ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr. Rashidul Haque Trainee Investigator (if any) _____

Application No. 93-006 Supporting Agency (if Non-ICDDR,B) _____

Title of Study Rapid diagnosis of pathogenic E. histolytica infection and significance of p...

Project status:
( ) New Study
(X) 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)
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) (Yes) No
   (b) Use of fetal tissue or aborths Yes (No)
   (c) Use of organs or body fluids 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
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
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 Ettee. 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.

Rashidul Haque
Principal Investigator

_____
Trainee

93-006
11.1.93

APPLICATION FOR PROJECT GRANT

1.	PRINCIPAL INVESTIGATOR	:	Dr. Rashidul Haque
	COINVESTIGATORS	:	Dr. S.M Faruque Dr. Kh. Zahid Hasan
	COLLABORATING INVESTIGATOR (Co-PI)	:	Dr. William A. Petri, Jr.Associate Professor of Medicine & Microbiology Department of Medicine University of Virginia Charlottesville, Virginia, U.S.A
2.	TITLE OF PROJECT	:	Rapid diagnosis of *Entamoeba histolytica* infection and significance of nonpathogenic and pathogenic *E. histolytica* infections
3.	STARTING DATE	:	February 1993
4.	DATE OF COMPLETION	:	January 1996
5.	TOTAL BUDGET REQUESTED	:	US$120,384
6.	FUNDING SOURCE	:	
7.	HEAD OF PROGRAMME	:	Associate Director Laboratory Sciences Division

R. Bradley Sack

8. ABSTRACT SUMMARY

E. histolytica is a protozoan parasite which is an important cause of dysentery/diarrhoea in developing countries. Recent work strongly supports the conclusion that there are two species of *E. histolytica*, one nonpathogenic and one pathogenic.

The importance of distinguishing pathogenic from nonpathogenic *E.histolytica* infection derives from the fact that infection by nonpathogenic *E. histolytica*

is more common (about 90%) and does not require therapy. Many patients are currently being needlessly treated with prolonged and potentially toxic courses of anti-amoebic drugs because there is no test available to diagnose pathogenic *E. histolytica* infection. Zymodeme analysis of *E. histolytica* which has been used in the past for research purposes is not practical for routine clinical laboratories. Results usually are not available for 4 days or more. There are no commercially available or practical and reliable methods for differentiating pathogenic from nonpathogenic strains for routine use in clinical laboratories. Current detection of *E. histolytica* infection depends on the morphologic identification of the parasite in stool by expert microscopy. This is an insensitive method and requires good skill but does not differentiate the pathogenic species of *E. histolytica* from the much more prevalent nonpathogenic species.

We propose to develop a stool detection test for pathogenic *E. histolytica* which could be used for differentiating pathogenic from nonpathogenic strains of *E. histolytica* for routine use in clinical laboratories.

Drs. Haque and Petri have developed an ELISA that sensitively and specifically detects in stool pathogenic *E. histolytica*. This assay has been applied to single stool specimens from 74 patients in Bangladesh. The ELISA assay for pathogenic *E. histolytica* was positive in 12/12 stool specimens with pathogenic amoeba subsequently cultured, in 0/22 stool specimens with nonpathogenic *E. histolytica*, and in 2/40 patients with other or no intestinal parasites. Sensitivity and specificity of the assay for pathogenic *E. histolytica* were 100% and 97% respectively. The major aim of the proposal is to confirm the validity of the test on a larger number of patients and in asymptomatic subjects, and to then adapt the assay into PVC strip /or latex

agglutination formats that can be easily used in the field. *E. histolytica* infection diagnosed as nonpathogenic by the assays will also be monitored longitudinally.

Successful completion of the project clinical laboratories will result in, for the first time, a rapid, simple and reliable test for diagnosing infections caused by the pathogenic *E. histolytica*, and thereby bring important new information on the diagnosis and clinical interventions of amoebic disease.

9. AIMS OF PROJECT

a) General aim

To test the applicability of recently developed diagnostic methods for pathogenic *E. histolytica* by testing stool specimens from 100 asymptomatic individuals infected with nonpathogenic *E. histolytica* and 100 patients infected with pathogenic *E. histolytica*, and adaption of the microtiter plate ELISA to a PVC strip ELISA or latex agglutination test.

b) Specific aims

1) To develop and test a monoclonal antibody-based ELISA for the detection of pathogenic *E. histolytica*.

2) Adaption of the microtiter plate ELISA to a PVC strip ELISA or latex agglutination to allow field detection of pathogenic *E. histolytica* infection.

3) To determine the sensitivity and specificity of monoclonal antibody-based ELISA and a PCR test to distinguish between pathogenic and nonpathogenic *E. histolytica*.

4) To confirm the harmless nature of infection by the nonpathogenic *E. histolytica* diagnosed as nonpathogenic by the recently developed immunoassay.

c) Significance

Infection with nonpathogenic *E. histolytica* does not require treatment; therefore a rapid diagnostic test that is specific for pathogenic *E. histolytica* infection will decrease the needless expense and toxicity of inappropriate treatment of nonpathogenic infection.

Successful completion of the proposed research will bring important new information on diagnosis and management of amoebic disease, by enabling for the first time the rapid and specific diagnosis of pathogenic *E. histolytica* infection, and by eliminating the costly and unnecessary treatment of nonpathogenic *E. histolytica* infection, if it is not a substantial risk.

10. ETHICAL IMPLICATIONS

Informed consent will be obtained from the subjects or their legal guardians. Because nonpathogenic *E. histolytica* have never been demonstrated to cause invasive amebiasis, and because the medical follow-up and surveillance that the subjects will receive is comprehensive, entry into the study should entail little or no risk to the subjects. This research is not going to cause any physical trauma. Confidentiality will be strictly maintained. The benefits of the study include the development of the ability to treat only pathogenic *E. histolytica* infection, with attendant decreased cost of medications and decreased risk of toxicities and the development of a rapid test to improve

the diagnosis of pathogenic *E. histolytica* infection, a major cause of morbidity and mortality.

