAGDK PSPFGQAAAGDK PSPFGQAAAGDKP GS KVAEAEK QKAAEATKVAEAEKQKAAEAT KVAEAEKQKAAEAT KLP<sup>SL</sup>SRSPKPAE PKPAE PKPAE PKPAE PKPAE PKPAE PKPAE PKPAE PKPAE PKPAE PKPAE PKPAE PAKAAAP PAKAAAP PAKAAAP PAKAAAP PAKAAAP PAKAAAP PAKAAAP PAKAAAP PAKAAAP PAKAAAP KL. The individual elements of the repeats as well as the residues deriving from restriction sites and adapter sequences (underlined) used for the fusion of the fragments are separated by short gaps.

**Expression and purification of recombinant antigens.** *Escherichia coli* XL1-Blue/pREP cells transformed with the respective plasmid constructs were induced for protein expression by using isopropyl-β-D-thiogalactopyranoside (Gerbu, Heidelberg, Germany) and harvested by centrifugation, and the proteins were purified under denaturing conditions using TALON metal affinity resin

(BD Biosciences, Palo Alto, CA) as recommended by the supplier. Protein concentrations were determined according to the methods of Bradford (2).

**Immunoblot assays.** To determine sensitivity and specificity, the recombinant antigens were serially diluted in 10 mM Tris-HCl (pH 7.5)–150 mM NaCl (Tris-buffered saline [TBS]), 10% glycerol and applied to nitrocellulose sheets as a line (10 µl/cm). Nonspecific binding sites were blocked by a solution of 1% Tween 20 in TBS. The sheets were then cut perpendicularly to the antigen lines in 0.4-mm strips and incubated with human serum diluted 1:200 in TBS and 1% bovine serum albumin for 1 h at room temperature on a shaker. The strips were washed three times for 10 min each with TBS, 0.1% Tween 20, incubated for 1 h with anti-human IgG conjugated to alkaline phosphatase (Dianova, Hamburg, Germany), and stained with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium as described previously (23). Antigen concentrations that led to a clear positive signal with sera from patients with Chagas' disease but not with negative-control sera or sera from patients with syphilis or leishmaniasis were determined as optimal and used in further experiments. Optimal concentrations varied between 100 µg/ml and 10 ng/ml depending on the antigen.

**TcBCDE ELISA.** Microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were coated with the TcBCDE antigen at a concentration of 10 ng/ml. To prevent nonspecific adsorption of this tiny amount of protein to the walls of plastic tubes or pipette tips, the dilution buffer phosphate-buffered saline (PBS) contained 2 µg/ml of bovine serum albumin. The plates were processed essentially as described previously (18) using 1% fat-free milk powder (Roth, Karlsruhe, Germany) in PBS as blocking solution. Upon drying overnight at 50°C, the plates were sealed with an adhesive plastic foil and stored in dark plastic bags at ambient temperature. Prior to the conduct of the assay, the serum samples were diluted 1:100 in blocking solution, and specific antibodies were detected with a goat anti-human IgG horseradish peroxidase conjugate (Dianova, Hamburg, Germany) in combination with 3,3',5,5'-tetramethylbenzidine as staining substrate (10). For evaluation of the results, the cutoff value was determined as the mean of all negative samples plus 3 standard deviations.

**Other diagnostic tests.** The commercial Chagas' disease-specific tests Chagas Stat-Pak (Chembio Diagnostic Systems, Medford, NY), Bioelisa Chagas (Biokit, Barcelona, Spain), and Wiener Chagatest-ELISA Recombinante version 3.0 (Wiener Laboratorios, Santa Fé, Argentina), which are all based on recombinant antigens, and the Bios Chile ELISA para Chagas III (Bios Chile, Santiago, Chile), which is based on whole extracts of *Trypanosoma cruzi* strains Tulahuen and Mn as antigens, were used according to the manufacturers' instructions.

A *Leishmania major*-specific ELISA was prepared from a crude extract of cultivated promastigotes essentially as described by Zeinali et al. (42).

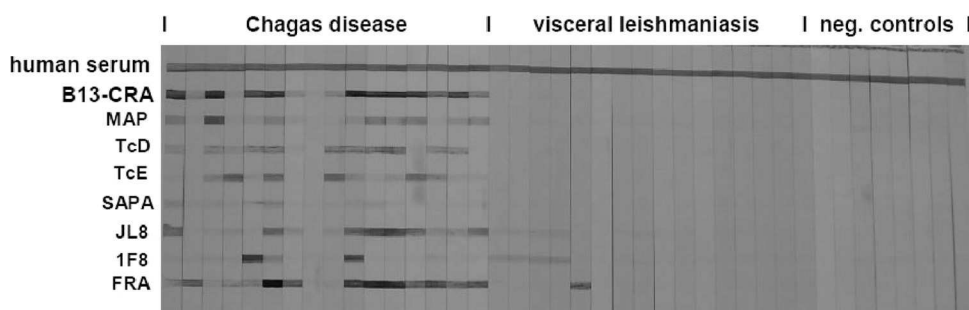


FIG. 1. Cross-reaction tests for the different recombinant antigens. The first line is a colorimetric reaction positive control (human serum). All antigens were tested with sera obtained from patients with Chagas' disease (lane 8 corresponds to a probably damaged hemolytic serum sample), visceral leishmaniasis, or with sera from healthy German persons as negative controls. Strong cross-reactions of the FRA and 1F8 antigens and weaker cross-reactions of JL8 antigen with visceral leishmaniasis patient sera are apparent.

**Nucleotide sequence accession number.** The nucleotide sequence for TcBCDE downstream of the histidine tag has been deposited in GenBank (accession number HM565960).

## RESULTS

**Production of oligomeric *T. cruzi* antigens.** A systematic *in silico* analysis of the *T. cruzi* genome revealed that many proteins contain highly conserved repetitive amino acid sequences in arrays of up to several hundred copies. The same repeats occur frequently in two or more related genes, possibly representing different alleles of the same gene in the heterozygous chromosomes of the diploid genome of *T. cruzi* (6, 13). As antibodies recognize these repeats most probably as defined tertiary structures, we sought to incorporate these structures in recombinant antigens by producing oligomeric forms of the amino acid repeats.

In order to predict the specificity of diagnostic antigens for *T. cruzi*, we searched in GeneDB for similar sequences in the genomes of *Leishmania braziliensis*, *Leishmania infantum*, and *L. major* by using the BLAST program. No sequences with significant similarity were detected for the antigens B13, CRA, TcD, TcE, and SAPA, whereas for other known tandem repeat antigens related sequences became clearly apparent. The antigens MAP and F8 occur with up to 50% identity, and the antigen FRA occurs with even 80% identity in different *Leishmania* species.

Oligomers of the coding sequences could be maintained stably only in *E. coli* when the repetitive character of the DNA was modified synthetically by incorporating different nucleotides in variable positions of codons. In this way, we succeeded in constructing 10 different oligomeric antigens, which are listed in Table 1. Details on the gene synthesis are described in Materials and Methods.

**Immunoblot evaluation of recombinant antigens.** Purified antigens were tested in immunoblot assays as described in Materials and Methods. The most reactive antigen was the dimeric FRA repeat, leading at a concentration of 1  $\mu$ g/ml to strong positive results with more than 95% of all infection sera. In contrast, TcMyo, a tetramer of a sequence occurring more than 170 times in the putative myosin gene of *T. cruzi*, had to be applied at a concentration of 200  $\mu$ g/ml to lead to a significant reaction with only a few of the patient sera (data not shown). Immunogenicity is not necessarily correlated with the

copy number of an antigen, but rather with the genetic disposition of the host. TcMyo may be highly immunogenic in one or more of the many other animal species infected with *T. cruzi*. In further immunoblotting experiments, the specificity of the antigens was determined using sera from leishmaniasis patients (Fig. 1). These tests revealed that not all of the antigens described in the literature are exclusively specific for *T. cruzi*, at least not if presented in oligomeric form. Some sera led to strong immunostaining of the synthetic FRA dimer and other sera interacted with 1F8 and JL8. A weak cross-reactivity was also observed with sera from cutaneous leishmaniasis patients (data not shown). As the leishmaniasis sera used in our study were from the Republic of Yemen, where Chagas' disease does not occur, coinfections can be excluded as a possible explanation of these results, pointing to the considerable similarity of the relevant antigens of *Leishmania* and *T. cruzi*.

Recombinant FRA antigen is used in several commercial diagnostic test kits for Chagas' disease, probably because of its high reactivity. As it was not clear to us whether the FRA antigen in such kits corresponds to the complete length of the repeat, or to subfragments only, we produced a trimer of the least conserved part of the repeat, named FRA2 (Table 1). The resulting antigen revealed strong reactivity with chagasic sera, but it was still cross-reactive with leishmaniasis sera, albeit to a lesser degree. For this reason we decided not to include recombinant FRA antigen in further tests.

**Establishment of a specific and highly sensitive ELISA.** In contrast to the signal strength observed in immunoblot assays, reactivity in the ELISA appeared to be too weak for some of the antigens. Concentrations of 10  $\mu$ g of protein/ml were needed for coating the microtiter plates with B13, TcD, and TcE in order to obtain clearly visible signals under standard test conditions. We supposed that the short polypeptides may not bind sufficiently strongly to the surface of the plastic plates, and therefore we constructed chimeric antigens of a bigger size. Only antigens which had proven not to cross-react with leishmaniasis sera were combined. Fusion of B13 with CRA on the one hand and TcD with TcE on the other hand led indeed to an increase of reactivity. Clear signals were obtained with protein concentrations of only 1  $\mu$ g/ml each in the coating buffer. This finding might be explained by better adsorption of the antigens to the microtiter plate, by adoption of an improved conformation in the absence of the histidine tag adja-



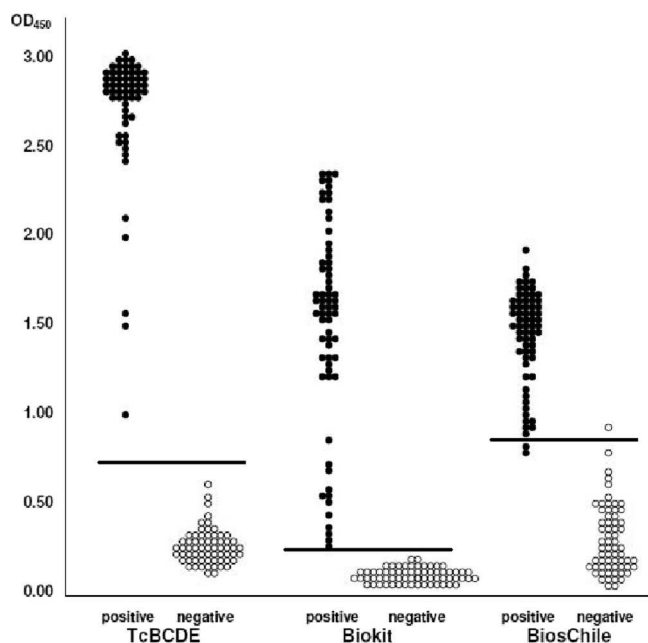


FIG. 2. Comparison of the TcBCDE ELISA with the Biokit Chagas ELISA and the Bios Chile Chagas ELISA. The same sets of 65 sera from *T. cruzi*-positive patients and of 65 negative-control sera from SEDES, Cochabamba, were tested under similar conditions (5  $\mu$ l of serum each for the reaction with antigen, and peroxidase-conjugated anti-human IgG as secondary antibody). Calculated cutoff values are indicated by horizontal lines.

cent to the second fusion partner, or by a combination of both effects.

Due to the unexpected increase of the reactivities of the B13-CRA and TcD-TcE fusion polypeptides, and in order to simplify antigen purification, we combined in the next step the two primary fusion proteins to a single polypeptide consisting of four antigenic repeat motifs. The final construct, designated TcBCDE, consisted of three repeats of the B13 antigen, three repeats of the CRA antigen, nine repeats of the TcD antigen, and six repeats of the TcE antigen. The fusion antigen showed excellent immunoreactivity with only 1 ng of antigen per well of a microtiter plate, i.e., a dilution of  $1:10^5$  of a 1-mg/ml stock solution was sufficient for a strong signal with Chagas' disease-positive serum.

The diagnostic efficiency of TcBCDE was tested first with sera stored at SEDES, Cochabamba, Bolivia, which had been tested by IHA (Polychaco, Buenos Aires, Argentina) and an immunofluorescent antibody test. Only sera positive in both tests were included as positive samples. Sera which had been clearly negative in these tests were used as controls. The reactivity of the TcBCDE ELISA was compared with two commercial diagnostic tests, Bioelisa Chagas (recombinant antigen) and Bios-Chile Chagas ELISA (*T. cruzi* crude extract antigen). The same set of 65 *T. cruzi*-positive and 65 negative-control sera was analyzed using 5  $\mu$ l of each serum sample. The results shown in Fig. 2 clearly indicate a higher reactivity of the TcBCDE antigen than to the antigens used in the two commercial test kits. Under the conditions used in this experiment, staining in the TcBCDE ELISA was very fast and had to be

stopped after 5 min. To facilitate handling, 1 to 2  $\mu$ l instead of 5  $\mu$ l of patient serum was used in further tests.

The specificity of the three different Chagas' disease-specific tests was evaluated with 35 serum samples from leishmaniasis patients from the Republic of Yemen and with 50 sera from Mongolian patients with syphilis. As neither Mongolia nor the Republic of Yemen are countries where Chagas' disease is endemic, cross-reactivity due to coinfections could be excluded. None of the leishmaniasis sera reacted in the TcBCDE ELISA or Bioelisa Chagas test, but five of them were positive in the Bios-Chile Chagas ELISA. The syphilis sera were negative in all three tests.

The issue of cross-reactivity was analyzed in more detail with 25 patient sera classified as inconclusive in Cochabamba, because they revealed either weakly positive results in the Bios-Chile Chagas ELISA or in alternative assays based on crude parasite extracts, such as IHA. We applied four different tests: the TcBCDE ELISA and Bioelisa Chagas employing recombinant antigens on the one hand, and Bios-Chile Chagas ELISA and an ELISA employing a self-prepared crude extract from *L. major* as antigen (42) on the other hand. While both of the assays that use recombinant antigen led to clearly negative results with all of these sera, 10 sera were positive in the Bios-Chile Chagas ELISA and 16 in the *L. major* ELISA (data not shown). As the reactivity with the two crude extract ELISAs appeared to be very similar, it can be assumed that many of the weakly positive results among the inconclusive samples may be caused by acute or treated *Leishmania* infections. Different forms of leishmaniasis occur in the area surrounding Cochabamba, even though there are no clear data on infection rates. *Leishmania*-specific tests are not performed in this area.

**Validation of the new ELISA.** In order to validate the sensitivity and specificity of TcBCDE as a diagnostic antigen, ELISAs were performed with additional sera collected from chagasic patients at INLASA, La Paz, and at the Percy Boland Maternal Hospital, Santa Cruz, Bolivia, in parallel with other diagnostic kits based on recombinant antigens, including the Wiener Chagatest Recombinante, Bioelisa Chagas, and Chagas Stat-Pak tests. Altogether, more than 200 serum samples were analyzed. The assays were performed using 10  $\mu$ l of serum (Wiener Chagatest and Bioelisa Chagas) or with 5  $\mu$ l of serum (Chagas Stat-Pak), as recommended by the suppliers. For the TcBCDE ELISA, only 1  $\mu$ l of serum was used. The results are summarized in the upper part of Table 2. With both sets of patient sera, one more sample each was classified as (weakly) positive by the TcBCDE ELISA. Assuming that the results with the other tests were correct, the TcBCDE ELISA must be considered at least equally sensitive and 99% specific. Alternatively, the results could be explained by a higher sensitivity of the TcBCDE ELISA.

