Attachment 1.

Date 23/1/83

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator P. Speelman

Trainee Investigator (if any)

Application No. 83-025(P)

Supporting Agency (if Non-ICDDR,B)

Title of Study "Amebic Antigen detection in stool specimens" (Pilot-study).

Project status:
( ) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

Circle the appropriate answer to each of the following (If Not Applicable write NA).

1. Source of Population:
   (a) Ill subjects Yes No
   (b) Non-ill subjects Yes No
   (c) Minors or persons under guardianship Yes No
2. Does the study involve:
   (a) Physical risks to the subjects Yes No
   (b) Social Risks Yes No
   (c) Psychological risks to subjects Yes No
   (d) Discomfort to subjects Yes No
   (e) Invasion of privacy Yes No
   (f) Disclosure of information damaging to subject or others Yes No

   N.A
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) (Yes) No
   (b) Use of fetal tissue or abortus Yes (No)
   (c) Use of ~~organs or body fluids~~ Stool samples (Yes) No
4. Are subjects clearly informed about:
   (a) Nature and purposes of study Yes No
   (b) Procedures to be followed including alternatives used Yes No
   (c) Physical risks Yes No
   (d) Sensitive questions Yes No
   (e) Benefits to be derived Yes No
   (f) Right to refuse to participate or to withdraw from study Yes No
   (g) Confidential handling of data Yes No
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes No

   N.A
5. Will signed consent form be required:
   (a) From subjects Yes No
   (b) From parent or guardian (if subjects are minors) Yes No
6. Will precautions be taken to protect anonymity of subjects Yes No

   NA
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ✓ Protocol (Required)
   ✓ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

We agree to obtain approval of the Ethical Review Committee for any changes involving the rights and welfare of subjects before making such change.

[signature]

Principal Investigator

Trainee

83-025 (P)
26/6/83

SECTION 1 - RESEARCH PROTOCOL

(Pilot-study)

1. TITLE: AMEBIC ANTIGEN DETECTION ON STOOL SPECIMENS.

2. PRINCIPAL INVESTIGATOR: Dr. P. Speelman

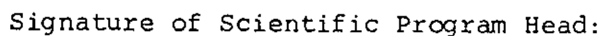
CO-INVESTIGATORS: Dr. I. Ljungström, Dr. G. Huldt, Dr. S. Grundy, Dr. M. Willcox, Ms. L. Lundin

3. STARTING DATE: September 1983

4. COMPLETION DATE: December 1983

5. TOTAL COST: US $ 1500.00

6. SCIENTIFIC PROGRAM: This protocol has been approved by the PATHOGENESIS AND THERAPY WORKING GROUP.

Signature of Scientific Program Head: 

Date: 19-6-83

7. ABSTRACT SUMMARY:

In spite of the introduction of new sensitive serological assays for detecting specific *E. histolytica* antibodies, the diagnosis of current *E. histolytica* infection is still largely dependent upon the identification by microscopic examination. There is therefore a demand for the development of more sensitive, more objective and less time consuming techniques for the detection of *E. histolytica*. Recently a new method for detecting *E. histolytica* antigens has been described using a multi-layer ELISA technique (1). During 1981 we did a pilot study to test the sensitivity and specificity of this technique. We found the method highly specific, but the sensitivity needed further improvement. Now the methodology has been further developed. So, a pilot study to test the clinical and epidemiological usefulness of this method seems indicated. We therefore plan to test 250 stool samples, 150 from patients infected with cysts and or trophozoites of *E. histolytica* and other Entamebae, 100 from patients without parasitic infectious. There is no direct involvement of patients in this study. The results of this study will demonstrate the clinical and epidemiological value of this new technique. If the new test if functionary well a major study might be set up.

8. REVIEWS:

(a) Research involving human subjects: ____________________

(b) Research Review Committee: ____________________

(c) Director: ____________________

SECTION II - RESEARCH PLAN

A. INTRODUCTION:

1. Objectives:

The objective of this study is to test the specificity and sensitivity of a new ELISA technique for detection of amebic antigen in fecal specimen.

