Simple Standardized Enzyme-Linked Immunosorbent Assay for Human Antibodies to *Entamoeba histolytica*

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A simple solid-phase enzyme-linked immunosorbent assay procedure for the detection of human antibodies to *Entamoeba histolytica* was developed which showed a high degree of correlation with the agar gel diffusion, counterelectrophoresis, and indirect hemagglutination methods, as well as with clinical data. The enzyme-linked immunosorbent assay is rapid (1 h 15 min, total incubation time), and the reported values are referenced to a positive control so that they correlate with levels of antibody sufficient to be detected by the gel diffusion methods. The enzyme-linked immunosorbent assay is highly reproducible, specific, and sensitive; it can be used qualitatively or quantitatively.

The diagnosis of invasive amebiasis, particularly extraintestinal infections, is being increasingly assisted by serology because of the availability and improved reliability of immunoassay procedures. Serological tests may play an important role in the diagnosis of clinical amebiasis including abscess, and some cases would have been undetected if not for serology (6, 7, 10, 11, 19, 20, 25). It has even been recommended that all patients with inflammatory bowel disease have a serological test for amebiasis (13).

The agar gel immunodiffusion (AGD), counterelectrophoresis (CEP), and indirect hemagglutination methods stand out among those which are now well accepted for use in the serodiagnosis of amebiasis. Although indirect hemagglutination has been used most often, especially in epidemiological studies (3, 9, 12, 15), AGD and CEP are felt to be somewhat more reliable for clinical amebiasis (5, 14, 18, 20, 25; P. Kelly, H. Hicks, J. Wofsy, and G. Healy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, C158, p. 301). These immunoassays detect 90 to 100% of amoebic liver abscess, 80 to 100% of symptomatic intestinal amebiasis, and 10 to 50% of asymptomatic cyst passers (9, 15, 17). Recently, enzyme immunoassays (enzyme-linked immunosorbent assay; ELISA) using microtiter plates or plastic tubes as the antigen carrier have been reported for the detection of human antibodies to *Entamoeba histolytica* (1, 2, 22, 24, 26). Our laboratory has developed a simple standardized semiquantitative ELISA procedure in which the reported results are related to the presence or absence of *E. histolytica* antibodies at levels sufficient to be detectable by gel diffusion methods, a level which appears to be clinically sig-

nificant. The assay has an objective, numerical read-out and is rapid, with a total incubation time of 1h 15 min, and the *E. histolytica* antigen is covalently coupled to small plastic disks (8, 8a, 8b, 16). Assay results were strongly correlated with titers obtained by AGD and CEP methods.

MATERIALS AND METHODS

The NIH 200 and HK 9 strains of *E. histolytica* were separately grown axenically in Diamond TP-S-1 medium for 3 days (4). They were harvested, washed three times with cold phosphate-buffered saline (pH 7.2), suspended in 9 volumes of cold phosphate-buffered saline, pooled in equal amounts, and sonicated. After high-speed centrifugation (40,000 × g, 30 min), the clear supernatant containing soluble antigens was harvested, diluted with 0.1 M carbonate buffer at pH 9.6, and mixed overnight under constant gentle agitation with the carrier plastic disk, which contain surface isothiocyanate groups. The antigen-coated disks were then washed, lyophilized, and stored at 4°C in the presence of desiccant until used.

The positive control human serum was a pool of sera from four patients containing relatively high level of antibodies to *E. histolytica*, initially demonstrated by AGD and CEP. It also served as a standard reference for test results. The negative control was a pool of human sera nonreactive for antibodies to *E. histolytica* by AGD, CEP, and ELISA.