In order to investigate the diagnostic potential of the TcBCDE ELISA for *T. cruzi* strains outside Bolivia, tests with several hundred sera collected from patients in Goiania, Brazil, were performed. These sera had been characterized thoroughly based on a *T. cruzi* crude extract ELISA (EIE Biomanguinhos; Fiocruz, Rio de Janeiro, Brazil), indirect hemagglutination (Wiener, Rosario, Argentina), and indirect immunofluorescence (bioMérieux conjugate with an in-house Y strain of *T. cruzi*) (A. O. Luquetti, unpublished

TABLE 2. Reactivity of the TcBCDE ELISA in comparison with different *T. cruzi*-specific immunoassays

Test	No. of samples with indicated reaction (total no. of samples from site)		
	La Paz, Bolivia (n = 130)	Santa Cruz, Bolivia (n = 85)	Goiania, Brazil (n = 381)
TcBCDE-ELISA			
Positive	77	65	164
Negative	53	20	217
Wiener Chagatest			
Positive	76	64	
Negative	54	21	
Bioelisa Chagas			
Positive		64	
Negative		21	
Chagas Stat-Pak			
Positive		64	
Negative		21	
Biomanguinhos crude extract ELISA			
Positive			165
Negative			216
Immunofluorescence			
Positive			165
Negative			216
Wiener hemagglutination			
Positive			165
Negative			216

results). A total of 165 samples positive in all three of these conventional serological assays and 216 samples negative in all three tests were analyzed with the TcBCDE ELISA. One of the samples positive in the conventional test was negative in the TcBCDE ELISA, corresponding to 99.3% sensitivity and 100% specificity (lower part of Table 2).

## DISCUSSION

### High immunoreactivity of the artificial TcBCDE antigen.

The TcBCDE antigen was found to be recognized by *T. cruzi*-specific human antibodies with remarkable affinity. Under the assumption that 0.1% of total IgG is specific for the antigen, the calculated interaction between 1  $\mu$ l of serum (0.05 pmol specific antibody) and 1 ng of antigen (0.05 pmol) corresponds to a molar relation of 1:1. In other recombinant ELISAs for Chagas' disease, the relation of specific IgG to recombinant antigen is instead 1:20 at the molar level. In the Bioelisa Chagas assay (7), for example, 10  $\mu$ l of serum (0.5 pmol specific antibody) is used with 75 ng of antigen (9 pmol).

Many of the commercial recombinant ELISAs for Chagas' disease are based on the fusion protein TcF (7). This antigen contains in part the same antigenic motifs as TcBCDE, but in a different context. TcF contains two repeats each of the antigens B13 (PEP2), TcD, TcE, and TcLo1.2 as direct fusions, i.e., not separated by spacer sequences as in TcBCDE. The CRA antigen is replaced by TcLo1.2, which occurs as a tandem

repeat in related forms in five different proteins of *T. cruzi*. The major differences between TcBCDE and TcF are the number of repeats of the antigens, which is considerably higher in TcBCDE than in TcF, on the one hand and the presence of proline-rich spacer regions between the individual antigens on the other hand. Obviously, the immunoreactive epitopes of (some of) the antigens are more accessible to the antibodies when presented in a larger protein.

Sera with high levels of *T. cruzi*-specific antibodies are detected equally efficiently with existing recombinant tests and the TcBCDE ELISA. However, the latter test has a greater potency to discriminate weakly positive samples from background. This can be demonstrated statistically by calculating the quotient of measured optical density values divided by the cutoff values. In the results shown in Fig. 2, only 3 of the 65 positive samples had a quotient smaller than 2.0 in the TcBCDE ELISA, whereas in the Bioelisa Chagas 9 samples were below this value. This potency facilitates diagnosis, because weakly positive results routinely need to be confirmed by alternative assays.

**Specificity of the TcBCDE antigen.** The high dilution of TcBCDE used for immunoassays minimizes contamination with bacterial antigens. This reduces greatly the cross-reactions with *E. coli*-specific antibodies, which are generally found in human serum (15, 41) and frequently cause background problems in immunoassays based on recombinant proteins. This may be an additional reason why the TcBCDE ELISA discriminates better between positive and negative results than alternative diagnostic methods available at present.

Cross-reaction of chagasic sera with other organisms is well known and occurs mainly for closely related members of the *Trypanosomatidae* family. While this can be tolerated for the analysis of donated blood—suspicious samples have to be eliminated anyway—misdiagnosis of patients may have severe consequences. Tests based on crude parasite extracts as antigens react frequently with sera from persons infected with different species of *Leishmania*, or with *Trypanosoma rangeli* (33).

Clear discrimination between Chagas' disease and leishmaniasis is a major problem, as the pathogens occur in parallel in many parts of Latin America. *Leishmania* strains found in the New World (*L. braziliensis*, *L. chagasi*, *L. mexicana*, and others) differ from the Old World strains *L. infantum* and *L. major* used in this study. However, with the exception of *L. braziliensis*, limited information is available for the genomic sequences of these New World species so far. Comparing *T. cruzi* sequences with *L. braziliensis* appears to be justified in any case, as up to 85% of all *Leishmania* infections in Bolivia are caused by *L. braziliensis* (11). Furthermore, Old World *Leishmania* and *L. braziliensis* revealed significant similarities in MAP and FRA antigens, as indicated in Table 1. Most probably, cross-reactivity can be avoided completely by selecting appropriate recombinant antigens for immunological tests. None of the antigens contained in TcBCDE (i.e., B13, CRA, TcD, and TcE) showed experimental cross-reactivity with sera from individuals infected with *Leishmania* or *T. rangeli* (37, 38).

**Diagnosis at affordable costs.** At present, diagnosis of Chagas' disease is normally performed only with donated blood, or when patients suffer from clinical symptoms. However, when symptoms such as cardiac or digestive dysfunctions become apparent, the efficacy of etiological treatment with the avail-

able drugs is rather poor. On the other hand, therapy is more promising if the infection is recognized early, i.e., long before the onset of clinical symptoms. Therefore, the only way to detect the infection in time would depend on screening of the endangered population in regular intervals, which demands diagnosis on a large scale.

The procedures described in this work exemplify how public bioinformatic data can be used to approach medical problems of developing countries in an inexpensive way. Neither the numerous genomic data nor the software programs needed for this work had to be bought, nor were they available only for a restricted number of persons. The results show the way forward to produce recombinant antigen for millions of assays at very low costs. Moreover, the small amounts of serum needed for this test may allow the use of blood collected by finger prick and dried on filter paper, thus reducing costs even more.

In the future, the test could be adapted to the specific needs in economically developing areas. Because most medical facilities in areas at risk are poorly equipped, work on a simple lateral flow device with TcBCDE as antigen is in progress.

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## Evaluation of Serological Tests To Identify *Trypanosoma cruzi* Infection in Humans and Determine Cross-Reactivity with *Trypanosoma rangeli* and *Leishmania* spp.<sup>▽</sup>

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**Five commercially available enzyme-linked immunosorbent assays (ELISAs), one in-house ELISA, and two hemagglutination assays were evaluated to determine their diagnostic accuracy for Chagas' disease in two studies. In study 1, ELISA kits showed 100% sensitivity, but specificities ranged from 82.84% to 100% when leishmaniasis cases were included and from 95.57% to 100% when leishmaniasis cases were excluded. Kits using recombinant antigens or synthetic peptides are more specific than those using crude extracts from *Trypanosoma cruzi* epimastigote forms. Kits evaluated in Panama, in study 2, showed 75% to 100% sensitivity and 97.12% to 100% specificity. These data were obtained by using a Western blot assay with *T. cruzi* trypomastigote excreted-secreted antigens as a reference test to confirm *T. cruzi* infection.**

Chagas' disease is caused by the protozoan *Trypanosoma cruzi*, which is transmitted to humans by blood-sucking triatomine bugs, by blood transfusion, through the placenta, or by ingestion of contaminated food. It is estimated that 9 to 12 million people on the Latin American continent are infected with *T. cruzi*. The prevalence of Chagas' disease among blood donors from different countries in South America differs within each country and locality (8, 22). However, in recent decades, several million persons have emigrated to developed countries, and this may lead to an increase in the prevalence of the infection in such countries (3, 8, 22). Indeed, infection with *T. cruzi* transmitted by blood transfusion or organ transplantation has recently been described in the United States (1, 5, 8, 9, 19).

Serological diagnosis of Chagas' disease is frequently based on tests such as enzyme-linked immunoassays (EIAs), indirect immunofluorescence assays, and indirect hemagglutination assays (IHAs), which usually employ *T. cruzi* epimastigote forms as the antigen. Provided that good-quality kits are selected and correct laboratory practices followed, good sensitivity can be achieved with any of the tests. Sensitivities on the order of 95 to 99% can be obtained, and these can be increased to 100% by using more than one test (8, 10, 15). The use of recombinant antigens and/or synthetic peptides has been proposed (17, 21) to improve specificity and sensitivity, which is essential if false-positive or false-negative results are to be avoided.

Several reports show that results can be inconclusive or

doubtful depending on the commercial diagnostic assay used for blood screening (5, 6, 7). The definition of inconclusive results differs with the commercial kit used, since reactions that are not clearly positive or negative are taken as inconclusive. Currently available kits are very effective at detecting blood donors presenting with high anti-*T. cruzi* antibody titers, but the results are often questionable when the kits are used for donors with low titers (7, 18). For the latter donors, it is not uncommon for a sample to be negative by one test when subjected to two or three tests (8). Some of these samples are known to be from genuine Chagas' disease patients, because they are confirmed by molecular biology methods (PCR) (7); other researchers have reported evidence that people infected with *T. cruzi* can have negative serology (16, 23).

Another factor that needs to be taken into consideration when one is using serological tests for Chagas' disease is cross-reactivity. Cross-reactivity between sera of patients infected with *T. cruzi* and sera of patients infected with *Leishmania* spp. in the serodiagnosis of Chagas' disease is well documented (2, 20). In some areas of endemicity in Central America and Brazil, where *T. cruzi* and the nonpathogenic protozoan *Trypanosoma rangeli* can be found infecting the same vectors and vertebrate hosts (12, 14), cross-reactivity has been the subject of discussion.

The aim of our study, which was divided into two separate studies (studies 1 and 2), was to compare the sensitivities and specificities of nine Chagas' disease assays for detection of anti-*T. cruzi* immunoglobulin G: six enzyme-linked immunosorbent assays (ELISAs), two IHAs, and one Western blot assay. Of these tests, the following seven are commercially available: three ELISAs manufactured with *T. cruzi* epimastigote antigens (ELISA Chagas III [BIOSChile-Ingenieria Genetica SA, Santiago, Chile], ELISACruzi [bioMérieux Brasil SA], and Chagatek ELISA [Laboratório Lemos SRL, Buenos Aires, Argentina; distributed by bioMérieux Argentina]), two

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TABLE 1. Numbers and percentages of positive cases obtained by six ELISAs for Chagas' disease diagnosis using the TESA blot as a reference test

Status of patients	No. of cases	No. (%) of positive cases by:						
		TESA blot (IMT) <sup>a</sup>	ELISA-IMT <sup>a,b</sup>	Chagas III (BIOSChile) <sup>b</sup>	ELISAcruzi (bioMérieux Brasil SA) <sup>b</sup>	Chagatek (bioMérieux Brasil SA) <sup>b</sup>	Chagatest Rec v3.0 (Wiener) <sup>c</sup>	Pathozyme Chagas (Omega) <sup>c</sup>
Study 1	187							
Chagas' disease patients	53	53 (100)	53 (100)	53 (100)	53 (100)	53 (100)	53 (100)	53 (100)
Subjects without Chagas' disease	134							
Healthy	45	0	0	0	1 (2.22)	0	0	0
VL <sup>d</sup>	13	0	12 (92.31)	12 (92.31)	11 (84.60)	11 (84.60)	0	0
CL <sup>e</sup>	8	0	6 (75.00)	1 (12.50)	7 (87.50)	6 (75.00)	0	0
<i>T. rangeli</i>	23	0	2 (8.69)	0	0	0	0	0
Other infections	45	0	0	0	4 (8.88)	0	0	0
Study 2 (Panamanians)	120	16 (13.33)	18 (15.00)	16 (13.33)	18 (15.00)	18 (15.00)	13 (10.83)	12 (10.00)
TESA blot positive	16	16 (100)	16 (100)	15 (93.75)	15 (93.75)	16 (100)	13 (81.25)	12 (75.00)
TESA blot negative	104	0	2 (1.92)	1 (0.96)	3 (2.88)	2 (1.92)	0	0

<sup>a</sup> Test prepared at the IMT.<sup>b</sup> ELISA prepared with *T. cruzi* epimastigote antigens.<sup>c</sup> ELISA prepared with *T. cruzi* recombinant proteins.<sup>d</sup> VL, visceral leishmaniasis.<sup>e</sup> CL, cutaneous leishmaniasis.

ELISAs prepared with recombinant *T. cruzi* antigens (Chagatest ELISA recombinant, version 3.0 [Chagatest Rec v3.0; Wiener Laboratories, Rosario, Argentina], and Pathozyme Chagas [Omega Diagnostics Ltd., Scotland, United Kingdom]), and two IHAs (HEMACruzi [bioMérieux Brasil] and Imuno-HAI [Wama Diagnóstica, São Paulo, Brazil]). The following two tests were prepared at the Instituto de Medicina Tropical, São Paulo, Brazil (IMT): ELISA-IMT, which was prepared with whole extracts of *T. cruzi* Y strain epimastigotes, and a Western blot assay, prepared with *T. cruzi* trypomastigote excreted-secreted antigens (TESA blot), as previously described (20). The TESA blot was used as a reference test (20, 21, 23). All commercial kits were used according to the manufacturers' instructions, and the test results were analyzed in accordance with the technical information provided for each assay. The cutoffs were calculated as described in the respective sections of each manual. For ELISA-IMT, the cutoff was calculated as

the mean optical density (OD) at 492 nm of the true-negative sera plus 3 standard deviations. The individual results were calculated as the ratio of the OD to the cutoff (see Fig. 1). A sample was considered positive if the ratio was equal to or greater than 1.0 and negative if the ratio was equal to or smaller than 0.99. Seropositivity rates for anti-*T. cruzi* antibodies in different tests, and their confidence intervals [CIs], were calculated using the mid-*P* 95% confidence interval (95% CI) using Epi Info (version 6.0) software (see Table 2). Samples that yielded discrepant results were tested at least twice on different days.

The guidelines of the Standards for Reporting of Diagnostic Accuracy were applied in this study.