2. Background:

The diagnosis of E. histolytica infections is still largely dependent upon the identification by microscopic examination of E. histolytica trophozoites and cysts. The usefulness of serology is limited; the long persistence of antibodies in sera of patients, particularly in areas where there is a high incidence of reinfection is an example of its limited usefulness.

The problems of stool microscopic examination in the diagnosis of E. histolytica are many. It is inefficient in non-dysenteric forms of amebic infection and requires conscientious well trained workers. Often multiple stool specimens are required. There are many substances which interfere with parasitologic examination of feces, such as bariumsulfate, antibiotics, laxatives and enema's. There is therefore a demand for the development of more sensitive techniques for the detection of E. histolytica which depend neither on the direct visualization of the parasite, nor on its physical integrity.

Recently a new method for detecting _E. histolytica_ antigens in fecal specimen have been described using a multi-layer ELISA technique. This technique involves coating plastic microtitre plates with a "catching antibody", specific rabbit anti-E. histolytica antibody. After adding the test material the presence of antigen is determined, using 2 additional heterologous antibody layers:

(a) Human anti-E. histolytica immunoglobulin as a developing antibody.

(b) Peroxidase conjugated sheep, anti human immunoglobulin antibody.

The specificity and sensitivity of the assay was investigated in collaboration with ICDDR,B. No cross reaction was observed with other intestinal parasities. Unfortunately, stools containing the cyst form of _E. histolytica_ gave _no_ reaction in the test. So the new method was found to be highly specific, but had a low sensitivity.

During the last year the method has been further developed by Dr. S. Grundy. Recently considerable evidence has been collected from work on normal and infected stool samples that the current design of the test is more sensitive for stool samples than the earlier one. It was found that in the presence of stool material the coating antibody is released from the plate and that there was same crossreaction between the rabbit antibody layer and the sheep immunoglobulin. These 2 problems have been resolved now. We therefore want to retest this method. People interested in details of the multilayer ELISA method are referred to Grundy's paper.

3. Rationale:

The methods available for the diagnosis of infection with E. histolytica have serious disadvantages. The use of a new ELISA to detect specific amebic antigen in feces may improve the accuracy of E. histolytica infections.

B. SPECIFIC AIM:

To test the usefulness of a new ELISA technique for detection of amebic antigen in fecal specimen.

Literature:

M.S. Grundy

Preliminary observations using a multilayer ELISA method for the detection of *Entamoeba histolytica* trophozoite antigens in stool samples.

Transactions of the Royal Society of Tropical Medicine and Hygiene. Vol 76 No. 3, 396-400, 1982.

C. METHODS OF PROCEDURE:

1. Collection of samples:

   1. 50 samples containing haematophagous or non-hematophagous trophozoites of E. histolytica.
   2. 50 samples containing cysts of E. histolytica
   3. 50 samples containing cysts or trophozoite forms of other Entamebae.
   4. 100 samples from patients without parasite infection.

11. Storage:

Samples will be stored in 3 parts (after examination in a wet mount preparation):

(a) one part in PVA, stored at + 4°C

(b) one part in a clean empty bottle, stored at + 4°C

(c) one part will be processed as follows:

- a 10-50% stool in PBS preparation will be made.
- this will be shaked on a whirly mix to get good homogenization.
- this solution will be pressed through a syringe which has a fine mesh gauze at the bottom.
- this stool preparation will be stored in 2 aliquots at -70°C.

III. Stools without cysts or trophozoites of Entamebae (control-stools) will be examined after concentration of stool with zinc sulfate to exclude the presence of cysts.

All PVA-stored samples will be examined by a experienced parasitologist; the results will be compared with the wet mount preparation result. The results of the examination of the PVA-stored material will be used as reference for comparison with the ELISA test. The results of ELISA on samples stored at $+4^{\circ}C$ will be compared with samples prepared in a special way and stored at $-70^{\circ}C$.

