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USE OF MICRO-ELISA FOR QUANTITATING ANTIBODY TO *TRYPANOSOMA CRUZI* AND *TRYPANOSOMA RANGELI**

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Abstract. Of 229 residents of a Panamanian village where both *Trypanosoma cruzi* and *Trypanosoma rangeli* are endemic, 52% had antibody to one or both species by the enzyme-linked immunosorbent assay (ELISA), 26% were positive by complement fixation, 17% were positive by direct agglutination, and 32% were positive on the basis of clinical impression. Although the sensitivity of ELISA makes it the procedure of choice for sero-epidemiologic studies, there does appear to be some serologic cross-reactivity between the two species. Sera from 55 Panamanians and 33 Bolivians were reactive with antigens of *T. cruzi* and *T. rangeli*, thus making definitive diagnosis by serologic methods impossible. Although the presence of antibodies to both species suggests serologic cross-reactivity, the possibility of dual infection must be considered also. Fifty-four Panamanians and 20 Bolivians had antibody to only one species.

Various modifications of the enzyme-linked immunosorbent assay (ELISA), originally described by Engvall and Perlmann,[1] have been used to demonstrate the antibody response in trypanosomiasis.[2–4] The usefulness of ELISA for measurement of antibodies to *Trypanosoma cruzi* has been established by Voller et al.[4] These authors demonstrated that the sensitivity and specificity of ELISA were equal to that of indirect immunofluorescent microscopy (IFA) and superior to that of complement fixation and direct agglutination. Their assessment, however, was carried out in the laboratory, using sera of predetermined reactivity by IFA and sera from patients with established xenodiagnoses. The present study was designed to evalute the ELISA under field conditions, and to determine whether or not the occurrence of *Trypanosoma rangeli* infection in the study population results in false positive assays.

MATERIALS AND METHODS

Study population and serum collection

The study area was a Panamanian village where both *T. cruzi* and *T. rangeli* are endemic. Two hundred and twenty-nine residents participated in the study. Sera from 52 confirmed cases of Chagas' disease, obtained from the Instituto Nacional de Laboratorios de Salud, La Paz, Bolivia, served as our positive controls. Negative control sera were from 13 United States military personnel in the Canal Zone.

For the evaluation of use of the ELISA under field conditions, blood was collected from finger punctures with the aid of non-heparinized capillary tubes and filter paper discs. The tubes were spun in an autocrit centrifuge connected to a portable generator and they were broken at the serum-cell interface. The serum, approximately 50 μl, was expelled into a tube containing 500 μl of phosphate buffered saline (PBS), pH 7.4, containing 0.5% Tween 20 and 0.02% sodium azide (PBS-Tween). Whole blood dried on the filter paper-discs was extracted in PBS-Tween to achieve a final dilution of 1:10.

Conjugate

The conjugate was the globulin fraction of a goat anti-human globulin serum labeled with alkaline phosphatase. Methods for preparation and determination of working strength have been described by Voller et al.[5]

Antigens

Antigens were prepared from strains of *T. cruzi* and *T. rangeli* isolated from cases of human infection in Panama. Epimastigotes from 10- to 13-day-old stock cultures were inoculated into a di-

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TABLE 1

ELISA titers for antibody to T. cruzi *and* T. rangeli *in 52 positive and 13 negative control sera*

T. cruzi	*T. rangeli* <10	10	20	40	80	Total
<10	13	0	0	0	0	13
10	1	0	0	0	0	1
20	2	0	1	0	0	3
40	1	0	0	0	0	1
80	1	0	2	2	0	5
160	5	2	4	2	1	14
320	5	1	5	3	1	15
640	3	1	1	1	1	7
≥1,280	1	1	2	2	0	6
Total	32	5	15	10	3	65

TABLE 2

Clinical diagnosis vs. antibody detection by ELISA for identification of infections with T. cruzi *(Tc) and* T. rangeli *(Tr) among Panamanian villagers*

Clinical diagnosis*		ELISA†				
Tc	Tr	Tc+ Tr−	Tc− Tr+	Tc+ Tr+	Tc− Tr−	Total
+	−	0	0	12	0	12
−	+	1	32	3	22	58
+	+	1	0	2	0	3
−	−	9	21	38	88	156
Total		11	53	55	110	229

