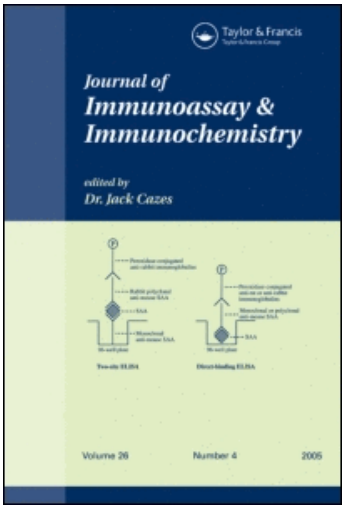

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Tsue-Ming Lin[a]; Charles M. Schubert[a]; David J. Kiefer[a]; Sandy Troy[a]; Robert Cort[a]; Joseph L. Giegel[a]; James H. Lin[a]; Larry Neill[a]
[a] Diamedix Corporation, Miami, Florida

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TWO RAPID AND SIMPLE ENZYME IMMUNOASSAYS FOR HUMAN ANTIBODIES TO *ENTAMOEBA HISTOLYTICA*

Tsue-Ming Lin, Charles M. Schubert, David J. Kiefer, Sandy Troy, Robert Cort, Joseph L.Giegel, James H. Lin and Larry Neill
Diamedix Corporation, 2140 N. Miami Avenue, Miami, Florida 33127

ABSTRACT

Two rapid and simple enzyme immunoassays (EIA) for antibodies to *E. histolytica* , the protozoa causing ambiasis, are described. In the rapid dot EIA, a qualitative procedure, antigens were dried as a small dot (3 mm in diameter) on a thin white opaque polystyrene strip and serum samples were assayed undiluted. The assay required 3 incubation periods, 1 to 3 minutes each, and was completed in 9 minutes, with a positive reaction revealed as a blue color (precipitate) on the antigen dot and negative as colorless. The developed color is stable for permanent record. In the Microwell EIA, a quantitative procedure, antigens were dried in the Microwells. The assay also consisted 3 incubation periods of 15 minutes each, and was completed in 50 minutes. The results in absorbance values were normalized to EIA units (EU). Both tests had good reproducibility, sensitivity and specificity; and highly correlated with 3 other serologic tests. Their reagents can be stored for more than a year. Both tests could be suitable for small and physicians' office laboratories, especially in developing countries. (KEY WORDS: Enzmye immunoassay, rapid EIA, antibody, amebiasis).

INTRODUCTION

The enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA) was first described in 1971 (1, 2). It has been continuously improved such that it is now the method of choice for qualitative and / or qualitative determination of a variety of antibody, antigen, hormones and drugs (3). We have previously reported a rapid, easy and inexpensive dot EIA for antibody to cytomegalousvirus (CMV), which tested undiluted serum samples on a CMV antigen dot on a thin polystyrene strip and took

less than 20 minutes to complete (4). This test procedure has also been successfully used for antibody to HIV by other workers (5). Our experience with other EIA procedures indicates that the assay for antibody to *Entamoeba histolytica* is faster than that for antidboy to CMV. *E. histolytica* infects 480 million people, causes invasive amebiasis in 48 million people and accounts for 40,000 to 110,000 deaths worldwide annually (6). We have adapted the rapid dot EIA for the detection of antibody to *E. histolytica* and found that the assay could be performed in 9 minutes. The technical findings from this dot EIA also made us substantially modify all 15 quantitative EIA systems for various infectious and immunological diseases, including amebiasis, which we had developed (7-12). These modified systems, using Microwells to solid phase antigens or antibodies, have been proven equivalent in perfomance, simpler to perform and cheaper to use than the previous EIA systems. We think that these two much improved EIA procedures for invasive amebiasis are very suitable for small and physician's laboratories, especially in rural areas and developing countries where ordinary laboratory tests are often not available or feasible. These test procedures, which could be easily adapted for the detection of other antigens or antibodies, are reported here.

MATERIALS AND METHODS

Antigens and Human Serum Samples. These materials have been described in detail elsewhere (12). Briefly, axenically cultured *E. histolytica* (NIH:200 and HK:9 strains) were washed, sonicated and extracted with 0.01M sodium phosphate-buffered saline, pH 7.2 (PBS). The extract was cleared with a high speed centrifugation (40,000 xg, 30 min) and stored frozen until use as antigens.