D. Significance:

From the result of this study it will be possible to determine whether this method of diagnosing *E. histolytica* infections is superior to the microscopic diagnosis.

E. Facilities required:

All required spaces are present and available.

F. Collaborative arrangements:

This will be a collaborative study between

ICDDR,B .. Dr. P. Speelman

National Bacteriological Laboratory: Dr. I. Ljungstrom
Dr. S. Grundy
Ms. L. Lundin
Dr. G. Huldt
Dr. M. Willcox

G. Data analysis will be performed in the following way:

Wet mount	PVA	ELISA on samples stored at +4°C	ELISA on same sample stored at -70°C
EH veg + rbc	...	...	...
EH veg - rbc	...	...	...
EH cysts	...	...	...
Troph. other amebae	...	...	...
Cysts other amebae	...	...	...
Negative	...	...	...

SECTION III - BUDGET

Detailed Budget:

1. Personnel services:

Name	Position	% effort	Project requirement US Dollar
Dr. P. Speelman	Principal Investigator	5%	500
Dr. G. Huldt	Co-Investigator	-	-
Dr. L. Ljungstron	Co-Investigator	-	-
Ms. L. Lundin	Co-Investigator	-	-
Dr. S. Grundy	Co-Investigator	-	-
Dr. M. Willcox	Co-investigator	-	-

Sub Total US $ 500

2. Supplies and materials: US Dollar

- Reagents (PVA, trichrome, PBS-tween, isotone saline etc.
- Stool cups
- Microtiter plates US $ 700
- Pipettes
- Tubes

Stool M/E 250 X 5 = Tk. 1000 US $ 50

Concentration techniques 100 X 10 = Tk. 1000 US $ 50

3. Transport Nil

4. Travel: Nil

5. Rent: Nil

6. Printing (Xerox) US $ 200

TOTAL US $ 1500

ABSTRACT SUMMARY FOR ETHICAL REVIEW COMMITTEE

1. This is a laboratory study without any direct patient involvement.

2. No risks.

3. N.A.

4. Samples will be coded to safeguard confidentiality

5. N.A.

6. N.A.

7. The potential benefit of this study is a more accurate diagnosis of amebiasis.

8. This protocol uses a part of those fecal specimens, brought to the laboratory for microscopic examination.

To Peter,
Best wishes
Steve.

TRANSACTIONS OF THE ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE, VOL. 76, NO. 3, 1982

Preliminary observations using a multi-layer ELISA method for the detection of *Entamoeba histolytica* trophozoite antigens in stool samples

M. S. GRUNDY
National Bacteriological Laboratory, S105 21, Stockholm, Sweden

Summary

A method for detecting *Entamoeba histolytica* trophozoite antigens in aqueous solution is described. This involves coating plastic microtitre plates with a 'catching antibody', specific rabbit anti-*E. histolytica* antibody (SRAE). After adding the test material the presence of antigen is determined using two additional heterologous antibody layers, one of 'developing antibody', in this case human anti-*E. histolytica* immunoglobulin (HAE), which is followed by a final layer of peroxidase conjugated sheep anti-human immunoglobulin antibody (SH-HRP). The specificity and sensitivity of the assay was investigated both in the model system and using stool samples from infected patients. In the model system, the test had a sensitivity equivalent to detection of approximately one amoeba per microscope coverslip (18 mm × 18 mm). Little cross reaction was observed with other intestinal parasites common to the area of Bangladesh from which the stool samples were taken. The possible use of this method in large scale screening of stool samples and in the detection of circulating antigens is discussed.

