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Development and Comparison of Enzyme Immunoassays for Diagnosis of Chagas' Disease Using Fixed Forms of *Trypanosoma cruzi* (Epimastigotes, Amastigotes, and Trypomastigotes) and Assessment of Antigen Stability for the Three Assays

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Three enzyme immunoassays (EIAs) for diagnosis of Chagas' disease were developed with fixed forms of *Trypanosoma cruzi* using a panel of 435 sera from the following groups: Venezuelan subjects positive by immunofluorescence ($n = 70$), Venezuelan healthy controls ($n = 85$), healthy Canadians ($n = 166$), and subjects with other parasitic diseases ($n = 114$). All assays achieved 100% sensitivity and reasonable specificity for amastigotes (97.6%), epimastigotes (98.3%), and trypomastigotes (99.3%). The fixed-trypomastigote assay was stable over 4 months at 4°C and room temperature. These data suggest that a fixed-trypomastigote EIA may be a suitable candidate for blood bank screening.

American trypanosomiasis, or Chagas' disease, is caused by the protozoan flagellate *Trypanosoma cruzi* (8). It is estimated that 16 to 18 million people are infected by *T. cruzi* in Latin America (22, 33).

A major effort to interrupt transmission in rural South America during the last 2 to 3 decades has dramatically altered the epidemiology of Chagas' disease (1, 20, 23, 35). At the present time, the major route for transmission in many regions in which Chagas' disease was previously endemic is blood transfusion. The migration of large numbers of asymptomatically infected individuals in recent decades has extended the risk of transfusion-associated Chagas' disease to all parts of the developed world (15, 16, 18, 19, 25, 34). To our knowledge, no jurisdiction in the developed world routinely screens blood and blood products for Chagas' disease.

Although many screening methods have been evaluated, the enzyme immunoassay (EIA) format has significant attractions in terms of its simplicity, low cost, and potentially excellent sensitivity (37). During the last 30 years, many different EIA-based assays using a wide range of antigens have been developed (2, 5, 7, 9, 10, 12–14, 24, 27, 28, 31, 36). Several studies have reported particularly promising results with fixed whole organisms (i.e., integral antigens) (5, 6, 10, 13, 14, 21). However, theses studies have been relatively small and have focused on epimastigote-form antigens.

Since the human immune system is exposed almost exclusively to trypomastigote- and amastigote-form antigens, we sought to determine which integral antigen preparation of *T. cruzi* offered the best combination of sensitivity and specificity for the diagnosis of American trypanosomiasis. We also assessed the stability of the fixed-form EIA under conditions of possible use (i.e., long-term storage at various temperatures).

We used a panel of 435 sera to evaluate the candidate assays. The subjects included Venezuelan blood donors ($n = 70$) defined as positive by immunofluorescence, indirect hemagglutination, and EIA. Negative controls included 85 Venezuelan blood donors and 166 nontraveling Canadian residents. Specificity was assessed by using sera from 114 subjects with other parasitic diseases: leishmaniasis ($n = 20$), ascariasis ($n = 6$), facioliasis ($n = 8$), malaria ($n = 23$), toxoplasmosis ($n = 17$), trichinosis ($n = 11$), filariasis ($n = 8$), cysticercosis ($n = 8$), and schistosomiasis ($n = 13$).

***T. cruzi* antigens.** Epimastigotes (Tulahuen and Brazil strains) were grown in LIT broth (11). Parasites were washed three times in 1 M phosphate-buffered saline (PBS; pH 7.4) and fixed in 2% formaldehyde (Fisher Scientific, Fair Lawn, N.J.)–PBS (pH 7.4) for 1 h at 37°C. To obtain trypomastigote and amastigote forms, Vero cell monolayers (ATCC CCL-81) were infected with epimastigotes and supernatants were harvested at either 7 or 15 days, respectively (7, 21). Harvested amastigotes were placed in LIT medium at 37°C for a further 48 h. Final purification was achieved with a column (17) or gradient (4) before formaldehyde fixation (1.5% trypomastigotes and 2% amastigotes) as described above. Organisms were counted in a Neubauer chamber, and final antigen preparations were produced by mixing the Tulahuen and Brazil strains 50/50. Antigens were stored in aliquots at −20°C until used. Morphology (Giemsa-stained preparations) was used as the principal criterion for assessing antigen purity: epimastigotes, 100% (yield, 1×10^8 to 1×10^9 per ml); trypomastigotes, 97%

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(yield, 0.6×10^7 to 7×10^7 per ml), and amastigotes, 100% (1×10^8 to 1×10^9 per ml) (data not shown).