11. BACKGROUND, RESEARCH PLAN AND BIBLIOGRAPHY

a) BACKGROUND

Entamoeba histolytica infects an estimated half billion people annually, and is exceeded only by malaria and schistosomiasis as the leading parasitic cause of death in children and adults (1). At the International Centre for Diarrhoeal Disease Research in Dhaka, Bangladesh, the majority of patients admitted with invasive amebiasis were children age 10 and younger, of whom 20% did not survive despite fluid resuscitation and anti-amoebic therapy (2). Fortunately, less than 10% of individuals infected with *E. histolytica* develop colitis or liver abscess (1). Asymptomatic infection can be explained at least in part by the existence of pathogenic and nonpathogenic species of *E. histolytica*. The presence of two distinct species (pathogenic and nonpathogenic) of *E. histolytica* has been demonstrated in over 3000 isolates by isoenzyme analysis (1,3,4), typing by monoclonal antibodies to surface antigens (5,6), internal antigen (23), ribosomal RNA sequence analysis (7), restriction fragment length polymorphisms (8,9) and Polymerase Chain Reaction (PCR) test (18). *E. histolytica* classified as nonpathogenic have never been documented to cause colitis or liver abscess (1,3-9). Pathogenic strains have been uniformly present in all cases of colitis and liver abscess examined, and can also cause asymptomatic colonization (as do the nonpathogenic strains). Earlier reports from 2 different laboratories that isoenzyme profiles of pathogenic and nonpathogenic *E. histolytica* could convert in vitro (10,11) may have resulted from inadvertent contamination of cultures, as isoenzyme

conversions have not been reproduced after exhaustive attempts by other investigators (12,13).

The importance of distinguishing pathogenic from nonpathogenic *E. histolytica* infection derives from the fact that nonpathogenic *E. histolytica* account for up to 90% of all infections, and most likely do not require therapy (1). Many children are being needlessly treated with prolonged, expensive and potentially toxic courses of anti-amoebic drugs because there is no test available to specifically diagnose pathogenic *E. histolytica* infection. Current detection of *E. histolytica* infection depends on the morphologic identification of the parasite in stool by expert microscopy. This is an insensitive and unreliable procedure which does not differentiate the pathogenic species of *E. histolytica* from the much more prevalent nonpathogenic species. While new monoclonal antibody [reviewed in (14)] and DNA probe assays (15) may detect *E. histolytica* in stool with similar sensitivity to microscopy, none of these reported tests distinguish pathogenic from nonpathogenic infection.

We have produced a monoclonal antibody (MAb) based ELISA to detect infection with pathogenic *E. histolytica* directly in stool. The ELISA is based on mAb to six non-overlapping epitopes on the galactose-specific adhesin from pathogenic *E. histolytica*. Monoclonal antibodies to epitopes 3-6 do not recognize the nonpathogenic adhesin, permitting differentiation of pathogenic from nonpathogenic *E. histolytica* (5). The ELISA uses microtiter wells coated with rabbit polyclonal anti-adhesin antibodies to "capture" the adhesin antigen from stool samples, and peroxidase-conjugated, pathogen-specific, murine mAb to adhesin epitopes 3 and 4 to detect only the pathogenic adhesin in the stool specimen. Our preliminary studies (conducted by Drs. Haque and Petri) have

shown that the ELISA was positive for 12/12 stools subsequently shown by culture and isoenzyme analysis to contain pathogenic *E. histolytica*. All 22 stools containing nonpathogenic *E. histolytica* were negative, and 2/40 stools with other or no parasites detected by microscopy were positive. The 2 stools that were positive by ELISA that did not have *E. histolytica* detected by microscopic exam may not be false positives as microscopy is an insensitive diagnostic technique (1). The ELISA in these initial studies has a sensitivity for pathogenic amoebae of 100% and a specificity of 97% (19).

The ELISA for glactose adhesin represents the first rapid and practical method for the detection of pathogenic *E. histolytica* infection in stool specimens. The ELISA has the potential to replace not only the current microscopic examination of stool for morphologic identification of *E. histolytica*, but also the cumbersome, lengthy culture and isoenzyme analysis presently required to differentiate the pathogenic from nonpathogenic species in stool. Performance of the assay by clinical labs could for the first time permit inclusion of knowledge of the pathogenicity of the organism into the decision of whether to treat the patient. While the ELISA has so far only been tested on a limited number of patients from Bangladesh, it is likely that it will also successfully detect pathogenic *E. histolytica* infection elsewhere. Previous work has shown that the pathogen-specific epitopes 3-6 are conserved on the adhesin from amoeba isolated in the United States, Mexico, India, South Africa and Thailand (5). A recently reported PCR test (18) which has only been tested with few laboratory isolates of pathogenic and nonpathogenic *E. histolytica* is entirely in accord with those of zymodeme analysis and this indicates that pathogenic and nonpathogenic *E. histolytica* are distinct in genomic DNA. Use of this PCR test will determine the genetic homogeneity of our clinical isolates.

The ELISA to detect pathogenic amoebae should not only be financially feasible but actually cost-saving: The cost of performing the ELISA test at ICDDR,B is estimated to be a maximum 10 cents U.S. per test including manpower if done in large scale intervention studies. The minimum cost of treatment for amebiasis in Dhaka (10 days of oral metronidazole, 750 mg three times a day) is US$3.00. Approximately 90% of amoebic infection has been found to be nonpathogenic; if our prospective study confirms that nonpathogenic infection does not require treatment then for the $1.00 cost of 10 tests on patients with amoebic infection, $27.00 would be saved by avoidance of 9 courses of therapy (with 9/10 infections predicted to be nonpathogenic).

b) RESEARCH PLAN

Study area

The proposed study will be conducted in Section-11, Mirpur (peri-urban slum area of Dhaka) and at the ICDDR,B Dhaka hospital. Section-11, Mirpur has around 980 households with a total of 5782 permanent residents, having 5.9 members in each family. The inhabitants here essentially fall in the lower and lower middle class socio-economic status (personal communication with Dr. A. Hall).

Study population

A population of approximately 1500 people >5 years of age in Section-11, Mirpur will be screened for the presence of *E. histolytica* infection by microscopy, stool culture and stool ELISA. Asymptomatic individuals who are positive for nonpathogenic *E. histolytica* infection as determined by culture with isoenzyme analysis will be invited to participate in the study. Informed consent will be obtained from the individual and legal guardian.

At the ICDDR,B cases of pathogenic *E. histolytica* infection will be recruited as a part of another study from Dhaka Hospital surveillance system and randomly selected patients with clinical dysentery - patients with a history of three or more bloody- unformed stools during the previous day, in whom known bacillary causes of dysentery are excluded.

Sample size

One hundred asymptomatic subjects infected with nonpathogenic *E. histolytica* and also 100 patients with clinical dysentery infected with pathogenic *E. histolytica* infection will be enrolled in the study.

The incidence of infection with *E. histolytica* in individuals > 5 years of age in section, 11, Mirpur of Dhaka city is approximately 10% by microscopy from a recent survey conducted by Dr. Andrew Hall (personal communication).

Required sample size to estimate the percentage of *E. histolytica* infection in this population will be:

$$N = \frac{Z^2 \cdot PQ}{E^2}$$

$$N = \frac{1.96^2 \cdot 0.1 \cdot 0.90}{0.02^2}$$

P = 0.1 (proportion of cases)
Q = 1-P
Z = 1.96 (95% confidence in the estimate)
E = 0.02 (allowable error 2%)

Therefore, a minimum of 150 individuals will be identified with amoebic infection by screening 1500 subjects. 90% of these infection have been

demonstrated by isoenzyme analysis to be nonpathogenic (16), even with loss of study subjects due to ineligibility for the study due to coinfection with other enteric parasitic pathogens and with loss due to outmigration from Dhaka (30%/ year maximum) there should be at least 100 subjects at the end of study.

