FIELD USE OF THE DOT-ELISA TEST FOR VISCERAL LEISHMANIASIS IN HONDURAS[1]

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INTRODUCTION

Serologic tests are universally accepted as an indispensable tool for diagnosing visceral leishmaniasis at health care facilities. Additionally, serodiagnosis is the most valuable single procedure in any surveillance program, since it permits rapid screening of large populations in remote areas without clinical evaluation, yields incidence data for the population, and provides an efficient means of early case detection *(1)*.

Several types of tests with a generally acceptable degree of reliability have been used as an aid in the diagnosis of visceral leishmaniasis. These include complement fixation, both with a nonspecific acid-fast bacterial antigen *(2)* and with a homologous parasite antigen *(3)*; direct agglutination *(4)*; and indirect fluorescent antibody *(5)*. Recently, the enzyme-linked immunosorbent assay (ELISA) utilizing soluble antigen has also been found effective *(6, 7)*.

However, efficient utilization of serodiagnostic tests in endemic areas is often difficult because the tests must be performed in a central laboratory, while the patients live in remote areas with poor means of transport and communication. Consequently, serologic methods are generally available only for those patients who come to a major hospital. In many cases this delays diagnosis for a hazardously long time, because the patient is not seen at a medical facility with adequate diagnostic capabilities until he is gravely ill. The development of serologic tests using dried blood specimens on filter paper has provided some improvement in this respect, particularly with regard to seroepidemiologic studies, since it has simplified the taking and processing of specimens and has eliminated the need for refrigeration during transport. However, extremely few primary health care facilities have this diagnostic service available from a central laboratory.

[1] This article will also be published in Spanish in the *Boletín de la Oficina Sanitaria Panamericana*, 1986.

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A test that could be performed at the village level without any laboratory facilities whatever, a true "field test," would be of inestimable value for improved patient care and for epidemiologic surveillance in endemic areas. At present, even when serodiagnostic services are available, the delays encountered between taking the specimen and obtaining a test result are considerable; and when a positive result is obtained from a patient who lives in a remote village that can be reached only by several hours travel on foot or horseback, the responsible physician or health authority is then faced with the problem of locating the patient and getting him to a medical facility that can confirm the diagnosis, which causes still further delays. So if testing could be done at the time the specimen was originally taken, the test would be of much more value; and, in the case of positive reactions, arrangements for overcoming transport or similar problems could be made immediately, so as to get the patient to a health care facility for confirmatory biopsies and, if necessary, immediate treatment.

Such a field test should have the following characteristics: (1) It should require only a small volume of blood or serum (an amount obtainable without venipuncture). (2) It should be inexpensive. (3) It should utilize materials and equipment that are not easily damaged or broken and that are easily transportable on foot or horseback. (4) It should require no electricity or facilities that may be unavailable at villages in developing countries. (5) It should not require closely controlled temperatures. (6) It should be read visually. (7) It should produce results within a few hours.

The Dot-ELISA technique of Pappas et al. *(8–9)* is a micro-ELISA utilizing a suspension of *Leishmania donovani* promastigotes dotted onto nitrocellulose filter discs as antigen. It is visually read, inexpensive, and portable; in addition, its sensitivity and specificity are equivalent to the standard ELISA and superior to the CF test *(9)*. Because this test seemed to meet the essential requirements and to be adaptable for field use, it was selected for a preliminary trial in a known endemic area in Honduras *(10–11)*.

The isolated rural localities where the work was performed are representative of those where the disease is encountered in the New World. The work thus constitutes a realistic trial under difficult conditions such as are likely to be encountered in other endemic areas.

MATERIALS AND METHODS

The Localities and Subjects Tested

Previously diagnosed visceral leishmaniasis patients who could be located provided a basis for selecting localities and subjects for testing. The 305 persons tested were classified into four groups as follows:

Group I consisted of nine former visceral leishmaniasis patients between four and eight years of age, who had two to seven years previously been parasitologically diagnosed and treated with meglumine antimoniate (Glucantime®).