In study 1, all ELISAs and the TESA blot had a sensitivity of 100% (95% CI, 94.5 to 100%) when evaluated with 53 samples collected from Brazilian blood donors with *T. cruzi* infection (18 to 60 years old; 60% male, 40% female) who had previ-

TABLE 2. Sensitivities and specificities of seven assays to detect *Trypanosoma cruzi* antibodies

Test	Study 1 ( <i>n</i> = 187)			Study 2 ( <i>n</i> = 120)	
	Sensitivity <sup>a</sup> ( <i>n</i> = 53)	Specificity <sup>a,b</sup>		Sensitivity ( <i>n</i> = 16)	Specificity ( <i>n</i> = 104)
		Including leishmaniasis cases ( <i>n</i> = 134)	Excluding leishmaniasis cases ( <i>n</i> = 113)		
TESA blot (IMT)	100 (94.5–100)	100 (97.8–100)	100 (97.4–100)	100 (82.9–100)	100 (97.2–100)
ELISA-IMT <sup>c</sup>	100 (94.5–100)	85.07 (78.3–90.4)	98.23 (94.3–99.7)	100 (82.9–100)	98.08 (93.8–99.7)
Chagas III (BIOSChile) <sup>c</sup>	100 (94.5–100)	90.30 (84.3–94.5)	100 (97.4–100)	93.75 (72.8–99.7)	99.04 (95.4–100)
ELISAcruzi (bioMérieux Brasil) <sup>c</sup>	100 (94.5–100)	82.84 (75.7–88.5)	95.57 (90.5–98.4)	93.75 (72.8–99.7)	97.12 (92.4–99.3)
Chagatek (bioMérieux Brasil) <sup>c</sup>	100 (94.5–100)	87.31 (80.9–92.2)	100 (97.4–100)	100 (82.9–100)	98.08 (93.8–99.7)
Chagatest Rec v3.0 (Wiener) <sup>d</sup>	100 (94.5–100)	100 (97.8–100)	100 (97.4–100)	81.25 (57.0–95.0)	100 (97.2–100)
Pathozyme Chagas (Omega) <sup>d</sup>	100 (94.5–100)	100 (97.8–100)	100 (97.4–100)	75 (50.1–91.5)	100 (97.2–100)

<sup>a</sup> Both sensitivity and specificity are given as percentages, with 95% CIs in parentheses. Confidence intervals were calculated using the mid-*P* 95% confidence interval (95% CI) with Epi Info 6.0 software.<sup>b</sup> Determined with sera from non-Chagas' disease patients.<sup>c</sup> ELISA prepared with *T. cruzi* epimastigote antigens.<sup>d</sup> ELISA prepared with *T. cruzi* recombinant proteins.

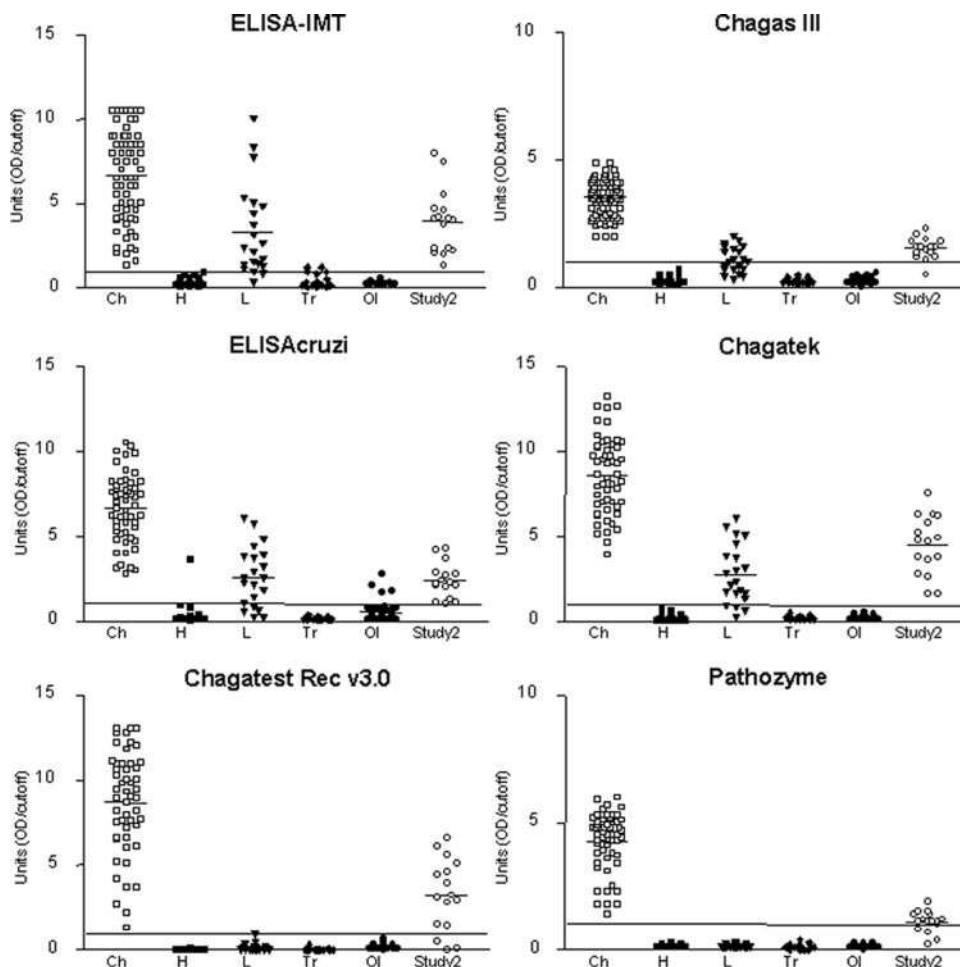


FIG. 1. Distribution of individual results of five commercially available ELISAs (Chagas III, ELISAcruzi, Chagatek, Chagatest Rec v3.0, and Pathozyme) and one in-house ELISA (ELISA-IMT) for diagnosis of Chagas' disease. The first five groups of data in each graph are results, from study 1, for 53 Brazilian individuals with *T. cruzi* infections (Ch), 45 healthy individuals (H), 21 patients infected with *Leishmania* spp. (L), 23 individuals infected with *Trypanosoma rangeli* (Tr), and 45 patients with other infections (OI). The last group of data in each graph (Study 2) corresponds to 16 TESA-blot-positive Panamanian cases. The horizontal line within each data group is the arithmetic mean. Each data point was calculated as the ratio of the OD to the cutoff. The horizontal line at 1.0 unit represents the cutoff.

ously been found positive by at least two commercial tests. This sensitivity was obtained for ELISAs manufactured with crude epimastigote antigens as well as with recombinant *T. cruzi* proteins (Tables 1 and 2; Fig. 1, Ch). Because the bioMérieux Brasil and Wama IHAs had low sensitivities (90.56%<sup>48/53</sup> and 86.79%<sup>46/53</sup>, respectively), they were excluded from subsequent analysis.

Specificity was evaluated with 134 samples collected from subjects without Chagas' disease, including (i) 45 healthy people (20 Brazilian blood donors and 25 Panamanians living in an area of endemicity), (ii) 21 patients infected with *Leishmania* spp. (13 with active visceral leishmaniasis, from an area in Brazil where Chagas' disease was not endemic, and 8 with mucocutaneous leishmaniasis, from Venezuela), (iii) 23 *T. rangeli*-infected Panamanians, and (iv) 45 patients with unrelated diseases, as defined by their respective clinical and laboratory diagnoses (5 with connective tissue diseases who were also positive for antinuclear antibodies, 5 with systemic lupus erythematosus, 5 positive for anti-streptolysin O, 5 with rheu-

matic fever, 5 with malaria, 5 with *Toxoplasma gondii*, 5 with *Toxocara canis*, 5 with *Schistosoma mansoni*, and 5 with *Taenia solium*) (Table 1). Patients infected with *T. rangeli* or *Leishmania* spp. were selected using epidemiological, clinical, and/or parasitological data (data not shown).

In study 1, the specificities (95% CIs) of EIAs prepared with *T. cruzi* epimastigote antigens, including results for *Leishmania* sp. cases, were as follows: for ELISA-IMT, 85.07% (78.3 to 90.4%); for Chagas III, 90.30% (84.3 to 94.5%); for ELISAcruzi, 82.84% (75.7 to 88.5%); and for Chagatek, 87.31% (80.9 to 92.2%) (Table 2; Fig. 1). A specificity of 100% (97.8 to 100%) was achieved with EIAs prepared with recombinant *T. cruzi* antigens (Chagatest Rec v3.0 and Pathozyme) (Table 2). When leishmaniasis cases ( $n = 21$ ) were excluded, the specificities of the kits (95.57 to 100%) indicated improved diagnostic performance (Table 2). No cross-reactivity with the 23 *T. rangeli* cases was observed in the ELISAs or the TESA blot, except for the ELISA-IMT, which cross-reacted with two cases (8.69%). These two patients had low titers (Table 1; Fig. 1, Tr).

No cross-reactivity was observed with samples from *T. rangeli* or *Leishmania* sp. cases or from cases with other infections by using the TESA blot (Table 1). However, 2.24% (3/134) of the samples (1 from a healthy individual, 1 from an individual infected with *Leishmania* spp., and 1 from an individual infected with *T. rangeli*) reacted, resulting in a faint band of 170 kDa in the TESA blot; this band can be confused with the 150- to 160-kDa band that is evident with sera of *T. cruzi*-infected individuals (data not shown).

Individuals infected with *T. rangeli* appear not to produce *T. cruzi*- or *T. rangeli*-reactive antibodies; they failed to show reactivity in an ELISA prepared with *T. rangeli* extracts (data not shown). These results, which agree with the findings of other studies, show that human infection with *T. rangeli* does not cause the production of enough cross-reacting antibodies to interfere with the serodiagnosis of Chagas' disease (13, 14). However, this does not exclude the possibility that exposure to *T. rangeli* may elicit a particular humoral and/or cellular immune response that confers some degree of protection against subsequent infection with *T. cruzi* (11).

The next step, in study 2, was to validate these assays by a retrospective study with 120 samples from individuals, aged 18 to 60 years, living in an area of endemicity in Mendoza, Panama. According to an earlier study, 2.91% and 6.80% of the children in this area are infected with *T. cruzi* and *T. rangeli*, respectively (13). In study 2, Chagas III gave positive results for 13.33% ( $n = 16$ ) of the samples. ELISA-IMT, ELISAcruzi, and Chagatek gave a positivity rate of 15% ( $n = 18$ ). Positivity rates for Chagatest Rec v3.0 and Pathozyme were 10.83% ( $n = 13$ ) and 10% ( $n = 12$ ), respectively (Table 1). The sensitivity, calculated using the TESA blot as a reference test, was 100% (82.9 to 100.0%) for the ELISA-IMT and Chagatek tests. The same result was not observed for the other tests; Chagas III and ELISAcruzi had a sensitivity of 93.75% (72.8 to 99.7%), Chagatest Rec v3.0 had 81.25% (57.0 to 95.0%), and Pathozyme had 75% (50.1 to 91.5%) (Table 2; Fig. 1, Study 2). In this study, the specificities (95% CIs) of tests were also variable, since cross-reactivity was observed for ELISA-IMT and Chagatek in two cases (98.08% [93.8 to 99.7%]), for Chagas III in one case (99.04% [95.4 to 100%]), and for ELISAcruzi in three cases (97.12% [92.4 to 99.3%]) (Tables 1 and 2).

In summary, our data, and those from other laboratories, indicate that commercial kits that use recombinant antigens or synthetic peptides are a more specific alternative to those that use complex crude extracts, although they sometimes have variable sensitivities (18, 21). Confirmatory tests with higher specificities have already been proposed as reference standards (3, 4, 20, 21, 23), but unfortunately none of these are commercially available.

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# Evaluation of the Abbott ARCHITECT Chagas prototype assay

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## Abstract

Serologic tests are established tools for the diagnosis of Chagas disease applied to support a safe blood supply in endemic countries. However, sensitivity and specificity of most commercially available enzyme-linked immunosorbent assays (ELISAs) are not regarded as adequate enough to rely on a single assay to determine the *Trypanosoma cruzi* infection status of a blood donor or a patient. The overall assay performance is driven by the general choice of antigens and the actual antigen cocktail provided in the test. In this report we describe key performance data of the Abbott ARCHITECT Chagas prototype assay in comparison to the well-recognized bioMérieux ELISA cruzi assay. The ARCHITECT assay demonstrated superior specificity (99.99% versus 99.93%) and sensitivity (99.85% versus 98.38%), along with excellent precision, thus showing the potential to serve as single assay to determine the *T. cruzi* status of a given blood unit or diagnostic specimen on a fully automated instrument platform.

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**Keywords:** Chagas disease; Recombinant antigens; Chagas IgG

## 1. Introduction

Chagas disease (American trypanosomiasis) is endemic in 21 Latin American countries (Shah et al., 2004). Approximately 18 million people are chronically infected with 50,000 new cases and 15,000 deaths still being reported annually (Anonymous, 2009). Vectorial transmission of the protozoan parasite *Trypanosoma cruzi* through triatomine insects is the best understood and primary mode of infection in endemic areas, but has been reduced by government and World Health Organization efforts to eliminate domiciliary vectors. Thus, transmission by blood transfusion, as the second most common route of infection in endemic as well as in non-endemic countries, has gained increasing attention (Leiby et al., 2000; Shah et al., 2004).

Most infected people, after a mild acute phase with nonspecific symptoms, enter the lifelong indeterminate phase that is characterized by a lack of symptoms, low parasitemia levels, and antibodies to a variety of *T. cruzi* antigens. Approximately 10% to 30% of persons with chronic *T. cruzi* infections, however, develop cardiac

(cardiomyopathy) or gastrointestinal (megacolon, megaesophagus) dysfunction as a consequence of the persistent presence of the parasite (Malan et al., 2006). As Chagas disease is untreatable in the chronic phase, chronically infected people serve as lifelong reservoir for transfusion transmission (Leiby et al., 2000).

Serologic tests for detecting antibodies to *T. cruzi* have been developed and are better suited for rapid, objective, and inexpensive diagnosis of the infection in contrast to parasitologic tests such as xenodiagnosis and hemoculture, which are highly specific but time-consuming, hard to interpret, and laborious while lacking sensitivity (Cheng et al., 2007). Serologic tests have already been used in the endemic countries for decades. Among those most widely used conventional assays are the indirect hemagglutination assay, the indirect immunofluorescence assay (IFA), and the enzyme-linked immunosorbent assay (ELISA) (Chang et al., 2006). Initially, no single test has been found to be sufficiently sensitive and specific to be designated as the sole screening assay, and the Pan American Health Organization as well as the World Health Organization (WHO) has requested laboratories to use at least 2 different methods for testing (Carvalho et al., 1993; Wendel and Dias, 1992; Médecins Sans Frontières, 2008). Based on their performance, it can be concluded that some of the current

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commercially available ELISAs appear to be suitable to justify their use for single-assay screening of blood donations with the bioMérieux ELISA cruzi assay showing a performance almost in the range of those 4 ELISAs being regarded as adequate (Otani et al., 2009). With either approach, it is deemed that antibody-positive units/donors, and thus *T. cruzi* parasite-positive patients, are optimally detected and removed from the donor pool versus deferring too many blood donors/units based on the insufficient specificity of certain tests, applied alone.

Using a cocktail of recombinant antigens for detection of IgG antibodies to *T. cruzi*, Abbott is currently developing the ARCHITECT Chagas assay on the fully automated, random access ARCHITECT system family. In this article we describe the key assay performance characteristics of the initial prototype assay, while further development of the assay is still in progress.

## 2. Materials and methods

### 2.1. ARCHITECT Chagas assay

The ARCHITECT Chagas assay is a qualitative fully automated 2-step chemiluminescent microparticle immunoassay (CMIA) used to determine the presence of antibodies to *T. cruzi*, the causative agent of Chagas disease, in human serum and plasma.

In the first step, sample, assay diluent, and paramagnetic microparticles co-coated with 4 chimeric *T. cruzi* recombinant antigens (FP3, FP6, FP10, and TcF) are combined (Chang et al., 2006; Cheng et al., 2007; Hoft et al., 1989; Houghton et al., 1999; Médecins Sans Frontières, 2008). The recombinant antigens used are detailed in Table 1.

After washing, murine acridinium-labeled anti-human IgG conjugate is added. After another wash cycle, pre-trigger and trigger solutions are added to the reaction mixture.

The resulting chemiluminescent reaction is measured as relative light units (RLUs). A direct relationship exists between the amount of antibodies to *T. cruzi* in the sample and the RLUs detected by the ARCHITECT *i* optical system. Results are calculated automatically based on comparison to an Index Calibrator. The cutoff value was determined by optimization of sensitivity and specificity performance.

