

EVALUATION ON SEROLOGIC TESTS FOR STUDIES ON CHAGAS' DISEASE¹

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Findings of high antibody prevalence rates to Chagas' disease in Oaxaca, Mexico (up to 79 per cent positive in an adult sample from one community) prompted studies directed at evaluating the tests used, the antibody detected, and the practice of collecting and storing blood specimens on filter paper. This article reports the results of these studies.

Introduction

A 1969 seroepidemiologic survey for parasitic antibodies in the State of Oaxaca, Mexico, detected antibody against *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, in 29% of the serum samples from one part of the state (1). This initial study was later expanded by indirect hemagglutination (IHA) testing of 4,023 persons residing in 60 communities located within a Pacific coastal zone of Oaxaca State. In a companion paper (2) we reported finding antibody prevalence rates that were remarkably high for Mexico, ranging up to a finding of 79% seropositive reactors among a sample of adults tested in one community.

¹ Also appearing in Spanish in the *Boletín de la Oficina Sanitaria Panamericana*, 1979.

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In order to better understand the origin and significance of the antibody found, three serologic tests for *T. cruzi* antibody, as well as cross-reactions involved in these tests, were evaluated in the laboratory. Using *T. cruzi* as antigen, specimens were subjected to two different paired tests and the serologic results were compared. The paired tests used were IHA and complement fixation (CF), IHA and direct agglutination (DA), and CF and DA. Cross-reaction in the IHA test for Chagas' disease were evaluated by testing antigens prepared from *T. cruzi*, *T. rangeli*, and *Leishmania mexicana* against sera from persons who had parasitologically confirmed *T. cruzi* and *T. rangeli* infections. In addition, filter paper blood eluates and sera were tested and compared in order to determine the effectiveness of filter paper strips for collecting and storing blood specimens in the course of field studies.

Material and Methods

Blood specimens were obtained by venipuncture without using anticoagulants. After clotting and retraction, specimens were centrifuged and processed aseptically, and the resulting sera were stored at -20° to -70°C. Filter paper blood samples were obtained by pricking each subject's finger and saturating both sides of a circular area

printed on a piece of filter paper.⁶ Efforts were made to collect the blood without squeezing the fingertip. The filter papers were air-dried at ambient temperature for 24 hours. During the field work (up to four weeks) they were stored at ambient temperature in airtight plastic bags containing a drying agent, silica gel. Thereafter, until processed in the laboratory for antibody (8-12 months after collection), the papers were retained in the airtight bags at 4°C.

Blood was subsequently eluted from the filter papers as follows: a 1 cm disc was cut out of the blood-filled circle with a paper punch. Each disc was placed in a 15 x 85 mm tube, and 0.2 ml of phosphate-buffered saline (PBS) at pH 7.2 was added. The discs were kept immersed in the saline solution between one and two hours at room temperature. Before a disc was removed from the tube, residual fluid was expressed from the disc by squeezing the disc against the side of the tube with a metal rod.

To compare IHA titers from sera with IHA titers from filter paper eluates, the same venous blood specimen used to prepare a serum was used to saturate a filter paper circle; such matched samples were obtained from the blood of 128 Oaxaca residents in 1971. The reproducibility of IHA test results was evaluated by retesting 44 of these filter paper blood eluates.

For comparison of the IHA and CF test, 150 sera were tested that had been collected in Oaxaca in 1969-1970. Results of the IHA and DA tests were compared by titrating 98 filter paper blood eluates collected in Oaxaca in 1972. The CF and DA tests were compared by examining 67 sera obtained in Oaxaca in 1973.

Comparative tests for antibodies to *T. cruzi* and *T. rangeli* were made with sera

from 27 patients in Chile who had parasitologically confirmed Chagas' disease; with sera from 12 persons in Panama who had parasitologically confirmed *T. rangeli* infections; with 41 sera from Mérida, Mexico; and with 140 of the sera obtained in Oaxaca in 1969-1970. Comparative tests for antibodies to *T. cruzi* and *L. mexicana* were made with 85 sera collected in Oaxaca in 1969-1970.