The incidence of infection with *E. histolytica* in patients with clinical dysentery >5 years of age at the ICDDR,B Dhaka Hospital is 8.5% (personal observation).

Required sample size to estimate the percentage of infection of *E. histolytica* using the above mentioned formula is:

$$N = \frac{1.96^2 \cdot 0.085 \cdot 0.0915}{0.02^2}$$

Therefore, a minimum of 170 patients infected with *E. histolytica* will be identified by screening 2000 patients. 80% of these infections have been documented to be infected only with pathogenic *E. histolytica*, which will give a sample size of approximately 135 patients.

The sensitivity and specificity of the pathogenic *E. histolytica* specific ELISA at present are 100% and 97% respectively. In order to determine the 95% positive predictive value of the pathogenic specific ELISA and assuming that sensitivity and specificity further decrease to 98% and 95% respectively, it is estimated that 100 stool specimens positive for pathogenic and 100 stool specimens positive for nonpathogenic *E. histolytica* will be required in the study.

To confirm the harmless nature of infection by the nonpathogenic *E. histolytica*, 100 subjects infected with nonpathogenic *E. histolytica* will

be required. With 100 subjects 95% confidence interval for a 0% incidence of disease will be 0 -3.6% (17).

Eligibility Requirements for Inclusion into the Study

1. Individuals must have asymptomatic infection with nonpathogenic *E. histolytica* from the community (as determined by isoenzyme analysis of the cultured amoebae from stools).

2. Patients must have dysenteric diarrhoea with pathogenic *E. histolytica* from the Hospital (as determined by isoenzyme analysis of the cultured amoebae from stools).

SURVEILLANCE

After enrollment of subjects infected with nonpathogenic *E. histolytica* a household morbidity surveillance will be instituted by the trained female Community Health Workers (CHW) using pre-coded form, who will visit the study participants twice a week to collect information about diarrhoeal diseases. Stool samples will be collected every three months routinely for one year and whenever they complain of diarrhoea. All stool specimens will be examined by microscopy, culture and isoenzyme analysis will be performed for each and every positive cultures of *E. histolytica* will be taken by using pre-coded forms. The CHW will collect rectal swabs and faecal specimens whenever they complain of diarrhoea. Stool samples from diarrhoeal episode will be examined for known causes of bacterial, viral and parasitic causes of diarrhoea.

Bacterial causes of diarrhoea that will specifically excluded include *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Aeromonas* spp., *Plesiomonas* spp., *C. difficile*, *B. fragilis* and *Vibrio* spp. all by standard

methodology of the ICDDR,B. *E. coli* strains will be picked from MacConkey agar and stored separately in vials containing trypticase soy broth with 15% glycerol at -20°C for later testing. These *E. coli* colonies will be probed for diarrhoeagenic properties with specific DNA probes available at the ICDDR,B. Parasites to be excluded will include *Giardia, Strongyloides, Cryptosporidia* and *Isospora*. MIF concentration method will be used for the cysts of *Giardia* and other gut protozoa, Bearmann method for *Strongyloides* and modified Ziehl-Neelsen stain for *Cryptosporidium* and *Isospora* will be used. In addition to this an ELISA for detection of Cryptosporidium and Giardia stool antigen will also be used. Rotavirus, adenovirus and astovirus will be detected by established ELISA methodology at ICDDR,B.

Definition of Invasive Amebiasis

The diagnosis of amoebic liver abscess will be established by clinical presentation, bacteriologically sterile abscess aspirate, response to specific anti-amoebic therapy with metronidazole, and a positive serologic test for anti-amoebic antibodies. Amoebic colitis or dysentery will be established by clinical presentation with diarrhoea, with stools positive for occult blood and *E. histolytica* cyst or trophozoites, consistent findings of colonic ulceration on sigmoidoscopy or colonoscopy, no other bacterial, viral or parasitic cause of diarrhoea found, and response to specific anti-amoebic therapy.

Treatment

Asymptomatic carriers of nonpathogenic *E. histolytica* infection will not be treated with metronidazole. If they develop infection with pathogenic *E. histolytica* or diarrhea or dysentery due to *E. histolytica* or other

bacterial, parasitic or viral infections, appropriate treatment will be given by physicians.

Laboratory Methods

Characterization of E. histolytica isolates

Zymodeme analysis, MAb based immunoassay and PCR test will be used for differentiation of pathogenic and nonpathogenic *E. histolytica* and for monitoring *E. histolytica* isolates from the study population. Validation of these tests will be done by using standard pathogenic and nonpathogenic *E. histolytica* isolates. Stool samples will be transported to the laboratory within three hours of collection where a direct smear will be prepared for microscopical examination to determine the presence of white and red blood cells in the stool wet preparation. Other protozoans or helminths, if present, will be recorded as well, and it will then be divided in three portions: 1 g will be cultured in Robinson's medium (3,4,16) at 37°C; 5 g will be preserved in polyvinyl alcohol fixative/formol-ether for future microscopical examination and for concentration methods; and 2 g will be preserved directly at -20°C for antigen detection ELISA. Positive cultures for *E. histolytica* trophozoites will be maintained in the laboratory for zymodeme characterization, IFAT, ELISA, and PCR test and then cryopreserved in liquid nitrogen.

Isoenzyme analysis of E. histolytica

The pathogenic and nonpathogenic zymodemes will be determined by isoenzyme electrophoresis according to the method described by Sargeaunt *et al.* (3,4) and Haque *et al.* (16). Three enzymes will be studied namely, hexokinase, phosphoglucomutase, and glucophosphate isomerase. Isolates with known zymodemes will act as control.

ELISA procedure

Polysterene 96-well microtiter plates (Corning Glass Works, Corning, NY) will be coated with 100 µl/well of a 1:2500 dilution of protein A-purified rabbit polyclonal antibodies against the glactose adhesin in 0.1 M bicarbonate buffer pH 9.6 overnight at 4°C. The plates after washing 3 times in PBS containing 0.05% Tween-20 will be blocked in 1% BSA (Sigma)-PBS-Tween for 1 hour at room temperature. Stool samples to be tested will be suspended 1:1 in PBS containing 2 mM phenyl methyl sulfonyl fluoride (PMSF) and 2 mM P-hydroxy mercuribenzoic acid (PHMB) and the stool suspension in 100 µl final volume added to the microtiter well. The microtiter plates will be incubated with the stool suspension overnight at 4°C or for 1-2 hours at room temperature with a 1:1500 dilution of a 4 mg/ml solution of peroxidase-conjugated anti-adhesin monoclonal antibodies 7F4 and 8C12 (directed against pathogen-specific epitopes 3 and 4 respectively of the adhesin 170 kDa subunit (5). The wells will be washed 3 times in PBS-Tween, followed by addition of substrate. Absorbance at 450 nm of the microtiter wells will be measured with an ELISA plate reader (Titertek Multiskan, Flow Laboratories, VA) after 10-45 min of development.