Introduction

In spite of the introduction of new, sensitive serological assays for detecting specific anti-*Entamoeba histolytica* antibodies (KESSEL *et al.*, 1965; GORE & SADUN, 1968; SAVANAT & CHAICUMPA, 1969; MORRIS *et al.*, 1970), the diagnosis of current, invasive *E. histolytica* infections is still largely dependent upon the identification, by microscopic examination, of *E. histolytica* trophozoites with ingested red blood cells in stool samples (ELSDON-DEW, 1969). One reason for the lack of usefulness of serology is the long persistence of antibodies in sera of patients after clinical cure, particularly in areas where there is a high incidence of re-infection. However, there are a number of problems associated with microscopic examination. Several agents are known to interfere with the integrity of the trophozoites (JUMPER, 1966) and, especially in countries where amoebic infection is relatively uncommon, large numbers of negative samples must be read, compared to the number of positive samples identified. This is an important point since microscopic examinations demand a high degree of concentration. In addition, since the output of parasites is not continuous, extra samples must be taken to confirm a negative report (DESPOMMIER, 1981). There is, therefore a demand for the development of more sensitive techniques for the detection of *E. histolytica* which depend neither on the direct visualization of the parasite, nor on its physical integrity (HEALEY, 1978).

This paper describes a method for detecting *E. histolytica* trophozoite antigens using a multi-layer ELISA technique in flat-bottomed microtitre plates. The stool samples used in the study were obtained from patients in Bangladesh, where multiple infection is common. The method was originally developed using a model system and the results compared with those obtained with a currently available kit based upon the method of ROOT *et al.* (1978). The specificity of the test was also investigated using stool samples from patients having evidence of other parasites, common to the population from which the positive samples were taken.

Materials and Methods

Preparation of E. histolytica *antigen*

E. histolytica strain HK9 were cultured axenically according to the method of DIAMOND (1968). After 96 hours the amoebae were harvested by centrifugation at 1000 *g* for 5 min (+4°C) and were then washed four times in PBS and homogenized by freezing and thawing three times between −20°C and +4°C.

Preparation of the normal rabbit serum immunosorbent column

Two grammes of freeze-dried CNBr-Sepharose 4B (Pharmacia) were swollen in 50 ml of 1 mM HCl for 15 min. The swollen gel was then washed with several aliquots of 1 mM HCl (200 ml/gm gel). After decanting off excess HCl the gel was mixed with 10 ml of a 20% solution of normal rabbit serum in coupling buffer (0·1M sodium bicarbonate buffer pH 8·5, containing 0·5 M NaCl). The suspension was then incubated overnight at +4°C in an end-over-end mixer. After incubation the suspension was allowed to stand for one hour at +4°C and excess liquid aspirated away. The remaining active groups on the gel were blocked by incubation of the suspension overnight at +4°C with 20 ml of 0·1 M glycine in coupling buffer. The gel was then transferred to a 10 ml syringe and washed alternately with coupling buffer and 0·1 M acetate buffer pH 4·0, containing 0·5 M NaCl. The gel was then washed with PBS and stored at +4°C until use.

Preparation of the E. histolytica *immunosorbent column*

The method of coupling *E. histolytica* protein to CNBr-Sepharose 4B was essentially the same as that described for the attachment of normal rabbit serum, except that 10 ml of a 5 mg/ml protein solution of *E. histolytica* freeze-thawed homogenate in coupling buffer containing 0·1% Triton X100 (Sigma), was used instead of the 20% normal rabbit serum. The washing and storage procedures were identical to those followed for the rabbit immunosorbent column.

*Preparation of specific rabbit anti-*E. histolytica *(SRAE) immunoglobulin*

Two New Zealand White rabbits were infected with 2×10^6 axenically cultured *E. histolytica* trophozoites intraperitoneally on days one, 14 and 21. Seven days after the final injection the rabbits were bled and the sera pooled. The pooled sera were then passed down the *E. histolytica* immunosorbent column, as prepared above. The column flow rate was 10 ml/hr, at +4°C. Elution of the SRAE was performed with 0·1 M glycine HCl buffer pH 2·4, and the pH of the eluate rapidly adjusted to 8·0 using 0·5 ml of 3 M tris: HCl buffer pH 8·0. The eluate was then dialysed overnight against PBS containing 0·02% sodium azide and stored at +4°C until use.

