Attachment 1.

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date 2/11/1989 6/11/89

Principal Investigator FPL VAN LOON

Trainee Investigator (if any) _____

Application No. 89-013

Supporting Agency (if Non-ICDDR,B) Netherlands Foreign Aid

Title of Study The role of Entamoeba Histolytica in the dysenteric syndrome in children and adults

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)
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 (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 N.A.
   (d) Sensitive questions Yes No N.A.
   (e) Benefits to be derived (Yes) No N.A.
   (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 Cttee. for review.

(PTO)

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

Principal Investigator

Trainee

89-013
6/11/89

SECTION I: RESEARCH PROTOCOL

1. Title : The role of Entamoeba Histolytica in the dysenteric syndrome in children and adults

2. Principal investigator : Dr FPL van Loon

Co-investigators : Dr D Mahalanabis
Dr R Huq
Mr AK Banik

Collaborating Investigator : Dr KN Jalan

3. Starting date : January 1990

4. Completion date : December 1990

5. Total direct cost : US $ 112962.

Funds are requested from Netherlands Foreign Aid (DGIS)

6. Scientific programme: This protocol has been approved by the Clinical Sciences Division

Signature of Division's Head

Date: 2.11.89

7. **Abstract summary:** Dysentery is caused by a range of microbes including bacteria and parasites. Clinically it presents with bloody diarrhoea and fever. Lack of reliable laboratory tests to assess severity and magnitude of amoebic dysentery in urban, intercity and rural settings has led to often improper diagnosis and mismanagement.

In a one-year case-control study we propose to assess the severity of *Entamoeba Histolytica* (EH) infections among over 1-year-old patients of either sex, presenting with dysentery at the Dhaka Clinical Research Centre of the International Centre for Diarrhoeal Disease Research, Bangladesh(ICDDRB). Cases will be diarrhoeal patients whose stool microscopy shows over 10 RBC's/hpf and haematophages EH trophozoites, and in whom known bacillary causes of dysentery are excluded. There will be three types of controls : patients with dysentery due to shigellosis, patients with dysentery not due to shigellosis, and patients with watery diarrhoea. The controls will be matched for sex and for age according to the following categories : 1-2 year (+/- 3 months), 2-5 year (+/- months), 5-15 year (+/- 1 year), > 15 year (+/- 3 year). Magnitude is here defined as the number of cases among this population. As prime indicator for disease severity stands the number of faecal haematophagous EH trophozoites / hpf present.

In the context of regional institutional collaboration, this will be a joint-project with Dr KN Jalan, Chief Coordinator, Kothari Centre for Gastroenterology, The Calcutta Medical Research Institute, Calcutta, India.

8. Reviews

(i) External Reviews : see attached copies plus our replies.

(ii) Research Review Committee : _____

(iii) Ethical Review Committee: _____

(iii) Director's signature: _____

SECTION II: RESEARCH PLAN

A. INTRODUCTION

1. Objective:

The objective of this study is to assess the following issues among dysenteric patients older than 1 year visiting the ICDDRB Dhaka Clinical Research Centre.

a. what is the magnitude of *Entamoeba Histolytica* induced illness.

b. how does the severity of the illness relate to prognostic/ risk factors including circulating antigen-immune complexes, iso-enzyme pattern of EH isolates serum antibody response, nutritional status, socioeconomic status.

c. what is the seasonality in the prevalence of amoebiasis.

2. Background

Within the dysenteric syndrome, bacillary dysentery, particularly shigellosis, is a disease of recognized major public health importance in terms of both morbidity and mortality. In contrast, the impact of amoebic dysentery on public health is less well-defined because of inadequate diagnostic tools available and resulting insufficient treatment (1-8). A recent ICDDRB study has described clinical distinctions between bacillary and amoebic dysentery(9). Hospital surveillance studies at our Centre using conventional detection methods assessed a 6 % infection

rate of amoebic dysentery, defined as bloody diarrhoea with haematophagous *Entamoeba Histolytica* trophozoites (10,11). It affects mostly children between 1 and 5 years and adults above 40 years. In 1988 *E histolytica* cysts or trophozoites were detected in 83 stool samples of 6094 inpatients (1.4%) and 82 stool samples of 2675 outpatients (3%) with clinical dysentery seen ted at the ICDDRB Dhaka Clinical Research Centre.

Recent amoebic dysentery screening methods such as serum antibodies and circulating antigen-immune complexes should, however, contribute to refining the diagnosis, to assessing the magnitude of disease in this population and to redefining individual or population treatment criteria (12-31).

Since this survey will cover one year, it will also allow for an assessment of the seasonality of amoebiasis among this population.

3. Rationale

Compared to bacillary dysentery, amoebiasis has been relatively neglected as an area of research. We feel that there is a need to improving tools for diagnosis of amoebiasis as to accurately assess both its magnitude in the community and its degree of disease severity in individual patients.

In the context of regional collaboration the study will be conducted in collaboration with the Kothari Centre of Gastroenterology, Calcutta.

B. **SPECIFIC AIMS:**

1. To assess whether isoenzyme patterns of EH isolates in stool, serum antibodies and circulating antigen-immune complexes could serve as indicators for amoebic disease severity.

2. To determine the role of nutritional and socioeconomic status as prognostic/risk factors for amoebic dysentery (32-34).

C. **METHODS OF PROCEDURE:**

Patients' selection and recruitment

Over a one-year period, all patients over 1 year-of-age of either sex presenting at the ICDDRB Dhaka Clinical Research Centre with clinical dysentery will be eligible for the study. The present triage criteria for referral to the shortstay treatment centre or inpatient area will be observed (10). If the patient qualifies as a case (see below), he/she will be invited to participate in the study and written consent of the patient or the legal guardian obtained. Ramifications into cases and controls will be made as follows (35-41).

