Date 26/2/91

ETHICAL REVIEW COMMITTEE, ICDDR,B.

rincipal Investigator Dr. S. Q. Akhtar Trainee Investigator (if any) CS

pplication No. 0091-002 Supporting Agency (if Non-ICDDR,B) _____

itle of Study The role of anaerobic microaerophilic bacteria in diarrhea
sistent diarrhoea in Bangladeshi population

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

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

Source of Population:
(a) Ill subjects (Yes) No
(b) Non-ill subjects Yes No
(c) Minors or persons under guardianship Yes No

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)

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)

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

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

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

S. Q. Akhtar
Principal Investigator

Trainee

INTERNATIONAL CENTRE FOR
DIARRHOEAL DISEASE
RESEARCH, BANGLADESH

Memorandum

TO : Dr. Moyenul Islam,
Acting Head, LSD.

FROM : Dr. S. Q. Akhter, LSD S.Q. Akhtar

February 26, 1991

SUBJECT : SUBMISSION OF PROTOCOL IN RRC.

I have received the copies of the external reviewers comments from your office.

I would like to request you to submit the protocol to RRC.

The present version of the protocol contains:

1. External reviewers comments,

2. Modifications suggested by the reviewers,

Thanking you.

Approved 6/3/91.

SQA:mh/S6:RRC01.MEM

91-002
7/3/91

APPLICATION FOR PROJECT GRANT

1a.	PRINCIPAL INVESTIGATOR	:	Dr. S. Q. Akhter
1b.	CO-INVESTIGATORS	:	Mr. D. Datta Mr. P.K.B. Neogi
2.	TITLE OF PROJECT	:	The role of anaerobic and microaerophilic bacteria in diarrhoeal and persistent diarrhoeal illness in Bangladeshi population.
3.	STARTING DATE	:	When project grant is available
5.	DATE OF COMPLETION	:	Two years from starting date
6.	TOTAL BUDGET REQUESTED	:	US$ 105,984
7.	FUNDING SOURCE	:	
8.	HEAD OF PROGRAMME	:	Dr. M. Moyenul Islam
9.	AIMS OF PROJECT	:	

a) General Aim

To assess the contribution of anaerobic and micro-aerophilic bacteria to the aetiology of certain types of diarrhoea such as persistent diarrhoea, acute diarrhoea, segmental necrotizing enterocolitis and pseudomembranous colitis.

b) Specific Aims

1) To quantify micro-aerophilic and anaerobic microfloras from jejunal fluids of patients with persistent diarrhoea and matched controls to study the aetiology and pathogenesis of persistent diarrhoeas.

2) To isolate and define the roles of micro-aerophilic bacteria such as *Campylobacter* species and anaerobic bacteria such as

Clostridium difficile, *Clostridium perfringens* types A & C and *Bacteroides fragilis* in the causation of diarrhoea by studying the following diarrhoeal stool specimens from:

a) Approximately 1550 ICDDR,B, surveillance patients during a 2 year period (1991-1993).

b) Approximately 80 children with severe persistent diarrhoea compared with 40 control children with mild persistent diarrhoea, and 40 control children with acute diarrhoea.

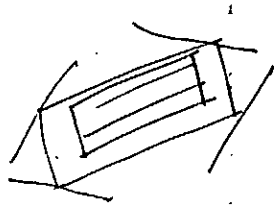
c) Post-mortem intestinal samples from cases where morphological evidences of segmental NEC and PMC were present.

c) Significance

Apart from the two well studied species of *Campylobacter* viz *coli* and *jejuni*, other species are also now known to be the cause of diarrhoea in different parts of the world. Among anaerobic bacteria, *Clostridium perfringens* and *Clostridium difficile* are established pathogens of the intestinal tract and *Bacteroides fragilis* has recently been implicated in diarrhoea in both animals and humans. The contributions of these organisms to diarrhoeal disease in humans in Bangladesh, have not been explored. This study aims to define the role of these organisms in diarrhoeal disease in this country.