Preparation of the Antigens

The *T. cruzi* and *T. rangeli* antigens were saline extracts of lyophilized epimastigotes of *T. cruzi* and *T. rangeli*, respectively; these epimastigotes were delipidized with benzene before extraction (1). The *T. rangeli* antigen was titrated against sera from rabbits that had been immunized with lyophilized *T. rangeli*. The antigen dilution giving the highest titers was used to test the sera described.

The *L. mexicana* antigen used in the DA test was made according to the method described for preparing *T. cruzi* antigen for this test (3). The antigen suspension consisted of formalin-fixed *L. mexicana* promastigotes that had been trypsinized. Three sera from patients with parasitologically confirmed *L. mexicana* infections were tested with this antigen and yielded titers $\geq 4,096$.

The serologic methods used in this study for the IHA (1), CF (1), and DA tests (3) have previously been described.

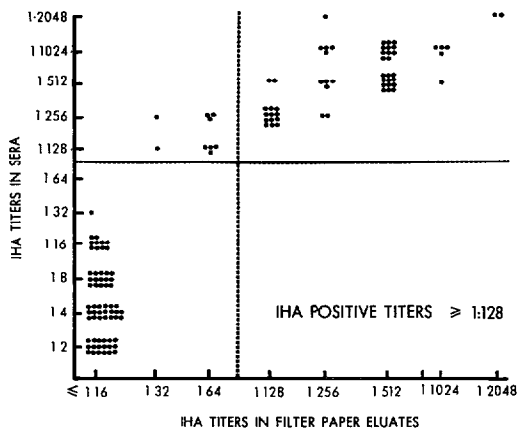
Results

Comparison of IHA Titers Obtained with Paired Filter Paper Blood Eluates and with Sera

Figure 1 depicts the IHA titers obtained by testing paired filter paper and serum specimens obtained from 128 people against *T. cruzi* antigen. Each pair of specimens consisted of a serum and a filter paper

⁶Ropaca No. 1023, Rochester Paper Company. (Use of trade names is for identification purposes only and does not constitute endorsement by the U.S. Public Health Service or the U.S. Department of Health, Education, and Welfare.)

Figure 1. A scatter diagram of indirect hemagglutination (IHA) titers obtained with 128 paired blood specimens: Serum titers plotted against filter paper eluate titers.



eluate prepared from the same venous blood sample. Since a filter paper eluate in 0.2 ml of buffer is considered equivalent to a 1:8 dilution of serum, filter paper titers below 8 cannot be detected in the IHA test. Nearly all filter paper eluate titers of 64 through 256 were one or two twofold dilutions below the corresponding serum titers. The differences in titers between paired specimens decreased, however, as the concentration of antibody increased; so that filter paper titers above 256 showed increasing agreement with serum titers. With a titer ≥ 128 taken to indicate a positive response, 55 specimens (43%) were positive by both methods, and 64 (50%) were negative

by both methods, resulting in 93% agreement between the methods. If a filter paper titer ≥ 64 had been considered positive, the titers for only two specimens would have been in disagreement, both specimens being negative by the filter paper method and positive by the serum method.

Reproducibility of IHA Results

Comparative findings obtained by titrating 44 filter paper eluates twice with *T. cruzi* antigen are shown in Table 1. Of the paired results, the titers of 34 specimens (77%) were the same or within one twofold dilution; eight specimens (18%) showed a difference of two twofold dilutions; only two specimens (5%) showed differences greater than two twofold dilutions. These results are within the range of acceptable laboratory variation.

Comparison of IHA and CF Tests

One hundred and fifty sera were tested against *T. cruzi* antigen with both of these methods (see Table 2). With CF titers of 8 or greater considered positive, 86 sera (57%) yielded a positive CF response. With IHA titers of 128 or greater considered positive, 91 sera (61%) yielded a positive response. Overall, 121 of the 150 sera were positive or negative by both test methods, resulting in 81% agreement between the two techniques.

Table 1. Reproducibility of the results of indirect hemagglutination (IHA) tests using filter paper eluates and *T. cruzi* antigen.

