Date 11/12/86

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator S.Q. AKHTAR

Trainee Investigator (if any) ___ 2

Application No. 86039P

Supporting Agency (if Non-ICDDR,B) ___

Title of Study Studies on the isolation rate of anaerobic bacterium C. difficile causing diarrhoea in Bangladesh.

Project status: Pilot Protocol
( ) 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)

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:
   (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 NA Yes No
7. Check documents being submitted herewith to Committee:
   NA 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)
   NA Informed consent form for subjects
   NA Informed consent form for parent or guardian
   NA Procedure for maintaining confidentiality
   NA Questionnaire or interview schedule *
   NA * 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)

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

S. Q. Al-Akhtar
Principal Investigator

JAN 4 - 1987

Trainee

86-039P
11/12/86

SECTION I - RESEARCH PROTOCOL

1. Title : Studies on the isolation rate of an anaerobic bacterium Clostridium difficile causing diarrhoea in Bangladesh.

2. Principal Investigator : Dr. S. Q. Akhtar

   Co-investigators : Drs. D. A. Sack, M.Q.K. Talukdar and Van Loon

3. Starting Date : Dec. 1986

4. Completion Date : June 1987

5. Total Direct Cost : $5000

6. Scientific Program Head : Dr. David A. Sack

This protocol has been approved by the Disease Transmission Working Group

Signature of the Scientific Program Head : David A. Sack

Date : 4-12-86

7. Abstract Summary:

The study plans to determine the frequency of isolation of C. dificile and its toxin from fecal specimens of patients with gastroenteritis. Preliminary studies performed at ICDDR,B have indicated the presence of this organism in stools of patients with antibiotic-associated diarrhoea and in animals treated with lincomycin and ampicillin. Tissue culture assay using WI-38, Y_1 adrenal, chinese hamster ovary (CHO) and HeLa cell lines will be performed to detect C. difficile toxin. Toxin detection will be attempted directly from stools and also from the recovered isolates. Tissue culture assay to detect cytopathic effect (CPE) on cell lines and neutralization with specific antiserum will be performed with individual specimen. The study population will comprise of 100 patients

with antibiotic-associated diarrhoea, 50 with chronic diarrhoea, 50 children under 1 yr of age with diarrhoea, 50 without diarrhoea and 50 diarrhoea patients above 5 yrs. of age without any antibiotic association to form the control group.

8. Reviews:

i) Ethical Review Committee : _____

ii) Research Review Committee: _____

iii) Director: _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective:

The main objective of this study is to determine whether anaerobic bacteria are responsible for diarrhoeal illness in Bangladesh. The incidence of C. difficile in clinical and pseudomembranous colitis, or as a whole in diarrhoeal illness in Bangladesh will be determined.

2. Background:

Toxigenic strains of C. difficile have been shown to be a major cause of antibiotic-associated pseudomembranous colitis (PMC) of man (1-5). Feces from the vast majority of PMC patients contain a cytopathic toxin which is neutralized by antitoxins of C. sordellii and toxigenic C. difficile in high concentration. Toxigenic strains of this organism have been isolated also at a high rate from feces of healthy neonates and infants (6-8) but rarely from those of healthy adults (6,9).

C. difficile was first isolated from the maconium and feces of the newborn infants in 1935 by Hall and O'Toole (6). The organism was named Bacillus difficilis because of the difficulty they experienced in studying it. Isolation of this organism has been facilitated by the development of a selective and differential medium - cycloserine-cefoxitin-fructose-agar (CCFA) (10). This medium has been reported to allow detection of as few as 2×10^3 colony-forming units (cfu) of C. difficile per ml of feces. A selective enrichment broth medium which combined enrichment and antibiotics to facilitate

detection of relatively small numbers of C. difficile in feces has very recently been reported (11). Taurocholate-containing media were reported to enhance spore recovery and is more sensitive than CCFA (12,13).

C. difficlie is now routinely identified in advanced clinical laboratories by culturing on selective media. Tissue culture assay of C. difficile cytotoxin is a recommended diagnostic test (14). C. difficile cytotoxin is detectable in broth cultures (15).

Although clinical presentation of antibiotic-associated diarrhoea and pseudomembranous colitis (PMC) has been known since late 19th century, it appears to have been more common since the introduction of antibiotic therapy. In particular, lincomycin and clindamycin have been associated with PMC, but most other antibiotics have also been increminated. The way in which the antibiotic precipitate the disease is not clear. The most favoured hypothesis is that they supress the normal intestinal flora and allow overgrowth by rapidly multiplying clostridium which have survived exposure to antibiotics or have been ingested after the antibiotic was discontinued.