The test procedure, other reagents, and accessory equipment to facilitate the assay have been described elsewhere (16). All reagents were stored at 4°C and were ready for use. In brief, 5 μl of test specimen was diluted with 500 μl of specimen diluent (6% bovine serum albumin) and incubated with an antigen-coated disk at 37°C for 30 min. The disk was washed and incubated with 500 μl of an anti-immunoglobulin G and anti-immunoglobulin M calf intestinal alkaline phosphatase conjugate mixture at 37°C for 30 min. It

was again thoroughly washed, transferred to a clean vial, and incubated at 37°C with 1 ml of the enzyme substrate solution (p-nitrophenyl phosphate, 1 mg/ml) for 15 min. The enzyme activity was stopped with 0.1 ml of 3 N NaOH, and the absorbance was read in a Digital 405 Photometer (Cordis Laboratories, Miami, Fla.) at a wavelength of 405 nm. Substrate reaction mixtures reading above the limits of linearity of the photometer (1.8) were appropriately diluted with tris(hydroxymethyl)aminomethane-saline wash solution and reread to obtain the true absorbance value. For reporting test results, the absorbance reading of the test sample was divided by that of the positive control and multiplied by 100 to yield a "percent of positive control absorbance" value. Use of this simple standardized value minimized the variations of absorbance readings due to test conditions and reagents from day to day. Tests of 50 serum samples could easily be completed for reporting in less than 2 h.

AGD and CEP assays were carried out with the test sets distributed by Cordis Laboratories. For titrations, serum samples were tested in duplicate, using twofold serial dilutions in phosphate-buffered saline. At the end of the incubation period, as specified in the manufacturer's instructions for use, the gel plates were dialyzed against phosphate-buffered saline and stained with amido black for final readings of the antibody titers (reciprocal of the dilution). The indirect hemagglutination test was carried out with reagents purchased from the ICN Corp. (Irvine, Calif.).

Human patient serum samples for evaluation of the present ELISA were obtained from Tulane Medical Center, New Orleans, La., from the Centers for Disease Control, Atlanta, Ga., and from the University of Antioquia (Colombia). Normal serum was procured from our healthy laboratory personnel. All serum samples were stored in small aliquots at -20°C when not in use. Patients from whom samples had been obtained were diagnosed clinically, microscopically, serologically, or by response to drug treatment. Some had well-confirmed invasive amebiasis, others were suspected of having it, and still others served as controls, as detailed below.

Statistical analysis followed the formula described by Snedecor and Cochran (21). Negative results from gel diffusion titration assays were given the titer at one dilution level below the lowest level tested in the titration system (23). When the number of negative results was large in comparison with that of positive results, we also analyzed positive results by themselves.

RESULTS

Reproducibility. Tests of five samples in eight randomized replicates revealed satisfactory reproducibility of the ELISA, with an average coefficient of variation of 5.5% (Table 1). Tests of day-to-day reproducibility demonstrated the improved performance when results were reported as percent of positive control rather than as absorbance readings, particularly for those ELISA values greater than 15% (Table 2). At the low values, very small differences in

TABLE 1. *Within-run reproducibility of ELISA for antibodies to E. histolytica*[a]

Test speci-men	Absorbance		% of positive control		Coefficient of variation (%)[b]
	Range	Mean	Range	Mean	
Eh-89[c]	2.94–3.63	3.30	105–130	118	6.4
Eh-91[c]	1.39–1.49	1.45	50–53	52	2.6
Eh-93[c]	3.18–3.90	3.59	114–140	129	6.6
Eh-99	0.22–0.26	0.24	7.9–9.3	8.4	5.1
Eh-102	0.45–0.53	0.50	16–19	18	7.0

[a] Each test specimen was assayed in eight randomized replicates.
[b] Same for absorbance and percent of positive control.
[c] From amebiasis patients; the other two were normal subjects from an endemic area.

absorbance readings disproportionately increased the coefficient of variation for both values. As will be described below, values less than 15% occurred in normal healthy subjects and were well below the cut-off point considered as indicating clinically significant levels of specific antibody to E. histolytica.