	Differences, in number of twofold dilutions, between the two titers obtained by testing the same eluates twice against <i>T. cruzi</i> antigen.				Total No. of eluates
No. of twofold dilution differences	0	1	2	> 2	
No. of filter paper blood eluates	19	15	8	2	44

Comparison of IHA and DA Tests

The titers obtained by testing 98 filter paper blood eluates with the IHA and DA techniques, using *T. cruzi* as antigen, are shown in Table 3. With titers of 128 or greater in both tests considered positive, only two of 42 specimens positive in the IHA test yielded negative results. Overall, 96 of the 98 sera were positive or negative by both test methods, so that there was 98% agreement between the two techniques.

Comparison of CF and DA Tests

Sixty-seven sera were tested by the CF and DA methods, using *T. cruzi* as antigen (Table 4). With a CF titer of 8 or greater considered positive, 48 of the 67 sera (72%) showed a positive CF response. In the DA tests, titers of 128 or greater were considered

Table 2. Comparison of indirect hemagglutination (IHA) and complement fixation (CF) results obtained by testing 150 sera with *T. cruzi* antigen.

		CF test ^b		Total
		+	-	
IHA test ^a	+	74	17	91
	-	12	47	59
Total		86	64	150

^aPositive IHA test: titer \geq 128.

^bPositive CF test: titer \geq 8.

Table 3. Comparison of indirect hemagglutination (IHA) and direct agglutination (DA) results obtained by testing 98 filter paper eluates with *T. cruzi* antigen.

		DA test ^b		Total
		+	-	
IHA test ^a	+	40	2	42
	-	0	56	56
Total		40	58	98

^aPositive IHA test: titer \geq 128.

^bPositive DA test: titer \geq 128.

Table 4. Comparison of complement fixation (CF) and direct agglutination (DA) results obtained by testing 67 sera with *T. cruzi* antigen.

		CF test ^b		Total
		+	-	
DA test ^a	+	42	9	51
	-	6	10	16
Total		48	19	67

^aPositive DA test: titer \geq 128.

^bPositive CF test: titer \geq 8.

positive, and 51 of the 67 sera (76%) yielded positive results. Overall, 52 of the 67 sera were positive or negative by both test methods, resulting in 78% agreement between the two techniques.

Cross-Reactivity between *T. cruzi*, *T. rangeli*, and *L. mexicana* Antigens

Comparison of *T. cruzi* and *T. rangeli* antigens: When tested by IHA, using *T. cruzi* as antigen, sera from 27 Chilean patients with Chagas' disease confirmed by xenodiagnosis all yielded titers of 128 or greater. These sera were also tested with an antigen prepared from *T. rangeli*. The IHA titers to *T. rangeli* ranged from 2 to 128, with most reacting at titers of 16 and 32. Only one of the sera showed a titer of 128, which is considered the lower limit for a positive titer against *T. rangeli* in the IHA test.

Twelve sera from children in Panama with parasitologically confirmed *T. rangeli* infections were tested by IHA, using both *T. cruzi* and *T. rangeli* antigens; all 12 yielded negative results.

Sera from 41 subjects in Mérida, Mexico, that yielded negative titers with the *T. cruzi* antigen also showed negative titers with the *T. rangeli* antigen. The *T. cruzi* titers were 16 or less, and the *T. rangeli* titers were 8 or less.

In addition, 140 of the sera obtained in Oaxaca in 1969-1970 were tested by IHA

with *T. cruzi* and *T. rangeli* antigens (Table 5). Some reactivity to *T. rangeli* antigen was found among the 70 sera positive with *T. cruzi* antigen, the titers with *T. rangeli* ranging from 4 to 256. All of the titers obtained with the *T. rangeli* antigen, however, were lower than those obtained with the *T. cruzi* antigen, and only four sera in this group (3%) yielded positive results with both antigens. All 70 of the samples seronegative with *T. cruzi* antigen were also seronegative with *T. rangeli* antigen.

Comparison of *T. cruzi* and *L. mexicana* Antigens: Eighty-five of the sera collected in Oaxaca in 1969-1970 were tested with *T. cruzi* antigen by IHA and with the *L. mexicana* antigen by DA (Table 6). Nine sera (11%) yielded positive titers with both *T. cruzi* and *L. mexicana* (128 or higher), and 33 were found negative in both tests. Of the remaining sera, 41 yielded positive results with *T. cruzi* but negative results with *L. mexicana*, and two were negative with *T. cruzi* but positive with *L. mexicana*.