Adaption of the microtiter plate ELISA to a PVC strip ELISA or latex agglutination

The conventional microtiter plate ELISA is convenient for doing multiple assays in a clinical laboratory, but is impractical for field use. In order to make more widely available the improvement in diagnostic ability that the pathogen-specific ELISA represents, we will attempt to adapt it to a latex agglutination or polyvinylchlorice (PVC) strip format.

Latex agglutination format

Our goal is to develop an easily read "all or nothing" test specific for pathogenic *E. histolytica* in stool that can be readily performed in the field by paramedical personnel. The latex agglutination format is straightforward and has been used successfully in stool specimens to detect fecal lactoferrin (24). We will attempt to adapt the ELISA for pathogenic *E. histolytica* to this format.

Latex beads (Bacto-Latex; Difco Laboratories, Detroit, MI) will be passively coated with purified anti-adhesin antibodies 7F4 and/or 8C12 (specific for pathogenic amoebae). The beads will first be centrifuged at 1,800 X *g* for 30 min, washed with 5 ml of glycine buffer (7.3 g of glycine and 10 g of NaCl/Liter, pH 8.2), and resuspended in 5 ml of glycine buffer to a final 1% suspension of beads. The anti-adhesin monoclonal antibodies (1 mg/ml final concentration) will be added to the beads, and the antibody-bead mixture incubated at 37°C for 1 h, after which the antibody-coated beads will be washed 3 times from unbound antibodies by centrifugation. The beads will finally be resuspended in 5 ml of glycine buffer containing 1% albumin and 0.05% azide and stored at room temperature for later use.

Stool samples will be tested with the latex particles only after the sensitivity and specificity of the agglutination assay for pathogenic amoebae has been confirmed with cultured amoebae. Stool samples from patients with pathogenic and nonpathogenic *E. histolytica* infection as well as stool samples from patients with other or no parasites detected will be tested with the latex bead agglutination test. The same samples used for the ELISA test (above) will be tested by latex particles, and determination of sensitivity or

specificity of the agglutination test will be done. If this is suboptimal in stool, we will reformulate the antibody coating procedure and/or the latex matrix used (see above).

Polyvinylchloride strip format

PVC strips are inexpensive, and have been successfully adapted by other investigators for the detection of total (not pathogen-specific) *E. histolytica* antigen in stool (20). The PVC strips coated with polyclonal anti-adhesin antibodies have the potential to be provided to health care personnel ready for use. The strips can be directly incubated in the stool specimens to absorb the adhesin, then washed and developed with pathogen-specific anti-adhesin mAb. We will work to optimize the PVC strip system to an assay that is easily read by the naked eye as a positive or negative result.

PVC strips will be coated with purified polyclonal anti-adhesin antibodies (50 µg/ml) in 0.1 M bicarbonate buffer, pH 9.6) overnight at 4°C. The strips will then be washed three times in PBS 0.05% Tween-20 (PBS-Tween) and blocked in 1% BSA-PBS-Tween. The strips can then be stored at room temperature in PBS-Tween containing 0.05% azide until ready for use. PVC strips will be incubated individually in vials containing 1 ml of sample containing amoebic antigen for 1 h, washed 3 times with PBS-Tween, and the bound antigen detected with phosphatase-conjugated pathogen-specific mAb (see ELISA methods, above). During the development of the test the strips will be read in a spectrophotometer to determine the absorbance at 445 nm; our goal will be to optimize the performance of the test so that it can be read by the naked eye as positive or negative for the presence of pathogenic *E. histolytica* in stool. Determination of the PVC strip assay system's sensitivity and

specificity will be as described above for the ELISA and latex agglutination tests. Stool samples will be assayed only after the test has been optimized for cultured amoebae. Problems with specificity or sensitivity, either with cultured amoebae or stool samples, will be dealt with by changing the antibody coating conditions for the PVC strips, by varying the amount or type be nonionic detergent used in the buffers, by using different pathogen-specific MAbs as capture or detection reagents and/or by using a different matrix strip to absorb the antibodies, such as polyvinylidene difluoride membranes.

Polymerase Chain Reaction (PCR) test

Two pairs of oligonucleotide primers, p11 (5'-GGAGGAGTAAGGAAAGTTGAC-3') & p12 (5'-TTCTTGCAAATTCCTGCTTACGA-3') and p13 (5'-AGGAGGAGTAGGAAAATTAGG-3') & p14 (5'-TTCTTGAAACTCCTGTTTCTAC-3') will be used for the detection of each sequence (18). The extracted DNA from cultured trophozoites of *E. histolytica* will be amplified for 30 cycles, the cycling conditions will be denaturation at 94°C for 1 min, annealing at 59°C for 90s, and polymerization at 72°C for 90s. One hundred base pair (bp) products will be amplified by using p11 and p12 primers from the genomic DNA of pathogenic strains (zymodemes). On the other hand, by using p13 and p14 primers, 101-bp PCR products will be detected only from *E. histolytica* isolates with nonpathogenic strains (zymodemes).

Analysis plan

All the data will be collected in pre-coded standard forms. The analysis of results will be performed at ICDDR,B by microcomputer.

ELISA results will be reported as the ratio of optical density at 450 nm of the well containing the stool sample over wells containing no stool. The normal range of the assay has been defined in the preliminary studies as the

mean±3 standard deviations of the optical density ratios for the 22 samples tested to date with nonpathogenic *E. histolytica* cultured from their stool. All stools from the cohort and hospital will be examined by microscopy, amoebic culture, ELISA and PCR. Because microscopic exam of stool is a relatively insensitive means to detect amoebic infection (1, personal observation), we expect that some stool specimens will have positive amoebic cultures but negative microscopic exam for cysts and trophozoites. Positive pathogenic *E. histolytica* specimens will be defined as those stools with *E. histolytica* cultured from the stool where the amoebae are shown to be pathogenic by isoenzyme analysis.

The sensitivity and specificity of the ELISA will be determined with respect to pathogenic *E. histolytica* in stool specimens, where:

$$\text{Sensitivity \%} = \frac{\text{True positive (TP)}}{\text{TP + False negative (FN)}}$$

$$\text{Specificity \%} = \frac{\text{True negative (TN)}}{\text{TN + False positive (FP)}}$$

The predictive value of the pathogenic specific ELISA will be estimated using the J Index (21), and the prevalence of pathogenic *E. histolytica* infection (22). The correlation between the optical density and the semi-quantitative number of pathogenic *E. histolytica* cyst or trophozoites seen in faeces will also be determined.