*Preparation of human anti-*E. histolytica *(HAE) immunoglobulin*

A pool of serum was prepared by mixing sera from patients with known positive titres of antibody against *E. histolytica* (passive haemagglutination, immunofluorescence and ELISA positive). The pool was then passed down a protein A-Sepharose column (Pharmacia, bed volume 10 ml) previously equilibrated with PBD, (flow rate 10 ml/hr, + 4°C). After extensive washing with PBS, bound protein was eluted using 0·1 M glycine: HCl buffer, pH 2·4. The pH of the eluate was then rapidly adjusted to 8·0 with 0·5 ml of 3 M tris: HCl buffer pH 8·0. The eluate was then dialysed overnight at +4°C against PBS containing 0·02% sodium azide, and stored at +4°C until use.

Preparation of sheep anti-human immunoglobulin-peroxidase (SH-HRP) conjugate

SH-HRP was prepared according to the method of NILSSON *et al.* (1981). The only modification was that, before conjugation, the sheep anti-human immunoglobulin preparation was absorbed by passage down the normal rabbit serum immunosorbent column prepared as described above. The resulting absorbed conjugate was stored lyophilized until use.

Preparation of the stool samples for ELISA

Stool samples were obtained from patients at the International Center for Diarrhoeal Disease Research, Bangladesh and examined under the microscope for parasites. For *E. histolytica* trophozoite identifications the samples were assigned a number indicating the number of parasites per cover-glass (18 mm × 18 mm), from 1+, (1-10 amoebae/cover-glass), 2+ (10-30 amoebae/cover-glass), 3+ (30-100 amoebae/cover-glass), 4+ (more than 100 amoebae/cover-glass). The stool samples were prepared for ELISA by taking cotton swabs and mixing them with the samples. The swabs (containing 0·2-0·4 gm stool sample, wet weight) were then placed in a 2·0 ml solution of PBS containing 0·05% Tween 20 (Kebo AB, Stockholm), and left frozen overnight at −20°C. The samples were then warmed to +4°C and allowed to stand for 15 min, allowing the large particles to settle. The supernatant was aspirated off and used as a stock solution for the ELISA.

Enzyme linked immunosorbent assay (ELISA)

ELISA was carried out in flat-bottomed microtitre plates (Greiner, Nurtinen, W. Germany). The final concentrations of the reagents used were obtained by titration, and were chosen to optimize the sensitivity of the test in the model system. Microtitre wells were coated with 100 µl of a 1:10 dilution of SRAE (20 µg/ml protein) in 0·05 M sodium bicarbonate buffer pH 9·6 at +4°C over night. The wells were then washed three times with 0·9% NaCl solution containing 0·05% Tween 20 (NaCl-Tween). Antigen solution (100 µl) in PBS containing 0·05% Tween 20 (PBS-Tween) was then added to each well and the plates incubated for three hours at +4°C. The plates were then washed three times in NaCl-Tween. 100 µl of a 1:10 dilution of HAE in PBS-Tween (200 µg/ml protein) was then added, the plates incubated for a further three at +4°C and then washed three times in NaCl-Tween. 100 µl of SH-HRP conjugate diluted 1:2,000 in PBS-Tween (9 µg/ml) was then added to each well, and the plates incubated overnight at +4°C. After washing the plates three times in NaCl-Tween, development was performed by adding 200 µl/well of a solution containing 0·08% 5-aminosalicylic acid (Merck, recrystalized using the method of ELLENS & GIELKENS, 1980) and 0·005% hydrogen peroxide, in 0·1M phosphate buffer pH 6·0, containing 1 mM $EDTANa_2$. The plates were incubated for 5 to 10 min at room temperature and the reaction stopped by the addition of 50 µl of 1 M NaOH solution. The absorbance at 450 nm was measured in a Titertec Multiscan Colorimeter (Flow Labs., Solna, Sweden). All absorbance values have been expressed against a blank containing the enzyme substrate alone.