Discussion

The U.S. Center for Disease Control (CDC) has used the IHA test in seroepidemiologic studies of parasitic diseases for a number of years because of the test's effectiveness in measuring the prevalence of antibodies to several parasitic antigens in

Table 6. Comparison of indirect hemagglutination (IHA) and direct agglutination (DA) results obtained by testing 85 Oaxaca sera against *T. cruzi* by IHA and against *L. mexicana* by DA.

		DA test ^b (<i>L. mexicana</i>)		Total
		+	-	
IHA test ^a (<i>T. cruzi</i>)	+	9	41	50
	-	2	33	35
Total		11	74	85

^aPositive IHA test (*T. cruzi*): titer \geq 128.

^bPositive DA test (*L. mexicana*): titer \geq 128.

large numbers of specimens. The IHA test has previously been used for seroepidemiologic studies of Chagas' disease in Brazil (4), Colombia (Kagan, unpublished), and Oaxaca, Mexico (1).

The use of filter papers greatly facilitates the collection and storage of blood samples in the field, because taking blood from a finger prick is much more acceptable to the population than venipuncture, and because transporting filter paper blood specimens is much easier than transporting sera, since filter papers do not require refrigeration. As already noted, in order to evaluate the filter paper method for field studies of Chagas' disease, we compared IHA titers in pairs of sera and filter paper blood samples from 128 persons. Except at the highest antibody concentrations, where the titers were about equal, titers from the filter paper eluates tended to be one or two fold dilutions lower than those from the sera. Overall, however, the observed loss in sensitivity was only 7% when titers of 128 or greater were considered positive. This indicates that collecting and transporting blood on filter papers can be a useful technique—and one well-suited to procurement of accurate results—when employed in epidemiologic studies for the detection of IHA antibodies to *T. cruzi*. Other investigators report that specimens of dried blood on filter paper have been of practical value for detecting Chagas' disease antibody by

Table 5. Comparison of indirect hemagglutination (IHA) results obtained by testing 140 Oaxaca sera with *T. cruzi* antigen and *T. rangeli* antigen.

	<i>T. rangeli</i> antigen ^b		Total
	+	-	
<i>T. cruzi</i> antigen ^a	+	66	70
	-	70	70
Total		136	140

^aPositive IHA test (*T. cruzi*): titer \geq 128.

^bPositive IHA test (*T. rangeli*): titer \geq 128.

IHA (5), CF (6), and indirect immunofluorescence (7, 8, 9).

The reproducibility of the IHA titrations was high; 77.3% of the titers obtained were either the same or within one twofold dilution, and 95.5% were within two twofold dilutions.

The IHA test for Chagas' disease is considered as sensitive or more sensitive than the CF test, the standard against which newer tests are evaluated. However, the IHA test is thought to give a larger (though still small) percentage of false positive reactions (10). In our first Oaxaca study (1), 73% of the results for 83 sera examined concurrently by both IHA and CF tests were in agreement; that is, the results of both tests were either negative or positive. In the study described here the overall agreement for 150 sera was 77%. Some of the results with specimens in these studies that yielded positive reactions by IHA but negative ones by CF may have been due to false positive reactions, or to the IHA test detecting antibodies that had persisted longer than those detected by CF.

The high correlation between the IHA and DA test results was unexpected. The fact that all but two of the 42 sera with a positive IHA titer also yielded a positive DA titer suggests that the DA test may be a useful serologic procedure for detecting antibodies to *T. cruzi*. However, the finding that 18% of the sera with a positive DA titer were negative in the CF test suggests the possibility of false positive DA reactions.

The specificity of the antibody detected in the Oaxaca sera with *T. cruzi* antigen was evaluated by testing the sera with antigens prepared from two other infectious agents, *L. mexicana* and *T. rangeli*. Dermal leishmaniasis (Chiclero's ulcer of the ear) caused by *L. mexicana* is found in some parts of southern Mexico, although it has not been found in the Pacific coastal region of Oaxaca. *T. rangeli* infections have not been reported from Mexico in man, ani-

mals, or vectors; but the parasite has been found as far north as Guatemala in *Rhodnius prolixus* (11). The distribution of *T. rangeli* is thought to be coterminous with the distribution of its principal vector, *R. prolixus*. Therefore, since *R. prolixus* has been in Oaxaca, *T. rangeli* may later be found in that area.