Assay results are presented as a ratio of the specimen signal (in RLUs) to the cutoff value (S/CO), where S/CO values of  $\geq 1.00$  S/CO are considered reactive for antibodies to *T. cruzi*, S/CO values from  $\geq 0.8$  to 0.99 are considered gray zone, and S/CO values  $< 0.8$  are considered nonreactive.

### 2.2. Specimens for specificity, sensitivity, and precision assessment

Samples were tested at Abbott GmbH & Co. KG (Wiesbaden-Delkenheim, Germany) as the internal site, and at Programa Nacional de Medicina Transfusional y Banco de Sangre (Guatemala, Guatemala) and at Fundação

Table 1

Recombinant antigens used in the Abbott ARCHITECT Chagas assay (based on Cheng et al., 2007)

Recombinant antigen and antigenic domain	Description <sup>a</sup>
TcF	
PEP-2 <sup>b</sup>	<b>GDKPSPFQA AA GDKPSPFGQA</b>
TcD <sup>b</sup>	<b>AEPKS AEPKP AEPKS</b>
TcE <sup>b</sup>	<b>KAAIAPA KAAIAPA KAATAPA</b>
TcLo1.2 <sup>b</sup>	<b>SSMP S GTSEEGSRGSSMPA</b>
FP3	
TcR27 <sup>c</sup>	Cytoplasmic protein
FCaBP <sup>d</sup>	Flagellar calcium-binding protein
FP6	
TcR39 <sup>c</sup>	Cytoskeleton-membrane protein
FRA <sup>b</sup>	Flagellar repetitive protein
FP10	
SAPA <sup>c</sup>	Shed acute-phase antigen
MAP <sup>c</sup>	Microtubule-associated protein

<sup>a</sup> Bold indicates repetitive sequences reported by others. Italics indicate protein segments fused into the recombinant antigen.

<sup>b</sup> Composed entirely of repeats.

<sup>c</sup> N-terminal and repeat segments included.

<sup>d</sup> Full-length, nonrepetitive protein.

<sup>e</sup> N-terminal, repeat, and C-terminal segments included.

Centro de Hematologia e Hemoterapia de Minas Gerais—Hemominas (Belo Horizonte, Brazil) as the external sites. A total of 10 284 specimens were evaluated to determine the specificity and sensitivity of the ARCHITECT Chagas assay.

The specificity population consisted of random blood donors: 1963 from German blood banks in Munich and Plauen (internal studies), 1846 from Guatemala, and 5420 from Brazil (both external studies). In addition, diagnostic specimens [400 from Horst-Schmidt-Kliniken (HSK) Wiesbaden, Germany—internal studies] were used for the specificity assessment (9629 specimens in total).

The following pre-characterized *T. cruzi* antibody-positive specimens were used for the sensitivity assessment: 147 specimens from Boca Biolistics, Coconut Creek, FL (origin Nicaragua—pre-characterized as *T. cruzi* positive using the Ortho *T. Cruzi* ELISA); 100 specimens (tested on the bioMérieux assay)/97 specimens (tested on the ARCHITECT assay) from Accacia Serum Products, Austin, TX (origin Caucasian—pre-characterized as *T. cruzi* positive using the Wiener Lab Chagas test ELISA recombinant 3.0); a collection of 101 specimens from different origins and vendors tested on the ARCHITECT assay only [20 specimens from Bolivia (vendor: Biocollections Worldwide, Miami, FL), 41 specimens from Argentina (vendor: Accacia Serum Products, Austin, TX), 14 specimens from Columbia (vendor: HemaCare, Ft. Lauderdale, FL), 8 specimens from the United States (vendor: Blood Systems, Tempe, AZ) as well as 18 specimens from Brazil (received per an agreement with Eduardo M Netto, MD, MPH, PhD, Hospital Univ.; Prof. Edgard Santos, Universidade Federal da Bahia, Salvador, Bahia, Brazil)] all tested internally and all pre-characterized as *T. cruzi* positive using the Chagas RIPA assay. In addition, 89 *T. cruzi* antibody-positive specimens

from Guatemala and 221 from Brazil were tested externally at the sites mentioned above. Six hundred fifty-five specimens were tested in total for the ARCHITECT Chagas sensitivity assessment, whereas because of sample volume restrictions, 557 were assessed on the bioMérieux ELISA cruzi assay.

The *T. cruzi* antibody-positive specimens represent a broad geographic range in Latin America, thus also covering the principal strain groups *T. cruzi* I and II. *T. cruzi* I is predominant from the Amazon Basin north into Central America and Mexico, whereas *T. cruzi* II is predominant in the southern cone of South America.

Forty specimens known to be *T. cruzi* antibody-positive (19 specimens randomly taken from the Boca Biolistics samples above and 21 specimens randomly taken from additional HemaCare specimens) were used to determine the dilutional sensitivity of the ARCHITECT Chagas assay versus the bioMérieux ELISA cruzi assay. Five different dilutions were tested: neat, 1:10, 1:100, 1:250, and 1:500.

For precision testing 4 different specimens, the ARCHITECT Chagas assay calibrator, the ARCHITECT Chagas-positive control, and a low-positive and a high-positive sample (non-recalcified human plasma positive for antibodies to *T. cruzi* diluted in human plasma negative for antibodies to *T. cruzi*) were used.

### 2.3. Testing algorithm

For evaluation of resolved relative sensitivity and resolved relative specificity, one replicate of each specimen was tested on the ARCHITECT Chagas assay and on the bioMérieux ELISA cruzi assay (bioMérieux Brazil). The bioMérieux ELISA cruzi assay was chosen for comparison as it is one of the ELISA assays predominantly used in target countries in Latin America.

In case of initial reactive or gray zone results on either assay, testing of the specimen was repeated in duplicate on the affected assay and the final specimen disposition was determined using 2 or 3 concordant test results out of the total 3 replicates. In case of 3 different test results, the specimen was concluded to be gray zone. Specimens with final gray zone results were excluded from sensitivity and specificity calculations.

Specimens used to evaluate specificity and sensitivity were considered discordant if they were reactive on ARCHITECT Chagas and nonreactive on bioMérieux ELISA cruzi or vice versa. Discordant specimens were further tested on the Wiener Lab Chagas test ELISA recombinant 3.0. If the results were discordant between the bioMérieux and Wiener Lab assay, then the samples were tested on the Abbott Chagas confirmatory immunoblot assay (in development) using 4 recombinant antigens. Final interpretation was performed using the 2 out of 3 rule applied on the non-ARCHITECT assays.

Commercial assays were performed according to their respective package insert instructions; and assays under development, according to their respective study brochure instructions.

### 2.4. Resolved relative specificity/sensitivity

Resolved relative specificity and sensitivity for the ARCHITECT Chagas and the bioMérieux ELISA cruzi assays were calculated as described below:

Res. Relative Specificity =

$$[\text{True Negative} / (\text{True Negative} + \text{False Positive})] \times 100\%$$

Res. Relative Sensitivity =

$$[\text{True Positive} / (\text{True Positive} + \text{False Negative})] \times 100\%$$

Final interpretation of discordant specimens was based on the results obtained for all assays described under testing algorithm.

## 3. Results

### 3.1. Precision

For precision testing, the ARCHITECT Chagas assay Calibrator and Positive Control as well as 2 different native patient samples (low and high positive) were used. The within-laboratory %CVs, which included within-run, between-run as well as between-day variance components, were below 5% CV in all cases. Specimens running at or close to the cutoff value showed slightly higher %CV values (between 4.04 and 4.14) compared to specimens running between 2.00 and 5.00 S/CO, which revealed %CV values between 2.69 and 3.33. However, there is no notable difference in the precision performance between native specimens and assay calibrator or control (for details, refer to Table 2).

### 3.2. Assay specificity and sensitivity

Of 10 284 specimens tested, 9629 had a final interpretation of *T. cruzi* antibody-negative based on the testing

Table 2  
ARCHITECT Chagas precision testing performed on i2000SR

Level	<i>n</i>	Mean	Unit	Within-laboratory SD	Within-laboratory %CV <sup>a</sup>
Calibrator	238	0.95	S/CO	0.039	4.14
Positive Control	225	2.07	S/CO	0.069	3.33
Low positive sample	241	1.43	S/CO	0.058	4.04
High positive sample	204	5.21	S/CO	0.140	2.69

Study design includes factors of run, day, and lot. Precision was assessed on a panel consisting of the ARCHITECT Chagas assay Calibrator and Positive Control as well as a low-positive and a high-positive sample containing antibodies to *T. cruzi* diluted in human plasma.

<sup>a</sup> Within-laboratory %CV contains within-run, between-run, and between-day variance components.

Table 3

Overall resolved relative specificity and sensitivity of the ARCHITECT Chagas assay versus the bioMérieux ELISA cruzi assay

## A) ARCHITECT Chagas assay results versus final interpretation

True Nonreactive	Gray zone	False Reactive	Grand Total	Resolved Relative Specificity	95% Confidence Interval
9625	3	1	9629	99.99%	(99.94–100%)
False Nonreactive	Gray zone	True Reactive	Grand Total	Resolved Relative Sensitivity	95% Confidence Interval
1	2	652	655	99.85%	(99.15–100%)

## B) bioMérieux ELISA cruzi assay results versus final interpretation

True Nonreactive	Gray zone	False Reactive	Grand Total	Resolved Relative Specificity	95% Confidence Interval
9620	2	7	9629	99.93%	(99.85–99.97%)
False Nonreactive	Gray zone	True Reactive	Grand Total	Resolved Relative Sensitivity	95% Confidence Interval
9	0	547	556	98.38%	(96.95–99.26%)

Resolved relative specificity of the ARCHITECT Chagas and bioMérieux ELISA cruzi assay was assessed on random blood donors from German blood banks in Munich and Plauen as well as from the Programa Nacional de Medicina Transfusional y Banco de Sangre (Guatemala, Guatemala) and Fundação Centro de Hematologia e Hemoterapia de Minas Gerais–Hemominas (Belo Horizonte, Brazil) as external sites accompanied by diagnostic specimens from the HSK in Wiesbaden, Germany.

Resolved relative sensitivity of the ARCHITECT Chagas and bioMérieux ELISA cruzi assay was assessed on pre-characterized *T. cruzi* antibody-positive specimens received from Boca Biolistics, Accacia Serum Products, Biocollections Worldwide, HemaCare, Blood Systems, as well as Universidade Federal da Bahia, accompanied by pre-characterized *T. cruzi* antibody-positive specimens from the external sites in Guatemala and Brazil (see above).

Resolved relative specificity and sensitivity performance of the ARCHITECT Chagas and bioMérieux ELISA cruzi assay were determined using the testing algorithm described under Materials and Methods.

algorithm used, whereas 655 specimens were determined to be *T. cruzi* antibody-positive.

### 3.3. Assay specificity

The ARCHITECT Chagas assay was able to detect 9625 of 9629 specimens as nonreactive, accompanied by 3 gray zone and 1 false reactive result, thus showing a resolved relative specificity of 99.99%. bioMérieux ELISA cruzi detected 9620 of 9629 specimens as nonreactive accompanied by 2 gray zone and 7 false reactive results, which corresponds to a resolved relative specificity of 99.93% (Table 3).

For ARCHITECT Chagas, a value of 21.4 SDs to the cutoff is obtained compared to 6.7 observed for the bioMérieux ELISA cruzi assay. The mean S/CO of the negative population was 0.04 for ARCHITECT Chagas versus 0.16 for bioMérieux ELISA (refer to Table 4). Details of the related histograms and sample distributions are shown in Fig. 1: The distribution of the ARCHITECT Chagas assay is tighter and most of the specimens had a substantially lower S/CO value.

### 3.4. Assay sensitivity

For the confirmed *T. cruzi* antibody-positive specimens, ARCHITECT Chagas detected 652 of 655 specimens as reactive, 2 as gray zone, and 1 as nonreactive; thus, the resolved relative sensitivity is 99.85%. The bioMérieux ELISA cruzi was evaluated on a lower number of *T. cruzi* antibody-positive specimens taken from the same specimen panel: 547 of 556 specimens were detected as reactive, whereas 9 specimens showed nonreactive results corresponding to a resolved

relative sensitivity of 98.38% (Table 3). For one *T. cruzi* antibody-positive specimen from Guatemala, the final interpretation using the bioMérieux ELISA cruzi assay (1 reactive, 1 gray zone, and 1 nonreactive result) was gray zone and therefore the specimen was excluded from the sensitivity calculation for this assay.

### 3.5. Assay sensitivity—assessing dilutions of *T. cruzi* antibody-positive specimens

Dilutions of 40 different *T. cruzi* antibody-positive specimens resulting in overall 200 neat or diluted samples were evaluated to compare the relative sensitivity of the ARCHITECT Chagas assay versus bioMérieux ELISA cruzi. For 30 specimens the ARCHITECT Chagas assay showed a superior dilutional sensitivity, that is, in 25 cases, at least one dilution was reactive versus nonreactive or gray zone and in 5 cases one dilution gray zone versus nonreactive. For the remaining 10 specimens, both

Table 4

SD to cutoff and other statistical specificity performance parameters of the ARCHITECT Chagas assay versus the bioMérieux ELISA cruzi assay

	Architect Chagas	bioMérieux ELISA cruzi
<i>n</i>	9629	9629
Mean S/CO	0.04	0.16
S/CO SD	0.045	0.124
SD to CO	21.4	6.7
Nonreactive	9625	9620
Gray zone	3	2
Reactive	1	7
Apparent specificity	99.99%	99.93%

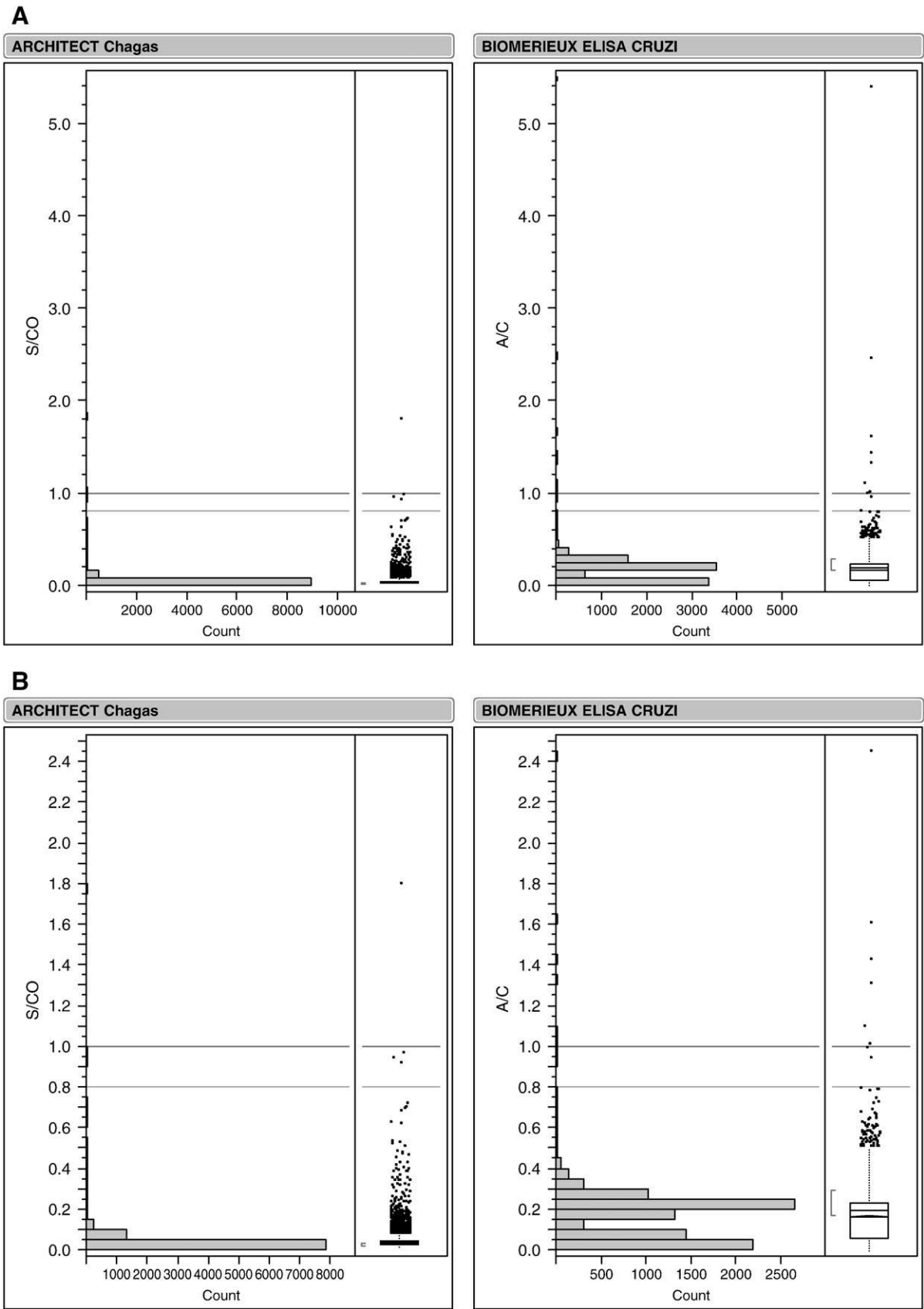


Fig. 1. Histograms and sample distributions (box plots and outlier analysis) of the specificity performance assessment of the ARCHITECT Chagas assay versus the bioMérieux ELISA cruzi assay: part A, diagram of all specimens; part B, zoom of area up to 2.5 S/CO and A/C, respectively.

assays detected the same dilutions as reactive and nonreactive, respectively.

