Attachment 1. 16

Date

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr. S.Q. Akhtar

Application No. 85-001(P)

Title of Study Role of Clostridium difficile in diarrhoea in Bangladesh

Trainee Investigator (if any)

Supporting Agency (if Non-ICDDR,B)

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 NA
   (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 NA
   (b) From parent or guardian (if subjects are minors) — Yes No NA
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)
   NA Informed consent form for subjects
   NA Informed consent form for parent or guardian
   NA 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.

NA

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

Saiyeda Rudsiya Akhtar
Principal Investigator

Trainee

SECTION I - RESEARCH PROTOCOL

1. Title : Role of Clostridium difficile in diarrhoea in Bangladesh

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

   Co-investigators : Drs. D.A. Sack, Van Loon, J. Wasserheit

   Adviser : Dr. D.A. Sack

   Consultant : Dr. J.G. Bartlett

3. Starting Date : As soon as budget is available (Expected March 1985)

4. Completion Date : Six months from the starting date

5. Total Direct Cost : US $ 5,000.00

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

This protocol has been approved by the Disease Transmission Working Group.

Signature of Scientific Program Head : _____

Date : _____

7. Abstract Summary

The study plans to determine the role of an anaerobic bacterium, Clostridium difficile in causing diarrhoeal illness in Bangladeshi population. Though sampling will be done on random basis, attempt will be made to include pseudomembranous colitis (PMC) patients, patients with antibiotic associated (AAC) and chronic diarrhoea. Fresh or frozen stool samples will be used for direct toxin detection by tissue culture assay. Presently this is the most reliable and sensitive assay for the examination of fecal specimens for cytotoxicity. Mouse Y_1 adrenal and HeLa cell lines will be used for the assay. One thousand samples will be analyzed through this study.

8. Reviews :

a. Research Involving Human Subjects : _____

b. Research Review Committee : _____

c. 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 — cyclose 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. difficile 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 [illegible] cycloserine-cefoxitin-fructose-agar (CCFA) [illegible] reported to allow detection of as few as 2 X 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

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(৫) [illegible]
খাদ্য নালী থেকে আক্রান্ত পাচক রস নেয়া হয়
এর জন্য আক্রান্ত একটু অস্বস্তি বোধ না
পারে, তবে কোন অসুবিধার কারণ নেই।

mediated, and recent publications from three groups indicated that C. difficile produces two distinct toxins (16-18). The toxin designated A and B, are antigenically distinct, large molecular weight proteins which are separated by anion-exchance chromatography. Both toxins cause actinmorphic 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 potent cytotoxin (18). 1,000-fold more cytotoxic than toxin A and responsible for most of the activity in the usual tissue 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 sutdy revealed that the antitoxins used for the detection of C. difficile by CIE react with other C. difficile 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 form 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).

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 20-25% diarrhoeal cases are not known. Exploring for new diarrhoeal etiology like C. difficile might aid our knowledge of unknown causes.

B. SPECIFIC AIMS

1. To determine the incidence of C. difficile in diarrhoeal cases in Bangladesh.
2. To assess the stool samples for the presence of C. difficile toxin by tissue culture assay.
3. To establish tissue culture assay for the detection of C. difficile toxin at ICDDR,B.

Methods of Procedure :

One thousand stool samples will be collected preferably from clinically suspected or colonoscopy positive PMC patients, from patients with antibiotic-associated diarrhoea and chronic diarrhoea. Responsible clinician will identify the patients. Patients from surveillance study will also comprise a part of the study population.

Tissue culture assay :

Standardization of the assay :

For the standardization of the assay, titration curves for C. difficile ATCC reference strains for stool samples from human and animal sources causing cytopathic effect (CPE positive) due to the presence of C. difficile will be plotted.

ATCC reference strain of C. difficile will be grown in cooked meat medium for 3-5 days at 37°C. After incubation culture supernatants will be filtered through multipore system (0.45 µm poresige). Filtrate will be considered as the standard toxin preparation. Serial 10-fold dilutions (10^{-1} to 10^{-6}) of this toxin preparation will be inoculated on both the cell lines. CPE for all the dilutions will be calculated and will be plotted against each dilution. Dilution causing 50% CPE will be calculated. Samples causing at least 50% CPE will be considered positive.

CPE positive stool samples from human and animal sources will also be standardized following some procedures. To obtain CPE positive animal

stool, guinea-pigs and rabbits (a set of 5 animals) will be fed with ampicillin and penicillin (50 mg/kg body wt/day), and clindamycin for 2 weeks. Stool will be checked for CPE from 8th day.

Direct toxin detection on tissue culture :

Attempt will be made to process the test samples within 4 hours of receipt. If not samples will be kept frozen (-40°C) until analyzed.

Two dilutions of the test samples : dilution which will give 50% CPE (end point for positive assay from the titration curve) and a dilution in between the end point and undiluted sample (dilution giving approx. 75% CPE), along with the undiluted sample will be subjected for the assay. Dilutions will be made in tissue culture maintainance medium.