Protein determination

Protein was determined using the method of LOWRY *et al.* (1951), with normal human serum of known nitrogen content as the standard.

Commercial ELISA kit

E. histolytica trophozoite antigen was also determined using the IMMUNOZYME *E. histolytica* reagent kit made from the Millipore Corp. (Goteborg, Sweden). The methods indicated by the manufacturers for the use of the kit were followed.

Results

Fig. 1 shows the results obtained in the model system, using aqueous *E. histolytica* trophozoite homogenate as the antigenic material. The homogenate was diluted in PBS-Tween and added to the microtitre wells previously coated with SRAE antibody (or normal rabbit immunoglobulin for the control). The sensitivity of the test under these conditions was 0·15-0·3 µg/ml *E. histolytica* trophozoite protein, equivalent to approximately one amoeba trophozoite per cover-slip with the HK9 strain. This compares favourably with the sensitivity of the commercial Millipore kit which showed a much lower sensitivity using the same antigen preparations (Table I). Additional controls using normal human immunoglobulin at the same concentration to replace the HAE frction gave similar results to those obtained when normal rabbit immunoglobulin was used to coat the plates (Fig. 1, control), suggesting little non-specific binding of either antigen or antibody under the experimental conditions used.

After testing the reagents in the model system, the assay was used to detect *E. histolytica* trophozoite

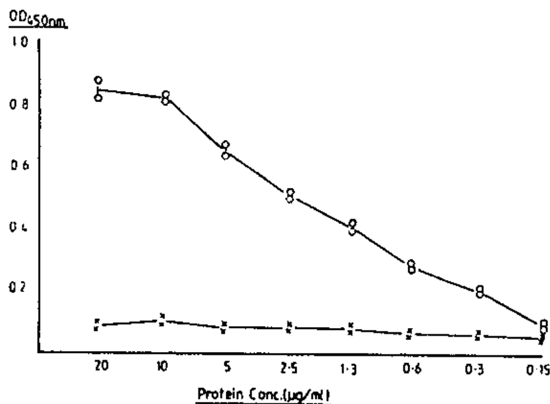


Fig. 1. The titration of axenically cultured *E. histolytica* trophozoite homogenate in ELISA, against SRAE (20 µg/ml) O——O, and normal rabbit immunoglobulin (20 µg/ml) x——x, duplicate values. The substrate incubation time was 5 min.

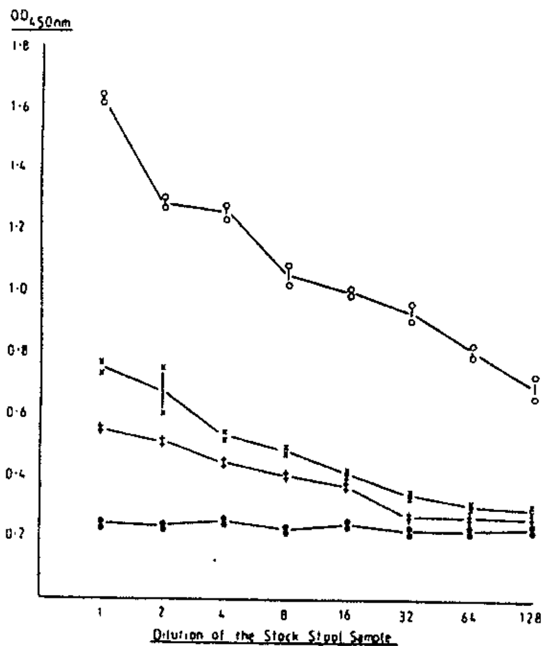


Fig. 2. The detection of *E. histolytica* trophozoite antigen in stool samples taken from human patients, duplicate values. The substrate incubation time was 10 min.
O—— O 4+ *E. histolytica* trophozoites in sample.
x —— x 2+ *E. histolytica* trophozoites in sample.
+ —— + 1+ *E. histolytica* trophozoites in sample.
● —— ● Sample negative for parasites.