Serum samples from healthy blood donors (considered normal), patients with amebiasis and those with other diseases were kindly supplied by the Centers for Diseases Control, Atlanta, GA; Tulane Medical Center, New Orleans, LA; and the University of Antioquia, Colombia (12). The patients were grouped according to clinical data (symptoms and response to drug treatments) and laboratory test results (microscopical stool exmaination and serological tests) as shown in Table 1. Those in the group F (suspected amebiasis) were from amebiasis endemic areas and diagnosed clinically by the original investigators without confirmation by laboratory tests. Additional serum samples from a group of 116 healthy blood donors were obtained locally and used in the Microwell test. A pool of serum samples from several amebiasis patients were used as a calibrator.

Rapid Dot EIA. The reagents and procedures described elsewhere were followed with minor modifications (4). An opaque white high impact polystyrene sheet (0.38 mm in thickness, 8 cm by 12 cm in area) was marked with black circles (6 mm in diameter) by the silk screening procedure using the Microtiter plate 96-well pattern. Three µl of cold antigen solution of appropriate concentration in 1 mM tris-saline, pH 7.2 was placed in the center of the circle, incubated in a moist chamber overnight at 4° C, and dried in a 55° C oven for 15 minutes. The antigens dried as a small dot of 3 mm in diameter. The sheets with dried antigen dots were stored in moisture-proof re-sealable bags with desiccant packets at 4° C. Alkaline phosphatase conjugated goat antibodies to human IgG and IgM, and the enzyme substrate (5-bromo-4-chloro-3-indolyl phosphate, toluiline salt) were dispensed with dropper bottles. The washing solution was 0.001M tris-saline containing 0.05 % Tween 20, pH 9.0 (instead of pH 7.4 used previously), dispensed with a squeeze bottle.

The assay was done at room temperature (20 to 25 C). A strip or card containing the required number of antigen dots was cut from a sheet and placed on the top of a bench or desk. A drop of serum or plasma sample was placed in the center of a circle containing an antigen dot for about 1 minute, then rinsed off with wash solution. The circle was flooded with the wash solution for 30 seconds and blotted dry with a paper towel. A drop of conjugate was placed inside the circle for 3 minutes, washed and blotted dry as above. A drop of substrate was then added. If the test serum sample contained specific antibodies to *E. histolytica*, blue color developed evenly on the antigen dot due to the deposit of insoluble end product of substrate hydrolyzed by the enzyme of conjugate. The strong reactors revealed blue color immediately after application of substrate drops, while the weak ones took 2 to 3 minutes. The final readings were made at 3 minutes and graded 0 to 4 according to the intensity of color, with 0 being no color and 4, the strongest. The substrate was rinsed off and blotted dry for permanent record. The color was reduced by rinsing, especially for the strong reactors, but will not fade during storage.

Microwell EIA. Wells of Immunolon I Removawell strips (12 microwells per strip, flat bottom, Dynatech, Va.) were used to solid phase antigen and for assay. One hundred µl of appropriately diluted antigen solution was placed in wells and aspirated after incubation for 2 hours at room temperature. The wells were then filled with 100 µl of saline containing 10 % normal goat serum for 1 hour at room temperature, aspirated and dried at 55° C for 15 minutes. The antigen-coated wells were stored in a re-

sealable moisture-proof bags with desiccant packets at 4° C as above. Alkaline phosphatase-labeled goat antibodies to human IgG and IgM (conjugate) and *p*-nitrophenyl phosphate (substrate) have been descirbed elsewhere (12). Both conjugate and substrate were also dispensed with dropper bottles and stored at 4° C as working solution when not in use. Wash solution was as described above.

The assay was also done at room temperature (20 to 25 C). An appropriate number of antigen-coated wells were removed from the storage bag after being warmed up to room temperature and placed in a holder (Dynatech, Va.). The wells are easily separated from each other. Test serum samples, a calibrator, a positive control and a negative control were diluted 1:21 by adding 10 µl of them into 200 ul of sample diluent (1 mM tris-saline containing 10 % normal goat serum and 0.05 % Tween 20, pH 7.2) in appropriate containers such as Microtiter plate wells. The 1:21 dilution was used because most Microtiter wells hold between 250 and 350 ul and most micropipettor could accurately deliver 10 ul of fluid. After mixing well, 100 µl of diluted sample was transferred into antigen wells and incubated for 15 minutes at room temperature. The antigen wells were washed 3 times quickly by shaking out the contents and filling with wash solution. After the third washing, the wells were drained thoroughly by inverting the wells on paper towels, with vigorous tapping. Two drops of conjugate were placed into the wells and incubated for 15 minutes at room temperature. The wells were again washed and drained thoroughly as above. Two drops (about 100 µl) of substrate were added to the wells and incubated for 15 minutes at room temperature, followed by addition of two drops of stop solution (1.5 M tri-sodium phosphate, pH 12). The color reactions were read at 405 nm against a reagent blank with a microtiter plate reader, which read the contents of the wells vertically. The absorbance value of the test sample was divided by that of calibrator and multiplied by 100 to obtain the EIA unit (EU) / ml value of test sample for interpretation. The absorbance values of calibrators were set at 1.2 to 1.8 by adjusting the conjugate concentrations. The cut-off points for negative and positive interpretation were 40 EU/ml and 50 EU/ml, respectively, with values between these two as equivocal. These cut-off points were based on the comparison with the previous EIA "CORDIA A" (12).