Group II was made up of 45 family members of these patients (44 siblings,

one mother), who constituted a high-exposure group.

Group III included a total of 244 children, ranging from six months to eight years of age, who resided in these and nearby localities and who were selected by random sampling done at clinics, schools, and homes.

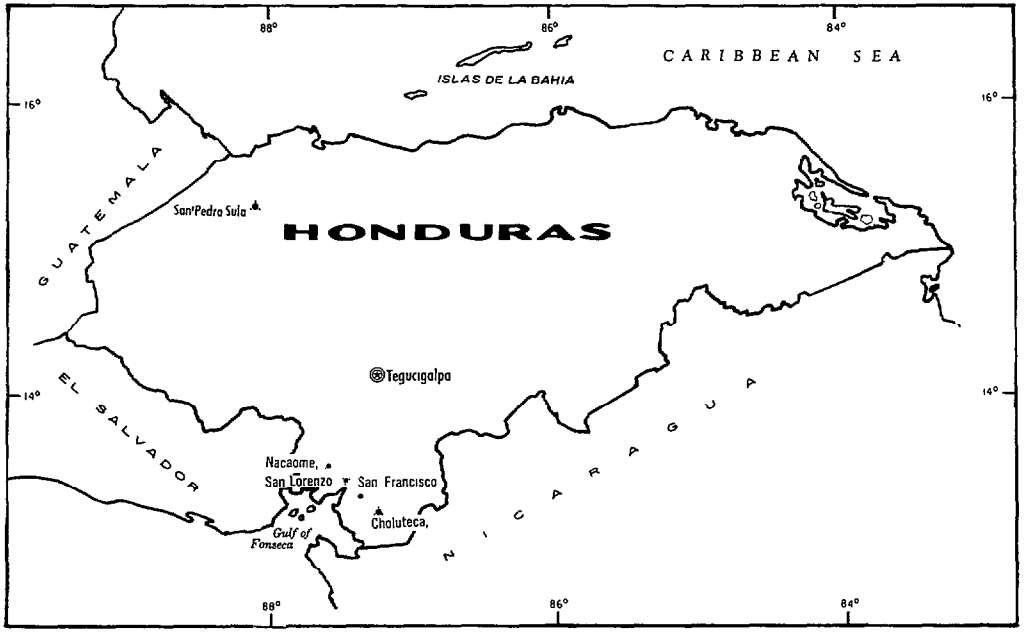
Group IV consisted of subjects with other hemoflagellate infections, including three children with parasitologically diagnosed cases of extensive and long-standing cutaneous leishmaniasis involving multiple disseminated lesions and four subjects with serologically diagnosed Chagas' disease.

Most of the localities involved were in the Southwestern section of the country, in a known endemic area (Figure 1). Six were in the municipality of San Francisco de Coray and three were in the municipality of Choluteca. Various small localities in the municipalities of Nacaome and San Lorenzo were also represented; and a few samples were also taken in Colonia Estados Unidos in the Capital (Tegucigalpa), where one former patient infected in San Francisco de Coray was residing.