Cases:

1. Patients with a history of three or more bloody, unformed stools during the previous 24 hours, admitted to the shortstay treatment area or inpatient area, in whom known bacillary causes of dysentery are excluded(see 3.).

2. Microscopy on wet mount stool sample showing > 10 RBC/hpf, haematophagous *EH* trophozoites.

3. Stool culture negative for *Shigella* spp, *Campylobacter*, *Salmonella*, *Plesiomonas shigelloides*, *Aeromonas hydrophila*, *Enterohaemorrhagic Escherichia coli* (42-44).

Controls-I:

1. Patients with a history of three or more bloody, unformed stools during the previous 24 hours, admitted to the short-stay or inpatient area.

2. Microscopy on wet mount stool sample showing > 10 RBC/hpf, but no *EH* cysts or trophozoites.

3. Stool culture positive for any *Shigella* spp.

Controls-II:

1. Patients with a history of three or more bloody, unformed stools during the previous 24 hours, admitted to the short-stay or inpatient area.

2. Microscopy on wet mount stool sample showing > 10 RBC/hpf, but no *EH* cysts or trophozoites.

3. Stool culture negative for any *Shigella* spp, whether or not positive for any other microorganism.

Controls-III:

1. Patients with a history of three or more watery diarrhoeal stools during the previous 24 hours, admitted to the short-stay or inpatient area.

2. Microscopy on wet mount stool sample showing < 10 RBC/hpf, <10 WBC/hpf, and whether or not *EH* cysts or trophozoites but no haematophages.

Prior use of antibiotics will exclude patients from the study.

On admission history and findings on physical examination will be recorded (appendix I). A day-to-day clinical record will be kept. On three occasions a fresh stool sample will be examined for *EH* trophozoites. A 5 ml serum sample will be collected on admission and two weeks later.

The magnitude of *EH*-induced illness is here defined as the number of cases among the above population.

Disease severity assessed on clinical grounds including degree of malnutrition and socioeconomic status will be correlated with the paired titers of serum antibodies and circulating antigen-immune complexes.

Laboratory examination

Gross and microscopic examination: On three occasions a wet mount of fresh warm stool specimens emulsified in saline will be examined for *EH* haematophages and red blood cells. For *EH* cysts formol-ether concentration method will be used routinely. Permanent preparation will be done by hematoxyline staining.

Bacteriology: The stool specimen from index cases will be plated immediately onto Mac Conkey and SS agar to avoid loss of shigella

if present. Stool will be cultured for *Shigella* spp, *Campylobacter*, *Salmonella* spp, *Plesiomonas shigelloides*, *Aeromonas aeruginosa*, *Vibrio cholerae* and *Entero-Haemorrhagic Escherichia coli*(EHEC) (42-45).

Storage: Stools from the admission and follow-up specimens of cases and controls will be frozen at -60^{0}C to identify viruses involved either as a copathogen or as a confounder.

Tissue specimens. In selected cases, adult patients will undergo rigid sigmoidoscopy after gut lavage (appendix II). Patients with fulminant disease will be excluded from this procedure. The endoscopy will allow to assess the distribution of the lesions in the lower gut. Tissue specimens obtained from suspect ulcers or mucosae will be preserved in formaldehyde and examined by independent pathologists.

Serology: Under strict sterile conditions, a 5 ml blood sample will be obtained for homologous serum antibodies and circulating antigen-immune complexes on admission and at a two-week follow-up.

Treatment

Cases. The age group of 1-5-years-old will receive metronidazol in a suspension (7.5 mg/kg body weight tid for 10 days), the older age group tinidazol in tablets(1 g once a day for 3 days for the 5-15 years, 2 g once a day for 3 days for the above 15 years)(50).

Controls. The control-I patients will receive nalidixic acid treatment, if needed to be changed according to the sensitivity pattern of the *Shigella* spp cultured. The control-II patients will be treated with antibiotics according to the stool culture and sensitivity pattern found. The control-III patients will receive rehydration therapy and antibiotics if and when required. The patients will be primarily treated by the ward physicians who will also decide about the duration of hospitalisation.

Prognostic/risk factors of interest

Primary:

1.1. The presence and titers of circulating *EH*-antigens(free or as immune-complexes).

1.2. The presence and titers of circulating antibodies during the acute phase.

Secondary (indicators of clinical severity)

2.01. The presence and numbers of haematophages in the stool.

2.02. The number of RBC's in the stool.

2.03. Duration of prehospital illness.

2.04. History of fever($T>38^{O}C$)

abdominal pain

tenesmus

nausea/vomiting

2.05. The body temperature

2.06. Blood WBC/mm^3x1000

2.07. Ht(%)

2.08. Liver size

2.09. Abdominal masses

2.10. The nutritional status (weight, height, mid arm circumference)

2.11. Socioeconomic status

Sample size calculation

1. Severe cases as defined above, we assume, will have circulating antigen in at least 70% of the patients. In controls-I we do not expect circulating antigen in more than 30% but likely even less. To detect a difference (a=.05, b=.1) the sample size will be 30 patients per group. In the controls-II and -III we expect an even lower proportion to have circulating amoeba antigen. Therefore the sample size will be even smaller.

2. If we expect in severe cases a rise in circulating antibody titer to occur in more than 70% of the cases, and in control-I in less than 30%, the sample size per group is about 30 patients.

3. On the assumption that 60% of the cases, 30% of the controls-I, and 15% of the controls-II and -III suffer from III degree malnutrition, the sample size will be 53 in each group.