Other Tests. The agar gel diffusion (AGD), counter-electrophoresis (CEP) and another EIA "CORDIA A" were performed with commercial test kits according to manufacturer's instructions for use (Cordis Laboratories, Miami, Florida), as previously described (12).

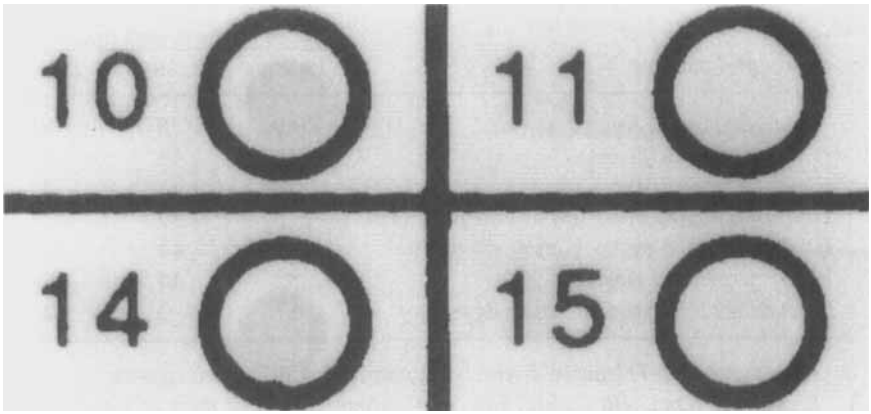

Figure 1. Photographic recording of rapid dot EIA results for antibodies to *E.histolytica* with two positive (no. 10 and 14) and two negative serum samples. The circles are 6 mm in diameter.

RESULTS

Rapid Dot EIA. The concentration of conjugate was the most critical parameter in the assay. A panel of serum samples with weak and negative reactivity in other assays for antibodies to *E. histolytica* was used to test conjugate so that the weak reactors showed color and the negative sera gave no color. Fig. 1 is a photograph of 2 positive and 2 negative test results.

The dot test results of 315 serum samples from normal and various diseases are summarized in Table 1. All 78 normal sera were negative and all 17 sera from well-documented amebic liver abscess and dysentery patients were positive in the assay. Ten percent of 50 cyst passers and about half of 96 suspected amebiasis cases were positive. Sera from all 15 patients with other intestinal parasites and all 17 with non-parasitic diseases were negative. Most of 42 normal blood donors in amebiasis endemic areas were negative. The dot test results of some of these serum samples were also compared with results of 3 other tests, as summarized in Table 2. The dot test results showed better agreements with CORDIA A (98 %) than with CEP (93 %) and AGD (94 %). The CEP revealed more negative while AGD gave more positive results

TABLE 1.

Rapid Dot EIA Test Results of 315 Serum Samples from Normal Subjects and Various Patients for Antibodies to *E. histolytica*.

Group of patients	No. of samples with dot EIA		
	Positive	Negative	Total
A. Normal blood donors	0	78	78
B. Non-parasitic diseases	0	17	17
C. Other intestinal parasites#	0	15	15
D. Blood donors in amebiasis endemic areas	3	39	42
E. *E. histolytica* cyst passers@	6	44	50
F. Suspected amebiasis	52	44	96
G. Amebic liver abscess and/or dysentery	17	0	17

13 patients with *Trichuris*, 7 with hookworms, 4 with *Ascaris*, 2 with *Endalimax nana*, and 1 with *Giardia*. 8 patients with 2 or more of these parasites.

@ Almost all of them had other intestinal parasites: *Entamoeba coli, Iodamoeba, Chilomastix, Trichomonas, Strogyloides* and those listed for Group C.