Specificity. Specificity was first studied by absorption experiments which followed the procedures described previously (16). The diluted human serum samples (0.5 ml) were preincubated for 30 min at 37°C with 25 μl of extracts of E. histolytica or the other antigens, with phosphate-buffered saline as control, before assay with the ELISA. The results (Fig. 1) clearly showed that the E. histolytica antigen was the only preparation able to significantly reduce the ELISA values of positive samples, indicating the immunological specificity of the present assay for antibodies to E. histolytica. The Toxoplasma gondii and rubella viral antigen preparations used were shown to be very potent in absorption experiments which used disks coated with these antigens and human serum specimens containing their respective antibodies. The E. histolytica antigen was inactive in these parallel experiments. The Trichinella spiralis antigen used was a concentrated total larval extract incorporated into a diagnostic kit that used immunodiffusion (Cordis Laboratories), and was known to possess relatively high specific antigenic activity.

Correlations between the ELISA and other serological test results. Serum samples from 236 patients with confirmed or suspected amebiasis or other diseases and from normal controls were assayed by the ELISA, CEP, and AGD methods. This was done to determine the correlation between the titers obtained by the methods and to establish cut-off points in the ELISA procedure which would be associated with positive reactions in CEP and AGD. Based

TABLE 2. *Day-to-day reproducibility of ELISA for antibodies to E. histolytica*

Test specimen[a]	% of positive control (absorbance reading) on day:				Coefficient of variation (%)
	1	9	10	31	
Eh-93[b]	125 (3.54)	127 (3.05)	131 (3.66)	123 (3.46)	2.7 (7.7)
Eh-89[b]	115 (3.26)	115 (2.76)	111 (3.11)	113 (3.17)	1.7 (7.1)
Eh-91[b]	52 (1.48)	53 (1.28)	53 (1.48)	52 (1.45)	1.1 (6.8)
A	48 (1.37)	49 (1.19)	43 (1.21)	46 (1.29)	5.7 (6.5)
Eh-102[c]	20 (0.58)	19 (0.46)	19 (0.52)	19 (0.53)	2.6 (9.4)
B	17 (0.47)	16 (0.38)	15 (0.41)	16 (0.44)	5.1 (9.1)
Eh-99[c]	10 (0.27)	11 (0.26)	8 (0.23)	8 (0.23)	16.2 (8.3)
C	7 (0.19)	7 (0.17)	6 (0.17)	6 (0.18)	8.9 (5.3)
Negative control	10 (0.28)	9 (0.23)	8 (0.21)	7 (0.20)	15.2 (15.5)
Positive control	100 (2.84)	100 (2.40)	100 (2.79)	100 (2.81)	0 (7.7)

[a] In the order of decreasing ELISA values. A, B, and C are dilutions of a positive serum.
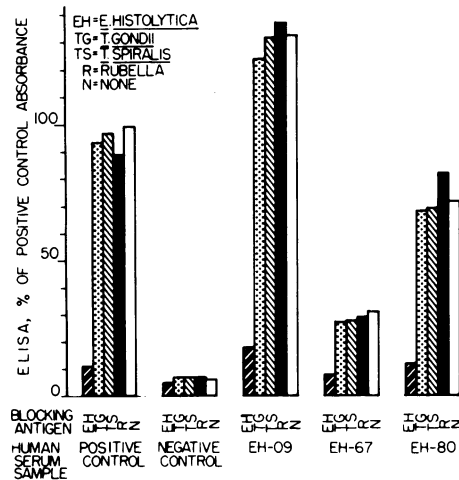[b] From amebiasis patients.
[c] From normal subjects in an endemic area.

FIG. 1. *Specificity of the ELISA assay for antibodies to E. histolytica. Five serum specimens were preincubated with E. histolytica, T. gondii, T. spiralis, rubella virus, or no antigen before being assayed by the ELISA procedure. Eh-09 and Eh-80 were from amebiasis patients. Eh-67 was from a normal subject in an endemic area.*

FIG. 2. *Comparison between the ELISA results and stained immunodiffusion (AGD) titer of 236 human serum samples. R_1, R_2 = Correlation coefficients of Pearson and Lee for all samples and for only AGD-positive samples, respectively.*

on these data (Fig. 2 and 3), ELISA values of ≥60% and <50% of the positive control absorbance were determined to be positive and negative, respectively, for significant antibody to *E. histolytica*. Values between these two levels were considered equivocal. Linear relationships were noted between the ELISA results on one hand and CEP and AGD titers of amoeba antibodies on the other. AGD showed greater sensitivity and higher correlation with ELISA than did CEP. All 151 of the samples negative by AGD were also negative by CEP, except 1. About 10% of samples negative by CEP showed positive reactions by AGD.