4. On the assumption that the lowest socioeconomic status (degree III) is present in 60% of the cases, in 30% of the controls-II, the sample size is about 53 in each group.

Proposed that there be twice as many controls-I, and trice as many controls-II and -III combined than cases, the over all numbers of patients studied will be as follows. Cases: 55 ; Controls-I: 110 ; Controls-II and -III :165.

Data analysis This is a descriptive case-control study of *EH*-induced dysentery. Based on the above surveillance data we are expecting to be able to enroll the 55 cases within one year. Since among the study population shigellosis and watery diarrhoea are more common than amoebic dysentery we are expecting to enroll the 110 controls-I and 165 controls-II and -III within the same period. Appropriate statistical tests for case-control studies will be applied. Discriminate analysis and linear regression for assessing disease severity will be performed in retrospect. .pa

Facilities required:

Existing facilities will be used. The assays of circulating immune complexes and homologous serum antibodies will be set up at the ICDDRB in collaboration with Dr KN Jalan, Chief Coordinator, Kothari Centre for Gastroenterology(KCG), The Calcutta Medical Research Institute, Calcutta, India. In the initial phase the assays will be run concurrently at the ICDDRB and KCG. Tissue specimens will be judged independently in both Centres.

Appendix I

History and physical examination

PATIENT'S NAME

STUDY #

FATHER'S /HUSBAND'S NAME

ADDRESS

PATIENT #

MAIN OCCUPATION

01=SERVICE [ADD]

02=BUSINESS 04=PROFESSIONAL

08=DAY LABOUR 16=MINOR 32=OTHER.

DATE OF INTERVIEW ¦_¦_¦_¦_¦_¦_¦

SEX 1=MALE ,2=FEMALE. ¦_¦_¦

AGE IN MONTHS ¦_¦_¦_¦

RELIGION ¦_¦

(1=Muslim,2=Hindu,3=Christian,4=Other.)

OUT / IN - PATIENT ¦_¦

(1=Out Patient,2= In Patient.)

FROM ¦_¦

(1=Urban, 2=Sub urban, 3= Rural)

FEVER IN ^{o}C ¦_¦_¦_¦ (>38^{o}C).

DURATION OF MAIN SYMPTOMS ¦_¦_¦_¦_¦_¦_¦

ABDOMINAL PAIN/ACHE OR ¦_¦

DISCOMFORT (O = neither,1 = ache, 2 = pain, 3 = both)

HUNGER PAIN ¦_¦ (1=yes,2=no)

CURRENT (during present illness)NO OF STOOL PER DAY ¦_¦_¦

CURRENT TYPE OF STOOL (1= watery, 2=loose,3=semiformed ¦_¦

4=formed 5=hard)

ACTUAL WEIGHT LOSS IN LAST SIX MONTHS(1=2Kgs,2=2-5kgs 3=5-10kgs

4= 10 kgs ¦_¦

PAST TREATMENT

ANTI AMOEBIC

ANTACIDS

ANTI HELMINTHIC

OTHER_____

FAMILY INCOME PER MONTH (in taka,1=500-1000,2=>1000-3500,

3=>3500-5500, 4=>5500-7500,5=>7500-10,000,6=>10,000.)

EDUCATION OF FAMILY HEAD (1=none,2=primary,3=secondary,4=higher

secondary,5=graduate, 6= post-graduate)

EDUCATION OF MOTHER (1=none,2=primary,3=secondary,4=higher sec-

ondary, 5=graduate, 6= post-graduate).pa

PROFESSION OF HOUSE HOLD HEAD (1=govt. service,2=private
service,3= business 4=skilled profession,5=other profession,6=day

labour,7=other_____).

TOTAL FAMILY MEMBERS ADULT_____ MINOR____ TOTAL_____.

TOTAL EARNING MEMBER_____

LIVING HOUSE STRUCTURE_____ (TYPE OF Wall & Roof;1

=bamboo,2=straw,3=tin,4=pacca,5=other).

NO OF LIVING ROOM _____

LATRINE STRUCTURE _____(1= Hanging latrine, 2=pit latrine,

3=definite site but no structure,4=sanitary latrine,5=no definite

site).

PHYSICAL EXAMINATION

GENERAL EXAMINATION

PULSE (beats / min)

BP (mm Hg)

WEIGHT(kg)

HEIGHT(cm)

MID ARM CIRCUMFERENCE(cm)

SKIN FOLD TRICEPS(mm)

SKIN FOLD BICEPS(mm)

SKIN FOLD SUB SCAPULAR(mm)

DEHYDRATION (0=absent,1=mild,2=moderate 3=severe)

PEDAL OEDEMA

JAUNDICE

LYMPHADENOPATHY(0=none,1=cervical,2=axillary,3=inguinal,4=generalised)

CARDIOVASCULAR SYSTEM(0=normal,1=abnormal_____)

RESPIRATORY SYSTEM (0=normal,1=abnormal_____)

CENTRAL NERVOUS SYSTEM(0=Normal,1=Abnormal_____)

JOINTS(0=Normal,1=Swelling,2=Other_____)

ENLARGEMENT OF LIVER(cms below costal margin in midclavicular line)

ABDOMINAL DISTENSION

SPLEEN PALPABLE

MASS(code site as above ; 0=no mass)

Laboratory INVESTIGATIONS

Blood

Hb g%

ESR mm / 1 hr

White blood cells / mm^3 . 1000

Cell differentiation (%)

N

L

E

M

B

Serum EXAMINATION

Circulating *EH* antigen/immune complexes

Circulating antibodies

TOTAL PROTEIN g%

ALBUMIN g%

GLOBULIN g%

BILIRUBIN mg%

ALK.PHOS u/L

S.G.O.T. u/L

S.G.P.T. u/L

AMYLASE u/L

CREATININE mg%

SUGAR mg%

Stool microscopy

WBC / hpf

RBC / hpf

(A)STOOL PROTOZOA

(1=cyst,2=troph,3=both)