TABLE 2.

Comparisons of Test Results of Rapid Dot and Microwell EIA with Three Other Immunoassays for Antibodies to *E. histolytica*.

Other Immunoassays		No. of serum samples with						
		Dot EIA			Microwell EIA			
Method	Results	Pos.	Neg.	Agree.	Pos.	Equiv.	Neg.	Agree.
CORDIA A	Pos.	74	3		60	1	2	
	Equiv.	1	7		1	3	0	
	Neg.	3	226	98 %	0	1	134	99 %
CEP	Pos.	42	1		31	0	0	
	Neg	10*	111	93 %	9**	2#	62	89 %
AGD	Pos.	50	8##		30	2	1	
	Neg.	2	104	94 %	2	0	61	97 %

* All 10 positive by AGD and 7 positive by CORDIA A.

** 7 positive by AGD, 6 positive by CORDIA A.

Both positive by AGD, 1 positive and 1 equivocal by CORDIA A.

7 negative by CEP and 5 equivocal by CORDIA A.

than the dot test. The positive samples with values greater than 100 EU/ml tended to give grade 3 or 4 reaction in the dot test (46 out of 47); while those less, grade 1 or 2 (24 out of 28).

Of the 315 serum samples listed in Table 1, 205 were from normal or various patients negative by all other 3 serologic assays (CEP, AGD and CORDIA A) for amebiasis. All except one of these 205 samples were negative by the dot test. Sixty-two samples were from well-documented amebiasis or suspected cases confirmed by all other serologic assays for amebiasis. All these 62 were positive by the dot test. These data indicate that the dot test has a 99.5 % specificity and a 100 % sensitivity. The high specificity of this test was further indicated in the negative results of all patients with other intestinal parasites and the low positive rate for the cyst passers and healthy blood donors in amebiasis endemic areas (Table 1). Almost all cyst passers also had other intestinal parasites (Table 1 footnote)

The test reproducibility of the dot test was very satisfactory. In within-run (triplicate) and run-to-run (5 runs) tests involving at least 4 coded CORDIA A positive sera, and at least 4 CORDIA A negative sera, all of the positive sera were always positive and all of the negative sera were always negative by the dot test. One lot of test set containing all reagents including antigen dots had been stored at 4° C, used periodically and were found still as good as freshly made after storage for at least 27 months..

Microwell EIA. The Microwell test results in EU / ml of 315 serum samples from various groups of patients are shown in Fig. 2. Some of these samples were also tested by the dot test above. Most sera from 186 healthy subjects, 11 patients with non-parasitic diseases and 7 with other intestinal parasites were negative by this test. The mean EU value of the normal group was at least 50 % less than the latter two groups which were from endemic areas. All samples from 26 well-documented amebiasis patients gave positive results, with EU values ranging from 60 to 170. About one quarter of 20 cyst passers and half of 65 suspected amebiasis were also positive; some of these positive values were as high as those seen with amebic liver abscess. Here again, the Microwell EIA results agreed better with AGD and CORDIA A (97 and 99 %, respectively) than with CEP (89 %)(Table 2). The descrepancies were found in 9 samples which were negative by CEP, but most of these 9 samples were positive by both Microwell EIA and AGD. Of the 161 samples tested by both Microwell and Dot EIA's, 58 were positive and 98 negative by both assays, a 97 %