In more detail, for 22 diluted samples, ARCHITECT Chagas was reactive, whereas bioMérieux ELISA cruzi was

Table 5  
ARCHITECT Chagas versus bioMérieux ELISA cruzi assay sensitivity on diluted *T. cruzi* antibody-positive specimens

Sample ID	DF	ARCHITECT Chagas			bioMérieux ELISA cruzi	
		RLU	S/CO	INT	A/C	INT
101	100	33526	0.82	Gray zone	0.26	Nonreactive
102	100	118461	<b>2.89</b>	<b>Reactive</b>	0.88	Gray zone
102	250	61413	<b>1.50</b>	<b>Reactive</b>	0.43	Nonreactive
104	10	33963	0.83	Gray zone	0.29	Nonreactive
105	10	130134	<b>3.18</b>	<b>Reactive</b>	0.83	Gray zone
106	10	161855	<b>3.95</b>	<b>Reactive</b>	<b>2.14</b>	<b>Reactive</b>
107	10	142554	<b>3.48</b>	<b>Reactive</b>	0.68	Nonreactive
108	10	88763	<b>2.17</b>	<b>Reactive</b>	0.36	Nonreactive
109	250	48202	<b>1.18</b>	<b>Reactive</b>	0.46	Nonreactive
110	100	38950	0.95	Gray zone	0.19	Nonreactive
111	100	86165	<b>2.10</b>	<b>Reactive</b>	0.46	Nonreactive
111	250	36261	0.89	Gray zone	0.19	Nonreactive
112	10	178425	<b>4.36</b>	<b>Reactive</b>	<b>1.43</b>	<b>Reactive</b>
113	100	54629	<b>1.33</b>	<b>Reactive</b>	0.30	Nonreactive
115	10	47017	<b>1.15</b>	<b>Reactive</b>	0.63	Nonreactive
117	10	73252	<b>1.79</b>	<b>Reactive</b>	0.49	Nonreactive
118	100	47486	<b>1.16</b>	<b>Reactive</b>	<b>1.08</b>	<b>Reactive</b>
119	100	38231	0.93	Gray zone	0.24	Nonreactive
120	10	143530	<b>3.50</b>	<b>Reactive</b>	<b>1.51</b>	<b>Reactive</b>
126	100	79979	<b>1.95</b>	<b>Reactive</b>	0.88	Gray zone
138	100	70675	<b>1.73</b>	<b>Reactive</b>	0.76	Nonreactive
138016	10	39051	0.95	Gray zone	0.30	Nonreactive
138019	0	224971	<b>5.49</b>	<b>Reactive</b>	<b>1.72</b>	<b>Reactive</b>
140454	10	54033	<b>1.32</b>	<b>Reactive</b>	0.37	Nonreactive
143535	100	42708	<b>1.04</b>	<b>Reactive</b>	0.42	Nonreactive
143536	10	149696	<b>3.65</b>	<b>Reactive</b>	0.73	Nonreactive
143538	10	184121	<b>4.49</b>	<b>Reactive</b>	0.57	Nonreactive
143539	10	182725	<b>4.46</b>	<b>Reactive</b>	<b>2.08</b>	<b>Reactive</b>
143541	10	61541	<b>1.50</b>	<b>Reactive</b>	0.30	Nonreactive
40220	10	65041	<b>1.59</b>	<b>Reactive</b>	0.40	Nonreactive
41438	10	218466	<b>5.33</b>	<b>Reactive</b>	<b>4.01</b>	<b>Reactive</b>
41441	10	176242	<b>4.30</b>	<b>Reactive</b>	<b>1.93</b>	<b>Reactive</b>
41648	10	148627	<b>3.63</b>	<b>Reactive</b>	<b>1.15</b>	<b>Reactive</b>
41649	100	49237	<b>1.20</b>	<b>Reactive</b>	0.34	Nonreactive
41651	100	62946	<b>1.54</b>	<b>Reactive</b>	0.56	Nonreactive
41652	100	55208	<b>1.35</b>	<b>Reactive</b>	0.39	Nonreactive
63221	10	145914	<b>3.56</b>	<b>Reactive</b>	0.77	Nonreactive
63224	10	117473	<b>2.87</b>	<b>Reactive</b>	0.59	Nonreactive
100714	0	59863	<b>1.46</b>	<b>Reactive</b>	<b>1.21</b>	<b>Reactive</b>
100715	10	47230	<b>1.15</b>	<b>Reactive</b>	0.53	Nonreactive
100716	10	165800	<b>4.05</b>	<b>Reactive</b>	0.83	Gray zone
100725	10	113129	<b>2.76</b>	<b>Reactive</b>	0.45	Nonreactive

DF = dilution factor; INT = interpretation (of result).

Forty specimens known to be *T. cruzi* antibody-positive (19 specimens randomly taken from Boca Biolistics specimens used for the assay sensitivity assessment, 21 specimens taken from additional HemaCare specimens) were used to determine the dilutional sensitivity of the ARCHITECT Chagas assay versus the bioMérieux ELISA cruzi assay. Five different dilutions were tested: neat, 1:10, 1:100, 1:250 as well as 1:500. Nonreactive results are not highlighted, gray zone results are shown in italics, and reactive results are shown in bold.

nonreactive at dilutions down to 1:250. For 4 diluted samples, ARCHITECT Chagas was reactive and a gray zone result was obtained with bioMérieux ELISA cruzi, and for 6 diluted samples, ARCHITECT Chagas was gray zone while bioMérieux ELISA cruzi was nonreactive. Two specimens (ID 102 and ID 111) gave different results for 2 consecutive dilutions. In all cases the preceding dilutions were reactive for both assays and the subsequent dilutions nonreactive for both assays. Table 5 shows the results of the discrepant dilutions as well as the last reactive dilution in case of the nondiscrepant specimens.

#### 4. Discussion

Although parasitologic tests such as xenodiagnosis and hemoculture and PCR have proven their application for acute-phase detection of Chagas disease due to being highly specific, they lack sensitivity for chronic-phase detection, apart from being labor-intensive and technically demanding. Therefore, serologic testing for specific IgG antibodies to *T. cruzi* is the most common method for diagnosing chronic infection by this protozoan parasite in both clinic patients as well as in blood donors. These tests demonstrate a range of specificities and sensitivities depending on the test method used and the choice of antigens used. The IFA, which is often used as 1 of 2 assays performed in Latin American laboratories, has been reported to lack sensitivity, be subjective, and show a high degree of cross-reactivity (Oelemann et al., 1998). Enzyme-linked immunosorbent assays are either based on whole parasites, semipurified antigenic fractions from insect-stage (epimastigote) parasites grown in liquid culture, or based on recombinant antigens/synthetic peptides (Chang et al., 2006). Assays using recombinant antigens have better reproducibility, lot-to-lot manufacturing consistency, and lower cross-reactivity to related diseases, such as *Leishmania*, when compared to assays using whole parasites or lysate preparations. Although the specificity of assays using recombinant antigens or synthetic peptides might be superior to lysate-based assays, the sensitivity of a test will greatly depend on the antigen cocktail used and might not be sufficient to detect all strains of *T. cruzi* and stages of infection (Caballero et al., 2007; Shah et al., 2004). A given test must be capable of detecting the varying antibody responses due to genetically diverse and polymorphous strains of *T. cruzi* in different patients. In the late 1980s, a number of highly conserved antigenic proteins of *T. cruzi* including tandemly repeated amino acid sequence motifs—a common phenomenon among protozoan parasites—were described (Hoft et al., 1989; Ibanez et al., 1988). It was already concluded that some of them (e.g., a sequence motif later designated as PEP-2) react with a wide collection of sera from chagasic patients, thus being present in parasites from widely different geographical regions (Ibanez et al., 1988). Among those antigens, there are identical or very similar sequences, which

had been given different names in the literature and many of those have been used in serologic assays for diagnosing *T. cruzi* infection in the form of synthetic peptides and recombinant proteins (da Silveira et al., 2001; Frasch et al., 1991). TcD, TcE, PEP-2 as well as TcLo1.2 were identified as key peptide epitopes for the development of a sensitive and specific diagnostic and blood screening reagent (Houghton et al., 1999, 2000). In this context it was also discussed that a multiepitope construct can minimize the problems of competition between different peptides for coating on the solid phase (da Silveira et al., 2001; Houghton et al., 2000).

For the ARCHITECT Chagas assay the chimeric recombinant antigens TcF, FP3, FP6, and FP10 were selected (Chang et al., 2006; Cheng et al., 2007). TcF represents a multiepitope construct including the key peptide epitopes TcD, TcE, PEP-2 as well as TcLo1.2. Overall, the 4 chimeric recombinant antigens contain 14 distinct antigenic regions that broadly represent all 3 morphologic forms in the life cycle of *T. cruzi*, that is, forms found in the insect vector and in the mammalian host. Moreover, these recombinant proteins also contain antigens recognized by antibodies present in people with acute *T. cruzi* infection as well as those with chronic Chagas disease (da Silveira et al., 2001).

Based on key performance data in this study, it was determined that the prototype ARCHITECT Chagas assay shows an excellent precision below 5% CV for multiple specimens near and slightly above the cutoff value of the assay, thus reflecting a good reproducibility at the clinical decision point.

As shown in the “WHO comparative evaluation of serologic assays for Chagas disease”, the specificity performance of the bioMérieux ELISA cruzi assay is already regarded as sufficient for a single-assay screening approach, while the sensitivity performance is at least in the confidence interval of those 4 assays being regarded as adequate for this approach with both parameters greater than 99% (Otani et al., 2009). In addition, a comparison study of 12 different commercially available diagnostic kits from the Brazilian Ministry of Health involving 4 well-recognized reference centers in Brazil concluded the sensitivity performance of the bioMérieux ELISA cruzi assay as being sufficient for diagnostic purposes (Ministério da saúde secretaria de vigilância em saúde departamento de vigilância epidemiológica coordenação geral de laboratórios de saúde pública, 2006).

The direct comparison of specificity and sensitivity data of the ARCHITECT Chagas assay versus bioMérieux ELISA cruzi, serving as reference assay for Latin America, showed superior performance characteristics for the ARCHITECT assay. In a population of specimens from Guatemala, southern Brazil, and Germany thus covering the principal strain groups *T. cruzi* I and II, the overall specificity of the ARCHITECT Chagas assay was determined to be 99.99% versus 99.93% of the bioMérieux ELISA cruzi assay. This represents 1 versus 7 false-reactive

results out of a population of 9629 specimens based on the testing algorithm used and is significantly different based on the calculation of respective confidence intervals. The sample population used for this study was predominantly blood donor specimens because this will be the major application of the assay during routine testing.

The ability to discriminate between reactive and nonreactive specimens is reflected by the SD to cutoff data of the specificity population. For ARCHITECT Chagas, a value of 21.4 was obtained compared with 6.7 observed for the bioMérieux ELISA cruzi assay, which again demonstrates a striking difference and improvement versus the reference assay. This is underlined by the respective histograms and sample distributions. The ARCHITECT Chagas assay shows a substantially tighter distribution at much lower S/CO value compared to the bioMérieux ELISA cruzi assay. The comparator assay also shows a tailing into the gray zone region. A clear separation between reactive and nonreactive specimens populations provides confidence in the conclusion of the Chagas antibody status of a given blood donor unit, and, together with a suitable sensitivity performance, may justify the use of single-assay screening of blood donations.

With regard to sensitivity the overall observed sensitivity was 99.85% versus 98.38% for the reference assay, again significantly different based on the confidence interval calculations. The origin of the specimens used for this study was Guatemala, (southern) Brazil, Nicaragua, Bolivia, Argentina, Columbia, and the United States. These data confirm that the cocktail of recombinant antigens provided in the ARCHITECT Chagas assay enables safe detection of a broad variety of different *T. cruzi* antibody-positive specimens. The inclusion of recombinant antigens known to be reactive with antibodies from both acute and chronic chagasic patients ensures detection throughout the different stages of infection (da Silveira et al., 2001). As clearly demonstrated with the dilution series of *T. cruzi* antibody-positive specimens with 30 specimens being superiorly detected and 10 specimens being equally detected, the ARCHITECT assay appears to be more sensitive at detecting low-titer positive specimens by showing reactivity in lower dilutions and by having an overall larger dynamic range. This is an important feature because many available kits are very effective at detecting blood donors presenting with high anti-*T. cruzi* antibody titers, but some show weaknesses when the kits are used for donors with low titers (Caballero et al., 2007).

In conclusion, the ARCHITECT Chagas assay, using chimeric recombinant antigens, showed better specificity and sensitivity than the well-established bioMérieux ELISA cruzi assay used for comparison. The utility of the fully automated ARCHITECT Chagas assay to be used in the diagnostic setting and for blood donor testing in endemic as well as non-endemic regions has been shown. The ARCHITECT Chagas assay, once it is commercially available, might be used as single test for routine testing in



high-prevalence areas, that is, Latin America, which would enable an economic testing strategy being highly required for the target countries.

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# His6-Tagged UL35 Protein of Duck Plague Virus: Expression, Purification, and Production of Polyclonal Antibody

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## Key Words

Duck plague virus • Polyclonal antibody • Protein expression • Protein purification • Recombinant protein • UL35 gene

## Abstract

**Objective:** Duck plague virus (DPV), the causative agent of duck plague (DP), is an alphaherpesvirus that causes an acute, febrile, contagious, and septic disease of waterfowl. UL35 protein (VP26) is a major capsid protein encoded by the UL35 gene, which is located in the unique long (UL) region of the DPV genome. To investigate the specific roles of VP26, the UL35 gene was amplified from the DPV DNA by polymerase chain reaction (PCR) and subcloned into pET-32a(+).