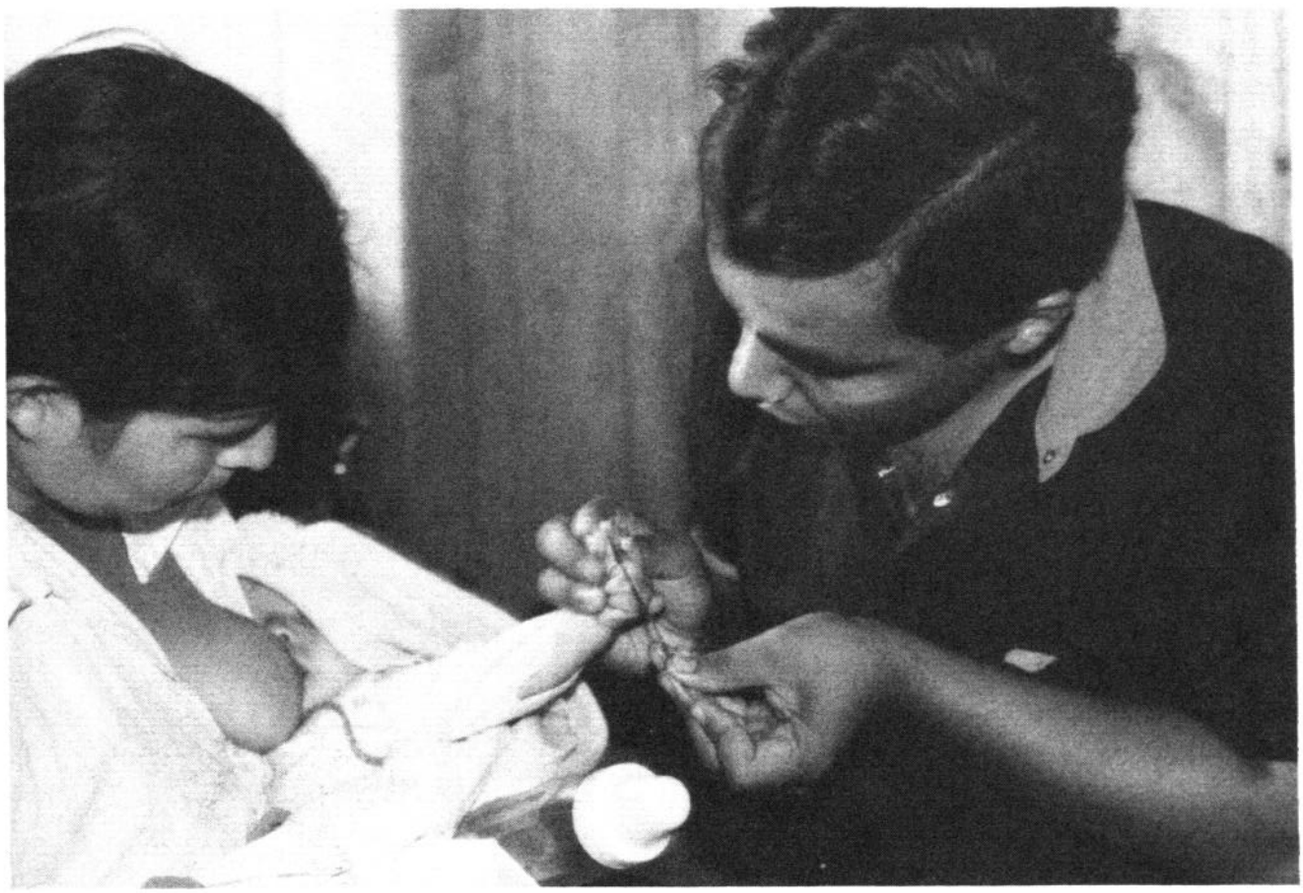
Blood Samples

Finger-prick blood samples (see photograph) were collected in 0.1 ml capillary tubes, after which the open end was sealed with plasticine modeling clay and the tube was immediately shaken down to remove any air bubbles. The specimens were kept at the ambient temperature for one to four hours to hasten coagulation and clot retraction, and were then stored on wet ice until processed.

FIGURE 1. A map of Honduras showing the location of the study areas.



One of the authors (Dr. Sierra) taking a capillary blood specimen from a young child. Capillary blood specimens obtained by finger-prick are much easier to obtain from young children than are venous blood specimens.



To extract the serum, the tube was nicked with a file and broken off at the bottom, just above the plasticine plug. The clot usually adhered to the plug and could be extracted intact while the tube was held nearly horizontal, leaving essentially clear serum in the tube. Any red cells remaining did not interfere with the test.

The sera were diluted with phosphate-buffered saline containing 1% bovine serum albumin. The plastic tip of a micropipette was inserted into the end of the capillary tube, and .01 ml of serum was removed. This was placed in .15 ml of diluent in a microtiter plate well (to achieve an initial 1:16 dilution), and .05 ml of this latter solution was transferred to a test well containing an antigen disc (see below) and an equal volume of diluent to provide a final dilution of 1:32 for the screening test.