10. ETHICAL IMPLICATIONS

Only stool specimens will be analysed for all studies except persistent diarrhoea study for which, in addition to stool, jejunal juice will be



analysed. The persistent diarrhoea study is a collaborative project with the Clinical Sciences Division (CSD), and the permission to obtain jejunal juice has already been given by the Ethical Review Committee through a separate grant application.

11. BACKGROUND

Because aerobic bacterial pathogens are relatively simpler to study, the vast majority of studies on aetiological investigations of diarrhoea, have been concerned with their isolation and characterisation. Due to the practical difficulties involved in the isolation and characterisation of micro-aerophilic and anaerobic bacteria, the studies on the role of these organisms in the causation of acute and chronic diarrhoea in humans, have lagged behind so far. It is important to study these organisms in Bangladesh both to fill the void in the aetiology of diarrhoea and to impart rational therapy to the patients.

C. jejuni has been isolated from diarrhoeal patients and controls in rural Bangladesh (1), hospitalised Bangladeshi patients in an urban treatment Centre (2) and Western travellers to Bangladesh with diarrhoea (3). Recent improvements in detection techniques have identified other species of *Campylobacter* such as *C. laridis*, *C. hyointestinalis*, *C. upsaliensis*, *C. foetus*, *C. cinaedi*, *C. fenalliae* as agents of diarrhoea in human beings in different parts of the world (4-15). There contribution to diarrhoea in Bangladeshi population has yet to be explored. *Campylobacter pylori* (recently named as *Helicobacter pylori*) has been implicated in the aetiology of gastritis (16) even though its role in gastritis-associated hypochlorhydria is controversial (17). A study conducted in the Gambia suggested that

significantly more children with malnutrition and chronic diarrhoea had serologic evidence of *H. pylori* infection than children with malnutrition alone and healthy controls (18). An interesting possibility exists that an impairment of the gastric acid barrier as a result of *H. pylori* infection could predispose to small bowel bacterial overgrowth and enteritis and may represent an important aspect of chronic diarrhoea and malnutrition. Alternatively, these organisms may, themselves, be involved in the causation of diarrhoea.

Other than micro-aerophiles, few anaerobes are also responsible for causing diarrhoeal diseases. *Clostridium difficile* has been documented as the causative agent of pseudomembranous colitis (PMC) and antibiotic-associated diarrhoea (AAD) (19). It has also been implicated in acute and chronic diarrhoeas (20, 21) and in necrotising enterocolitis (22). Two toxins (A&B) are implicated in the pathogenesis of diarrhoea associated with this organism. Toxin A (enterotoxin) causes secretion by damaging the mucosa. In addition, toxin A seems to exert a long term effect on the mucosa increasing susceptibility to damage by small amount of toxin A and acting synergistically with toxin B (cytotoxin) to cause intestinal pathology. Diagnosis is made by demonstration of cytotoxin in faeces or by culturing of the organism and demonstration of its cytotoxin production. However, laboratory findings should be interpreted with caution, as there is a high percentage of asymptomatic carriage in infants and children (23). Some adults also do harbour the organism and their toxin without any disease (24, 25). A limited study carried out at ICDDR,B, has shown the presence of this organism in AAD (26). In this country where antibiotic abuse is very common, the role of *C. difficile* should be thoroughly studied.

Clostridium perfringens type A and type C are also known to cause gastrointestinal disorders. Type A is involved in food-borne illness (27). The pathogenesis is due to an enterotoxin which is released during the sporulation of the organisms in the intestine or food. Type C was responsible for extensive outbreaks called Darmbrand in Germany and enteritis necroticans (Pig-bel) in Papua New Guinea. The pathogenesis of this syndrome is due to B-toxin. This organism is also a suspected cause of necrotising enterocolitis (28) and should be sought in all the above suspected diseases in Bangladesh and especially during festival seasons when outbreaks of diarrhoea are common after meat consumption. This disease affects children more than adults and may be fatal.