**Methods:** The resultant prokaryotic expression vector, pET-32a(+)/UL35, includes an amino-terminal His6 as a fusion partner. *Escherichia coli* BL21 (DE3) competent cells were transformed with the construct and protein expression was subsequently induced by the addition of isopropyl-β-D-thiogalactopyranoside to the culture medium. Protein lysates were submitted to SDS-PAGE to evaluate recombinant protein expression. **Results:** The band that corresponded to the predicted protein size (33 kDa) was observed on the SDS-PAGE gel. The recombinant His6-tagged VP26 fusion protein was expressed at a high level in an insoluble form (inclusion bodies) and constituted about 24% of the total cellular pro-

tein. Then, the fusion protein was purified to near homogeneity using single-step immobilized metal affinity chromatography on a nickel-nitrilotriacetic acid affinity resin, yielding about 620 mg per liter culture. After purification, New Zealand white rabbits were immunized with purified His6-tagged VP26 in order to raise polyclonal antibody against this recombinant protein. Using the resultant sera, Western blot analysis showed that the recombinant protein was recognized by the polyclonal antibody. **Conclusion:** Thus, the polyclonal antibody prepared here may serve as a valuable tool to study the functional involvement of VP26 in the DPV life cycle.

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## Introduction

Duck plague (DP), or duck virus enteritis, is an acute, febrile, contagious, and septic disease of waterfowl (duck, goose, and swan) caused by DP virus (DPV). The typical characteristics of DP are vascular injury, tissue hemorrhage and digestive tract and lymphatic organ damage. Duck-producing areas of the world where afflicted by the

The first four authors contributed equally to this work and should be considered as first author.

DPV and have suffered considerable economic losses in the commercial duck industry due to high mortality, decreased egg production of ducks and the different lethality in wild waterfowl [1–9]. In China, the virulence of DPV has recently increased in geese but declined in ducks [10]. Presently, however, the molecular characteristics of DPV remain largely unknown.

DPV is composed of a linear, double-stranded DNA genome with 64.3% guanine-plus-cytosine content, which is higher than any other reported avian herpesvirus in the subfamily Alphaherpesvirinae [11]. Although DPV was previously grouped in the subfamily Alphaherpesvirinae [12, 13], it was classified as an unassigned virus in the Herpesviridae family according to the Eighth International Committee on Taxonomy of Viruses [14]. At present, studies on the genomic organization and nucleotide sequence of DPV lag behind other members of the Herpesviridae family because most of the previous studies have focused on the epidemiology and prevention of this disease. To the best of our knowledge, neither the molecular structure of the genome nor the restriction enzyme map of DPV DNA has been published up to now [4]. To date, the majority of reported DPV sequences are limited to single open reading frames (ORFs) in the unique long (UL) region, which includes UL6 [4, 15], UL25, UL26, UL26.5, UL27, UL28, UL29, and UL30 [16, 17]. However, no reports have been published concerning the DPV UL35 gene. Therefore, very little is known about the DPV UL35 gene or its encoding protein, VP26.

Recently, a DPV CHv-strain genomic library was constructed successfully for the first time [18] and the UL35 gene was discovered in our laboratory. In an effort to elucidate the function of VP26, we constructed a recombinant expression plasmid that drives expression of the DPV VP26 fused to His6. This plasmid was used to transform *Escherichia coli* BL21 (DE3) cells, which were subsequently induced to express the recombinant protein (His6-tagged VP26). His6-tagged VP26 was purified to near homogeneity using single-step immobilized metal affinity chromatography (IMAC) on a nickel-nitrilotriacetic acid ( $\text{Ni}^{2+}$ -NTA) affinity resin. The purified protein was then used as an antigen for the production of polyclonal antibody specific for His6-tagged VP26.

## Materials and Methods

### *Virus, Strains, Plasmids, Enzymes, and Other Materials*

The DPV CHv strain is a high-virulence field strain of DPV that was isolated and preserved in the authors' laboratory. *E. coli* strain DH5 $\alpha$ , *E. coli* BL21 (DE3), cloning vector pMD18-T, and

expression vector pET-32a(+) were purchased from Invitrogen. Yeast extract and tryptone for bacterial medium preparation were obtained from Promega. Fetal bovine serum (FBS), antibiotics, and isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) were bought from Novagen. Restriction enzymes, DNA ligase, Ex *Taq* DNA polymerase, DNA molecular weight markers, and protein molecular weight markers were obtained from TaKaRa; other reagents were offered by the Avian Diseases Research Center of the Sichuan Agricultural University. All reagents were of the highest purity that was commercially available. Purification of His-tagged protein was performed on a  $\text{Ni}^{2+}$ -NTA resin (Qiagen). Here, *E. coli* strain DH5 $\alpha$  was used as the cloning host for the UL35 gene encoding VP26 because of its very high transformation efficiency. For the expression of His6-tagged VP26, the *E. coli* strain BL21 (DE3) was used. This strain has the advantage of being deficient in both the lon and ompT proteases and harbors the T7 bacteriophage RNA polymerase gene, which permits the specific expression of heterologous genes driven by the T7 promoter [19–21].

### *Preparation of DPV DNA*

DPV was propagated in primary duck embryo fibroblasts that were grown in Dulbecco's MEM (D-MEM; Gibco-BRL) supplemented with 10% FBS at 37°. For virus infection, D-MEM supplemented with 2–3% FBS was used. Viral particles were harvested when the cytopathic effect reached 75%. Cell lysates containing DPV were subjected to three freeze-thaw cycles and were then stored at –70° until use. The presence of DPV was confirmed by both electron microscopy and polymerase chain reaction (PCR), as described previously [15, 22].

To isolate the nuclear DPV DNA, 5–10 ml DPV-containing cell lysate was centrifuged at 5,000 rpm for 10 min at 4°. The supernatant was decanted, and the cell pellet was resuspended in 2–5 ml NET (0.1 M NaCl, 1.0 mM EDTA, pH 8.0, and 10 mM Tris, pH 8.0). The cell suspension was centrifuged at 5,000 rpm for 10 min at 4°. Then, the supernatant was discarded and the pellet was redissolved in 750  $\mu$ l NET, 7.5  $\mu$ l proteinase K (10 mg stock), and 50  $\mu$ l of 15% sarcosyl. The sample was incubated at 37° to promote proteinase K activity. The cell lysate was then centrifuged at 12,000 rpm for 30 min at 4°. The nuclear DNA-containing supernatant was transferred to a fresh tube and submitted to RNase treatment at 37° for 1 or 2 h. DNA was extracted first with one volume of phenol (750  $\mu$ l), then with phenol/chloroform/isoamyl alcohol (25:24:1), and finally with chloroform. DNA was precipitated with two volumes of prechilled absolute ethanol and stored at –20° for future use.

### *Primer Design and PCR Amplification of the DPV UL35 Gene*

The full-length DPV UL35 gene (GenBank accession No. EF643558) is composed of 354 base pairs and contains a complete theoretical ORF for a 117-residue UL35-like protein. The primers for the PCR-based amplification of this ORF were designed using the biological software Oligo6.0 and Primer5.0 and were synthesized by TaKaRa, with an expected amplified fragment of 354 bp. The upstream primer 5'GGATCCATGTCTAATTCTGGAG-GTTCA anneals with the first 21 nucleotides of the UL35 sequence and introduces an upstream *Bam*HI restriction site (underlined). The downstream primer 5'AAGCTTTTATCGCT-GATCGTCTGG is complementary to the final 18 nucleotides of the UL35 sequence and introduces a *Hind*III restriction site (underlined). These restriction sites were included to facilitate the subsequent cloning procedure.

The UL35 gene was amplified by PCR from the genome of the DPV CHv strain, using DNA, which was purified as described above, as the template. PCR was performed in a 25- $\mu$ l reaction volume, as described previously [22]. PCR amplifications were carried out using the following reaction cycles in a commercial PCR system (2700; GeneAmp): initial denaturation at 95° for 5 min followed by 32 consecutive cycles of denaturation at 94° for 50 s, annealing for 40 s at 56°, and extension at 72° for 40 s, and then a final extension at 72° for 10 min, using Ex *Taq* DNA polymerase. The amplified product (354 bp) was analyzed by electrophoresis on a 2% (w/v) agarose gel stained with 0.5  $\mu$ l/ml ethidium bromide. After the PCR-amplified product had been validated as the intended product, it was purified using a PCR gel purification kit (Qiagen) according to the manufacturer's instructions. The purified PCR product was then submitted to ligation into a pMD18-T vector.

#### *Construction of Cloning Plasmid pMD18-T/UL35*

The purified PCR product was digested with *Bam*HI and *Hind*III restriction enzymes, purified and ligated into the correspondingly digested cloning vector pMD18-T at 16° overnight using T4 DNA ligase to generate a recombinant cloning plasmid named pMD18-T/UL35 (fig. 1), using standard cloning methods [23]. Competent *E. coli* DH5 $\alpha$  cells were transformed with the ligation mixture by the heat shock method and used for propagation of the cloning plasmid. The transformants were cultured at 37° on Luria-Bertani (LB) solid medium (1.0% sodium chloride, 1.0% tryptone, 0.5% yeast extract, and 1.5% agar) for 16 h and in LB liquid medium (as above but without agar) at 37° for 12 h, supplemented with 100  $\mu$ g/ml ampicillin when plasmid maintenance was required. After mini-scale isolation of plasmid DNA using the modified alkaline lysis method [24], the presence of the appropriate insert in the obtained plasmid was verified by PCR and restriction analysis. One clone was then selected and sent to TaKaRa for sequencing.

#### *Construction of the pET-32a(+)/UL35 Expression Plasmid*

After its sequence had been confirmed, pMD18-T/UL35 was digested with *Bam*HI and *Hind*III restriction enzymes and submitted to electrophoresis through a 1% agarose gel, then the DNA band corresponding to the digested insert (ORF) was cut and purified from the agarose gel and directionally ligated into the previously *Bam*HI/*Hind*III-digested and dephosphorylated expression vector pET-32a(+), downstream of the T7 Lac promoter and His6 tag and upstream of the T7 terminator, to construct the recombinant prokaryotic expression vector pET-32a(+)/UL35 using T4 DNA ligase (fig. 2). Cloning at the *Bam*HI site in pET-32a(+) results in a His6 fusion to the N-terminus of the cloned gene. This fusion tag permits purification of the produced protein by metal chelate chromatography on a nitrilotriacetic acid agarose matrix charged with nickel ions. The ligation mixture was transformed into competent *E. coli* DH5 $\alpha$  cells. Positive clones with a gene insert in the plasmid were first evaluated by PCR and then reconfirmed by restriction digestion and DNA sequencing. Each identified positive colony was grown in LB medium containing ampicillin (100  $\mu$ g/ml). The plasmid pET-32a(+)/UL35 was isolated from the bacterial cells and used to transform the competent *E. coli* strain BL21 (DE3) cells for the purpose of recombinant protein expression.

#### *Expression of the Recombinant Protein*

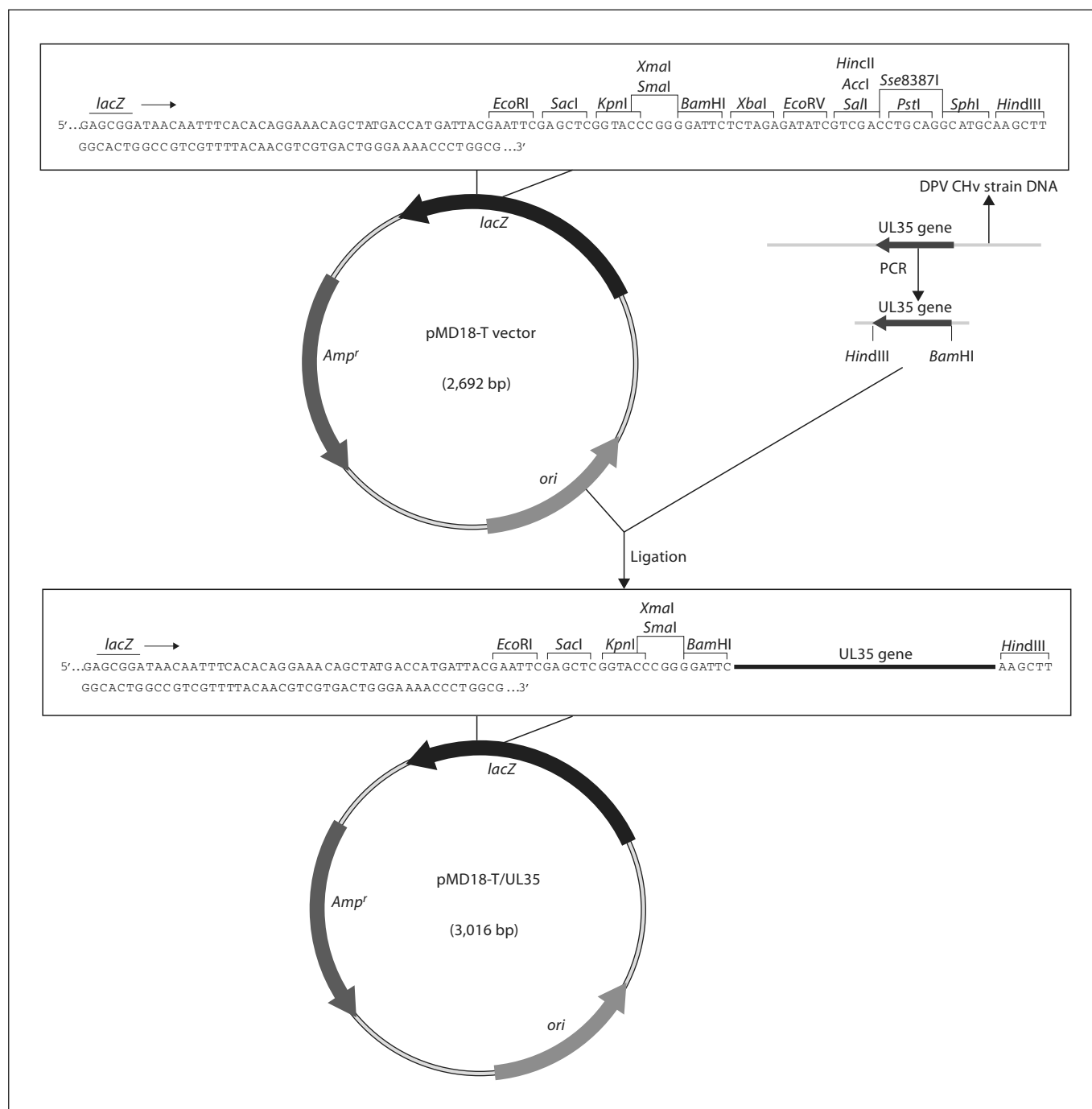
For recombinant protein expression, *E. coli* strain BL21 (DE3) cells were transformed with the plasmid pET-32a(+)/UL35 and selected on LB solid medium containing ampicillin (100  $\mu$ g/ml). The transformants were inoculated into 5 ml of culture medium in test tubes and allowed to grow overnight at 37° with constant agitation (200 rpm). These cultures were used to inoculate 100 ml LB containing ampicillin (100  $\mu$ g/ml) and were submitted to vigorous shaking in a fermenter. When the cultures initially reached logarithmic phase (at OD<sub>600</sub> of 0.5–0.6), expression of the recombinant fusion protein, His6-tagged VP26, was induced by the addition of IPTG (final concentration 1.0 mM) with further growth at 37° for 4 h. After induction, the cells were harvested by centrifugation at 6,000 rpm for 10 min at 4° and lysed in 5 $\times$  SDS-PAGE loading buffer (0.313 M Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, and 0.05% bromophenol blue, with 100 mM DTT). Then, the cell lysates were boiled for 15 min, centrifuged at 12,000 rpm for 10 min, submitted to 12% SDS-PAGE, and then analyzed by Coomassie brilliant blue R-250 staining. Briefly, the gel was stained with Coomassie brilliant blue R-250 overnight and destained in 6% acetic acid until a clear background was reached to assess the expression level of the recombinant fusion protein by densitometric scanning using BandsScan4.5 software [25]. The uninduced control culture and the vector control culture were analyzed in parallel.