Antigen Preparation

Dot-ELISA antigen preparation has been described in detail elsewhere *(8)*. Antigen was prepared at Walter Reed from *L. donovani* (WR 311) promastigotes, which were dotted on nitrocellulose filter paper in 1 μl volumes (2.5×10^4 parasites). These "dots" were placed in the center of 6 mm diameter circles marked on the filter paper, and

antigen discs were cut out with a punch as needed in the field.

The Test Procedure

The Dot-ELISA was performed in standard 96-well flat-bottom microtiter plates, essentially in the manner described by Pappas et al. *(8)*, the only significant modification being the use of pH 7.4 phosphate-buffered saline (PBS) in place of triethanolamine-buffered saline (TBS). The PBS was transported as a 10X concentrate to conserve space and weight, and was diluted with distilled water in Honduras. Regarding storage in the field, the antigen, bovine serum albumin, control sera, conjugate, and substrate were kept in an insulated container and were protected against high temperatures with wet ice or other coolants. However, the recommended temperature of 4°C was seldom maintained because of high ambient temperatures that at times reached 30–34°C. The tests were conducted at ambient temperatures that reached 28–30°C.

The shaking of the microtiter plates during the various reactions was accomplished manually by moving the plates with a rotary motion in small circles on the surface of a table, instead of with the mechanized shaker described in the original account of the procedure. Antigen discs were cut from the nitrocellulose papers and were placed in the wells of the microtiter plates just before each test run.

The test procedure can be summarized as follows:

1) Blocking: Add .075 ml of 5% BSA[4] in PBS to each well containing an antigen disc. Shake the microtiter plate for one minute, incubate for 30 minutes, and aspirate off.

2) Antibody reaction: Place 0.1 ml of test serum (diluted 1:32 in PBS-1% BSA) in well, shake for one minute, incubate for 30 minutes, aspirate off.

3) Washing: Wash discs by adding 0.1 ml of .05% NP-40[5] in PBS. Shake for one minute and aspirate. Repeat twice, then allow the solution to stand for 10 minutes on the third wash.

4) Conjugate reaction: Add .05 ml of affinity-purified horseradish peroxidase-conjugated goat antihuman IgG (gamma chain-specific),[6] optimally diluted 1:100 in PBS-1% BSA to each well. Shake for one minute, incubate for 30 minutes, and aspirate. Wash three times, as above.

5) Substrate development: Add .05 ml of the precipitable substrate 4C1N[7] to each test well, shake for one minute, incubate for 30 minutes, and aspirate. Wash two times in PBS (without BSA) and a third time in distilled water. Aspirate and air-dry.

Microvolumes of sera and other reagents were measured with an adjustable-volume pipette[8] with plastic disposable tips. However, the volumes of the wash solutions were not critical, and were not precisely measured; they were delivered into the wells with a pasteur pipette, estimating the 0.1 ml required for washing. All the solutions were withdrawn from the wells with a pasteur pipette connected to a 20 ml syringe by a length of quarter-inch diameter rubber surgical tubing, an arrangement that facilitated aspiration of the wells in rapid succession.

[1] Bovine serum albumin, fraction V, Sigma Chemical Co., St. Louis, Missouri, USA.

[5] Nonidet P-40 (a nonionic surfactant), Calbiochem-Behring Corp., La Jolla, California, USA.

[6] Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland, USA.

[7] 4-chloro-1-naphtol, a peroxide-chromogenic precipitable substrate (Kirkegaard and Perry Laboratories), was prepared by adding equal volumes of substrate solution and then diluting 1:3 in PBS.

[8] Gilson pipetman (.001 ml – 0.2 ml).

Results

Positive reactions appeared as blue-purple dots on the white nitrocellulose discs. In some instances the dots became more distinct as the filter-paper dried. There were virtually no equivocal reactions, the dots being either present or absent. Positive control sera were positive in all the runs, while negative control sera and reagent controls were unreactive.