A series of *C. perfringens* related gastroenteritis occurred over a period of several months among elderly, chronic care patients in a psychiatric hospital (29). Sporadic diarrhoea due to *C. perfringens* has also been reported (30,31,32).

Post-mortem examination of patients who died of invasive diarrhoea in our Centre suggested segmental necrotising enterocolitis in about 20% of cases, a few of whom also had evidence of pseudomembrane formation (33). Also, the affected loops of small bowel was distended in these cases (34). A significant number of patients also had received multiple antibiotics including broad spectrum antibiotics. Various aetiological agents including the beta toxin of *C. perfringens* have been proposed for this fatal complication (35). Therefore, there is an urgent need to study the role of *Clostridia* in the aetiology and pathogenesis of this disease condition.

Enterotoxigenic *Bacteroides fragilis* has been associated with diarrhoea in lambs, calves, pigs and foals. Recently this organism has been isolated from children and adults with acute and chronic diarrhoea (36). The information on the pathogenic mechanism of this organism is only preliminary and is thought to be mediated by an enterotoxin. The toxin induces fluid accumulation in ligated ileal loop of lamb, and when injected into ligated caecum of adult rabbit, viable bacteria produced fatal enteric disease. In a preliminary survey of 38 diarrhoeal patients at ICDDR,B, enterotoxigenic *B. fragilis* was isolated from one patient which produced fatal enteric disease in rabbit. A thorough investigation of the role of this organism in both acute and chronic diarrhoea is necessary.

RESEARCH PLAN

a) Aetiology and pathogenesis of persistent diarrhoea in collaboration with Clinical Sciences Division

About 80 children aged 3 months to 3 years with severe persistent diarrhoea will be studied. The controls for the study include 40 children with mild persistent diarrhoea and 40 children with acute diarrhoea.

A single jejunal fluid specimen and 3 different stool specimens will be studied from each patient and control as soon as after admission. Again, after the treatment regimen is completed, a single jejunal fluid and a single stool specimen will be studied from each patient and control.

Definition of Severe Persistent Diarrhoea

1) Duration of diarrhoea for more than 14 days but less than 6 weeks
2) Requires prolonged I.V. maintenance (i.e. more than 48 hrs)
3) Stool output more than 100 ml/kg/day during the initial 48 hrs of observation

4) Duration of diarrhoea after admission more than 6 days in spite of supportive treatment and diet manipulation (but without antimicrobials used)

Definition of Mild Persistent Diarrhoea

1) Duration of diarrhoea for more than 14 days but less than 22 days
2) Does not require I.V. maintenance beyond first 24 hrs
3) Diarrhoea does not last beyond 4 days after admission on supportive treatment and diet manipulation (but without antimicrobial therapy)

Only those patients who will fulfill all the set criteria for the different groups will be included in the study. For example, a patient should require I.V. maintenance for 48 hrs, pass stool 100 ml/kg/day during observation period, have diarrhoea persisting for 6 days after admission and have total diarrhoea duration between 14 and 42 days to qualify for the severe persistent dirrhoea group. All patients will be followed up to discharge, and those acute diarrhoea control patients who develop persistent diarrhoea will be treated accordingly during analysis.

Justification of Controls for Persistent Diarrhoea Study

One problem in designing this study is the difficulty in obtaining data from suitable controls. We propose to include mild diarrhoeal patients as controls. We postulate that these patients are at the tail-end of normal distribution of patients with an acute attack of diarrhoea. In addition, for

each case, an age-matched child admitted with acute watery diarrhoea will be identified concurrently and included in the acute diarrhoea controls.

b) Anaerobic and micro-aerophilic bacterial pathogens associated with diarrhoea in patients enrolled in ICDDR,B Dhaka Hospital Surveillance study

A surveillance system has been set-up at ICDDR,B in 1979 in which every 25th patient seen is entered into the programme for in-depth clinical, microbiological and demographic work-up. Stool samples from 4% of these patients will be tested for the bacterial pathogen mentioned. Approximately 1550 patients will be studied.

c) Study of intestinal contents collected during postmortem examination of fatal cases of diarrhoea at ICDDR,B. Approximately 50 cases are expected per year.