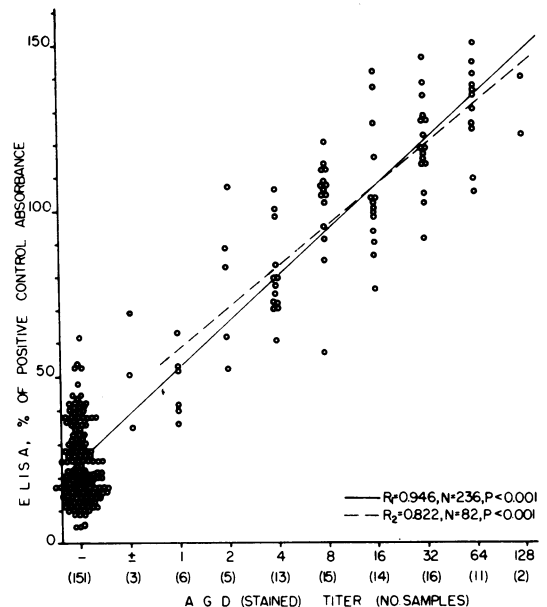Indirect hemagglutination assay titers of amoeba antibodies were obtained for 99 of these samples. Their correlation with the ELISA values was also statistically significant (Fig. 4), although not as high as those seen above with CEP and AGD. It has been suggested that indirect hemagglutination detects different immune systems from CEP and AGD for detection of amoeba antibodies (5, 18, 20, 25).

Test results with clinically well-documented specimens. Of 23 specimens obtained from the Centers for Disease Control and Tulane Medical School, 20 were collected from patients
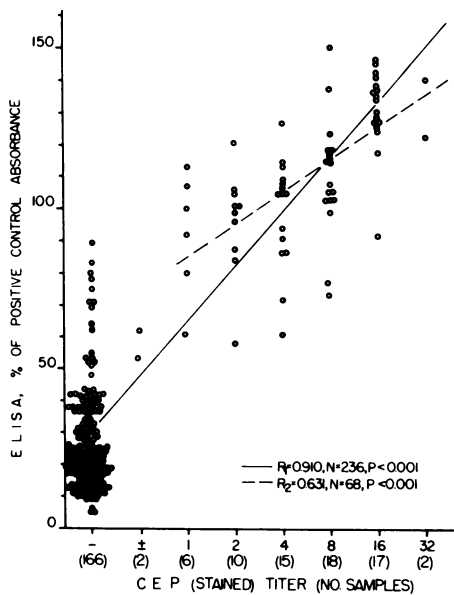
FIG. 3. Scatter diagram showing correlation between the ELISA results and CEP titers of 236 human samples for antibodies to E. histolytica. See Fig. 2 for explanation of R_1 and R_2.
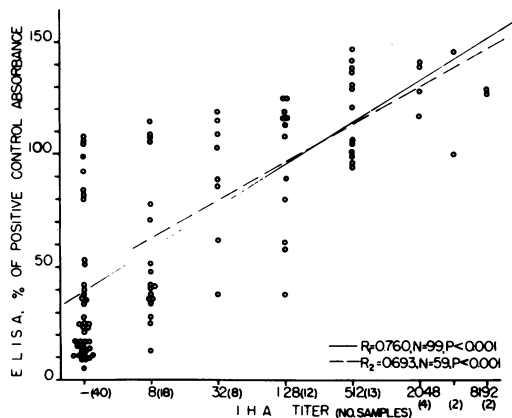

FIG. 4. Comparison between the ELISA results and indirect hemagglutination titer of 99 human serum samples for antibodies to E. histolytica. See Fig. 2 for explanation of R_1 and R_2.

who had clinically diagnosed liver abscess, dysentery, or both, which were confirmed microscopically, serologically, by drug treatment, or by a combination of these to be caused by E. histolytica. The remaining three were from normal healthy subjects who were further confirmed as serologically negative and served as controls. These 23 samples were coded and studied with results from ELISA, as well as from CEP and AGD, at various times of incubation as specified in the instructions to these test sets.