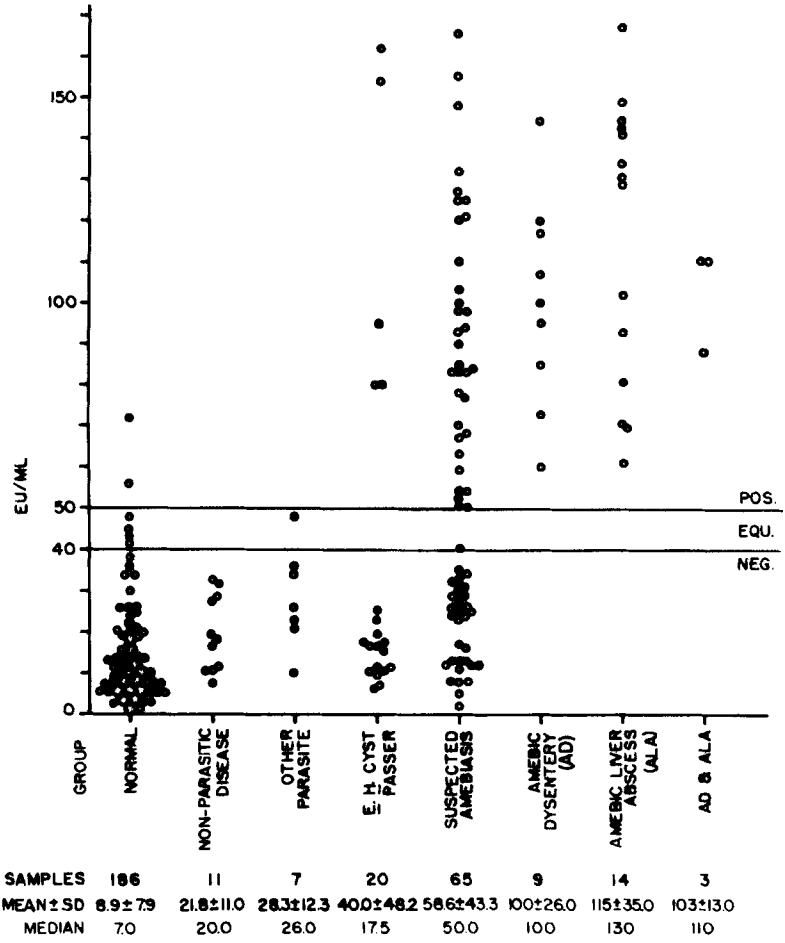

Figure 2. Microwell EIA values of normal and various patient serum samples for antibodies to *E. histolytica*. One small circle, one test sample; one large circle, ten test samples.

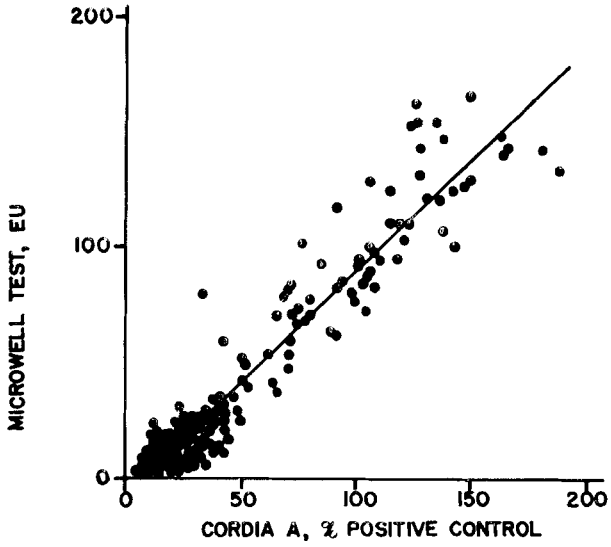

Figure 3. A scatter diagram showing correlation (r=0.957, p<0.001) between CORDIA A and Microwell assay results of 202 serum samples for antibody to *E. histolytica.*

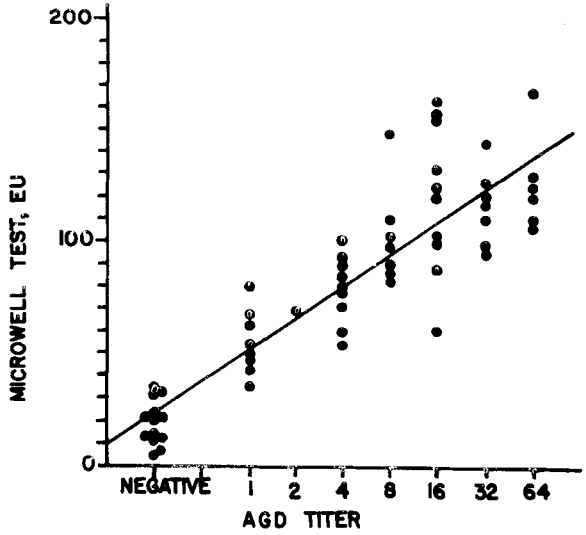

Figure 4. A scatter diagram showing correlation (r=0.883, p<0.001) between antibody titers by agar gel diffusion and Microwell assay results of 60 serum samples for antibody to*E. histolytica* .

TABLE 3

Reproducibility of Microwell Assay for Antibody to *E. histolytica.*

Test sample	Assay results, EU/ml							
	Within-run, n=6				Run-to-run, n=5			
	Range	Mean	SD*	% CV	Range	Mean	SD*	%CV
A	59-73	65	4.5	7.0	65-74	69	3.6	5.2
B	57-71	64	4.9	7.7	64-70	64	3.3	5.1
C	67-78	73	4.0	5.5	71-80	75	3.8	5.1
D	7.5-10.1	8.3	1.0	--**	6.7-12	9.6	2.1	--**
E	1.8-3.7	2.8	0.6	--**	1.9-6.5	4.5	2.1	--**
F	5.2-13.1	7.9	2.7	--**	5.1-12	7.9	2.7	--**

* SD, standard deviation.