C. difficile is a normal member of the indigenous, anaerobic bacterial flora in the bowel of adult human and infants but it is usually numerically insignificant and represent less than 0.1% of the cultivable flora found in healthy subjects. After various antibiotic therapies, C. difficile may prolifirate at the expense of the repressed normal bowel flora and produce toxin which is believed to be involved in the pathogenesis of PMC. This disease is believed to be toxin mediated, and recent publications from three groups indicated that C. difficile produces two distint toxins (16, 18).

The toxin designated A and B, are antigenically distinct, large molecular weight proteins which are separated by anion-exchange chromatography. Both toxins cause actinomorphic changes with fibroblast cells in tissue culture, although titers are substantially different and results with other assays of biological activity show considerable variations. Toxin B is a potent cytotoxin (18), 1,000-fold more cytotoxic than toxin A and responsible for most of the activity in the usual tissue culture assay. It is also lethal to mice (17). Toxin A appears to be more active in assays of enteric disease in experimental animals, suggesting a paramount role in the clinical expression of the disease. Toxin A has been observed to cause fluid accumulation in rabbit ileal loops and has been called an "enterotoxin." Two toxins are immunologically distinct molecules and are acid and heat labile. Molecular weight of toxin A ranges from 440,000 to 500,000, and of toxin B is 360,000 to 470,000.

Enzyme immunoassay for detection of antibody to toxins A and B of C. difficile has been developed (19). The ELISA assays should provide a sensitive, specific and practical method to define the prevalence of antibody to C. difficile toxins. These assays could be readily applied to human sera to examine and study the immune response of patients with C. difficile induced disease. Enzyme immunoassays for detection of C. difficile toxins A and B were developed with use of a double-sandwich microtiter plate format. The specificities of the toxin A and B ELISAs were 98.6% and 100% respectively (20). This could serve as a substitute for the tissue culture cytotoxicity assay.

Recently counterimmunoelectrophoresis (CIE) has been proposed as a method for detecting the toxins (21,22). But further study revealed that the

antitoxins used for the detection of C. difficile by CIE react with other C. difficle antigens in addition to the toxins produced by the bacterium (23). The potential of CIE for detecting cross-reacting antigens and, therefore, for yielding false positive results has been confirmed in a recent clinical study. Epidemiological study of outbreaks of C. difficile toxin-positive diarrhoea in day care centre using polyacrylamide gel electrophoresis technique has also been performed. The study indicated the importance of searching for C. difficile in day care centre children who develop diarrhoea and that the electrophoretic patterns of isolates may be used as an epidemiological tool to study the potential chain of transmission of C.difficile (24). Recently, polyacrylamide gel electrophoresis-electroblot technique has been applied for immunochemical fingerprinting of C. difficile without information about their origin. The PAGE-electroblot technique indicated that it could greatly aid investigations into the epidemiology of C. difficile infections (25). A bacteriophage and bacteriocin typing system for C. difficile has been developed recently for use in studies of the epidemiology of colitis induced by C. difficile (26). The typing system was used to determine whether antibiotic-associated colitis in hamster results from overgrowth of C. difficile that was already part of the hamster's bowel flora, by acquisition of C. difficile from the environment, or from both mechanisms. The result suggested acquisition of C. difficile from the environment (27).

A fluorescent antibody test applied to stool smears detected 81% of C. difficile positive stools from patients with antibiotic-associated diarrhoea (8), but many cross-reactions were noted between antisera

raised against C. difficile and other clostridia. Studies to detect C. difficile in feces by direct gas liquid chromatography has been performed and it was concluded that gas chromatographic detection of volatile fatty acids or p-cresol in feces are not satisfactory screening tests for the presence of C. difficile (29, 31). (A table on Background information is added at the end to show the rate of isolation in other laboratories). C. difficile was isolated also in Bangladesh from antibiotic-associated diarrhoea patients through pilot studies performed by the principal investigator at ICDDR,B. An abstract on this work was accepted by the ASM and was given a press release. A manuscript has already been submitted for publication in international journal. Antibiotic induced diarrhoea in animals has also been experienced in ICDDR,B studies and the manuscript is in preperation.