antigens in stool samples obtained from human patients. Initially, four samples were taken and titrated in ELISA, and the results are shown in Fig. 2. The positive stools used in this test were positive for *E. histolytica* trophozoites under the microscope, but negative for the cyst form and other parasites. The control stool was negative for all parasites. From the results it can be seen that the stool samples positive using microscopic examination were also positive in the ELISA, and showed a dose-response curve as compared with the control sample. Additional controls included repeating the experiment using plates coated with normal rabbit immunoglobulin, and replacing the HAE with normal human immunoglobulin. No reactions above that for the normal control stool in Fig. 2 were obtained.

Table II shows the results of a preliminary study of the specificity of the test. Several stool samples were screened in ELISA as undiluted stock solutions, each sample having been first examined under the microscope, and the parasites contained identified. Little reactions was apparent with either the cyst forms of *E. coli*, *Endolimax nana* or *Entamoeba histolytica*, or with the vegetative forms of *E. coli* or *E. nana*. Samples 12 and 13 (Table II) were originally obtained from patients in whose stools *E. histolytica* trophozoites had been found, but who had been treated with Metronidazole three days before the ELISA assay determination. The samples from these two patients three days after treatment were found to be negative for *E. histolytica* trophozoites both in the ELISA and by microscopic examination.

Discussion

A method has been described for detecting *E. histolytica* trophozoite antigen in stool samples. The principle of this method is similar to that used for detecting rotavirus antigens (SIMHON *et al.*, 1979), in which microtitre plates were coated with antibody. From the results using the model system (Fig. 1) the test is able to detect 0·15–0·3 µg/ml *E. histolytica* protein concentrations, equivalent to approximately one amoeba/microscope cover-slip, with only low levels of non-specific reaction in the controls. This compares favourably with the commercial kit currently on the market (Table I). Despite employing the same basic principle, there are several differences in reagents and technique which may explain the increased sensitivity of the test using the microtitre plates. The kit uses a rabbit immunoglobulin preparation as the catching antibody, but in the method described here the catching antibody is purified by immunosorbent chromatography. In addition, the kit uses one less antibody layer, which may also contribute to the difference in sensitivity. One other major difference between the two methods is the conjugate. The method described in this paper uses a peroxidase conjugate prepared using a new technique (NILSSON *et al.*, 1981). These conjugates have a high enzymic activity relative to immunoglobulin, and show very low non-specific interactions.

The use of protein concentration as a measure of the sensitivity of the test is not ideal in this context, since some of the antigens may not be proteins and some of the proteins may not be antigens, and the test is specifically designed to detect antigens. It is however, the most convenient measure available when dealing with such complex systems.

The specificity of the assay was studied by comparing the results obtained using stool samples taken from a number of patients, each sample containing other parasites common to the area from which the positive samples were taken and of a structure which might cross-react with *E. histolytica* trophozoites (Table II). This preliminary study suggested that with these reagents and method of sample preparation,

Table I—The sensitivity of the commercial ELISA kit for the detection of *E. histolytica* trophozoite antigens. The antigen preparation used was homogenized axenically cultured *E. histolytica* trophozoites

E. histolytica trophozoite protein conc. μg/ml	Test	Positive control	Negative control
20	++	++	±
2	±	++	±
0·2	±	++	±
0	–	++	–

Table II—The reactivity of ELISA of a number of stool samples containing a variety of parasite forms common to the same area of Bangladesh