The data in this study support the evidence presented in the relatively few other studies concerning serologic cross-reactions between *T. cruzi* and *T. rangeli* (11, 12, 13, 14, 15). These studies indicate that *T. rangeli* infections in man do not elicit antibodies that cross-react with *T. cruzi*; yet, conversely, infection in man with *T. cruzi* produces antibodies that do cross-react at low levels with *T. rangeli*. Our study of 12 sera from subjects with parasitologically confirmed *T. rangeli* infections did not detect any antibody to either *T. rangeli* or *T. cruzi*. However, one of 27 sera from subjects with parasitologically confirmed *T. cruzi* infections yielded a titer of 128 against *T. rangeli* antigen, and sera from four subjects that yielded positive titers with *T. cruzi* antigen also yielded positive titers with *T. rangeli* antigen. To evaluate these cross-reactions further, cross-absorption techniques should be carried out with immune sera from animals infected with each of the parasites, as well as with sera from people who have had naturally acquired infections.

The findings of both this report and our 1972 study (1) are consistent with the experience of others indicating that serologic cross-reactions occur between *T. cruzi* and some *Leishmania* strains (16). In our previous study none of the 52 sera positive with *T. cruzi* antigen yielded positive results when tested with the nonindigenous *Leishmania* strain *L. donovani*. In the study described here, however, where *L. mexicana* antigen was used in the DA test, nine of the 50 sera that were positive with *T. cruzi* were also positive with *L. mexicana* antigen (Table 6). Of the 35 sera that yielded a negative

reaction with *T. cruzi* antigen, two yielded a positive response in the DA test with *L. mexicana*. Because the presence of *L. mexicana* infection in the area of Oaxaca where the sera had been collected cannot be ruled out, the reactions obtained from some or all of the 11 sera that showed positive titers with *L. mexicana* antigen may reflect the presence of antibodies to *L. mexicana*.

In the DA test for leishmaniasis with an antigen prepared from *L. donovani*, a titer of 32 or greater is considered positive (17). Further studies of sera from persons with parasitologically confirmed *L. mexicana* infections are needed so that the sensitivity and specificity of serologic tests for *L. mexicana* can be evaluated.

ACKNOWLEDGMENTS

The field studies reported here were conducted under a program for collaborative research between Benito Juárez University of Oaxaca and the University of California (San Francisco). Research was supported in part by grants from the National Institutes of Health, U.S. Public Health Service—A1-10051 (University of California International Center for Medical Research) and HD-06922.

The authors wish to thank Drs. Fernando Galindo, Mario Pérez Ramírez, and Hugo Sarmiento Díaz of Benito Juárez University, and Gerardo Varela of the Institute of Health and Tropical Diseases in Mexico City for their help and encouragement.

SUMMARY

A comparative evaluation has been made of three serologic tests used for epidemiologic studies of Chagas' disease in Oaxaca, Mexico. The results of this evaluation, concerned with the techniques of indirect hemagglutination (IHA), direct agglutination (DA), and complement fixation (CF), were as follows:

The reproducibility of IHA test results was demonstrated by testing the same sera twice by IHA against *Trypanosoma cruzi* antigen. The results of these tests showed 95% agreement. In addition, 81% agreement was obtained when blood samples were tested against *T. cruzi* by IHA and CF. Similar testing of samples by IHA and DA yielded 98% agreement, while 78% agreement was obtained with samples tested by DA and CF.

When *T. rangeli* antigen was used, sera tested by IHA showed few or no positive results. Cross-reactivity was found in 11% of 85 sera tested against *Leishmania mexicana* antigen by DA and against *T. cruzi* by IHA.

Sera from 128 subjects were tested against *T. cruzi* antigen by IHA, and the results were compared to those obtained using eluates from filter paper strips containing blood specimens from the same subjects. Agreement between the two sets of results was obtained in 93% of the cases, indicating that collecting and transporting blood specimens on filter paper can serve as a useful and accurate technique in epidemiologic studies concerned with IHA antibodies to *T. cruzi*.

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