E.HISTOLYTICA

G.LAMBLIA

(B)STOOL HELMINTHS

(1=larvae, 2=ova 3=both)

A.LUMBRICOIDES

A.DUODENALIS

T.TRICHIURA

S.STERCORALIS

T.SOLIUM

E.VERMICULARIS

T.SAGINATA

OTHER

STOOL CULTURE

(0=-ve,1=EHEC,2=Campy 3=Salm,4=Shig,5=Yers,6=Cholera,7=Aeromonas

Appendix II

Gut lavage, lower endoscopy and biopsy:

In selected cases, adult patients will be invited to undergo a gut lavage with a solution of the following composition :

PEG-4000 or mannitol	80 mmol/l
NaCl	25 mmol/l
Na_2SO_4	80 mmol/l
KCl	10 mmol/l
$NaHCO_3$	20 mmol/l.

Appendix III

Laboratory procedures.

The following procedures will be established at the ICDDR,B:

1. Zymodemes characterization using cellulose acetate electrophoresis or starch gel electrophoresis (Sargeaunt 87). To isolate E. histolytica, fresh stools will be inoculated into Robinson's medium (Robinson, 1968) and inoculated for between 48 and 72 hours. Trophozoites are harvested when they are about 5×10^4 or more organisims and then lysed by freezing and thawing in the presence of enzyme stabilizers (Sargeaunt and Williams, 1978). After removing cell debris by centrifugation, drops of supernatant will be frozen in liquid nitrogen to prepare beads. These beads are then unfrozen when required and applied to plates in cellulose acetate electrophoresis or are soaked into cotton threads for starch gel electrophoresis. To distinguish between zymodemes, isoenzyme electrophoresis will be performed for the enzymes glucose phosphate isomerase (EC. 5.3.1.9), phosphoglucomutase (EC. 2.7.5.1), hexokinase (EC. 2.7.1.1) and 1 malate: NADP+ oxidoreductase (oxalacetate decarboxylating). Both cellulose acetate and starch gel electrophoresis will be undertaken but every effort will be made to use only cellulose acetate electrophoresis because of its implicity and other advantages.

2. The detection of antigen in polyethylene-glycol-precipitated circulating immune complex (CIC). Serum samples will be collected from cases and control groups. CIC will be prepared by precipitation with 2.5% polyethylene glycol (PEG) as it is described by Vinayak and others (1986). Briefly, 0.2 ml of serum will be mixed with PEG in veronal buffer saline, pH 7.6 and incubated overnight at 4^0C the samples will then centrifuged at 2,000 x g for 20 min at 4^0 C. The precipitate will be washed once with 2.5%-PEG in veronal buffer saline containing 0.01 EDTA. Finally, the precipitates will be dissolved in 0.2 ml of veronal buffer saline by incubation at 37^0 C for one hour.

E. histolytica antigens will be prepare from axenically grown E. histolytica and antisera against the whole amebic extract will be raised in rabbits using established procedures. Finally, ELISA procedure will be used for this purpose as it is describved by Vinayak and others (1986) or Ghandi and others (1988).

3. Determination of antiamebic antibodies will be performed by cellulose acetate membrane precipitin test and agar gel diffusion test. Cellulose acetate membrane precipitin test for detection of antiamebic antibodies will be used as it is described by Stamm and Phillips (1977). For this purpose cellulose acetate membrane strips will be in use. Results will be available within 4 hours using this test. Since a positive agar gel diffusion is rare in

the absence of active invasive amebiasis and the test is useful for the confirmation of successful treatment, in that it does not usually remain positive for long, this test will be used parallely with the cellulose acetate membrane precipitin test.

The serological assays will initially be carried out in parallel at the Kothari Centre of Gastroenterology in Calcutta. Isolates will be preserved in liquid nitrogen at -196^{0} C and subcultured within two days before transfer (by hand) in Robinson's medium.

References:

1. Jalan KN, Maitra TK. Amebiasis in the Developing World. *In*: Amebiasis, Human Infection by Entamoeba histolytica. JI Ravdin, John Wiley & Sons, NewYork 1988, p 535-555.

2. Sargeaunt PG, Williams JE. The epidemiology of *Entamoeba histolytica* in Mexico City. A pilot survey I. Transactions of the Royal Society of Tropical Medicine and Hygiene 1980;5:653-6.

3. Sargeaunt PG, Williams JE, Bhojnani R, Campos JE, Gomez A. The epidemiology of *Entamoeba histolytica* in a rural and an urban area of Mexico. A pilot survey II. Transactions of the Royal Society of Tropical Medicine and Hygiene 1982;2:208-10.

4. Guerrant RL. The global problem of amebiasis : current status, research needs, and opportunities for progress. Rev Inf Dis 1986;2:218-27.

5. Anand BS, Tuteja AK, Kaur M, Alam SM, Aggarwal DS, Baveja UK. Clinical and Epidemiological Aspects of *Entamoeba histolytica* Cysts Passers: Final Report. Indo-UK Workshop on Diarrhoeal Diseases, 9-13 January 1989, Calcutta India.

6. Thong YH. *Clinical Review.* Primary amoebic meningoencephalitis: fifteen years later. Med J Aust 1980;1:352-4

7. Overbosch D, Stuiver PC, Van der Kaay HJ. Hepatic Amoebiasis. Current concepts and a report of 25 cases in the Netherlands. Acta Leidensia, 1983;51:3-17.