The correlations between the ELISA values and CEP and AGD titers were very high (Fig. 5), with correlation coefficients of 0.811 and 0.911, respectively (Fig. 5A and C). Essentially similar correlations were seen when CEP and AGD were examined after staining, except that the gel diffusion titers increased in many cases. All three specimens from normal healthy subjects were negative for specific antibodies to E. histolytica by ELISA. There was no significant difference in ELISA values among those with liver abscess only, dysentery only, and both diseases (Table 3). Reading of the CEP results immediately after the electrophoresis or 60 min later gave two- to fourfold lower titers in most cases and failed to detect 10% of the positive reactions, which appeared the following day.

In all the quantitative ELISA tests which our laboratories have developed with the same format, for other infectious and immunological dis-
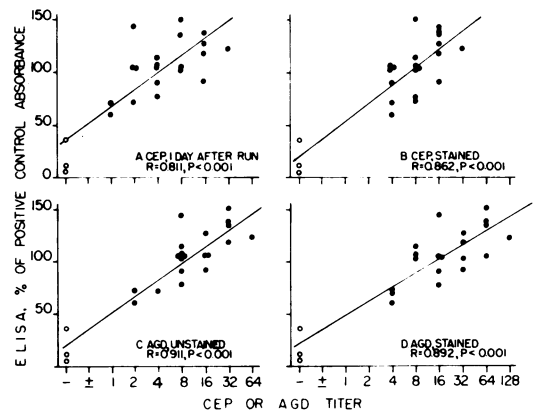

FIG. 5. Correlation between the ELISA and the CEP or AGD tests on serum samples from 20 well-documented amebiasis patients (●) and three normal control subjects (○). These samples were obtained from the Centers for Disease Control or Tulane Medical School. R, Correlation coefficient of Pearson and Lee. −, Negative; ±, positive in one of duplicate tests.

TABLE 3. Comparison of ELISA values of serum samples from three groups of well-documented cases of invasive amebiasis

Group	No. tested	ELISA values (% of positive)		
		Mean ± SD[a]	Range	Median
Liver abscess	7	99 ± 29	73–151	103
Dysentery	8	116 ± 25	72–143	123
Liver abscess + dysentery	5	105 ± 17	77–123	105

[a] The differences in mean value among the three groups are not significant statistically by the Student t test. SD, Standard deviation.

eases, a standard curve was required for each assay, with absorbance readings from a serially diluted positive control being used to convert absorbance values to units of antibody activity (8a, 8b, 16). For comparison purposes in the present study, the positive control was arbitrarily assigned a value of 1,000 U of antibodies to *E. histolytica* per ml and was used to establish a similar standard curve, using fivefold serial dilutions (1,000, 200, 40, and 8 U/ml). As in the other assays, the standard curve relating absorbance readings and unit values yielded a straight line after logarithmic transformation. From this standard curve, the antibody activity, in units per milliliter, of each of the 23 well-documented specimens was obtained. When these values were plotted against CEP and AGD titers, the scatter diagram (Fig. 6) looked similar to that shown in Fig. 5. Similar analysis of the 82 AGD positive samples shown in Fig. 2 revealed equivalent results, with a correlation coefficient of 0.838 between antibody activity (170 to 5,750 U/ml) and AGD titers (1 to 128) of these samples. This value is close to that shown in Fig. 2.
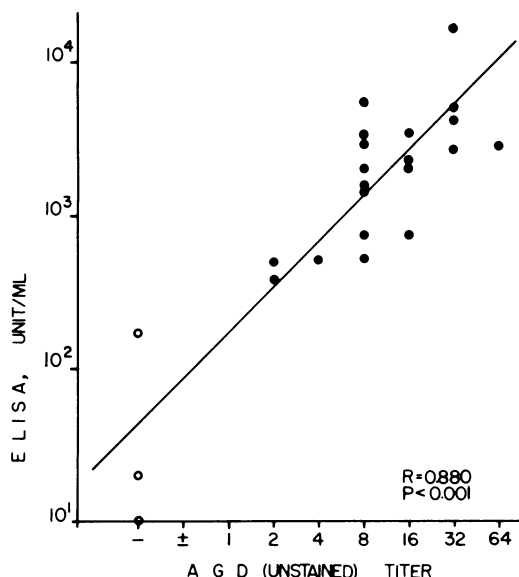
Samples from 20 healthy normal laboratory