The sensitivity and specificity of PVC strip ELISA or Latex agglutination and PCR test will also be determined with respect to pathogenic *E. histolytica* in stool specimens using the above mentioned statistics.

Prevalence of both pathogenic and nonpathogenic forms of *E. histolytica* will be determined from the data obtained from the hospital and community. Follow-up data from the cohort of nonpathogenic *E. histolytica* infection will be used to calculate the incidence rate of invasive amoebiasis and other causes of diarrhoea in the cohort if any.

FACILITIES REQUIRED

Existing facilities in the Parasitology and Molecular Biology Laboratory will be used. No new PCR machine will be needed.

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5) Haque R, Hall A, Tzipori S. Cellulose acetate electrophoresis of isoenzymes from *E. histolytica* isolates. In: Programme and Abstracts of the Symposium on Amoebiasis, New Delhi 14-16 December 1990.

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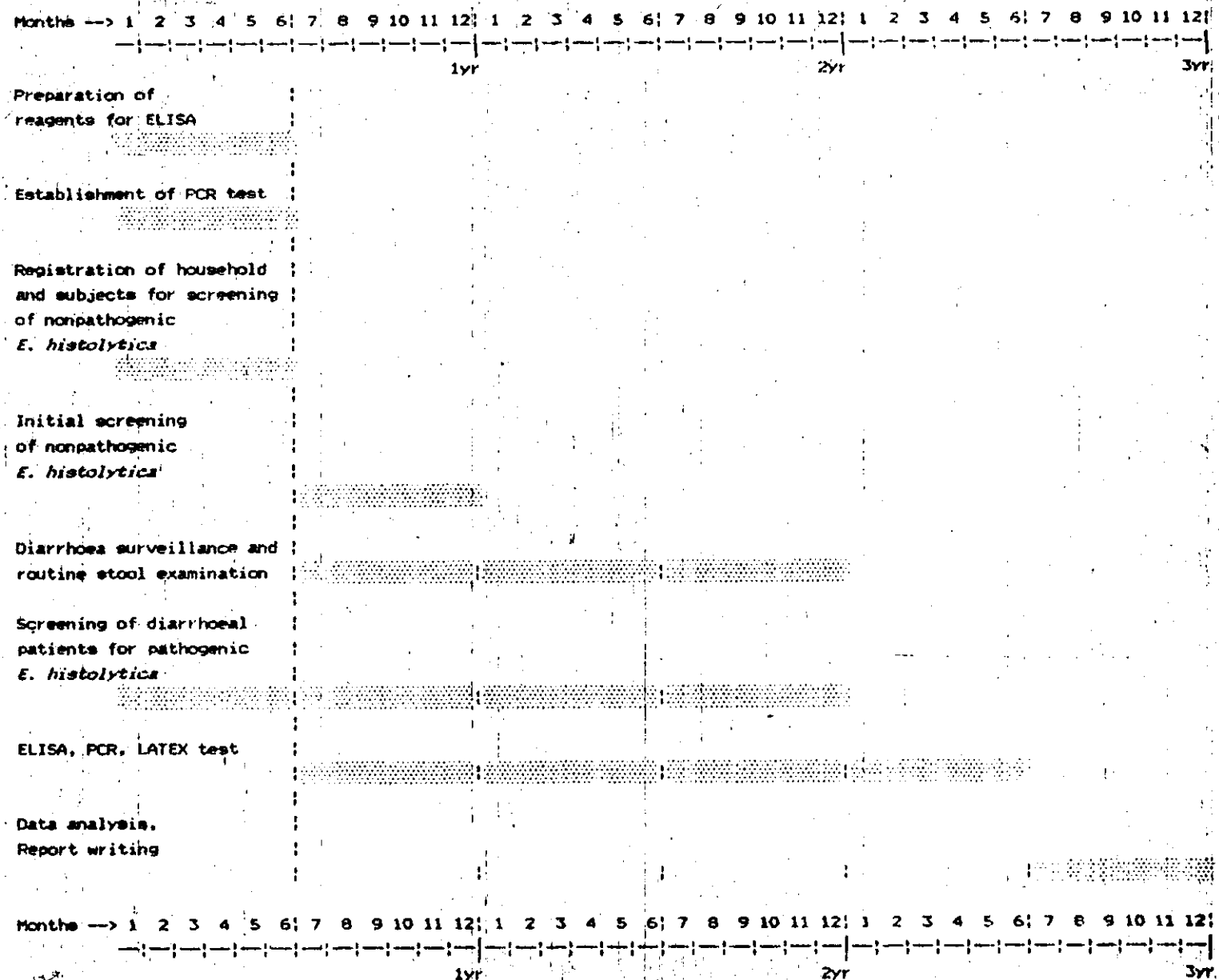
13. FLOW CHART OF THE WORK PLAN



14. ITEMIZED SPECIFIC TASKS FOR EACH LISTED INVESTIGATOR

The proposed project represents a collaboration between Dr. Rashidul Haque at the ICDDR,B and Dr. William Petri, Jr. at the University of Virginia.

Dr. Rashidul Haque

- Collaborate with Clinical Sciences Division, ICDDR,B for enrollment of patients, collection of clinical information and specimens.
- Collaborate with field staff to ensure proper enrollment and collection of specimens.
- Collaborate with Dr. WA Petri to develop PVC strip ELISA or latex agglutination.
- Maintain an accurate record of all clinical and laboratory information.
- Ensure that careful processing, storage and cataloging of study samples is maintained
- Perform ELISA, PVC strip ELISA or latex agglutination and other methods required.
- Collect and write-up results.

Dr. WA Petri

- Collaborate with Dr. Haque to develop PVC strip ELISA or latex agglutination.
- Collaborate with Dr. Haque to coordinate and discuss study protocols and results.

Dr. SM Faruque

- PCR test for pathogenic and nonpathogenic *E. histolytica*

Dr. Kh Zahid Hasan

- Collaborate with Dr. Rashidul Haque in matters relating to the epidemiological aspect of the study.