** All test results of these three samples were negative.

agreement. Three samples were weak positive by the Dot and negative by the Microwell EIA; while two others gave reverse results.

The Microwell EIA and CORDIA A values are highly correlated (correlation coefficient of Pearson and Lee, $r = 0.957$, $p<0.001$) as shown in Figure 3. Using the same serum pool as calibrator (Microwell) and Positive Control (CORDIA A), the Microwell EU values were on average 10 % lower than the CORDIA A (% of Positive Control) values. The cut-off points for negative and positive reactions for the Microwell EIA were determined based on their corresponding values in CORDIA A in this comparison. There was also a significant correlation between the Microwell values and the AGD titers, with $r = 0.86$ ($p < 0.001$), as shown in Fig. 4. The AGD titers are the reciprocal of the highest dilutions of test samples which still gave positive reaction in the assay. Thus, these correlation data of Microwell EIA values with both CORDIA A values and AGD titers demonstrated the quantitative nature of the Microwell EIA.

Based on the data from 186 healthy subjects and 57 patients with clinically and serologically proven amebiasis, the Microwell test has a 100 % sensitivity and a 99 % specificity. As in the Dot EIA, the specificity of Microwell EIA was further shown in the non-positive results of all 7 patients with other intestinal parasites listed in Fig. 2 and of three quarters of cyst passers; almost all the latter also had other intestinal parasites as listed in Table 1. In within-run (6 replicates) and run-to-run (5 runs) tests of 6 sera, three serum samples were all positive, with coeffcient of variations (CV)

between 5.1% and 7.7 % and an average CV less than 7 % (Table 3). Three other serum samples were all negative. Several lots of test sets containing all reagents including antigen-coated wells were found still good after storage at 4° C for at least one year.

DISCUSSION

The rapid Dot EIA showed a performance equivalent to serveral other serologic tests for antibodies to *E. histolytica*. It is a rapid procedure because it takes less than 9 minutes to complete; it is simple because it requires no laboratory equipment and instrument (except forceps, for holding a strip or card of antigen dots) and can be performed by non-technical personnels in the physicians' office; it is flexable because any number of samples can be tested, especially with the aid of a multi-channel pipettor to apply a number of samples and reagents at the same time; it is economical because the solid phase plastic is very cheap, no laboratory equipment and instrument required and no training needed, and small amounts of reagents are used; and finally, it is convenient to keep the record because the finished strips with developed dots can be easily marked and stored in a record book and the developed blue color does not fade in storage. The same procedure had been developed for IgG antibodies to cytomegalovirus (CMV), human immunodeficiency virus, rubella virus and pseudorabies virus (4,5, unpublished data).

The Microwell EIA had an almost identical procedure as CORDIA A (12). The latter used plastic discs with isothiocyanate groups to covalently bind antigens and had 30 minutes longer total incubation time. The Microwell EIA had antigens physically bound to polystyrene, yet it performed the same as the disc. Surprisingly, the background readings in the Microwell procedure were lower than those in the disc, and this may account for the different cut-off points between these two procedures. A number of Microtiter well EIA for amebiasis antibody have been reported (12 - 17). Almost all of them have some features which make them not suitable for small and physicians' office laboratories. These disadvantages include long incubations of assay, necessity of reagent preparation before and/or during assay, uncertain shelf life of reagents, lack of strandarization of test results, various equipment for assay, test serum titration, etc.

The fact that the reactions in all the three incubation periods of these ELISA are concentration dependent makes the assay easier. So long as the volume of diluted

sample, conjugate and substrate are adequate to cover the antigen surface, it is their concentrations which determine the reaction rate and therefore the test results. For this reason, both conjugate and substrate can be dispensed with dropper bottles. An additional feature in the Microwell procedure is that the microwell reader read the wells vertically so that it measures the product of concentration and solution depth. In the event that the solution is too strong beyond the linearity of the reader, portions or half of the solution can be moved to other clean wells. Both these and original wells are read again. The sum of their readings will be that of the original undivided well. To correct some possible changes in meniscus due to a low level of solution inside the well, two or more drops of distilled water can be added.

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