Samples collected from segmental necrotizing enterocolitis (NEC) and pseudomembranous colitis (PMC) would be the test samples. Control samples would be those collected from other post-mortem cases than NEC and PMC cases. [Post mortems are regularly carried out at ICDDR,B under a separate protocol, No.89-011, P.I. : Dr. M. Moyenul Islam].

d) Interpretation of significance of pathogens

There is no healthy control group against which the isolation rate of pathogens can be compared when studying the hospital surveillance patients. Since ICDDR,B is a Centre entirely devoted to the treatment of diarrhoea patients, it is not possible to recruit healthy controls. However, we hope to clarify the aetiological relationship of various pathogens by comparison of their isolation rates in acute diarrhoea, dysentery, and chronic diarrhoea and in relation to the presence or absence of other well-established pathogens.

The aerobic and viral pathogens of the patients enrolled in this protocol are studied through two other protocols. [ICDDR,B Hospital Surveillance Study, P.I. Dr. A.N. Alam; role and characteristics of diarrhoeagenic *E. coli* in clinical and epidemiological investigations, P.I. Dr. M. J. Albert and role of enteric viruses in diarrhoeal disease in Bangladesh, P.I. Ms. L. Unicomb).

LABORATORY PROCEDURES

Jejunal fluid samples will be transported from the hospital to the laboratory for culture under liquid paraffin sealing to prevent oxygen diffusion.

Direct Gram staining of the faecal specimens would be done.

Stool and jejunal fluid will be cultured for anaerobic and micro-aerophilic bacteria on selective and non-selective media and aerobic pathogenic bacteria will be characterised following standard methods. For the study of jejunal fluid, 0.1 ml of undiluted fluid and 10 fold serial dilutions from 10^{-1} to 10^{-5} made in sterile heart infusion broth will be plated onto plain blood agar and selective media and incubated anaerobically. The dilutions will be inoculated onto Rogosa SL agar and incubated micro-aerophilically for lactobacilli. The samples will also be inoculated onto blood agar and MacConkey agar for enumeration and characterisation of aerobic flora under a seperate protocol. Stool and jejunal fluids will be cultured micro-aerophilically on blood agar for campylobacters; for this 0.1 ml of jejunal fluid and a suspension of stool in normal saline will be spot inoculated on a membrane filter (0.65 µm pore size) placed over a blood agar plate. Test specimens will be cultured on neomycin blood agar and on neomycin egg yolk agar for the isolation of *C. perfringens* type A and C, CCFA agar for

C. difficile and on PINN medium for the isolation of *Bacteroides fragilis.* Incubation for the isolation of micro-aerophilic and anaerobic organisms will be performed in a jar with BBL GasPak Micro-aerophilic and Anaerobic Systems respectively. For anaerobic incubation, disposable anaerobic indicator (BBL GasPak Anaerobic System) will be used in each jar for the confirmation of complete anaerobiosis. Plates for micro-aerophilic organisms will be incubated for 72 hrs, except those for *Helicobacter pylori*, which will be incubated for a further 2 days before declaring the culture negative. The medium for *C. difficile* will be incubated for 72 hrs and the media for the remaining anaerobes for upto 5 days.

Selective enrichment in cooked meat with cycloserine (500 µg/ml) and then plating on *C. difficile* agar for *C. difficile* and also selective enrichment in cooked meat with neomycin (100 µg/ml) and then plating on neomycin blood agar for *C. perfringens* would be attempted.

Specimens from post-mortem cases will also be cultured for pathogenic micro-aerophilic and anaerobic bacterial flora following the procedure mentioned earlier. A small segment of bowel from the autopsy cases along with luminal contents will be collected in a sterile bottle under liquid paraffin seal. Luminal contents will also be aspirated in a sterile bottle for subsequent toxin assay on tissue culture monolayers.