Polystyrene 96-well plates (Immulon 2; Thermo Labsystems, Franklin, Mass.) were coated overnight with (per well) 100 μl of fixed *T. cruzi* antigen (10^6 epimastigotes, 10^5 amastigotes, or 10^5 trypomastigotes per ml) at 4°C in 1 M sodium carbonate buffer (pH 9.6). Plates were incubated with blocking buffer (PBS–5% bovine serum albumin–0.1% Tween [Sigma]) for 1 h at 37°C. Sera were diluted 1:400 in blocking buffer, added in duplicate to wells (100 μl/well), and incubated for 1 h at 37°C. The assays were completed with horseradish peroxidase-conjugated goat anti-human immunoglobulin G for 30 min at 37°C (100 μl/well; Perkin-Elmer Life Science, Boston, Mass.) and then with 3,3′, 5,5′-tetramethylbenzidine (100 μl/well) for 10 min at room temperature. The reaction was stopped with IN sulfuric acid (H_2SO_4; 50 μl/ml) and read at 450 nm (Titertek Multiskan MCC/340; Labsystem and Row Laboratories, Finland). Results from the healthy control sera were used to establish cutoff values that yielded optimal sensitivity and specificity for each assay.

After assay optimization, negative control values were low and tightly clustered in all three fixed-organism EIAs. Mean optical density (OD) values for the Canadian negative control sera were 0.09 ± 0.07, 0.07 ± 0.03, and 0.09 ± 0.05 for the epimastigote, trypomastigote, and amastigote assays, respectively. Sera from Venezuelan negative controls had significantly higher background noise in the amastigote ($P < 0.03$), trypomastigote ($P < 0.0001$), and epimastigote ($P < 0.0001$) assays. Among the healthy control sera, a linear model indicated a significant difference among the three antigens ($P < 0.0001$). Tukey's post hoc tests revealed the following order from least to most reactive: trypomastigote < epimastigote = amastigote (Table 1). As expected, ODs for sera from subjects with other parasitic diseases were more variable: epimastigote, 0.09 ± 0.19; trypomastigote, 0.07 ± 0.17; and amastigote, 0.12 ± 0.21. For this group, the linear model indicated that the ODs did not differ among antigens ($P = 0.14$). The mean ODs for the known positive sera were 1.98 ± 0.32, 1.65 ± 0.42, and 1.79 ± 0.49 for the epimastigote, trypomastigote, and amastigote assays, respectively. Analysis of variance showed significant differences between the mean ODs among the three assays ($P < 0.0001$). Tukey's post hoc tests revealed the following order from most to least reactive: epimastigote > trypomastigote = amastigote (Table 1).

Cutoff values between ODs of 0.20 to 0.40 yielded excellent sensitivity (100%) with a range of specificities: epimastogote, 86.2 to 98.3%; amastigote, 84.8 to 97.6%; and typomastigote, 93.8 to 99.3% (Table 1). When specificity was assessed at a cutoff OD of 0.40, the trypomastigote-based assay performed best with both healthy patient sera (100%) and sera from patients with other parasitic diseases (98.2%). The presumed false-positive reactions (five, two, and seven false-positive results for the epimastigote, trypomastigote, and amastigote assays, respectively) occurred with sera from subjects with known protozoan or helminth infections and one healthy control (Table 1).

Our first hypothesis—that a simple EIA based upon fixed, whole organisms could be sensitive, specific, and practical for field application—proved to be correct. Using readily accessible materials and straightforward techniques, we were able to generate large numbers of essentially pure epimastigote, trypomastigote, and amastigote forms. These assays performed well with our panel of sera, confirming the earlier observations of several groups (5, 10, 13, 14). As noted above, these early studies focused almost exclusively on epimastigote antigens. Only Araujo and Guptill (6) tested amastigote forms using a small number of positive and negative sera, and no sera from subjects with other parasitic diseases were included.

The second of our initial hypotheses—that fixed amastigote or trypomastigote antigens would be superior to epimastigote antigens in the EIA format, proved to be largely incorrect. The trypomastigote-based assay was slightly better than the other two assays in terms of specificity, and this advantage was accentuated at lower arbitrary cutoff values (Table 1). These data suggest that there is extensive sharing of antigens among the three forms of the parasite (3). It is also possible that the in vitro culture systems used by us and by others do not generate the antigenic diversity that may exist in the different forms in natural infection (26, 32). We found it relatively simple to generate large numbers of morphologically homogeneous trypomastigote forms, and the diagnostic potential for trypomastigote antigens has not been well studied. Indeed, we could

TABLE 1. ODs and specificity estimates for serum groups in EIAs based on formalin-fixed *T. cruzi* amastigote, epimastigote, or trypomastigote forms