* Based upon clinical symptomatology and positive complement fixation test for *T. cruzi*, established by positive xenodiagnosis for *T. rangeli*.
† Positive defined as ELISA titer ≥1:10.

phasic culture medium.[6] The parasites were harvested at maximum growth phase following procedures outlined by Vattuone and Yanovski,[7] and were lyophilized. The dry residue was reconstituted in carbonate-bicarbonate buffer, pH 9.6, at a concentration of 200 mg/ml. Working strength of the antigen was determined by titrating positive and negative control sera in wells coated with serial dilutions of antigen (50–1,000 μg/ml). The highest dilution of antigen which gave maximum titer with the positive control serum but which produced no coloration with the lowest dilution or the negative control serum was used as the working strength.

ELISA

Wells of polystyrene microtiter plates (Linbro MVC-98) were filled with 200 μl of antigen diluted in carbonate-bicarbonate buffer, pH 9.6. After an overnight incubation at 4°C, the plates were washed three times with PBS-Tween. Two hundred microliters of test serum, diluted to 1:10 in PBS-Tween 20, was added to a single well. One plate could be used to screen 96 sera. The plates were incubated at room temperature for 2 hours and then washed an additional three times. Two hundred microliters of conjugate, diluted 1:800 in PBS-Tween, was added to each well and incubation was continued for 2 hours at room temperature. Plates were washed three more times and 200 μl of para-nitrophenyl phosphate in 10% diethanolamine buffer (5 mg/ml) was added. After 15 min at room temperature the reaction was stopped by the addition of 50 μl 3M NaOH. Intensity of color development was assessed visually as 0, 1+, 2+, 3+ or 4+. All sera exhibiting a reaction of ≥2+ were considered positive and were titrated, in 200-μl volumes, through dilutions of 1:10 to 1:1280. PBS-Tween was used as the diluent.

For the evaluation of ELISA in the field, plates were coated with antigen in the laboratory and transported to the village in a container of wet ice. The field version was exactly the same as described above but plates were incubated at ambient temperature.

Ancillary tests

Methodologies of direct agglutination (DA) and complement fixation (CF) for quantitating antibody to *T. cruzi* have been described elsewhere.[7,8] In addition to serologic tests, xenodiagnosis[9] and microscopic examination of blood smears were used in establishing the clinical diagnosis.

RESULTS

Specificity and sensitivity of the ELISA were optimal when the conjugate was used at a dilution of 1:800 and the antigens were used at 1:3,000 and 1:200 for *T. cruzi* and *T. rangeli*, respectively. These dilutions of antigen represent 30 μg protein/well in the *T. cruzi* assay and 120 μg protein/well in the *T. rangeli* assay.

The distribution of ELISA titers for the 52 positive control sera and the 13 negative controls is given in Table 1. Although 33 of the positive controls had antibody to both species, the *T. cruzi* titer was higher in all but one case.

Of the 229 Panamanian villagers, 119 had antibody to either one or both species. Results are

TABLE 3

Distribution of ELISA titers for antibodies to T. cruzi *and* T. rangeli *in sera of Panamanian villagers*

T. cruzi	*T. rangeli*								Total
	<10	10	20	40	80	160	320	≥640	
<10	162	32	19	8	7	3	0	0	231
10	4	1	4	2	1	0	0	0	12
20	2	3	6	3	2	1	1	1	19
40	2	0	5	5	3	4	0	0	19
80	3	7	4	5	1	0	2	0	22
160	2	2	0	1	2	1	3	0	11
320	3	0	0	0	3	3	0	0	9
≥640	0	0	1	0	2	0	0	1	4
Total	178	45	39	24	21	12	6	2	327

compared to clinical impressions in Table 2. Although there were no false negative assays for *T. cruzi*, 14 of the 15 cases confirmed by clinical diagnosis also had antibody to *T. rangeli*. Of the 61 cases of *T. rangeli* confirmed by xenodiagnosis, 22 were false negative in the ELISA and six had antibody to *T. cruzi*. Three of these six cases were confirmed as dual infections. The ELISA picked up 68 infections with either one or both species which had been missed clinically. Distribution of titers for 327 sera representative of the 229 villagers is given in Table 3. Sixteen sera reacted with *T. cruzi* only, 69 sera were specific for *T. rangeli*, 80 sera had antibody to both species, and 162 were negative.