3. Rationale:

The role of an obligate anaerobic spore-forming bacterium, C. difficile in diarrhoeal illness, specifically in pseudomembranous colitis, antibiotic-associated diarrhoea, even in their contacts has been established. The organisms is now routinely identified in advanced clinical laboratories by culturing on selective media. Bacterial toxins have been directly detected from stool samples. Uptil now, the etiology for 20-30% diarrhoeal cases are not known. Exploring for new diarrhoeal etiology like C. difficile might aid our knowledge of unknown causes.

Isolation of this bacterium from antibiotic-associated diarrhoea patients at ICDDR,B through two previously performed pilot studies indicated that C. difficile is a cause of diarrhoeal illness in Bangladesh. This finding demands further study.

B. SPECIFIC AIMS

1. To determine the incidence of C. difficile in patients with gastroenteritis and to investigate the role of this organism in diarrhoeal illness in Bangladesh.
2. To assess the stool samples for the presence of C. difficile toxin by tissue culture assay.

C. METHODS OF PROCEDURE:

Patient selection criteria:

a. One hundred antibiotic-associated diarrhoea patients, the onset of diarrhoea occuring during the course of antibiotic treatment for other complications, or if diarrhoeal illness become more severe due to antibiotic administration as a control of gastraintestinal complications.

b. Fifty patients with chronic diarrhoea (more than 3 weeks) with no established diarrhoea pathogens.

c. Fifty children under 1 year with diarrhoea from ICDDR,B and IPGM&R.

d. Fifty children under 1 year without diarrhoea from ICDDR,B and IPGM&R.

e. Fifty diarrhoea patient above 5 years of age without any antibiotic association to form the control group.

Collection of Stool Samples:

Catheter sample will be collected in all cases; 2-3 ml of the stool samples will be collected in small vials and will be immediately frozen for direct toxin assay. Second part of the catheter drawn stool sample (2-3 ml) will be directly inoculated into cooked meat medium for enrichment purposes. Rest of the sample will be collected under liquid paraffin for direct inoculation and for keeping stock.

Direct toxin assay:

Attempt will be made to test all fecal samples directly for C. difficile cytotoxin within 4 hrs of receipt of the sample. Samples that cannot be tested immediately will be stored at -40°C until examined. Ten percent suspensions

of fecal specimens will be prepared in 0.05 M phosphate-buffered saline (pH 7.0), and will be centrifuged at 10,000 rpm for 10 min. The supernatant will be sterilized by filtration through membrane filter with a 0.45-μm poresize. 0.1 ml of serial 10-fold dilutions of stool filtrates will be added to tissue culture monolayers. The tissue cultures will be observed for the characteristic (actinomorphic/rounding) cytopathogenic effect (CPE) hourly for 8 hr and again at 24, 48 and 72 hr. Cytotoxicity is defined as the demonstration of CPE in at least 50% of the cells. All positive filtrates will be retested and neutralized by the addition of 0.1 ml of a 1:25 dilution of C. difficile antitoxin. The antitoxin will be purchased from the Anaerobic Laboratory, Virginia Polytechnique Institute. Neutralized cell cultures will be examined at the same intervals as the non-neutralized cells. A toxin standard (from the Anaerobic Laboratory, Virginia Polytechnic Institute) will be used, a positive control and a CPE negative fecal filtrate from an asymptomatic volunteer will be used as a negative control.

Examination of feces for C. difficile

Fecal samples collected under liquid paraffin will be directly inoculated on CCFA (10), for the recovery of the vegetative form of the organism, on TCCFA (12) for the recovery from spore-form. 1-2 ml of the fecal samples will be heated at 80°c for 20 min and the heated samples will be inoculated on blood agar plates. Characterisitic spore formation is best observed on blood agar plates. Inculated media will be incubated anaerobically at 37°c in an anaerobic chamber or jar for 24-72 hr. C. difficile on CCFA, TCCFA or blood agar plate will be identified by characterisitic features in culture, Gram-stain and fluorescence. The API 20A (Analytab Products, Montreal, Canada) anaerobic system will also be used for confirmation of the identity of all isolates as C. difficile.

The enrichment medium, cooked meat carbohydrate-selective broth (II) will be initially incubated with the sample in screw capped tubes for 4-6 hrs. at 37°c and then will be inoculated on CCFA and TCCFA. Enrichment broth will be incubated upto 72h and subsequent subculture will be made at 24, 48 and 72h. Identical culture condition and identification criteria will be maintained for enrichment broth as well.