[Flow chart 2 in a later page gives details of identification]

Suspected colonies on selective plates for anaerobic bacteria will be grown in Robertson's cooked meat medium, glycerol added to 50% concentration and stored at -70^0C for further characterisation.

Recovery of the desired organisms from their spores would also be attempted.

Alcohol shock for spores:

To obtain large numbers of spores, Clostridium and Bacillus cultures are incubated in cooked meat broth for 5 days at 37^{O}C. The broth should be prereduced for at least 18 hrs in an anaerobic jar before inoculation.

An aliquot (0.5 ml) of a cooked meat broth culture and 0.5 ml of absolute ethanol would be incubated at room temperature for 1 hr; mixed at approximately 15 minutes intervals. The alcohal treatment kills vegetative cells but spores should remain viable.

Aliquots (0.1 ml) of the incubated samples would be inoculated onto *C. difficile* agar (CDA) and Blood agar with neomycin for cultures of *C. difficile* and *C. perfringens* from spores. Cultures would be incubated maintaining identical anaerobic condition. Spore cultures would be characterised following standard morphological and biochemical criteria.

1) Tissue Culture Assay

C. difficile isolates will be inoculated into cooked meat medium and incubated for upto 96 hrs for optimal toxin production. Supernatants from the broth will be used for the detection of toxin following Chang's procedure (37,38). HeLa, Y_1 adrenal and CHO cell lines will be used for the detection of cytopathic effect (CPE) produced by toxigenic isolates. Neutralisation of toxins with specific antisera will be attempted.

Luminal contents from post-mortem cases will also be subjected to tissue culture assay. Any morphological change produced by these luminal contents will be taken into account.

2) Gas-liquid chromatography

Gas-liquid chromatographic (GLC) analysis of the fatty acids, from the metabolic end products of anaerobic bacterial growth will be used for the identification of anaerobic bacteria.

C. difficile, *B. fragilis* and *C. perfringens* type A and C will be grown in cooked meat medium and the short chain fatty acid profiles from these growth will be analysed by GLC, in comparison with reference strains.

3) Animal model

B. fragilis isolates, will be subjected to reversible ileal tie in adult rabbit (RITARD) model for the confirmation of their virulence properties (39). Isolates positive in this assay will be considered toxigenic.

4) Serotyping

Neomycin Blood Agar and Neomycin egg yolk agar will be used for the selective isolation of *C. perfringens*. Typical colonies will be serotyped (Flow chart 2).

C. perfringens will be typed by slide agglutination method with commercially available antisera for identification of types A and C.

5) *Campylobacters*

Campylobacter isolates will be characterised upto species level following differential reactions for their identification.

C. jejuni, *C. coli*, *C. laridis*, *C. upsaliensis*, *C. foetus*, *C. cinaedi*, *C. fennelliae* and *Helicobacter pylori* will be differentiated based on catalase, urease and H_2S production; hippurate hydrolysis; nitrate reduction; growth at different temperatures (25^oC, 37^oC, and 42^o), in 1% glycine and in anaerobic environment; and susceptibility to nalidixic acid and cephalothin (15).

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12. PUBLICATIONS OF INVESTIGATORS

Publication of Dr. S.Q. Akhtar

1. Akhtar SQ. Isolation of *Clostridium difficile* from diarrhoea patients in Bangladesh. J. Trop. Med. Hyg. 1987; 90:189-192.

2. Akhtar SQ. Application of Biken Test (modified Elek Test) for sampling of heat-stable enterotoxin of *Escherichia coli* isolated in Bangladesh. Biken Journal. 1986; 26:73-75.

3. Akhtar SQ. Antimicrobial sensitivity and plasmid mediated tetracycline resistance in *Campylobacter jejuni* isolated in Bangladesh. Chemotherapy. 1988; 34:326-331.

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13. FLOW CHART 1

Activities of the laboratory.

First year

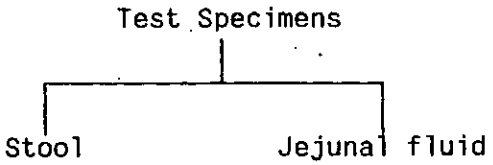
Stool culture and quantitative culture of jejunal fluid for anaerobic and micro-aerophilic bacteria.