8. Cameron JAM, Collins JC. Intestinal Obstruction by Amoebic Granulomata. J Royal Army Med Corps 1942;3:140-2.

9. Speelman P, McGlaughlin R, Kabir I, Butler TC. Differential Clinical Features and Stool Findings in Shigellosis and Amoebic Dysentery. Trans Roy Soc Trop Med Hyg.1987;81:549-51

10. Stoll BJ, Glass RI, Huq MI, *et al* Surveillance of patients attending a diarrhoeal disease hospital in Bangladesh. Br Med J 1982:285:1185-8

11. Wanke C, Butler T, Islam M. Epidemiological and clinical features of invasive amebiasis in Bangladesh: a case-control comparison with other diarrheal diseases and postmortem findings. Am J Trop Hyg 1988;38:335-41.

12. Martinez-Palomo A. The pathogenesis of amoebiasis. Parasitology Today 1987;4:111-8

13. Pillai S, Mohimen A. A Solid-Phase Sandwich Radioimmunoassay for *Entamoeba histolytica* Proteins and the Detection of circulating antigens in Amoebiasis. Gastroenterology 1982:83:1210-6

14. Gandhi BM, Irshad M, Acharya SM, Tandon BN. Amebic Liver Abscess and Circulating Immune Complexes of *Entamoeba Histolytica* Proteins. Am J Trop Med Hyg 1988;39:440-4

15. Mohimen A, Maitra TK, Mehra S, Jalan KN. Significance of Complex In *Entamoeba histolytica* Infection. Indo-UK Workshop on Diarrhoeal Diseases, 9-13 January 1989, Calcutta

16. Diamond LS, Harlow DR, Cunnick CC. A new medium for the axenic cultivation of *Entamoeba histolytica* and other Entamoebae. Trans Roy Soc Trop Med Hyg 1978;4:431-2.

17. Diamond LS. Axenic cultivation of Entamoeba histolytica: progress and problems.Arch Invest Med 1980;supl 1:47-54.

18. Diamond LS. Techniques of axenic cultivation of Entamoeba histolytica Schaudinn, 1903 and *E. Histolytica*-like Amebae. J Parasitol 1968;5:1047-56.

19. Cox JC, Nairn RC.Assessment of Amoebisasis:serodiagnosis by immunofluorescence with lyophilized entamoebae. Technical Methods 1975:1018-9.

20. Grundy MS. Preliminary observations using a multi-layer ELISA method for the detection of *Entamoeba histolytica* trophozoite antigens in stool samples. Trans Roy Soc Trop Med Hyg 1982;3:396-400.

21. Kessel JF, Lewis WP, Pasquel CM, Turner JA. Indirect Hemagglutination and complement fixation tests in amebiasis. Am J Trop Med Hyg 1965;4:540-50.

22. Pillai S, Mohimen A, Mehra S. A Sensitive Unlabelled-Antibody ELISA Technique: Its Application to the Detection of Class Specific *E Histolytica* Antibodies and *E Histolytica* Antigens( ):256-61.

23. Mohimen A, Maitra TK, Jalan KN, Mehra S. A specific solid-phase assay for the detection of immune complexes containing *Entamoeba histolytica* antigens. J Immunol Methods 1989;117:39-44.

24. Mohimen A, Maitra TK, Jalan KN. Use of Periodate Activated Sepharose 4B Immunoabsorbent in Purification of Anti *Entamoeba histolytica* Antibody. Indian J Exp Biol 1985;23:644-6.

25. Gelderman AH, Keister DB, Bartgis IL, Diamond LS. Characterization of the deoxyribonucleic acid of representative strains of *Entamoeba histolytica*, *E histolytica*-like amebae, and E Moshkovskii. J Parasitol 1971;4:906-11.

26. Gelderman AH, Bartgis IL, Keister DB, Diamond LS. A comparison of genome sizes and thermal-denaturation-derived base composition of DNAs from several members of *Entamoeba* (histolytica group). J Parasitol 1971;4:912-6.

27. Diamond LS, Mattern CFT, Bartgis IL, Viruses of *Entamoeba histolytica* I. Identification of Transmissible Virus-Like Agents. J.Virol 1972;2:326-41.

28. Mattern CFT, Diamond LS, Daniel WA. Viruses of *Entamoeba histolytica* II. Morphogenesis of the Polyhedral Particle($ABRM_2$-HK-9), HB-301,and the filamentous Agent $(ABRM)_2$-HK-9. J Virol 1972;9:342-58.

29. Mirelman D, Bracha R, Wexler A, Chayen A. Changes in Isoenzyme patterns of a cloned culture of non-pathogenic *Entamoeba histolytica* during axenization. Inf Imm 1986;3:827-32.

30. Sargeaunt PG. The reliability of Entamoeba histolytica Zymodemes in Clinical Diagnosis. Parasitology Today 1987;3(2):40-4

31. Sargeaunt PG. Zymodemes of *Entamoeba histolytica*. Parasitology Today 1987;5:158.

32. Diamond LS, Harlow DR, Phillips BP, Keister DB. *Entamoeba histolytica*: iron and nutritional immunity. Arch Invest Med 1978;suppl 1:329-38.

33. Murray MJ, Murray A, Murray CJ. The salutary effect of milk on amoebiasis and its reversal by iron. Br Med J 1980;6:1351-2.

34. Diamond LS. Amebiasis: Nutritional Implications. Rev Infect Dis 1982;4:843-50.

35. Miller JH. Clinical Parasitology: Introduction and collection of specimens. *In:* Lennett EH. Manual for Clinical Microbiology 3rd ed. Washington DC. American Society for Microbiology 1980, p 669-773.