DETAILED BUDGET

			Year 1	Year 2	Year 3
a) Personal cost					
PI	80%	NOB US$	6,743	7,585	7,585
Coinvestigator (1)	20%	NOB	1,885	2,425	2,425
Coinvestigator (2)	5%	NOC	-	-	-
Research Officer	100%	GS-V	2,784	5,568	3,250
Lab Technician	100%	GS-III	3,597	3,597	1,798
Lab.Attendant	100%	GS-I	1,980	2,376	2,376
CHW (2)	100%	GS-I	3,960	4,752	1,188
		US$	20,949	26,303	18,632
b) Operating cost					
Chemicals and media	-	US$	6,500	4,000	2,000
Supplies and others	-		3,000	3,000	1,500
Transport	-		1,500	1,500	1,000
Maintenance, telex, etc.			400	400	400
Photocopying and mimeography			500	500	500
Cost for bacteriological tests			1,500	1,500	500
Cost for E.coli testing			500	400	400
Cost for virological test			500	1,000	500
Computing	-		0	2,000	2,000
Travel	-		0	0	3,000
		US$	14,400	14,300	9,300
c) Capital expenditure	-		6,500	0	0
		US$	42,349	40,603	27,932

TOTAL: 1st year	US$	42,349
2nd year		40,603
3rd year		27,932
Total for 3 years	US$	110,884
Overhead 25% of operating cost		9,500
GRAND TOTAL:	US$	120,384

Justification of the budget

1. The cost of salaries for the staff required to undertake the work come to $68,861, 50.8% of the total.

2. Large volume of media will be required each week in order to culture *E. histolytica*. Primers will be needed for PCR test.

3. One Helena Cellulose acetate electrophoresis apparatus is already available, but it is very old and should be replaced by a new one.

4. One cryobank will be needed to store in liquid nitrogen isolates of *E. histolytica*.

5. One visit of Dr. Petri to Bangladesh to coordinate and discuss study protocols and results.

RH:mh/H4:EHIS.PRT

Abstract summary for ERC:

1. *E. histolytica* infection is more common in population > 5 years of age. The major aim of this study is to test the applicability of recently developed simple diagnostic method for pathogenic *E. histolytica* infection in human. Therefore, we have selected two such groups of population: a) patients with pathogenic *E. histolytica* infection from the ICDDR,B hospital and b) asymptomatic subjects with nonpathogenic *E. histolytica* to be selected randomly from the community.

2. Entry into the study should entail no potential risks - physical, psychological, social, legal or other to the study subjects. Invasive techniques are not involved, only stool specimens will be collected from the study population.

3. This research is not going to cause any physical trauma. Nonpathogenic *E. histolytica* have never been demonstrated to cause invasive amoebiasis, however a regular medical follow-up and surveillance will be carried out.

4. Confidentiality will be strictly maintained by coding each subject.

5. Although there is no potential risk to the subjects:

a) Signed informed consent will be obtained from the subject/ legal guardian of the subject.

b) No information will be withheld from the subject.

c) There is no potential risk or privacy to the subject. However if any subject develop diarrhoea appropriate treatment will be given.

6. An interview will be taken by the Community Health Workers using pre-coded form in the community. Time required for the interview is about 5-10 minutes.

7. The benefits of the study include the development of the ability to treat only pathogenic *E. histolytica* infection, with attendant decreased cost of medication and decreased risk of toxicities and the development of rapid and reliable test to improve the diagnosis of pathogenic *E. histolytica* infection, a major cause of morbidity and mortality in the developing countries. The cohort studied will also directly benefit from the medical care that they receive during the study period.

8. The medical records of the patients from ICDDR,B hospital will be used for this study only. No other body fluids, the fetus etc will be utilised.

CONSENT FORM

HOUSEHOLD ID:
SUBJECT'S ID:
FATHER'S NAME:
ADDRESS:

STUDY: Rapid diagnosis of pathogenic *E. histolytica* infection and significance of pathogenic and nonpathogenic *E. histolytica infection.*

(Statement to be read to the subjects or legal guardian when consent is obtained).

Diarrhoea is a very common illness in Bangladesh. *E. histolytica* is one of the cause of dysentery/ diarrhoea. There are two species of *E. histolytica*, one of which does not cause diarrhoea. ICDDR,B in collaboration with University of Virginia, U.S.A has developed a rapid method for detection of pathogenic *E. histolytica* and is testing this method for its applicability. In order to do this we are interested to conduct a study in your community for a period of one year. We would like to collect stool samples only for our study purpose.

If we find that you or your child are infected with asymptomatic nonpathogenic *E. histolytica* infection then you will be enrolled in this study. If you are become sick or develop diarrhoea we will try to give necessary treatment.

If you agree to participate in the study, then please sign your name or give your thumb impression. You may withdraw yourself or your child from the study at any time you wish.

Signature of Investigator

Signature or left thumb print of participant/ guardian

Date:

STOOL MICROSCOPY & PARASITE CULTURE FORM

1. Name : _____

3. I.D. number : __/__/__/__/

4. Date collected : __/__/__/__/__/__/

4. Why sample collected : __/ (1=screen; 2=routine 1; 3=routine 2 4=routine 3; 5=diar1;6=diar2)

5. Consistency :__/ (1=formed;2=loose;3=liquid;4=Watery)

6. Visible blood :__/ (0=no ; 1=yes)

7. Mucus :__/ (0=none;1=trace;2=mod;3=many)

8. Pus cells :__/ (0=none;1=few; 2=mod; 3=many)

9. RBC's :__/ (0=none; 1=few; 2=mod; 3=many)

10. Yeasts :__/ (0=none;1=few; 2=mod; 3=many)

11. Fat globules :__/ (0=none;1=few;2=mod;3=many)

12. Giardia cysts :__/ (0=none; 1=few;2=mod;3=many)

13. Giardia-trops :__/ (0=none;1=few;2=mod;3=many)

14. E. histolytica -cysts:__/ (0=none;1=few;2=mod;3=many)

15. E. histolytica-trops :__/ (0=none;1=few;2=mod;3=many)

16. E.his-trop +RBC :__/ (0=no; 1=yes)

17. Other protozoa 1 :__/ (0=no; 1=E.coli;2=E. nana;3=I.butsch; 4=E.hart;5=C.mesnili;6=B.hom;7=T.hom 8=E.hom;9=other_ comment_____)

18. Other protozoa 2 :__/

19. A. lumbricoides :__/ (0=no; 1=yes)

20. T. trichiuria :__/ (0=no; 1=yes)

21. Hookworm :__/ (0=no; 1=yes)

22. Other helminths 1 :__/ (0=no; 1=S.stercorlis; 2=H. diminuta 3= E. vermicularis).

23. Other helminths 2 :__/

24. Smear examined by :__/ (1=S.A;2=R.H;3=Other)

25. Culture for E.h :__/ (0=no; 1=yes)

26. Culture for other :__/ (0=no; 1=B.hom; 2=E.coli; 3=E.nana)

(cont.)