To increase the production of the recombinant protein, culture conditions for expression were optimized in terms of different temperatures (30, 34, and 37°), concentrations of IPTG (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.5, and 2.0 mM), and durations of induction (0, 1, 2, 3, 4, 5, 6, and 7 h, and overnight). Protein expression was assessed by SDS-PAGE, as described above.

For the initial experiments, which were designed to determine the solubility of the recombinant protein, log phase cultures were induced with 1 mM IPTG for 5 h at 34°, and approximately 4 g of wet weight cells from 1 liter of culture were harvested by centrifugation at 6,000 rpm for 10 min. The cells were left overnight at –20° and were then suspended in 20 ml lysis buffer of 20 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl, 1.0 mM phenylmethyl sulfonylfluoride (PMSF), and 1.0 mg/ml lysozyme. The suspension was incubated for 30 min at 4° with stirring and was then pulse sonicated on ice (30 s working and 30 s resting on ice, Vibrocell VCX600 sonicator, 600 W max; Sonics and Materials) until the sample was clear. Sonication was performed to release intracellular proteins. The resulting cell lysate was centrifuged at 12,000 rpm for 30 min. The clear supernatant (soluble fraction) was collected and the remaining pellets (insoluble fraction), which contained the inclusion bodies, were dissolved in deionized water and stored at –20° until use. Soluble and insoluble fractions were then analyzed in parallel by 12% SDS-PAGE.

#### *Purification of the Recombinant Protein*

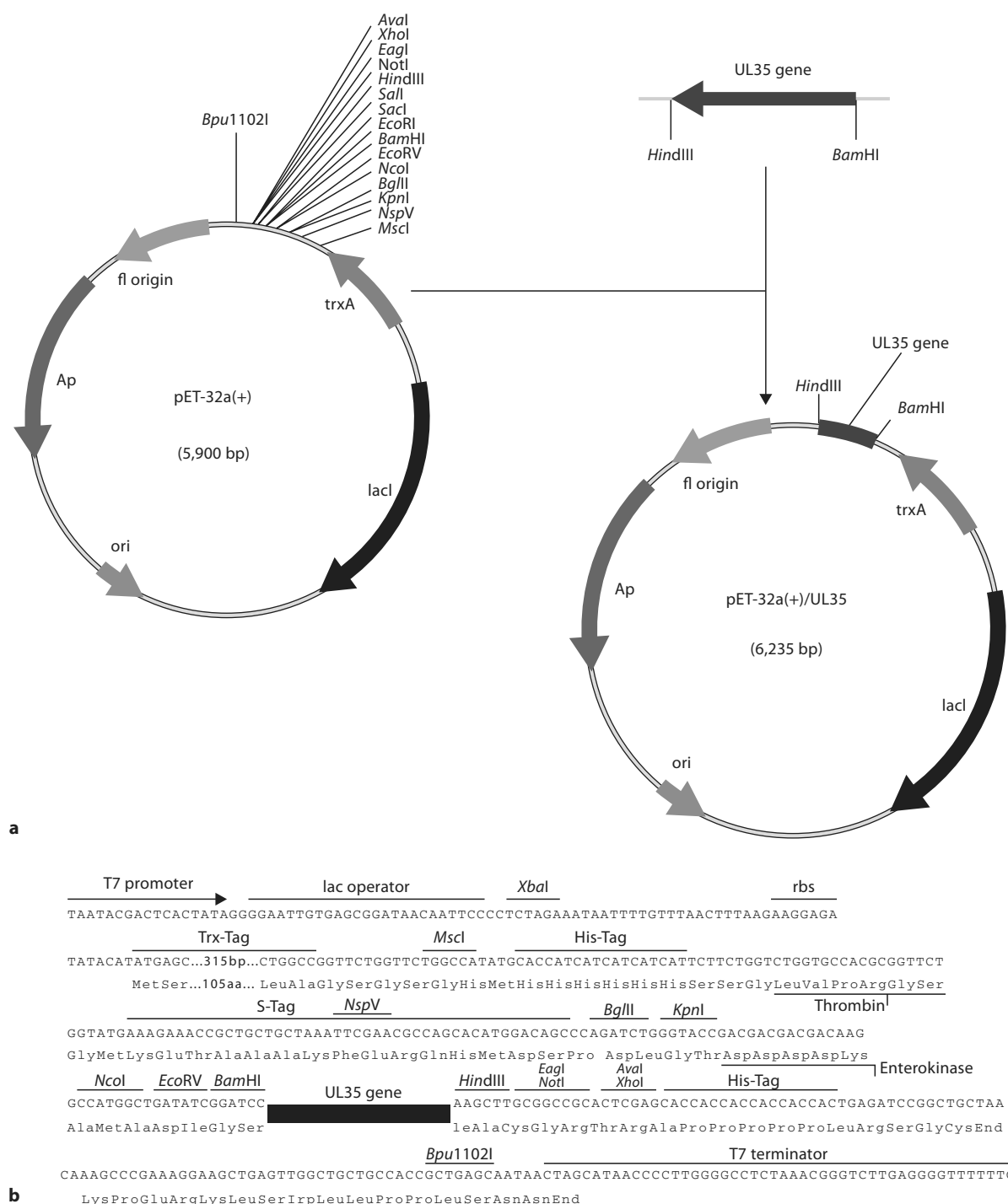
The pellets of the insoluble fraction (crude extract) were resuspended in extraction buffer (0.5 M NaCl, 2 M urea, 1 mM DTT, 2% Triton X-100, and 20 mM Tris-HCl, pH 7.9), sonicated for 30 s at 4°, and centrifuged at 15,000 rpm for 30 min. The pellets were washed twice with washing buffer (0.5 M NaCl, 2 M urea, 20 mM Tris-HCl, pH 7.9), resuspended in regeneration buffer (0.5 M NaCl, 6 M urea, 20 mM Tris-HCl, pH 7.9) and incubated at room temperature (25°) for 30 min. The incubated mixture was then centrifuged at 15,000 rpm for 10 min, and the resultant supernatant was submitted to further purification.



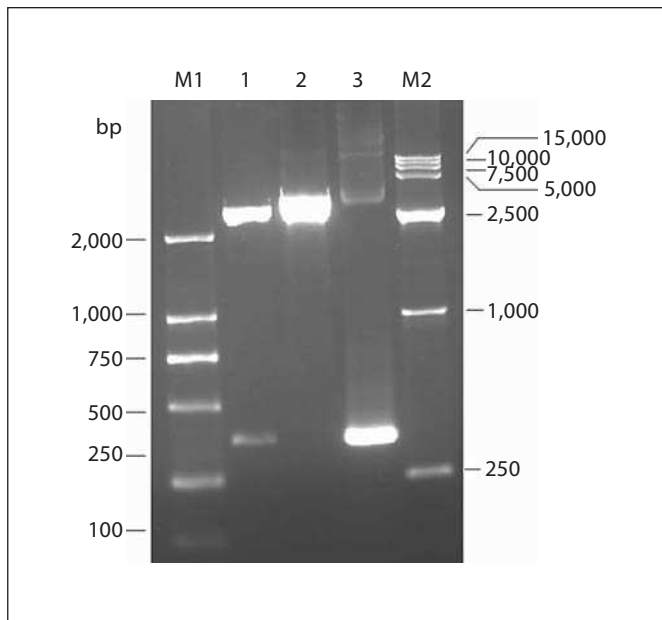
**Fig. 1.** Schematic diagram of the UL35 ORF cloned into the pMD18-T cloning vector.

The recombinant His-tagged VP26 was purified from the supernatant obtained above by IMAC on  $\text{Ni}^{2+}$ -NTA affinity resin following the manufacturer's instructions with modifications. A glass column (20 ml capacity) was packed with  $\text{Ni}^{2+}$ -NTA resin matrix. The column was equilibrated with 4 bed volumes of IMAC buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.5 mM

PMSF, and 10 mM imidazole). The chromatography flow rate, which was driven by gravity, was 0.5 ml/min. The supernatant (crude extract) was loaded onto the  $\text{Ni}^{2+}$ -NTA agarose resin column preequilibrated with IMAC buffer. The column was washed successively with 3 bed volumes of IMAC buffer and 5 bed volumes of IMAC buffer containing 20 mM imidazole. The protein



**Fig. 2.** Construction of the recombinant expression plasmid pET-32a(+)/UL35. **a** Schematic diagram of the UL35 ORF cloned into the pET-32a(+) expression plasmid. **b** Detailed structural features of the constructed recombinant expression plasmid pET-32a(+)/UL35. The recombinant protein consisted of Trx, His tag, thrombin cleavage site, S tag, and enterokinase cleavage site.



**Fig. 3.** Characterization of the recombinant plasmid pMD18-T/UL35 by restriction digestion and PCR-based amplification. M1 = DNA marker of 2,000 bp; 1 = pMD18-T/UL35 digested with *Bam*HI and *Hind*III; 2 = pMD18-T/UL35 digested with *Bam*HI; 3 = product amplified from pMD18-T/UL35 (ORF 354 bp); M2 = DNA marker of 15,000 bp.

impurities devoid of histidine tails were removed from the agarose resin at a flow rate of 2.0 ml/min at 4°. The fusion protein was eluted with IMAC buffer containing 8 M urea and 100 mM imidazole at a flow rate of 1.0 ml/min at 4°. Five-milliliter fractions were collected and analyzed by SDS-PAGE to identify the fusion protein and to assess the level of homogeneity. The protein concentration was estimated by the method of Bradford using bovine serum albumin (BSA) as the standard (0–12 µg) [26]. Fractions that contained the purified His6-tagged VP26, which was in a denatured state, were pooled and dialyzed against PBS buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, 140 mM NaCl, and 2.7 mM KCl) containing 1 mM PMSF with progressively lower concentrations of urea (4, 2, and 0 M) for a total time of 72 h. The purified recombinant protein was stored at 4° for use within 1 week or at –70° for use after a longer time.

#### *Production of Polyclonal Antibody against the Recombinant Protein*

The purified recombinant protein was used for raising antibody in New Zealand white rabbits. Blood (1.5 ml) was collected prior to immunization by bleeding the rabbits from the marginal vein of the ear. This serum served as a negative control. Afterwards, the rabbits were then intradermally injected with a mixture of 500 µg of purified recombinant protein mixed with an equal volume of Freund's complete adjuvant (Promega) on the back and proximal limbs (100 µl/site). After 2 weeks, the rabbits were twice boosted intramuscularly with 500 µg of purified re-

combinant protein and incomplete Freund's adjuvant at a 1-week interval. Subsequently, each rabbit was intravenously immunized with 100 µg of the purified recombinant protein. Two weeks after the last injection, the rabbit with the best reactivity toward His6-tagged VP26 was sacrificed, and the antiserum was harvested from the arteriae carotis and stored at –80° until further use.

#### *Purification of the Antiserum*

First, the rabbit IgG fraction was precipitated from the harvested rabbit polyclonal antiserum by ammonium sulfate precipitation. Then, by using a DEAE-Sepharose column (Bio-Rad), the IgG fraction was purified by ion exchange column chromatography following the manufacturer's instructions. The purified IgG fraction was analyzed by 12% SDS-PAGE.

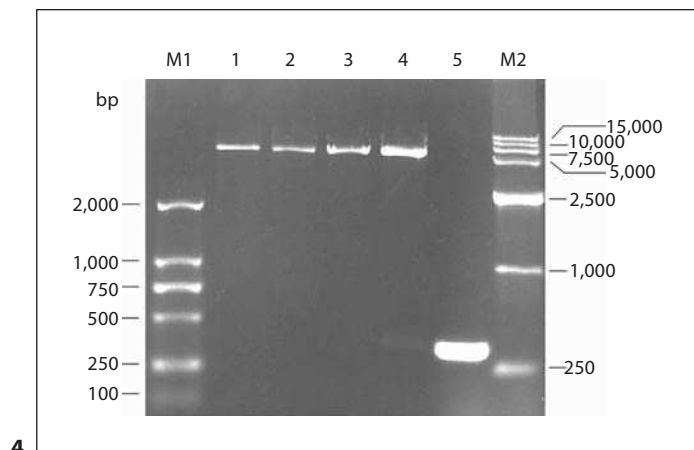
#### *Western Blot Analysis*

To characterize the antigenicity of the recombinant fusion protein, His6-tagged VP26, Western blot analysis was performed according to a standard procedure [27] using the purified rabbit anti-His6-tagged VP26 IgG. After the proteins had been separated by 12% SDS-PAGE, the gel was immersed in transfer buffer (0.24% Tris-HCl, 1.153% glycine, and 15% methanol, pH 8.8) and electrophoretically transferred onto nitrocellulose membrane (Bio-Rad) pre-equilibrated in transfer buffer using a Mini Trans Blot electrophoretic transfer cell (Bio-Rad) at 80 V for 1.5 h. The membrane was incubated in blocking buffer (5% BSA in PBS buffer) for 1 h at 37° or overnight at 4°. After three washes (10 min each) with 1× PBS buffer, the membrane was incubated with rabbit anti-His6-tagged VP26 IgG at a dilution of 1:100 with 1% BSA in PBS overnight at 4°. The membrane was then washed three times with 1× PBS containing 0.1% Tween-20 (PBST), and was further incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (Boster) at a dilution of 1:5,000 for 1 h at 37°. The membrane was then washed three times with PBST and three times with PBS and developed with diaminobenzidine substrate buffer. Color development was terminated by thorough washing in distilled water.

## **Results and Discussion**

#### *Gene Amplification, Cloning, and Sequencing*

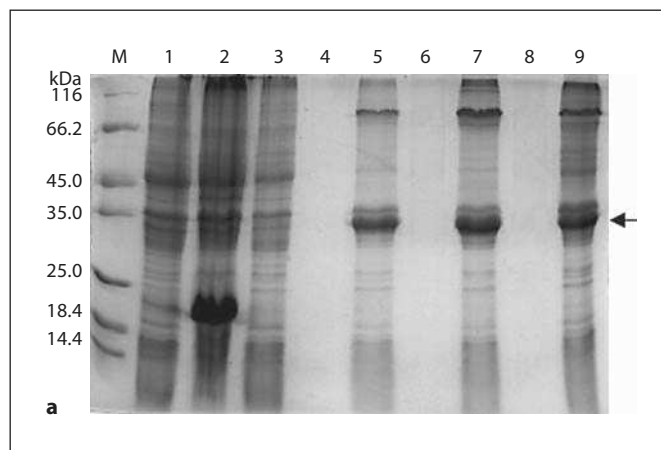
On the basis of the constructed DPV CHV-strain genomic library [18], primers were designed to amplify the UL35 gene using DPV DNA as a template. *Bam*HI and *Hind*III sites were designed in the primers to facilitate subsequent cloning. The PCR product (354 bp; data not shown) was digested with *Bam*HI and *Hind*III restriction enzymes and the ORF was inserted into the vector pMD18-T between the *Bam*HI and *Hind*III sites to construct the cloning vector pMD18-T/UL35. Then, the recombinant plasmid was confirmed by DNA sequencing and restriction digestion (fig. 3). The sequencing result showed that there were no nucleotide errors in the synthetic UL35 gene fragment (data not shown).