36. Wolfe MS. Intestinal Parasites. *In* : Goodman & Gilman(ed)1985 p 427-31

37. Knight R, Wright SG. Progress report. Intestinal protozoa. Gut;19:940-53.

38. Mathews HM. Parasitic Disease: Testing with Filter-Paper Blood Spots. Laboratory Management 1981;2:55-62.

39. Diamond LS. Medical Protozoology and Helminthology. Ed US Naval Medical School, National Naval Medical Center. 1965(revised) p 55-59.

40. Allan AVM, Ridley DS. Further observations on the formol-ether technique for faecal parasites. J Clin Pathol 1970;23:545-6.

41. Speelman P, Ljungstrom I, Lundin L, Huldt G, Willcox MC, Grundy MS. ELISA for Detection of *Entamoeba histolytica* Antigens in Faecal Specimens. *In* : Thesis, University of Amsterdam, 1986.

42. Edwards PR, Ewing WH. Identification of Enterobacteriaceae, 3rd Ed 1972, Minneapolis, Burgess Publishing Co.

43. Manual for Laboratory investigations of acute enteric infections. WHO/CDD 1983;83.3

44. Blaser MJ, Glass RI, Huq MI *et al*. Isolation of Campylobacter fetus subspecies jejuni from Bangladeshi children. J Clin Microbiol 1980;12:744-7

45. Sherman P, Soni R, Petric M, Karmali M. Surface Properties of the Vero Cytotoxin-Producing *Escherichia coli* 0157:H7. Inf Imm 1987;8:1824-9.

46. Davis GR, Santa Ana CA, Morawski SG, Fordtran JS. Development of a lavage solution associated with minimal water and electrolyte absorption or secretion. Gastroenterology 1980;78:991-5.pa

47. Stamm WP, Phillips EA. A cellulose membrane precipitin(Cap) for test amoebiasis. Transactions of the Royal Society of Tropical Medicine and Hygiene. vol 71, no 6, 1977. 490-492.

48. Katzenstein D, Rickerson V, Braude A. New concepts of amebic liver abcess derived from heoatic imaging, serodiagnosis and hepatic enzymes in 67 consecutive cases in San Diego. Medicine 61:237, 1982.

49. Vinayk VK, Prakash O, Talwar GP, et al Evaluation of gel diffusion preciptin test for amebiasis Ind J Med Res 62;1317, 1974.

50. Ataxpa TOU, Damisah M, Whittle HC, Greenwood BM: Amebiasis in northern Nigeria. An evaluation of the gel diffusion test. Ann Trop Med Parasitol 72:123, 1978.

51. Turner JA. Drug Therapy of Gastrointestinal Parasitic Infections. Am J Gastroenterol 1986;81:1125-37.

52. Neal RA. Antiamoebic activity of drugs given singly and in combination against axenically grown *Entamoeba histolytica*. Arch Invest Med 1978;9:387-92.

ICDDR,B
1988 BUDGET PROPOSAL (IN US $)

DIVISION NAME: CLINICAL SCIENCE DIVISION
PROTOCOL/BRANCH NAME:
NAME OF P. I./BRANCH HEAD/DIVISION HEAD:DR. FPL VAN LOON
BUDGET STARTING DATE:
PROTOCOL NO: COMPLETION DATE:
DONOR NAME : GRANT AMOUNT:

EXPENSE CATEGORY		Column A	Column B	Column C
A/C Code	Description	Refer to Page No.	Actual Jan. - June '87	Estim. Whole Yr 1987	Proposed 1988
3100	Local Salaries	02			27984
3200	Intl. Salaries	08			29400
3300	Consultants	14			1440
3500	Travel Local	15			848
3600	Travel Intl.	16			3160
3700	Supplies & Mat.	18			2730
4000	Other Costs	19			1900
4800	Inter Deptl. Ser.	21			29000
	Total Direct Operating Cost				96462
0300	Capital Expenditure (P.22)				16500
	TOTAL DIRECT COST				112962

Reply to the issues raised by the reviewers.

a. Professor Mathan :

1. We have followed Professor Mathan's advice to contrast the study groups differently.

   The cases will be defined as before.

   There will be three types of controls :

   1. patients with dysentery due to shigellosis.
   2. patients with dysentery due to any other bacillus, or in whom no pathogen could be identified.
   3. patients with watery diarrhoea.

2. The collaboration with Dr Jalan is deliberately sought for his great expertise on EH clinic and serology.
3. Gut lavage has become a routine procedure in EH colitis diagnostics at the KCG having been performed over the years without complications. At the ICDDRB it won't be performed in fulminant cases.
4. The questionnaire that for that matter was framed on an existing one from KCG has considerably been condensed and simplified.
5. An updated budget has been added.

b. Dr Ackers

When funding becomes available both clinical and laboratory parts of the study can be conducted.

1. Cases are unequivocally defined as

1. Patients with a history of three or more bloody, unformed stools during the previous 24 hours, admitted to the shortstay treatment area or inpatient area, in whom known bacillary causes of dysentery are excluded(see 3.).

2. Microscopy on wet mount stool sample showing > 10 RBC/hpf, haematophagous *EH* trophozoites.

3. Stool culture negative for *Shigella* spp, *Campylobacter, Salmonella, Plesiomonas shigelloides, Aeromonas hydrophila, Enterohaemorrhagic Escherichia coli.*

2. EH trophozoites will be looked for in a fresh warm stool sample emulsified in saline. EH cysts will be sought for using the formol-ether concentration method.

3. The zymodemes are studied according to Sargeaunt (30,31).

c. Dr RL Guerrant

Because stool samples will be examined for the presence and number of RBC's, there is no need for the guiaic test.

We will see off from storage of stool samples.