Hospital surveillance	.....	750	stools
Persistent diarrhoea study	.....	632	stools
Persistent diarrhoea study (Quantitative culture)	.....	276	jejunal fluid
Intestinal tissue from post-mortem cases	.....	50	

Second year

Hospital surveillance	.....	800	stools
Intestinal tissue from post-mortem cases	.....	50	

FLOW CHART 2



- Stool specimens are directly inoculated on selective media,
- Jejunal fluids are directly inoculated along with 5, 10-fold serial dilutions, 10^{-1} to 10^{-5} for quantitation of bacteria

Anaerobic Culture

(in BBL Gaspak system)

PINN medium for *B. fragilis*	*C. difficile* agar for *C. difficile*, cooked meat broth with cycloserine.	Neomycin Blood agar, cooked meat broth with neomycin (100 µg/ml) and neomycin egg yolk agar for *C. perfringens*.
Non-spore forming Gram-negative bacillus, catalase + Indole -	Green fluorescent colonies distinct odor Gram-positive bacillus	Gram-positive spore forming bacilli,
Fatty acid analysis by GLC	Lecithinase-rhizoidal or pin head colony	Lecithinase +
Detection of toxigenic isolates by RITARD model.	Fatty acid profile by GLC	Serotyping by specific antitoxin
	Detection of toxigenic isolates by tissue culture assay.	
	Neutralization of toxin B for confirmation	

14. ITEMIZED SPECIFIC TASKS FOR EACH LISTED INVESTIGATOR

Sequence of tasks

Culture and testing of specimens	....	18	months
Analysis of data and writing report	....	6	months

Investigators

S. Q. Akhtar D. Datta	Anaerobic/micro-aerophilic culture
S.Q. Akhtar P.K.B. Neogi	Tissue culture assay

15. DETAILED BUDGET

A. Personnel

Name	Level/Step	Salary annum	Total Salary in 2 years
S.Q. Akhtar	NO.B, 13	12,000	24,000
D. Datta	GS.4	2,580	5,160
		Sub-Total =	29,160

B. Operating Costs

Bacteriology	
Total stool culture for 2 years	30,486
Total jejunal fluid culture for 2 yrs.	15,168
Stock of all cultures	2,000
Sub-Total =	47,654
Identification of *C. difficile* (from stool)	3,715
" of *C. perfringens*(from stool)	955
" " *B. fragilis* (from stool)	7,075
" " *Campylobacter* (from stool)	2,075
Identification of *C. difficile* (from J/F)	1,500
" " *C. perfringens* (from J/F)	500
" " *B. fragilis* (from J/F)	1,500
" " *Campylobacter* (from J/F)	1,250
Sub-Total =	18,570
Cost for RITARD Model for 100 rabbits (@ US$ 30/assay)	3,000

The identification cost mentioned includes media/reagents for presumptive identification and also GLC, tissue culture assay and serotyping for confirmation.

C.	Capital Equipment	
	Refrigerator (Westinghouse)	600
	Eppendorf microfuge (for stock)	1,000
	UV lamp	1,000
D.	Travel	2,000
E.	Computing/Publication/Reprints	3,000
	GRAND TOTAL	US$ 105,984

16. JUSTIFICATION FOR BUDGET

Anaerobic bacteriology cell needs its separate freezer. Presently all are stored in the same freezer and there is a shortage of space.