There were significant differences in the rates of positivity of the four groups screened (Table 1). In Group I, eight of nine (88.9%) of the parasitologically diagnosed visceral leishmaniasis patients reacted positively at the screening dilution of 1:32. And in Group II, the family members with presumably increased exposure to infection, 13 of 45 (28.9%) reacted positively. In contrast, only 8 of 244 (2.3%) of the apparently healthy children from the general population in the area reacted positively.

A noteworthy number of positive responses were also obtained from the subjects with related protozoan infections (Group IV). That is, three out of four subjects with serologically diagnosed cases of Chagas' disease and one out of three children with extensive long-standing disseminated lesions of cutaneous leishmaniasis also yielded a positive response.

Sera from the visceral leishmaniasis patients in Group I, as well as sera from the cutaneous leishmaniasis and Chagas' disease patients in Group IV that responded positively, were then fully titrated to determine their end-points (Table 2). All of the nine previously diagnosed and treated visceral leishmaniasis patients in Group I were reactive at titers that ranged from 1:512 to 1:8,192. The one Group I serum that was inexplicably nonreactive in the screening test yielded an end-point titer of 1:1,024 when titrated. The reciprocal titers exhibited by sera of Group IV patients with other hemoflagellate infections ranged from 2,048 to 32,768. These latter titers were surprisingly high, especially since all had yielded titers of only 512 with homologous species antigen.

TABLE 1. Results of Dot-ELISA testing of the four groups screened for visceral leishmaniasis in Honduras.

Group	Diagnostic category	No. tested	No. positive	% positive
I	Subjects diagnosed as having visceral leishmaniasis and treated for it	9	8	88.9
II	Family members of treated subjects	45	13	28.9
III	Random sample of children in high-exposure areas	244	8	2.3
IV	Subjects with hemoflagellate infections:			
	1) Cutaneous leishmaniasis with multiple disseminated lesions	3	1	33.3
	2) Chagas' disease	4	3	75
Total		305	33	10.8

TABLE 2. End-point titers of sera from Group I and Group IV subjects that gave a positive screening test reaction.

Patient No.	Age (in years)	Sex	Clinical diagnosis[a]	Years since diagnosis	End-point titer
1	4	M	VL	2	8,192
2	5	F	VL	3	512
3	6	F	VL	4	4,096
4	6	F	VL	4	2,048
5	6	M	VL	4	2,048
6	7	F	VL	5	512
7	8	M	VL	5	4,096
8	8	F	VL	6	512
9[b]	8	M	VL	7	1,024
10	8	F	CL	>1	32,768
11	47	M	CD	NT[c]	4,096
12	63	M	CD	NT	4,096
13	60	F	CD	NT	2,048

[a] VL = visceral leishmaniasis; CL = cutaneous leishmaniasis; CD = Chagas' disease
[b] This subject yielded a negative reaction upon initial Dot-ELISA screening at a dilution of 1 32
[c] NT = Not treated.

DISCUSSION AND CONCLUSIONS

The localities where this trial was carried out appear representative of those where visceral leishmaniasis is found in the New World, and the conditions of the trial constituted a realistic testing under difficult conditions likely to be encountered in other endemic areas. The antigen discs and reagents proved to be stable over extended periods of time, and high ambient temperatures (up to 28°C) prevailing during the assay did not appear to adversely affect the results.

The degree of nonspecificity of the test, as indicated by the positive reactions seen in sera from one subject with multiple disseminated lesions of cutaneous leishmaniasis and from three of four subjects with Chagas' disease, is probably about the same as for any serologic test utilizing promastigotes as antigen. This specificity could possibly be improved by use of amastigotes as antigen, an improvement that has been demonstrated in the indirect fluorescent antibody test for cutaneous leishmaniasis *(13)*. However, attaining some improved specificity this way would not be of great practical importance, since the purpose of serologic screening is to select individuals for definitive clinical evaluation. In the case of both cutaneous leishmaniasis and Chagas' disease, early diagnosis is beneficial, and the differential diagnosis is not difficult. Hence, from the point of view of primary medical care in developing countries, the incidental diagnosis of Chagas' disease should be regarded as a serendipitous advantage rather than a drawback.