D. SIGNIFICANCE

Pseudomembranons colitis, antibiotic associated diarrhoea, necrotizing enterocolitis, even C. difficile induced diarrhoea through acquisition of dessiminated vegetative or spore form has been reported to be fatal, specially in neonates. Correct diagnosis is essential for therapeutic measures and vancomycin is usually used. Commonly used antibiotics have shown to deteriorate the cases.This study should provide significant knowledge and make contribution towards new diarrhoeal etiology. Through this protocol an anaerobic laboratory will be established that will provide scope for exploring other anaerobes causing diarrhoeal illness. Other anaerobic bacteria than C. difficile have also been reported to be involved in causing diarrhoea.

E. FACILITIES REQUIRED

Arrangements have been made.

F. COLLABORATIVE ARRANGEMENTS

Collaborative arrangements with Dr. J.G. Bartlett of Division of Infectious Diseases, School of Medicine, Johns Hopkins University, a pioneer researcher in exploring the role of C. difficile in antibiotic-associated diarrhoea and pseudomembranous colitis, has already been made. He has assured of all possible help and cooperation and been sending us research materials, literatur for methodologies etc.

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সমস্ত মেডিকেশনের বৃত্তান্ত গোপন রাখা হবে।

যদি আপনি বাচ্চাকে গবেষণায় অন্তর্ভুক্ত করতে রাজী থাকেন, তবে অনুগ্রহপূর্বক আপনি নীচে নাম স্বাক্ষর করবেন অথবা টিপ সই দেবেন।

গবেষণাকারীর স্বাক্ষর

রোগী বা অভিভাবকের স্বাক্ষর অথবা টিপসই

তারিখ

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Tissue Culture Assays for *Clostridium difficile* Toxin

Patient category	No. positive no. tested
Antibiotic-associated PMC	
Larson *et al.* (1978)	9/9
R. H. George *et al.* (1978)	8/8
Keighley *et al.* (1978)	16/16
Bartlett *et al.* (1980)	137/141
Delmee and Wauters (1981)	24/25
W. L. George *et al.* (1982b)	29/35
Antibiotic-associated diarrhea without established PMC	
Bartlett *et al.* (1980)	193/710
Gilligan *et al.* (1981)	15/59
Lishman *et al.* (1981)	9/52
Delmee and Wauters (1981)	33/62
Aronsson *et al.* (1981)	280/827
Gilligan *et al.* (1981)	15/61
W. L. George *et al.* (1982b)	29/138
Diarrhea unrelated to antibiotics	
Bartlett *et al.* (1980)	9/562
Gilligan *et al.* (1981)	2/100
Keighley *et al.* (1978)	0/28
W. L. George *et al.* (1982b)	0/17
Antibiotic exposure without diarrhea	
Bartlett *et al.* (1980)	2/110
Lishman *et al.* (1981)	4/53
W. L. George *et al.* (1982b)	3/12
Healthy adults	
Bartlett *et al.* (1980)	0/60
Lishman *et al.* (1981)	0/27
Meuwissen and Rietra (1980)	1/421
Healthy neonates	
Viscidi *et al.* (1981)	12/45
Borriello (1979)	10/19
Kim *et al.* (1981)	9/21
Sherertz and Sarubbi (1982)	10/37
Gurwith *et al.* (1981)	9/47
Donta and Meyers (1982)	17/401
Meuwissen and Rietra (1980)	17/121

[FROM: Human Intestinal Microflora in Health and Disease. Edited by David J. Hentges.]

ICDDR,B
1986 BUDGET PROPOSAL
(In US $)

AREA DESCRIPTION

Program Name:.....

Project/Protocol/Branch Name:.....

Principal Investigator/Branch Head/Program Head:.....

Budget Code:..... Estimated Beginning Date:.....

Protocol No:..... Estimated Ending Date:.....

EXPENSE CATEGORY		*Column A Total Project Cost	Column B Actual Jan-Sept. 1985	Column C Estimated Whole Yr. 1985	Column D Proposed 1986
A/C No.	Description	Refer Page			
3100	Local Salaries	2	1,100			1,100
3200	Intl. Salaries	8				
3300	Consultants	14				
3500	Travel Local	15				
3600	Travel Intl.	16				
3700	Supplies & Mat.	18	2,650			2,650
3800	Other Costs	19	250			250
4800	Inter Deptl. Ser.	20	1,000			1,000
	Total Direct Operating Cost					5000
0300	Capital Expenditure Refer Page 21			**	**	
	TOTAL DIRECT COST					

* Refers to entire life of project.
** For Finance use only.

Budget86.1-3
AZIZ-5.