The specificity of ELISA is compared to that of the more conventional techniques of CF and DA in Table 4. When an ELISA titer of ≥1:10 was considered definitive evidence of infection with *T. cruzi*, 45 sera were false negative by CF and 72 sera were false negative by DA. Conversely, 22 and 30 sera were false positive by CF and DA, respectively. When clinical impression was considered definitive evidence of infection, two villagers were false negative by CF and 12 villagers were false negative by DA (Table 5).

No serious problems were encountered in our attempts at performing the ELISA in the field. Results of field specimens are compared to results of the laboratory assay in Table 6. There were some minor fluctuations in titers, but of the 27 villagers who participated discrepancies of positivity occurred in only two instances. Case no. 7 was negative for *T. cruzi* in the field assay, but had a 1:20 titer in the laboratory version. Case no. 8 was negative for *T. rangeli* in the field assay but positive at 1:20 in the laboratory.

DISCUSSION

Xenodiagnosis is the most specific method for identification of humans infected with *T. cruzi*. Nevertheless, this technique is useful only during a transient state of disease, and it is laborious, time-consuming, and dangerous. Furthermore, xenodiagnosis requires facilities for maintaining colonies of the vector and it is not suited for large scale epidemiologic surveys involving both human and animal populations. If control of *T. cruzi* infection, either through vector control or immunoprophylaxis, is to become a reality, we must devise a means for identifying foci of disease and for assessing the immune status of the indigenous population. This means must be specific, sensi-

TABLE 4

Comparative specificities of ELISA, complement fixation (CF), and direct agglutination (DA) for detecting antibody to T. cruzi

ELISA	CF+ DA+	CF+ DA−	CF− DA+	CF− DA−	Total
+	12	39	12	33	96
−	0	22	30	179	231
Total	12	61	42	212	327

TABLE 5

Clinical diagnosis vs. antibody detection by complement fixation (CF) and direct agglutination (DA) for identification of infections with T. cruzi

Clinical diagnosis	CF+ DA+	CF+ DA−	CF− DA+	CF− DA−	Total
+	2	11	1	1	15
−	9	38	26	141	214
Total	11	49	27	142	229

TABLE 6

Comparison of ELISA titers of assays performed in the field with titers of assays performed in the laboratory

Serum no.	Field assays				Laboratory assay by phlebotomy	
	Capillary blood		Filter paper			
	T. cruzi	*T. rangeli*	*T. cruzi*	*T. rangeli*	*T. cruzi*	*T. rangeli*
1	40	20	160	20	40	40
2	10	10	20	10	10	40
3	10	10	10	10	40	40
4	80	80	160	40	40	160
5	40	40	40	20	80	40
6	<10	40	<10	20	<10	80
7	<10	40	<10	10	20	40
8	20	<10	10	<10	40	20
9	20	<10	20	<10	10	<10
10	20	40	10	20	40	80
11	160	40	20	20	320	160
12–27	<10	<10	<10	<10	<10	<10

tive, inexpensive, simple to perform, and capable of handling large numbers of sera under the most adverse environmental conditions. The ELISA, as described, shows the most promising potential for fulfilling these requirements.

The controlling elements of specificity and sensitivity in all assays designed to detect circulating antibody are the quality and quantity of the antigen. In the ELISA, use of insufficient amounts of antigen in the coating process results in false positive assays. Similarly, coating wells with crude antigens, such as saline soluble extracts of whole organisms, increases the chance of serologic cross-reactivity. This cross-reactivity occurs as a consequence of shared antigens between related or unrelated species and results in false positive assays. Therefore, it is paramount that we examine the study population for concurrent medical problems which may influence the specificity of the assay.