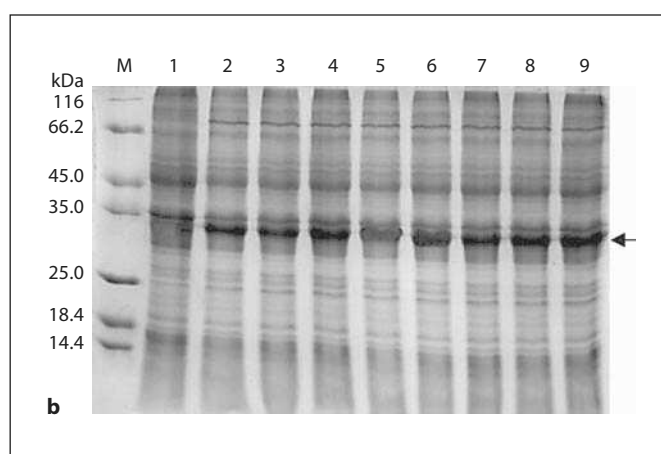
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**Fig. 4.** Characterization of the recombinant plasmid pET-32a(+)/UL35 by restriction digestion and PCR-based amplification. M1 = DNA marker of 2,000 bp; 1 = pET-32a(+); 2 = pET-32a(+) digested with *Bam*HI and *Hind*III; 3 = pET-32a(+)/UL35 digested with *Bam*HI; 4 = pET-32a(+)/UL35 digested with *Bam*HI and *Hind*III; 5 = product amplified from pET-32a(+)/UL35; M2 = DNA marker of 15,000 bp.

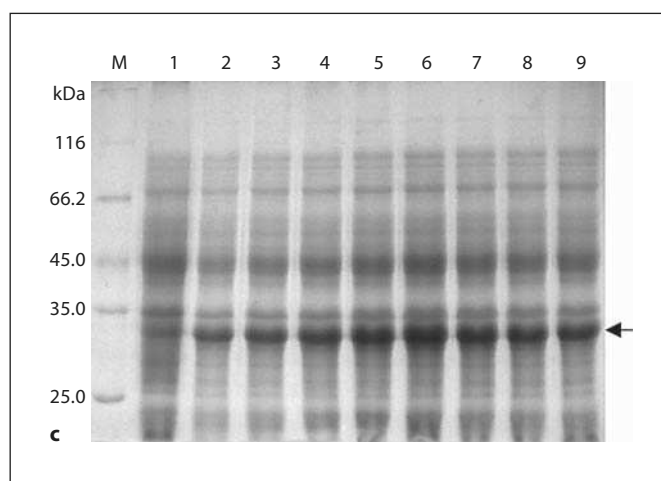
**Fig. 5.** Expression analysis and optimization of the expression conditions of the His6-tagged VP26 fusion protein. **a** Coomassie-stained SDS-PAGE gel (12%) analysis for the expression and optimization of the temperature for His6-tagged VP26 fusion protein. M = Protein molecular weight marker; 1 = total protein from *E. coli* BL21/pET-32a(+) before induction; 2 = total protein from *E. coli* BL21/pET-32a(+) after induction; 3 = total protein from *E. coli* BL21/pET-32a(+)/UL35 before induction; 4, 6 and 8 = soluble fractions of total protein from *E. coli* BL21/pET-32a(+)/UL35 after induction at 30, 34, and 37°, respectively; 5, 7 and 9 = insoluble fractions (inclusion bodies) of total protein from *E. coli* BL21/pET-32a(+)/UL35 after induction at 30, 34 and 37°, respectively. **b** SDS-PAGE analysis for optimization of the concentration of IPTG for His6-tagged VP26 fusion protein expression. M = protein molecular weight marker; 1–9 = total protein from *E. coli* BL21/pET-32a(+)/UL35 after induction with the following concentrations of IPTG: 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.5, and 2.0 mM, respectively, at 34°. **c** SDS-PAGE analysis for optimization of the duration of induction for His6-tagged VP26 fusion protein expression. M = protein molecular weight marker; 1–9 = total protein from *E. coli* BL21/pET-32a(+)/UL35 after induction with IPTG (1.0 mM) for 0, 1, 2, 3, 4, 5, 6, and 7 h and overnight, respectively, at 34°. Since an equal amount of sample was loaded into each lane, it is possible to compare the expression levels among lanes. The highest level of expression was observed for 5 h after induction at 34° with 1 mM IPTG. Arrows indicate the position of the fusion protein.



**a**



**b**



**c**

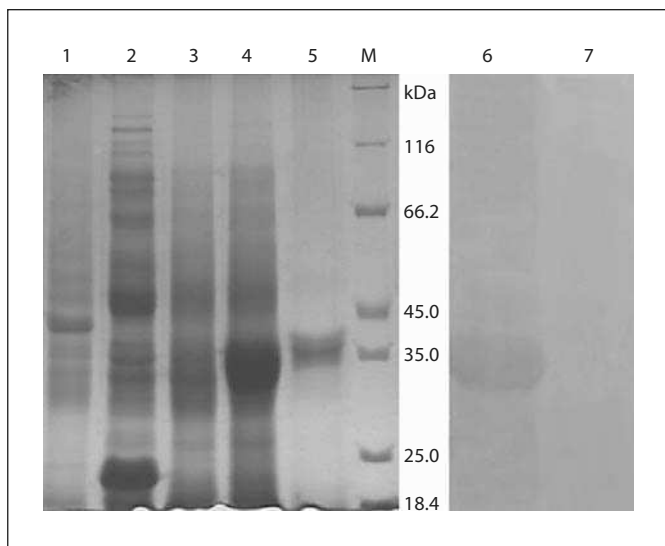
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### Construction of the Expression Plasmid

The prokaryotic expression vector pET-32a(+), which features a high stringency T7 lac promoter, His6 tag, and T7 terminator, has been recognized as one of the most powerful tools for producing recombinant proteins in

*E. coli* [28]. The UL35 gene fragment, which was obtained by digestion of pMD18-T/UL35 with *Bam*HI and *Hind*III, was directionally inserted downstream of the His6-tag sequence in the pET-32a(+) plasmid to construct the expression plasmid pET-32a(+)/UL35. The initial transfor-





**Fig. 6.** Purification and Western blot analysis of the purified recombinant His6-tagged VP26 fusion protein. M = Protein molecular weight marker; 1 = total protein from *E. coli* BL21/pET-32a(+) before induction; 2 = total protein from *E. coli* BL21/pET-32a(+) after induction; 3 = total protein from *E. coli* BL21/pET-32a(+)/UL35 before induction; 4 = total protein from *E. coli* BL21/pET-32a(+)/UL35 after IPTG induction (1.0 mM) at 34° for 5 h; 5 = purified recombinant His6-tagged VP26 fusion protein using a single chromatographic step of IMAC on Ni<sup>2+</sup>-NTA agarose; 6 = rabbit anti-His6-tagged VP26 IgG reacted with His6-tagged VP26; 7 = negative control serum reacted with His6-tagged VP26.

mation was carried out with competent *E. coli* DH5 $\alpha$  cells for the purpose of screening. The positive colonies were identified by PCR and restriction digestion (fig. 4). After confirmation, a positive clone was submitted to DNA sequencing and the result confirmed that the UL35 gene was in frame with the N-terminal His6 tag within the pET-32a(+) multiple cloning sites (data not shown).

#### *Expression of the Recombinant Protein*

After sequence confirmation, the recombinant plasmid pET-32a(+)/UL35 was introduced into the expression host *E. coli* BL21(DE3). A positive transformant was used for the subsequent induction. Initially, the induction of His6-tagged VP26 expression was carried out at 37° for 4 h by the addition of 1.0 mM IPTG. A distinct band of approximately 33 kDa of molecular weight, corresponding to the expected size of the His6-tagged VP26 fusion protein (fig. 5a, lane 9), was observed. This result confirmed that the ORF was properly expressed in the transformed *E. coli* BL21 (DE3) cells. Expressed protein

was not detected in the negative control *E. coli* BL21 (DE3) cells (fig. 5a, lane 3) nor was it found without induction of *E. coli* BL21 (DE3) cells transformed with the pET-32a(+) vector (fig. 5a, lane 1). Analysis of the densitometry scan of the 12% SDS-PAGE gel showed that the IPTG-induced band constituted approximately 24% of the total proteins in the induced cell extract (data not shown).

The relative distribution of the expressed recombinant protein in the soluble and insoluble fractions of the supernatant and the pellet (see Materials and Methods) of the cell lysate was examined after sonication. The recombinant protein was predominantly expressed in the insoluble fraction, in the form of inclusion bodies (fig. 5a, lanes 5, 7 and 9), indicating that little or no soluble proteins are formed. We then tried to optimize the expression conditions as described in Materials and Methods by utilization of different durations of induction, concentrations of IPTG and induction temperatures. The optimal induction of His6-tagged VP26 protein expression was obtained by growth in the presence of 1.0 mM IPTG (fig. 5b, lane 6) for 5 h (fig. 5c, lane 6) at 34° (fig. 5a, lane 7).

#### *Purification of the Recombinant Protein*

Generally, high-level expression of recombinant proteins in *E. coli* often results in the formation of insoluble and inactive aggregate known as inclusion bodies [29, 30]. They develop as a result of misfolding or partial folding of polypeptides with exposed hydrophobic patches and the consequent intermolecular interactions [31]. Therefore, purified inclusion bodies must be resolubilized by strong denaturants, such as 6 M guanidine hydrochloride or 8 M urea, which promote the disruption of intermolecular interactions and complete unfolding of the protein [32, 33]. The denaturation solution is removed by dilution or a buffer exchange step [34, 35], which permits renaturation of the proteins.

Histidine tags have become common fusion partners for recombinant proteins to facilitate purification using IMAC. For purification of the recombinant His6-tagged VP26 fusion protein from the insoluble fraction, the dissolved protein (inclusion bodies) was used as starting material and subjected to His tag purification using a single chromatographic step of IMAC on Ni<sup>2+</sup>-NTA agarose, as described. After elution with IMAC buffer containing 8 M urea and dialysis against PBS containing progressively lower concentrations of urea (4, 2, and 0 M), a clear band corresponding to a molecular mass of about 33 kDa was seen on the SDS-PAGE gel following Coomassie blue staining (fig. 6, lane 5). This procedure allowed to harvest ~620 mg of nearly homogeneous protein (98%, accord-

**Table 1.** Purification of the recombinant His-tagged VP26 from 4 g of wet weight *E. coli* cells

Purification step	Volume ml	Total protein <sup>a</sup> mg	His-tagged VP26, mg	Protein concentration <sup>b</sup> , µg/ml	Purity %	Purification factor, fold	Cumulative yield, %
Crude extract <sup>c</sup>	10	54.75	13.14	1,314	24	1	100
IMAC <sup>d</sup>	3	1.90	1.86	620	98	4	14

<sup>a</sup> Total protein was isolated from 1 liter of culture medium after induction with 1.0 mM IPTG at 34° for 5 h.

<sup>b</sup> Protein concentration indicates the concentration of His-tagged VP26. Protein concentrations were estimated by the Bradford method using BSA as standard.

<sup>c</sup> The pellets containing the insoluble fraction (crude extract) obtained from 1 liter of culture medium after centrifugation and sonication.

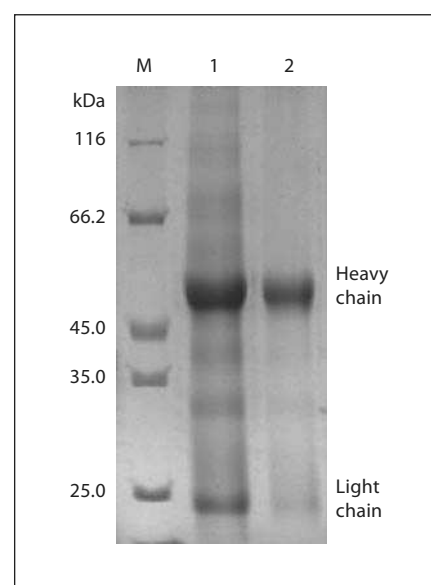
<sup>d</sup> The purified recombinant His-tagged VP26 using IMAC on Ni<sup>2+</sup>-NTA affinity resin.

ing to gel densitometry analysis) per liter of culture medium (1 liter bacterial cell culture produced about 4 g of wet weight cells in our study). The purification is summarized in table 1.

#### *Purification of the Antiserum and Antigenicity Analysis of the Recombinant Protein*

After 4 injections had been given to 6 New Zealand white rabbits, the serum containing anti-His6-tagged VP26 polyclonal antibody with the stronger specificity was collected. The rabbit anti-His6-tagged VP26 IgG, with 55 and 22 kDa of the heavy and light chains, was firstly precipitated by ammonium sulfate precipitation (fig. 7, lane 1) and then purified by ion exchange column chromatography (fig. 7, lane 2). Western blot analysis showed that the purified His6-tagged VP26 was recognized by the rabbit anti-His6-tagged VP26 IgG and showed a specific signal at 33 kDa, which is the expected size of the fusion protein (fig. 6, lane 6). Importantly, no positive signal was observed when using the negative control serum (fig. 6, lane 7), indicating that the recombinant protein induced an immunological response and that the antiserum had a high level of specificity. Based upon these results, this antiserum was deemed suitable to characterize the structure, molecular mechanism, and functional involvement of the VP26 protein in the DPV life cycle.

Previous studies of the herpes simplex virus (HSV) UL35 protein of the subfamily Alphaherpesvirinae have documented that the UL35 gene acts as the real late gene  $\gamma_2$ , which encodes a small nucleocapsid protein that is located on the hexon of the nucleocapsid. This protein interacts with the viral DNA of the nucleocapsid. It is nonessential for the formation of the viral capsid and viral propagation in cell culture, but it is essential for viral



**Fig. 7.** SDS-PAGE analysis of the purified rabbit anti-His6-tagged VP26 IgG. M = Protein molecular weight marker; 1 = rabbit anti-His6-tagged VP26 IgG obtained by ammonium sulfate precipitation; 2 = rabbit anti-His6-tagged VP26 IgG obtained by ion exchange column chromatography.

replication in the nervous system. Furthermore, the absence of this protein will result in a 100-fold reduced yield of infectious virus in the trigeminal ganglion [36–38]. This protein is located at the exterior of the icosahedral capsid, which indicates that VP26 may serve as a core that connects the capsid with the external tegument and envelope in the late stage of viral assembly [37]. The sites of this protein that are exposed on the outside of the capsid also reveal that VP26 is in a favorable position to couple

the capsid and its functional ligand, such as tegument [39]. Similarly, research on the homolog of VP26 suggests that the smallest capsid protein, which is located at the most exterior region of the capsid, may play an important role in the tegumentation and/or uncoating of the virion in the course of infection and in the regulation of the interaction between the capsid and tegument as well as cytoskeletal proteins [40]. Recent studies have also revealed that VP26 of HSV-1 associates with ribosomes and may regulate the host cell translation [41]. Based upon the important role played by HSV VP26 during HSV infection, we suggest that VP26 of DPV may play a similar role in the course of DPV infection. Importantly, the exact roles of DPV VP26 remain undefined.

## Conclusion

In conclusion, the UL35 gene that encodes VP26 was amplified by PCR from the genome of the DPV CHv strain. It was cloned, and the recombinant protein was

expressed and purified from *E. coli*. Western blot analyses suggest that the polyclonal antibody raised against the recombinant protein reacted with the purified recombinant protein, indicating that this specific antibody may serve as a good tool for future studies of VP26, e.g. analyses of subcellular localization, and may permit elucidation of the structure, molecular mechanism, and functional involvement of VP26 in the DPV life cycle.

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