17. BUDGET SUMMARY

First year

Media/reagents/tests	....	....	50,410
Personnel	....	....	14,580
Freeze and microfuge tubes	....	....	1,600
Computing/Publication/Reprints	....	....	1,500

Second year

Media/reagents/tests	....	....	18,814
Personnel	....	....	14,580
Computing/Publication/Reprints	....	....	1,500
Travel	....	....	2,000
Total cost (for 2 year)		US$	105,984

Q1:ps:SQANAROB.PRO

VANDERBILT UNIVERSITY
NASHVILLE, TENNESSEE 37232 TELEPHONE (615) 322-7311

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FAX 343-6160

FAX COVER SHEET

Date: 2/11/91

To: Dr. Moyenul Islam
Acting Head
Laboratory Sciences Division
International Centre for Diarrhoeal Disease Research, Bangladesh

880-2-883116

From: Martin J. Blaser
Vanderbilt University Medical Center
Department of Medicine/Division of Infectious Diseases
A-3310 Medical Center North
Nashville, TN 37232-2605

Number of pages including cover sheet: 2

Comments:

Project title: The role of anaerobic and microaerophilic bacteria in diarrhoeal and persistent diarrhoeal illness in Bangladeshi population.

Principal Investigator(s): .....

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 to 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: .....

Position: .....

Institution: .....

..... 2-10-91

Signature Date

Project title: The role of anaerobic and microaerophilic bacteria in diarrhoeal and persistent diarrhoeal illness in Bangladeshi population.

Principal Investigator(s): .....

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 to 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: S. J. Borriello

Position: Head of Group

Institution: Clinical Research Centre (MRC)

S. J. Borriello
Signature

29/1/91
Date

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 they are justified.

(Use additional pages if necessary)

The only minor points I wish to make are:

1. For *H. pylori* primary isolation it is essential to use fresh blood agar.

2. For *C. difficile* primary isolation they may find it easier to differentiate *C. difficile* from the other bacteria present if they use blood agar (not egg yolk) with cycloserine and cefoxitin. If a UV lamp is available they will also be able to screen the plates under longwave UV, detect the fluorescence characteristic of *C. difficile*. Some workers couple alcohol shock to select for spores with subsequent culture on selective agar. Some sensitivity is lost, but following this procedure *C. difficile* is about the only thing that grows.

Could you pass on these comments to the authors.

QIMR Phone No: (07) 253 6222
QIMR Fax No: (07) 252 5499
International Fax No: 61+7+252 5499

Apertis operiis

QUEENSLAND INSTITUTE OF MEDICAL RESEARCH

FACSIMILE TRANSMISSION

DR MOYENUL ISLAM, LABORATORY SCIENCES DIVISION

TO: INTERNATIONAL CENTRE FOR DIARRHOEALDISEASE RESEARCH

ORGANIZATION/INSTITUTION: BANGLADESH

COUNTRY: BANGLADESH **CITY:** DHAKA

FAX NO: 880-2-883116.

FROM: Glawrence **Date:** 12.2.91

NO. PAGES: 4 (Including this page) **COST CENTRE:** 01 531

MESSAGE:

Review of protocol as requested. Please confirm receipt.

Yours sincerely

Brunston Terrace, Herston, Brisbane, Qld., Australia 4006.
Telephone: (07) 253 6222, Telex: QIMRBR, AA145420, Cables: LEPTOSPIRA, Fax: (07) 252 5499

Project title: The role of anaerobic and microaerophilic bacteria in diarrhoeal and persistent diarrhoeal illness in Bangladeshi population.

Principal Investigator(s): .....

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 to 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 G LAWRENCE

Position: SENIOR RESEARCH FELLOW

Institution: QUEENSLAND INSTITUTE OF MEDICAL RESEARCH

Signature

10.2.91.
Date

Report on The role of anaerobic and microaerophillic bacteria in diarrhoeal and persistent diarrhoeal illness in Bangladeshi population.

This application addresses some an important problem the possible role of anaerobic and other organisms. Standard methods of dealing with stool specimens do not demonstrate the possible role of anaerobic organisms because of the inability of the techniques to reveal the presence or the numbers of the anaerobes present. In particular the role of Clostridial food poisoning in acute diarrhoea in domestic circumstances has always been of interest to me because as it has always seemed that this possibility has been largely ignored. Enteritis necroticans is also important because of its high case mortality and the availability of an effective vaccine. Other anaerobes may be important too and will only be discovered when they are looked for.

Although the aims of the project are laudable I have great reservations about the design of the study, its practability and the cost.