We could consider determination of cytotoxins in cases suspect of *Cl difficile* infection.

Summary for the Research and Ethical Review Committees.

Lack of reliable laboratory tests to assess severity and magnitude of amoebic dysentery in urban, intercity and rural settings has led to improper diagnosis and management to a large extent. Therefore there is a need for a validation of the newer serum tests such as the determination of circulating antigens of Entamoeba Histolytica (EH) - the causative agent of amoebiasis - and antibodies against established parameters of disease severity such as the number of blood cells and blood ingested EH protozoa in the stool and related to clinical findings. After such a validation in a proper case-control hospital-based study involving the most serious patients these tools would become available for studies in slums and field. We therefore propose in a one-year case-control study to assess the severity of *Entamoeba Histolytica* (EH) infections among over 1-year-old patients of either sex, presenting with dysentery at the Dhaka Clinical Research Centre of the International Centre for Diarrhoeal Disease Research, Bangladesh(ICDDRB). Cases will be diarrhoeal patients whose stool microscopy shows over haematophages EH trophozoites and 10 RBC's/hpf, and in whom known bacillary causes of dysentery are excluded. There will be three types of controls : patients with dysentery due to shigellosis, patients with dysentery not due to shigellosis, and patients with watery diar-

rhoea. The controls will be matched for sex and for age according to the following categories : 1-2 year (+/- 3 months), 2-5 year (+/- months), 5-15 year (+/- 1 year), > 15 year (+/- 3 year). Magnitude is here defined as the number of cases among this population. As prime indicator for disease severity stands the number of faecal haematophagous EH trophozoites / hpf present. The study will not interfere with the care and treatment by the ward physician-in-charge who will also decide about the duration of the patient's admission. On two occasions, on admission and two weeks later, 5 ml of blood will be drawn for serology. On selected cases sigmoidoscopy will be done if the clinical condition so requires, but not in fulminant disease. The study will be conducted in collaboration with the Kothari Centre for Gastroenterology (Research Coordinator : Dr KN Jalan) where extensive expertise on amoebiasis already exists.

roject Title: The role of Entamoeba Histolytica in the dysenteric syndrome in children and adults

rincipal Investigator(s): Dr FPL Van Loon

ummary of Referee's Opinions: **Please see the following table to valuate the various aspects of the proposal by checking the ppropriate boxes. Your detailed comments are sought on a eparate, attached page.**

	Rank Score		
	High	**Medium**	**Low**
uality of Project		✓	
dequacy of Project Design		✓	
uitability of Methodology		✓	
easibility within time period	✓		
ppropriateness of budget	✓		
otential value of field of knowledge		✓	

ONCLUSIONS

support the application:

a) without qualification [ ]

b) with qualification

- on technical grounds [✓]

- on level of financial support [ ]

do not support the application [ ]

ame of Referee: V.I. Mathan ..... Signature: ....

Date: ...04-7-89...

sition: ..Professor of Medicine + Gastroenterology...

stitution: ...C.M.C. Hospital, Vellore, India 632004.

Detailed Comments

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel thay are justified.

(Use additional pages if necessary)

This study aims to look at the role of entemoeba histolytica (EH) in patients with dysentery over the age of one year, who present to the treatment centre of the ICDDRB at Dhaka, assess the severity of the illness and look at additional diagnostic modalities such as antibodies and circulating antigen immune complexes. The role of EH in the Dhaka situation has been relatively little studied while there is considerable information on this available from an adjacent country with similar environmental factors in Calcutta. It would therefore be appropriate and of high priority to have a detailed study of entamoeba histolytica at the ICDDRB. However, I do have certain specific comments and suggestions regarding the technical details of this study as follows.

1. Three groups of subjects are defined. (i) EH patients. (ii) Shigellosis patients, and (iii) Watery diarrhoea. These three groups are necessary for looking at the antibody responses and at the circulating immune complexes. But since one of the major aims of the study is to look at the diagnostic criteria and clinical illness of the disease, it may be more appropriate to have two major groups - (i) patients with dysentery as defined by the criteria of cases and (ii) a control group of age, sex etc., matched patients with watery diarrhoea. The patients with dysentery could then be divided into three groups. One, those in whom EH were detected; two, those in whom shigellae were detected and three, a group in which other pathogens or no pathogen was detected, but who clinically had invasive diarrhoea. It would enable determination of the contribution of EH to the bloody diarrhoea syndrome and the clinical severity between groups can be compared. A further sub-group of this will provide the material for deciding the efficacy of the other investigations, the antibodies and the immune complexes which are proposed. Since EH detection is at low frequency, the study design could be such that when a specified number of EH positive patients have been included the study could be terminated. This would provide adequate numbers of the other cases.

2. It is most appropriate that Dr. K.N. Jalan is a collaborating investigator. In his laboratory the techniques for amoebic antibody determination and the techniques for the immune complex determination have been established and standardised. His help would be most valuable in the successful completion of the study especially since he has considerable clinical experience with these syndromes.

3. However, I do have reservations about the cleansing solution approach, where the patient drinks 4 litres of the cleansing solution. This is the standard preparation for colonoscopy which is used now all over the world. However, there is a potential risk in using this in patients with clinical invasive amoebiasis since some of them might have amoebic ulcers which are penetrated through the muscularis and there is a potential risk of perforation if this solution is used in them. An alternative approach would be to use a rigid sigmoidoscope for examining the lower large bowel, obtain smears from suspected ulcers or mucosae and also obtain rectal biopsies (this is proposed in any case) and examine them for the presence of amoebiasis. The published literature would suggest that the yeild would be similar in this case. In the case of the colonic washout, examining the very large volume of fluid which will be expelled would be a major difficulty.