The low number of positive reactions among the general population in Group III was probably due in part to the use of IgG-specific HRP-conjugated

second antibody, which has been shown to reduce the number and titers of false positive reactions due to related protozoan diseases and interfering hyperglobulinemia (primarily IgM) observed in populations residing in the tropics *(9)*. It is not possible at this time to determine whether the positive reactions in Group III represented clinically inapparent infections with visceral leishmaniasis, or were due to subclinical Chagas' disease or other related protozoan infections.

The nine Group I subjects (all with past visceral leishmaniasis infections that had been treated two to seven years before) had titers that were considerably lower than those encountered in active disease cases—the latter sometimes ranging up to 1:524,288 *(8)*. These lower titers probably resulted from declines in antibody levels after treatment, although the temporal response of antibodies to effective treatment is not yet known. The possibility that such long-lasting persistence of antibody indicates treatment failure cannot be ruled out.

A definitive diagnosis of visceral leishmaniasis can only be made by demonstration of the parasites, either by direct examination or by culture, usually of biopsy material from the bone marrow or spleen. Although splenic biopsy is now accepted as a safe and reliable procedure for diagnosis and assessing treatment efficacy *(14)*, it is not entirely without risk and normally should not be done outside a medical facility. It also requires skilled, medically qualified operators, and should be performed only when there is a strong clinical suspicion of infection.

Within this context, serology provides a means of screening that permits this relatively time-consuming and expensive biopsy procedure to be limited to those patients with a high probability of infection. Additionally, serodiagnosis is undoubtedly the most valuable single procedure in any program for surveillance of visceral leishmaniasis, since it permits screening of populations in remote areas with coverage of a large number of subjects in a short time without need of clinical evaluation.

The Dot-ELISA technique appears to be a practical and economical one for rapid screening at the village level. Only .05 ml of patient serum is required, an amount easily acquired by finger-prick. The test is cheap, costing less than five cents (US) per test well, and can be performed by personnel without a high level of technical training. All the material required can be transported easily on foot or horseback, and no electricity or facilities not readily available at the village level are required. We believe that this test can be of great value in areas endemic for visceral leishmaniasis anywhere in the world.

Acknowledgments

We are grateful for the unstinting support and assistance provided by Dr. Omar González, Chief, Division of Vector Control, Ministry of Health of Honduras, and by Dr. Hugo Villegas, Country Representative of the Pan American Health Organization, which made this trial possible. The Chagasic sera were kindly furnished by Dr. Carlos Ponce of the Central Laboratory of the Ministry of Health. The technical assistance of Mrs. Mary T. Nowicki and Mr. John Stiteler is gratefully acknowledged. Financial support was received from the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases.

Summary

The dot enzyme-linked immunosorbent assay (Dot-ELISA), a rapid, visually read microtechnique for serodiagnosis of human visceral leishmaniasis, was field-tested in a known endemic area in the Republic of Honduras. Of 305 individuals screened at a serum dilution of 1:32, positive reactions were observed in eight of nine parasitologically diagnosed visceral leishmaniasis patients who had received treatment, 13 of 45 family members of patients (a high-risk group), and eight of 244 randomly selected children in the endemic area. Cross-reactions were observed in one of three children with parasitologically confirmed cutaneous leishmaniasis and three of four adults serologically positive for Chagas' disease. End-point titrations performed on the visceral leishmaniasis sera gave reciprocal titers ranging from 512 to 8,192, which are lower than those usually encountered in untreated active cases.

This test does not require electricity, and all materials are easily transportable on foot. It is rapid, simple to perform, and inexpensive, yet sensitive and relatively specific under field conditions. It could prove to be a valuable tool for primary health care facilities and for epidemiologic surveys in the many endemic areas where no serologic testing capability currently exists.

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