PERSONNEL REQUIREMENT-(LOCAL STAFF) 1986

	No.of Positions	No. of Man Months	$ Amount
A. Direct Project/Protocol/Branch Staff at 1.1.1986 Sourced from Page 3			
Add:			1,100
B. New Recruitments Sourced from Page 4			
C. Staff allocated from other area Sourced from Page 5			
(i) Sub-Total			
Less:			
D. Separations Sourced from Page 6			
E. Staff allocated to other area Sourced from Page 7			
(ii) Sub-Total			
(i)-(ii) TOTAL			* 1,100

*Agrees with Page 1 A/C No.3100 Column D

NEW RECRUITMENT-(LOCAL STAFF)-1986

Job Designation	Level	Start Date	No. of Posit.	***No. of Man Months	**Rate Per Month	$ Amount
	GS 5	15.12.86	1	6	190	1,100
		TOTAL	* 1	* 6	190	*1,100

** See annexure A for a list of rates.

*** Calculate man month from the month of joining upto the end of December 1986 i.e., if joining month is March 1986 then man month will be 10 for one person. If no. of persons is more than one, say 4 then man month will be (10x4)=40.

* AGREES WITH PAGE 2 ROW B

SUPPLIES AND MATERIALS-1986

A/C Code	Item Description	$ Amount
3701	Drugs (used for medication in the hospitals and field stations)	
3702	Glassware (Bottle, beaker, cylinder, petridish, aluminium seal, slides, stopper, tube etc.)	
3703	Hospital Supplies (bandage, gauze, blade, bowl, catheter, cotton, needle, syringe, solution, leukoplast, towel etc.)	100
3704	Stationery and Office Supplies (Battery, book register, binders, files, pencil, fastener, paper, ribbon, stapler etc.)	200
3705	Chemicals and Media (Acid, reagent, dextrose, sodium, bactoagar etc.)	
3706	Materials for Uniform (Cloth, button etc. required for making uniforms)	
3707	Fuel, Oil and Lubricants (Diesel, mobil, petrol, Kerosene etc.)	
3708	Laboratory Suplies (Aluminium foil, bag, blade, brush, cap, container, film X-Ray etc.)	
3709	Housekeeping Supplies (Aerosol, battery, wiping cloth, duster, lock and key etc.)	
3710	Janitorial Suppies (Bleaching powder, brush, detol, detergent, insecticide, soap etc.)	
	(Contd. to Page No. 18)	

SUPPLIES AND MATERIALS-1986

(Contd. from Page No. 17)

A/C Code	Item Description	$ Amount
3711	Tools and Spares (Automobile spares, tyres, tubes, battery, stores required for maintenance services etc.)	
3712	Non-stock Supplies (Materials not normally kept in stock and purchased only against specific requisitions)	1,500
	Sub-Total	1,500
3713	Freight and other Charges Add 30% to above sub total	400 450
	TOTAL	2,650

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Note: For rates please contact Supply Ext.260 (add 10% to rates for inflation)

OTHER COST-1986

A/C Code	Accounts Description	$ Amount
3800	**Repairs and Maintenance** (Maintenance and repairs of vehicles, equipments, furniture and building)	100
3900	**Rent, communication and utilities** (Postage, telephone, telegram, electricity etc.)	50
4100	**Bank charges**	
4200	**Legal and professional expenses** (Professional membership fee, legal fee, audit fee etc.)	
4300	**Printing and Publication** (Printing of forms, books, journals, reprints etc.)	100
4400	**Entertainment, Hospitality & Donation** (Guest house accommodation, donations, hospital food, lunch, refreshment etc.)	
4500	**Service Charges** (Porter, labour, washing, laundry and other misc. exp.)	
4600	**Staff Development and Training** (Training course fee, training materials, stipend,scholarship, subsistance paid to the staff)	
	TOTAL	250

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**INTERDEPARTMENTAL SERVICES-1986

A/C Code	Service Area	$ Amount
4801	Computer	
4802	Transport Dhaka	600
4803	Transport Matlab	
4804	Water Transport-Matlab	
4805	Transport Teknaf	
4806	Xerox and Mimeograph	100
4807	Pathology	
4808	Microbiology Tests	
4809	Biochemistry	
4810	X-Ray	
4811	I.V. Fluid	
4812	Media	200
4813	Patient hospitalisation study	
4814	Animal Research	
4815	Medical Illustration	100
4817	Telex	
4818	Out Patient care	
4830	Transport Subsidy	
	TOTAL	* 1,000

** See annexure B for rates.

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