4. The questionnaires etc., which have been appended are adequate but they are rather extensive. Whether this detailed information is necessary is doubtful. The longer the questionnaire, the less likely they are to be filled in accurately.

5. The details of the budget were not appended. I am therefore unable to comment on the budget.

V. I. Mathan, M.D., Ph.D., F.R.C.P., F.A.M.S.,
Professor of Medicine & Gastroenterology
Head, Wellcome Research Unit &
Department of Gastroenterology
Christian Medical College Hospital
Vellore 632 004, India

Project Title: The role of Entamoeba Histolytica in the dysenteric syndrome in children and adults

Principal Investigator(s): Dr FPL Van Loon

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 ☑ – only as noted in attached sheet
- on level of financial support ☐

I do not support the application ☐

Name of Referee: Dr R. Guerrant Signature: Richard L. Guerrant M.D.

Date: 29 August 1989

Position: Professor of Medicine, Head Division of Geographic Medicine.

Institution: University of Virginia School of Medicine

Detailed Comments

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel thay are justified.

(Use additional pages if necessary)

Hopefully this proposal will help determine the role of E. histolytica relative to other causes of diarhea and dysentery by keeping denominator data on all consecutive cases, including those with Shigella, Campylobacter, Salmonella, Plesiomonas, Aeromonas EHEC, Vibrio etc.

In addition, the subgroup comparisons as outlined can be done to relate illness severity and etiology to immune complexes, serum antibody, isoenzyme pattern, nutritional status, etc.

I assume fecal leukocytes will be recorded, as well as gross (with microscopic) blood. Will guiaic be done?

Were any of the previously noted cases negative for occult or gross blood in the stool?

What period will the fecal samples be stored before cultures for Yersinia, Vibrio EHEC, C. difficile etc.? Will cytotoxin be sought (with specific antiserum neutralization) for C. difficile colitis?

Project Title: The role of Entamoeba Histolytica in the dysenteric syndrome in children and adults

Principal Investigator(s): Dr FPL Van Loon

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	NOT	SEEN	
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 ☐

Name of Referee: Dr JP Ackers Signature: [signature]

Position: Senior Lecturer Date: 1 Aug 1989

Institution:

J. P. ACKERS
DEPARTMENT OF MEDICAL PROTOZOOLOGY
LONDON SCHOOL OF HYGIENE AND TROPICAL MEDICINE
KEPPEL STREET
LONDON WC1E 7HT

For Van Loon to [illegible]

Detailed Comments

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel thay are justified.

(Use additional pages if necessary)

The aims of this study are important, and in many respects coincide with an investigation proposed by Dr Andrew Hall with which I am associated. It is not appropriate for me to comment on organisational matters within ICDDR,B, but I would hope that if both studies are funded it will be possible to run them collaboratively, or even combine them.

The three objectives defined on page 3 of the application are all important and I should like to know the answer to each of the questions posed, particularly the first ('What is the magnitude of E.histolytica induced illness?'). My reservations relate not to the objectives of this project but to certain features of the study design.

1 I am not very clear about the definition of a case. In the abstract summary (Page 2. para. 7) and on Page 6, it is implied that a case is any patient with bloody diarrhoea in whom a known bacillary cause has been excluded; but in the procedure (Page 5, para. 2) it is stated that haematophagous trophozoites of E.histolytica must also be present. Depending on the study design and objectives either definition of a case might be appropriate, but they are obviously not the same.

2 Some of the details in the proposal suggest to me that the Principal Investigator is not very familiar with the techniques needed to study E.histolytica; for example on Page 7 it is stated that the formol-ether concentration method will be used to look for E.histolytica trophozoites in stool specimens. While this concentration method is very useful for increasing the chance of detecting small numbers of cysts (and should certainly be carried out routinely), trophozoites are generally destroyed by this procedure and are best detected using wet-film examination of very fresh, warm specimens emulsified in saline.

3 I am also concerned that there is no mention of where or how the zymodemes are to be determined, and I do not understand what is meant by "Amoeba zymodeme titre" (item IX on the Laboratory Findings form).

These criticisms should not detract from what I see as a valuable study, but one which needs to be rewritten with the help of expert parasitological advice - which I am sure is available in Dhaka. I am particularly pleased to see that it is intended to investigate further Dr Jalan's interesting but still controversial serum antigen detection test.

The amoebic dysentery syndrome in adults and children

CONSENT FORM
(Translation of the Bangla original)

The International Centre for Diarrhoeal Disease Research, Bangladesh would like to carry out research on amoebic dysentery.

Although rarely fatal, amoebic dysentery is a serious disease that can linger on or frequently relapse over long years. Hitherto however, insufficient means have been to our disposal to assess the degree or extent to which an individual patient may suffer from the disease. Therefore we need to compare patients with various forms of diarrhoea, redefine amoebic dysentery on the basis of clinical features and up-to-date laboratory findings and characterize its different stages.

Because you (your child) are(is) suffering from diarrhoea (dysentery-like or watery), we are inviting you(r child) to participate in the study. You(r child) will receive the medical care as is usual in our Treatment Centre. On top of that we will ask you(r child) to have 5 ml of blood drawn on admission and two weeks thereafter. In addition your (child's) stool will be repeatedly checked for germs as well.

The investigations will allow us to more precisely determine how seriously ill you(r child) are(is) so that we can tailor your (child's) treatment more accurately.

All records of your (child's) treatment will be kept confidential. If you would disagree your (child's) treatment will in no way be hampered or challenged. If you agree to the proposal that you(r child) should participate in the study, then please sign here.

Signature of the investigator

Finger print/Signature

patient('s guardian)

Wittness

Date

Date