I have no argument with the selection of cases/ specimens on page two, except that post mortem samples may be an unreliable source of material for studying enteritis necroticans as type A organisms may well grow type C in that situation so that the type C presence is masked, the administration of antibiotics may also contribute to the problem. Are surgical specimens available? Or would it be possible to collect specimens from admissions with the clinical syndrome abdominal pain and bloody diarrhoea?

In the research plan page 8 and the section on laboratory methods the understanding of the problems assocated with the identification and enumeration of Clostridia seems to be deficient. There are a number of difficulties: firstly type A food poisoning is caused by the multiplication of the organism in the food that causes the episode and subsequent sporulation. It is necessary to show the number of Clostridia in the stool or gut contents and use the high count of the organism as evidence for its pathogenic role. For this reason immediate determination of organism numbers is important and selective indicating media have been designed for this. Neomycin blood agar is not selective enough to suppress the GNBs. Hauschild, Can J Micro 25,953-963, 1979 or de Vos,N., Eur J Clin Micro, 1, 267 271,1982 describe media, we use the de Vos SPM medium. The number of spores as well as vegetative forms might also give useful information.

The tests for speciation are also open to question. We do not use GLC much because, in part, access to one is not easy. My colleague responsible for this area says that simple tests colonial morphology, Gram stain, lecithinase and lipase, spore stain, stormy clot and DNAase are used initially then gelatinase,nitrate reduction, indole, esculin and reversed camp, if appropriate cells are available, for further confirmation. ANAII (Innovative diagnostics) and ATB 32A (API) might be used if there were any problems, but would not be necessary very frequently in a project like this. It does not appear from examining the manual that GLC would supply a simple and definite speciation of C perfringens. Direct Gram staining of the faeces would also be advisable because it gives some check on quality control and might give valuable information.

While on the laboratory procedures why are lactobacilli being sought in the jejunal fluid?

Finally the isolation of Type C Clostridium perfringens. One of the major problems with diagnosing enteritis necroticans is in the isolation of Type C strains and in the separation of them from the ubiquitous Type As. They look identical. Therefore it is necessary to test quite a large number of colonies from clinical material to ensure that type C organisms are not being missed. In cases that have had antibiotics or where the specimens have been taken a long time after the initiation of the disease the causative type may not be present in large numbers. A number of approaches may be suitable: selective enrichment in cooked meat with neomycin and then plating on a suitable selective medium such as above where 10 or so colonies can be picked. It is probably very important to obtain fresh specimens from very early in the disease if possible, post mortem is probably too late.

The section on the actual typing of the strains is not correct, there is no commercially available agglutinating sera for typing available. Typing is a toxicological test carried out with the Wellcome antisera in mice or in the skins of depilated rabbits. This is time consuming and very expensive. A DNA probe is being developed in Thailand and an immunodiffusion test has been used in Australia to avoid these problems.

The proposal does not state the availability of gas jars, if they have to be purchased, and a lot would be needed, they are very expensive. A flexible plastic anaerobic chamber with glove ports is very desirable if much anaerobic work, particularly enumeration is to be done. It protects the cultures and avoids the difficulty and expense of repeatedly opening the jars. If a supplier of gas mixture (N_2,H_2 and CO_2) is available this system is much cheaper to run than the large number of gaspaks that would be required. The amount of work proposed seems more than one could expect one laboratory person to accomplish.

So, although this proposal addresses some important problems the methods to be used are unlikely to provide the answers needed. Because of the indicated existence of many cases of segmental enteritis in the hospital it is important that the cause of this disease in Bangladesh be established, as well as the possible importance of type A Clostridium perfringens, other anaerobes and Campylobacter being investigated.

Answer to -

Dr. S.P. Borriello's comments:

For *C. difficile*, blood agar with cycloserine and cefoxitine will be used.

UV lamp will be procured for the detection of fluorescence characteristic of *C. difficile*.

Alcohol shock to select for spores with subsequent cultlure on selective agar will be done.