Sample number	Contents by microscopic examination	ELISA, absorbance at 450 nm 10 min undiluted stock solution
1	Control PBS.	0·18, 0·19
2	4+ *E. histolytica* trophozoites.	1·64, 1·62
3	2+ *E. histolytica* trophozoites.	0·77, 0·74
4	1+ *E. histolytica* trophozoites.	0·54, 0·55
5	Negative	0·20, 0·23
6	Negative	0·21, 0·22
7	Negative.	0·22, 0·24
8	*E coli* trophozoites, *E. nana* trophozoites, *E. histolytica* cysts, *E. coli* cysts, *E. nana* cysts.	0·25, 0·22
9	*E. coli* cysts, *E. histolytica* cysts, *E. nana* cysts.	0·23, 0·24
10	*E. coli* trophozoites, *E. coli* cysts.	0·21, 0·24
11	*E. nana* trophozoites, *E. coli* cysts.	0·22, 0·25
12	Patient treated with Metronidazole 3 days earlier, stool negative.	0·21, 0·23
13	Patient treated with Metronidazole 3 days earlier, stool negative.	0·24, 0·24

little interference occurred in the presence of *E. coli* cysts or trophozoites, *Endolimax nana* cysts or trophozoites, or with *Entamoeba histolytica* cysts. The apparent lack of reactivity with the cyst form of *E. histolytica* is interesting. Several factors may be responsible. The cyst surface may be antigenically distinct from the trophozoite-derived material used to immunize the rabbits for preparing the catching antibody and, as the cysts are less likely to have been lysed during the preparation of the stool samples, no reaction would occur with this experimental design.

Clearly this lack of reactivity with the cyst form has disadvantages as the relationship between cyst passers and the manifestation of clinical symptoms has yet to be defined. On the other hand, a test such as described above may help to resolve this situation, in that the stool samples of cyst passers may be examined to determine whether they are excreting lysed trophozoites in significant quantities, and this may then be related to clinical manifestations of invasion by the amoebae. For epidemiological purposes, however this test must be modified so that cysts can also be detected. Further studies are in progress on this point.

The development of the reagents for use in this technique was performed using homogenized *E. histolytica* trophozoites as the antigen. During the course of this development several factors were identified which ensured the specificity of the test. The incubation temperature of +4°C for plate coating and all subsequent incubations until substrate addition was found to be very important. Increasing the temperature for these procedures increases the level of non-specific binding. Why this is so is unclear, but it may be in part related to the interactions of the immunoglobulin molecules with the plastic surface, since temperature is known to affect such physical processes. Secondly, the purification of the catching antibody, the SRAE, by immunosorbent chromatography was found greatly to increase the sensitivity of the assay, compared to the situation if either whole serum or purified immunoglobulin fractions were used to coat the plates. Any loss of antibody activity as a result of this purification procedure was more than compensated for by the removal of much of the other immunoglobulin in the preparation which would otherwise compete with the specific antibody for the limited number of binding sites available on the plastic. This is probably the major reason for the difference in sensitivity between this assay and the commercial kit. The purity of the developing antibody (HAE) was found to be less important. Whilst using whole anti-*E. histolytica* human serum did give a quite high level of non-specific binding, much of this could be reduced by purification of the immunoglobulin fraction using immunosorbence on protein A-

Sepharose. No additional benefits were found by purifying this immunoglobulin fraction further.

From a clinical point of view, the use of pooled human anti-*E. histolytica* immunoglobulin as the developing antibody is very relevant for the diagnosis of infections. The patients from which the pool was prepared all had clinical amoebiasis and, as such, their sera contained antibodies against those antigens particularly relevant to clinical infections in humans. In addition, since this method has shown that human antibody can be used in such a context, it should be possible to adapt the technique to detect circulating antigen and specific immune complexes in the sera and body fluids of patients. Such observations may be a more significant measure of current invasion than serum antibody titres. From a practical point of view, however, if the technique is to be used for the routine detection of antigen, it would be better to replace the human antibody with that from a more easily accessible species, e.g., goat or sheep.

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