25. CONCENTRATION METHOD :__/ (0=not done; 1=done)

Wt bottle +fixative + stool _____
Wt bootle + fixative _____
Total drops in test tube _____
Sample drops examined _____

26. Giardia cysts :__/ (0=no;1=few;2=mod;3=many)

27. E.histolytica cysts :__/ (0=no;1=few;2=mod;3=many)

28. Other protozoan cysts 1 :__/
(0=no; 1=E.coli;2=E. nana;3=I.butsch;
4=E.hart;5=C.mesnili;6=B.hom;7=T.hom
29. Other protozoa 2 :__/ 8=E.hom;9=other_comment_____)

30. A. lumbricoides :__/__/__/__/__/ (0=none;1-99998=epg)

31. T. trichiuria :__/__/__/__/__/ (0=none;1-99999=epg)

32. Hokworm :__/__/__/__/__/ (0=none;1-99999=epg)

33. S. stercoralis :__/__/__/__/ (0=none;1-9999=epg)

34. H. diminuta :__/__/__/__/ (0=none;1-9999=epg)

35. E. vermicularis :__/__/__/__/ (0=none;1-9999= epg)

36. Smear examined by :__/ (1=S.A; 2=R.H; 3=Other)

37. Cryptspoidium slide made :__/ (0=n0; 1=yes)

38. Crypto in stained smear :__/ (0=no; 1=yes)

Laboratory investigations on *E. histolytica* positive stool specimens.

1. Name :_____
2. I.D number :__/__/__/

3. Zymodeme :__/ (1=PZ; 1=NPZ)
4. IFAT :__/ (0=Neg; 1=Pos)
5. ELISA (cultured amebae) :__/ (0=Neg; 1= Pos)
6. ELISA(cultured amebae OD): __/__/__/__/ (indicate value)
7. ELISA (stool specimens) :__/ (0=Neg; 1=Pos)
8. ELISA (stool OD) :__/__/__/__/ (indicate value)

9. PCR (cultured amebae) :__/ (1=PZ; 2=NPZ; 3=PZ+NPZ)
10. PCR (stool specimens):__/ (1=PZ; 2=NPZ; 3=PZ+NPZ)

PZ = Pathogenic
NPZ= Nonpathogenic

Recruitment form

1. Name :_____
2. Father's name :_____
3. Address :_____
4. Household no :__/__/__/
5. ID number :__/__/__/
6. Age in years :__/__/__/__/
7. Sex : (1=male;2=female)
7. Date of recruitment :__/__/__/__/__/__/
5. Main occupation : (1=student; 2=service; 3=house wife ;4= business; 5= unemployed)
9. Fever if any ^{0}C :__/__/
10. Abdominal pain/ache or any discomfort :__/(0=no;1=ache; 2-pain; 3=both)
11. Current type of stool:__/(1=formed, 2=Loose; 3=Watery)
12. Any abdominal complain during the last three months: :__/ (0=no; 1=ache, 2=pain; 3=both)
14. Total family members :__/__/

Diarrhoea Report Form

1. Name :_____

2. I.D No. :__/__/__/

3. Date of recruitment :__/__/__/__/__/__/

4. Date of visit : dd/mm/yy

_____/_____/_____/_____/_____/_____/_____

5. Diarrhoea began (dd/mm/yy)?

6. Has [ ] been vomiting (0=no;1=yes)

7. Any fever (0=no;1=yes)

8. Still has diarrhoea (0=no; 1=yes)

9. How many stools in the last 24h? (No)

10. Type of stools since last visit?: A

11. Has taken any medicine from outside?: B

12. Any other illness now?: C

13. Stool collected (0=no; 1=yes for diarrhoea ; 2= yes for routine)

14. Any drug treatment started? D

(A: 1= normal, formed; 2= loose; 3= liquid ; 4= watery) (B: 0=none; 1=doctor/clinic; 3=homeopath/herbal; 4= other) (C: 0=none; 1=resp.tract infection; 2=measles; 3=skin inf; 4=cold; 5= referred elsewhere; 6=other) (D: 0=no; 1=MNZ; 2=AMP;3=NDX;4=referred elsewhere; 5=other)

Reviewer - 1

My sincere apologies for the lateness of this reply but I have been involved in duty travel.

My comments follow:

1) There is now a large body of evidence to suggest that E.histolytica exists as non-pathogenic and pathogenic forms. This distinction appears to be increasingly viewed as non-controversial - even by those who once argued most strongly against it. I am concerned, therefore, that the General Aim seeks to re-open this debate. While little in science or nature is absolute, the balance of evidence would seem to suggest that this aim is most unlikely to be satisfied.

2) Precisely because there is now a strong perception that there are clinically distinct strains of Entamoeba, there is an urgent need to describe the differing natural histories of the two strains. This would be a much more worthwhile aim for the study, and would require only a change of approach rather than of design.

3) I understand that Dr Haque has already evaluated a MaB in collaboration with the London School of Hygiene. Would there be any merit in including this reagent in the present trial since its parameters are presumably now well known? Given the current problems with diagnosis of entamoebiasis, a broad range of diagnostic approaches would seem desirable.

4) The attempt to screen out all non-Entamoeba pathogens with a battery of tests is unlikely to succeed. Many, perhaps most, patients are likely to have multiple species infections, and it will be impossible to desegregate the contribution of these to the Entamoeba infection simply because the actual pathogen was not detected by the tests employed. These is also the possibility of synergy and antagonism. For example, severe Trichuris infection (which seems not to be included in the list for screening but can cause dysentery) has been described as potentiating the pathogenicity of Entamoeba and other pathogens by creating lesions which permit entry into the mucosa.

5) A minor technical point is that PVA is unsuitable for Giardia if a concentration method is employed. The PVA will preserve trophozoites, but they will not survive subsequent concentration techniques. Formalin may be a better choice.

6) My overall view is that a rather more basic study focusing on the natural history of Entamoeba infection and reinfection, but using the sophisticated diagnostic tools proposed, would be of more immediate value.

I hope these comments are of some use, despite their lateness.

Yours sincerely,

Reviewer-2

Title: Role of nonpathogenic Entamoeba histolytica infection in Bangladeshi children and rapid diagnosis of pathogenic E. histolytica infection

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project	X		
Adequacy of Project Design		X	
Suitability of Methodology		X	
Feasibility within time period		X	
Appropriateness of budget			X
Potential value of field of knowledge	X	X	

CONCLUSIONS

I support the application:

a) without qualification ☐

b) with qualification ☒

- on technical grounds ☒
- on level of financial support ☒

I do not support the application ☐

Reviewer-2

The objective of this study is to test a recently described ELISA based method for identification of pathogenic *Entamoeba histolytica* and to modify its application to a PVC strip ELISA or latex agglutination for field detection of amoebae. The authors propose to use a PCR test to distinguish between pathogenic and nonpathogenic amoebae.