Serum group (*n*)	Mean OD ± SD (range)[a]	% Specificity at OD cutoff:	
		0.20	0.40
Healthy Canadian subjects (166)			
Amastigote	0.09 ± 0.05 (0.025–0.45)	97.6	99.4
Epimastigote	0.09 ± 0.065 (0.034–0.55)	92.8	99.4
Trypomastigote	0.07 ± 0.03 (0.03–0.31)	99.4	100
Healthy Venezuelan blood donors (85)			
Amastigote[b]	0.12 ± 0.06 (0.055–0.25)	90	100
Epimastigote	0.121 ± 0.04 (0.06–0.26)	95.3	100
Trypomastigote	0.108 ± 0.03 (0.07–0.29)	97.65	100
Subjects with other parasitic diseases (114)			
Amastigote	0.12 ± 0.21 (−0.04–2.040)	65.8	94.7
Epimastigote	0.085 ± 0.19 (−0.045–1.86)	75.4	96.5
Trypomastigote	0.07 ± 0.17 (−0.03–1.64)	85.1	98.2
Chagas' disease-positive Venezuelan blood donors (70)			
Amastigote	1.79 ± 0.5 (0.832–2.77)		
Epimastigote	1.98 ± 0.32 (1.143–2.62)		
Trypomastigote	1.65 ± 0.42 (0.65–2.28)		

[a] Values are expressed as means ± standard deviation (SD). The following numbers of presumed false-positive reactions occurred using an OD cutoff of 0.40: amastigote-based assay, four subjects with cutaneous leishmaniasis, one with ascariasis, one with fascioliasis, and one who was healthy; trypomastigote-based assay, one subject with cutaneous leishmaniasis and one with schistosomiasis; and epimastigote-based assay, two subjects with cutaneous leishmaniasis, two with malaria, and one who was healthy.

[b] Only 10 sera from healthy Venezuelan blood donors were tested in the amastigote-based assay.

only find a single trial that used a crude trypomastigote extract antigen in an EIA format (7).

Since antigen stability and assay consistency are major concerns for many of the reported Chagas' disease assays, we sought to determine the stability of the fixed trypomastigote assay over time under realistic storage conditions. Half of the wells of EIA plates were precoated and stored with desiccant sachets at 4°C or room temperature for up to 4 months. At monthly intervals, the unused wells were coated with frozen stock trypomastigote antigen, and the EIA procedure outlined above was completed for precoated and freshly coated wells in parallel. Using multiple replicates of pooled negative and positive sera, we observed excellent maintenance of reactivity without upwards drift in the negative controls over the 4-month period. Indeed, the positive samples had generally higher absorbance values in the precoated wells than in the freshly coated wells on plates stored either at 4°C (2.39 ± 0.235 versus 1.52 ± 0.326, respectively; $P < 0.006$) or at room temperature (2.13 ± 0.169 versus 1.60 ± 0.234; $P < 0.0001$), respectively. Compared with the freshly coated wells, there was also less variation in the precoated wells stored at either 4°C (coefficient of variation [CV] for positives, 21.5 versus 9.8; CV for negatives, 87.0 versus 23.6) or room temperature (CV for positives, 14.6 versus 8; CV for negatives, 88.7 versus 19.4). Similar assay stability has recently been reported for up to 12 months and at temperatures up to 50°C by Rebeski et al. in a whole, fixed *Trypanosoma congolense* EIA (29, 30).

Several limitations of the present study should be acknowledged. Most important, the positive sera used were known to be reactive in a battery of other serodiagnostic tests. As a result, our estimates for assay sensitivity are almost certainly artificially high. This fact may also have contributed to the very high signal/noise ratios we observed. Although several of the presumed false-positive sera were obtained from subjects with cutaneous leishmaniasis, no visceral leishmaniasis samples were included in our panel of samples from subjects with other parasitic diseases. Finally, two different *T. cruzi* strains were used to generate the antigens we used, but all of the Chagas' disease-positive sera were obtained from a single geographic region.

In conclusion, we believe that formalin-fixed, whole-organism antigens have several advantages compared with most other antigen preparations reported to date for use in the EIA format. These include ease of preparation and quantification (e.g., hemocytometer) and stability for prolonged periods of time at a range of temperatures. We have shown that fixed, whole-organism EIAs based on epimastigote, trypomastigote, or amastigote forms of *T. cruzi* can have excellent sensitivities and specificities. These assays were reproducible and robust under various storage conditions. The trypomastigote-based test had advantages in terms of specificity in our initial evaluation. As with all novel assays for *T. cruzi*, the performance of these assays in the field will determine their eventual clinical utility.

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