FIG. 6. *Comparison between the ELISA, as reported in arbitrary units of antibody activity, and the AGD tests on serum samples from the 20 well-documented amebiasis patients (●) and three normal subjects (○) studied in Fig. 5. Readings from the positive control (1,000 U/ml) and its three fivefold serial dilutions were used to establish a standard curve, from which the values (units per milliliter) for test samples were obtained by extrapolation with their absorbance readings. Note the similarity of this scatter diagram to Fig. 5C.*

volunteers were all negative by CEP, AGD, and ELISA, with a mean ELISA value of 16.3% and a range between 5 and 39% (Table 4). Specimens from 19 patients with nonparasitic diseases, 30 patients with other intestinal parasites, and 50 patients passing *E. histolytica* cysts showed a low incidence of positive reactors for antibodies to *E. histolytica* by ELISA. In most cases, positive reactions by ELISA in these patients were correlated with positive reactions by the gel diffusion methods. The average ELISA values of these groups were close to those of 150 samples from a miscellaneous group of patients which were negative by CEP or AGD; the differences were not significant statistically. These data further support the specificity of this ELISA for antibodies to *E. histolytica*.

DISCUSSION

The present ELISA was shown to be satisfactorily reproducible and specific and to be equivalent to CEP and AGD in detecting amebiasis antibodies. The latter assays have been well accepted in clinical laboratories because of their reliability and simplicity (10, 18, 20; Kelly et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, C158, p. 301). Both CEP and AGD take 1 day or longer for final readings which are relatively subjective, whereas the present ELISA requires less than 2 h for tests of 50 samples and yields a

TABLE 4. *ELISA for antibodies to E. histolytica in human serum samples from patients without invasive amebiasis or negative by CEP or AGD*

Sample donor	No. examined	ELISA results			
		Values (mean ± SD[a])	No. found		
			Positive	Equivocal	Negative
Healthy normals	20	16.3 ± 8.3	0	0	20
Patients with nonparasitic diseases	19	25.9 ± 11.2	0	1[b]	18
With other intestinal parasites[c]	30	30.2 ± 21.4	3[b]	0	27
E. histolytica cyst passer	50	35.8 ± 25.7	7 (6)[d]	3 (1)[d]	40
CEP- and AGD-negative miscellaneous	150	22.2 ± 11.5	0	3	147

[a] SD, Standard deviation.
[b] All these samples were positive by CEP or AGD.
[c] Five with *Trichinella*; 5, *Trichuris*; 2, *Trichomonas*; 2, *Giardia*; 2, *Endolimax nana*; 3, hookworm; 5, *Ascaris* and others; 10, *Trichuris* and others; 7, hookworm and others; 1, *E. nana* and others.
[d] Parentheses indicate number of samples positive by CEP or AGD.

numerical value. The procedure is simple, the reagents are safe and ready for use, and the objective absorbance readings are converted to the final values by a simple division. This ELISA is thus very suitable for clinical laboratories, since the number of samples submitted for amebiasis testing is usually small and the reagents are ready for use.

Reporting the results in relation to the stable positive control reduces variations due to test conditions which may occur from day to day, from laboratory to laboratory, and from performer to performer. Based on data from the ELISAs for other infectious and immunological diseases, using the same format and many of the same reagents, the stability of the test sets of the ELISA for amebiasis antibodies should be at least 1 to 2 years (16). The linear relationship between the ELISA values and the antibody levels determined by CEP and AGD titration indicated that the ELISA assay can be used semiquantitatively.

ACKNOWLEDGMENTS

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