The authors have recently described a monoclonal antibody based ELISA to detect pathogenic *E. histolytica* from stool specimens (reference 16). The test had a sensitivity of 100% and a specificity of 98%. This is a major contribution in the field as it offers for the first time a rapid diagnostic method to distinguish pathogenic and nonpathogenic *E. histolytica*. I fully support a large scale prospective study to determine the sensitivity and specificity of the ELISA based assay and modification of its usage for field applications. This aspect of the study, I believe, will bring important new information on diagnosis and clinical intervention. What is unclear is whether the latest agglutination and PVC Strip format would have the same sensitivity as the ELISA assay. Since this aspect of the study has to be worked out the short term benefit of the study may not be realized. Nevertheless, this is an important area of research that should be encouraged. The PCR test on the other hand, is very vague and it is left to the imagination as to how the oligonucleotide primers were chosen and as to the source of the genomic DNA from pathogenic amoebae. Also it is unclear why PCR test is needed in addition to the ELISA, latex agglutination and PVC strip assays. This aspect of the study does not integrate with the previous sections and no justification for its usage is given.

The budget of salary support is reasonable but operating cost seems rather high. Chemical, supplies and bacteriological test could be reduced by 30%

In summary, I fully support the use and application of the MAb-based ELISA test to advance its usage and modification for large scale testing for its sensitivity and specificity for the diagnosis of pathogenic and nonpathogenic *E. histolytica*.

Title: Role of nonpathogenic Entamoeba histolytica infection in Bangladeshi children and rapid diagnosis of pathogenic E. histolytica infection

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

	Rank Score		
	High	Medium	Low
Quality of Project			✓
Adequacy of Project Design			✓
Suitability of Methodology			✓
Feasibility within time period			✓
Appropriateness of budget		✓	
Potential value of field of knowledge	✓		

CONCLUSIONS

I support the application:

a) without qualification ☐

b) with qualification ☐

- on technical grounds ☐
- on level of financial support ☐

I do not support the application ☑

This grant proposes to follow 100 children with nonpathogenic E. histolytica infection and develop new technology for antigen detection in diagnosis of intestinal amebiasis. Monoclonal antibodies specific for pathogenic E. histolytica are used for diagnosis. Development of new methods for diagnosis of E. histolytica infection is important and worthy of support. However, there are significant problems in the proposed research that should be addressed prior to any funding being considered.

The first specific aim makes no sense and is not adequately approached experimentally. With thousands of cases evaluated, invasive amebiasis has never been found during nonpathogenic infection. Lack of a control group is unacceptable. This is necessary for comparison of symptoms and evaluation of height or weight as inferred in the protocol. This portion of the proposal should be eliminated.

There is no evidence that these investigators have ever performed stool cultures and zymodeme analysis, nor are sufficient details provided to assure us of this capability. Development of alternative methods for antigen detection, and especially PCR, are entire projects in their own right. No preliminary data is offered with any of these methods. Inadequate rationale (in fact none) is provided for adding PCR to the antigen detection methodology, which was apparently so successful. Again, the authors have no preliminary data or published expertise in this area.

The statistical analysis provided is totally insufficient. Specific dependent and independent variables were not identified, and it appears that consultation with a clinical epidemiologist would be necessary for analysis of data.

As pathogenic infection is so infrequent in this study, it seems that an alternative population should be utilized for study of methods to differentiate pathogenic from nonpathogenic infection. Perhaps a population of subjects presenting with acute diarrhea would be more appropriate.

The budget and travel expenses are not justified.

Response to reviewers comments

Thanks for the critical reviews of the reviewers which have helped us greatly to improve our proposal further. The protocol has been revised in the light of the comments sent by the reviewers.

Reviewer - 1

We strongly believe that *E. histolytica* exists in two forms: nonpathogenic and pathogenic. Our work during the last two years was aimed at developing simple immunoassays to distinguish these two forms. It is true as the reviewer thinks that the distinction between pathogenic and nonpathogenic *E. histolytica* appears to be increasingly viewed as non-controversial, but the controversy refuses to die. We disagree with the comment of reviewer -1 that the General Aim of our study seeks to re-open this debate. Although there is a pressing need to confirm that nonpathogenic *E. histolytica* does not lead to invasive amebiasis; especially in the light of the existing controversies and unconfirmed report of laboratory conversion of nonpathogenic amebae to pathogenic, the major aim of our proposal is to confirm the validity and applicability of the recently developed test in our laboratory on a larger number of patients and asymptomatic subjects infected with pathogenic and nonpathogenic *E. histolytica* respectively.

Although there is now strong perception that there are now clinically distinct strains of *E. histolytica*, there are no commercially available or practical and reliable methods for differentiating pathogenic from nonpathogenic strains for routine use in clinical laboratories. This proposal was developed with the goal towards developing a rapid and reliable Ag-detection test of pathogenic *E. histolytica* in stool specimens. Further, we propose, here, to confirm harmless nature of nonpathogenic *E. histolytica* diagnosed as nonpathogenic by the developed immunoassay. This will allow application of this simple test to diagnose pathogenic amebic infection and it's field use in the developing countries where the disease is endemic. Natural histories of the two strains are not subject of research of our proposed study, it could be undertaken when simple and reliable diagnostic methods for detection of pathogenic and nonpathogenic *E. histolytica* are available.

Not only the monoclonal antibody 20/7 from London School of Hygiene and Tropical Medicine will be used, but also the recently reported PCR test will also be performed to compare the results with that of the ELISA. And this will obviously would carry some merit towards our goal.

We do agree that certain percentages of our proposed study population may have multiple species of infections, and so do we

have decided to exclude such subjects from our study, and, we will also screen out subjects with severe helminthic infections like *Trichiuris*.

10% Formalin along with PVA will be used in the study.

Yes, we also agree with the last comments of Dr. Bundy, but our specific aims are not to study *E. histolytica* infection and reinfection, rather to establish simple diagnostic assays to distinguish pathogenic *E. histolytica* from nonpathogenic, which should be useful to clinicians towards a proper diagnosis and treatment of amebic infection.

Reviewer - 2

The reviewer has commented on the importance of conducting this study which he thinks will bring important new information on diagnosis and clinical intervention of amebic disease. Reviewer - 2 has fully supported the use and application of MAb - based ELISA to advance its usage and modification for large scale testing for its sensitivity and specificity for the diagnosis of pathogenic *E. histolytica*.

We, would, however try to confirm that the latex agglutination and PVC strip format would have closely similar sensitivity and specificity as the ELISA by our proposed study.

Reviewer -2 has not supported the use of PCR test, while reviewer -3 has supported that fully. We assume that considering the current problems with diagnosis of *E. histolytica* a broad range of diagnostic approaches should be undertaken in this study which would be helpful to validate our simple immunoassay more precisely. We have selected a recently reported PCR test which is entirely in accordance with those zymodeme analysis which is similar to our immunoassay to be performed in this study.

Reviewer - 3

The reviewer has supported that the development of new methods for diagnosis of *E. histolytica*, according to him, is an important area of research in this field. But, as the reviewer is critical with the less number of pathogenic infections in the proposed study, we have changed our research proposal accordingly: we have included similar number of patients with diarrhoea infected with pathogenic *E. histolytica*.