Although *T. rangeli* is reported to be nonpathogenic for man, it does occur in many of the same areas of Central and South America where *T. cruzi* is endemic. Since other investigators have reported that these two species share common antigens and cross-react serologically,[10] we must contend with the possible occurrence of false positive *T. cruzi* assays as a result of infection with *T. rangeli*. In the study population of 229 Panamanian villagers, 55 persons had antibody to both species. Only three of these cases were confirmed, clinically, as instances of dual infection. It is possible that demonstration of either one or both species was missed by xenodiagnosis, since this technique is dependent upon parasites circulating in peripheral blood. Thirty-three Bolivian sera, from confirmed cases of Chagas' disease, had antibody to *T. rangeli*. However, with the exception of one case, the *T. cruzi* antibody titer was at least 2-fold higher. Unfortunately, we do not know the incidence or distribution of *T. rangeli* infections in that country.

If we accept seropositivity to both species as a consequence of cross-reactivity, rather than dual infection, we must explain why 11 Panamanians and 19 Bolivians had antibody to *T. cruzi* only. *T. cruzi* presents itself as a very potent immunogen and elicits a profound antibody response to both major type specific antigens and minor group specific determinants.[11,12] If some of these minor determinants are structurally similar to determinants by *T. rangeli*, we can expect the appearance of cross-reacting antibody at some time during the course of disease. Other investigators have reported that the antibody response early in the course of Chagas' disease is directed toward an antigen specific for *T. cruzi*.[13] Apparently, nonspecific antibodies to other species, including *T. rangeli*, occur only after a prolonged antigenic challenge associated with chronic disease. On the other hand, *T. rangeli* appears to be a poor immunogen. Of 58 confirmed cases, 22 showed no evidence of an antibody response (Table 2). In most cases where a measurable response did occur, it appeared to be directed toward major species-specific determinants. Antibody to minor determinants shared by *T. cruzi* occurred in only four of the confirmed cases.

In spite of this problem of false positive assays, the ELISA, by virtue of its superior sensitivity, must be considered preferable to CF and DA. The CF test was positive in 13 of 15 cases with a clinical diagnosis of *T. cruzi* (Table 5). However, when the ELISA result was considered definitive of infection, there were 45 false negative CF tests (Table 4). Since most of these sera were from individuals who had been CF-positive 1–3 years earlier, we knew that we were dealing with chronic cases and we suspected that circulating antibody had fallen below the level measurable by the less sensitive CF technique. Of the 22 sera which represented false positive CF tests, all were from villagers who either had a positive xenodiagnosis for *T. rangeli* or who had antibody to *T. rangeli* by ELISA. Thus, the CF test, in addition to having an inferior sensitivity, is also less specific than the ELISA.

The DA procedure was positive in only three of the 15 confirmed cases of Chagas' disease. When the ELISA result was considered definitive of disease, there were 72 false negatives and 30 false positives. The false negatives were attributed to chronic infections with a decline in circulating antibody below the level of sensitivity of the DA method. The false positives converted to negative following treatment with 2-mercapto-ethanol and were considered the consequence of nonspecific agglutinins.

In contrast to CF, DA, and xenodiagnosis, ELISA is readily adaptable for performance in the field under the most adverse environmental conditions. In 27 duplicate sera, assayed in the field and in the laboratory, there were only two discrepancies with respect to positivity. Minor differences in titer could be attributed to errors in preparation of the initial 1:10 dilution when working with the capillary tube and filter paper specimens.

In summary, a good diagnostic test for Chagas' disease is a top priority need in rural health centers throughout Central and South America. The ELISA, which has a sensitivity for detection of humoral antibody at the nanogram level appears to be the most promising assay for fulfillment of this need. The technique is economical and it can be performed in the most primitive of environmental conditions. Additionally, the ELISA can be adapted to monitor the immune status and frequency of infection in animals of endemic areas. The major problem facing ELISA is one of specificity and, as with any immunodiagnostic assay, its applicability is limited whenever results are influenced by concurrent medical problems. Since the controlling element of specificity of the ELISA is the antigen, we must direct our efforts toward development of assays which use species-specific, or even strain-specific, antigens.

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