Y_1 adrenal and HeLa cell lines will be used for the assay. The cell lines will be grown in F-10 and minimal essential medium supplemented with 10% fetal calf serum. The cell monolayers will be prepared by placement of 0.2 ml of cell suspension into each well of a 96-well flat bottomed microtiter tissue culture plate. Plates will be incubated at 37°C in 5% CO_2 until monolayer is formed (2 days). The assay for C. difficile toxin will be performed by placing 50 µl of test samples onto tissue culture monolayers.

The test samples will comprise the supernatant of liquid stool or an aqueons extract of solid stools prepared by adding an equal volume of phosphate-buffered saline (PBS). These will then be centrifuged at

10,000 rpm for 10 minutes. The supernatant will be carefully taken out for the assay.

- Two parallel sets of samples will be analysed : One set will be sterilized through millipore filtration (0.22 µm pore size), other set by the addition of antibiotics (gentamycin 40 µg/ml, penicillin 100 µg/ml).

Since millipore filtration is an expensive and time taking procedure we would like to evaluate, whether this can be substituted by antibiotic addition.

The stool supernatants will be incubated with the cell monolayers at 37°C in 5% CO_2 for 48h. The monolayers will be examined at 12h intervals during the incubation period. Those monolayers showing no morphological change after 48 hrs. will be considered negative. These monolayers showing actinomorphic changes (rounded cell with radiationg processes) in at least 50% of the cells, will be considered positive. Cytotoxicity is defined as the demonstration of CPE in at least 50% of the cells. Neutralization assay will be performed with the CPE positive samples. Monolayers showing toxic changes such as cell lysis or cell fusion (other than characteristics cytopathic effect-CPE) will also be subjected for neutralization assay.

Neutralization assay

Confirmation of the presence of C. difficile toxin in patients' specimen requires the neutralization of cytotoxic activity with specific antibody. Positive samples at a dilution of 1:10 will be mixed with a 1:10 dilution

of antiserum to C. difficile or C. sordellii toxin (equal volume). (The antitoxin will be purchased from the Anaerobic Laboratory-Virginia Polytechnique Institute). The mixture will be then incubated at room temperature for 30 minutes and 100 ul of the mixture will be assayed as described for toxin assay. Specimens causing no morphological change of the tissue culture cells in the presence of C. difficile/ C. sordellii antitoxin will be considered positive.

Stool samples causing cytopathic effect (CPE) on tissue culture monolayers, neutralizable by C. difficile/C. sordellii antitoxin will be confirmed for C. difficile toxin positive.

This study will detect toxin only. Toxin titers from the test samples will not be determined. So CPE positive samples, neutralized with antitoxins will only be recorded.

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 detoriate 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

Dr. Bartlett has assured of all possible help and cooperation.

Abstract Summary

The study plans to determine the role of an anaerobic bacterium, Clostridium difficile in causing diarrhoeal illness in Bangladeshi population. Though sampling will be done on random basis, attempt will be made to include pseudomembranous colitis (PMC) patients, patients with antibiotic associated (AAC) and chronic diarrhoea. Fresh or frozen stool samples will be used for direct toxin detection by tissue culture assay. Presently this is the most reliable and sensitive assay for the examination of fecal specimens for cytotoxicity. Mouse Y_1 adrenal and HeLa cell lines will be used for the assay. One thousand samples will be analyzed through this study.

Test samples willbe obtained from ERC and RRC approved studies. No separate sampling will be done for this study. No colonoscopy will be done for this study. No consent form is needed.

This is a laboratory based study.

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SECTION III - BUDGET

A. DETAILED BUDGET

1. PERSONNEL SERVICES

		% or No. of days	Project Requirement Taka	Dollar
Dr. S.Q. Akhtar	Principal Investigator	40%	30,000.00	-
To be named	Research Officer	100%	36,000.00	-
		Sub total Tk.	66,000.00	-

2. SUPPLIES AND MATERIALS

Toxins/antisera		300.00
Millipore filter		300.00
Glassware (vials, test tubes, millipore systems)		500.00
Tissue culture seed		200.00
Media/chemicals for tissue culture		700.00
Microtiter plates/tips etc.		250.00
	Sub total	$ 2250.00

3. EQUIPMENT

4. PATIENT HOSPITALIZATION

None

5. OUTPATIENT CARE

None

6. ICDDR,B TRANSPORT

None

7. TRAVEL AND TRANSPORTATION OF PERSONS

None

8. TRANSPORTATION OF THINGS

None

9. RENT, COMMUNICATION AND UTILITIES

None

10. INFORMATION SERVICES (LIBRARY & PUBLICATION)

None

11 PRINTING AND REPRODUCTION

None

12. OTHER CONTRACTUAL SERVICES

None

13. CONSTRUCTION, RENOVATION AND ALTERATION

None

B. BUDGET SUMMARY

		Taka	Dollar
1.	Personnel Services	66,000.00	-
2.	Supplies and Materials		2,250.00
3.	Equipment	-	-
4.	Patients Hospitalization	-	-
5.	Outpatient Care	-	-
6.	ICDDR,B Transport	-	-
7.	Travel and Transportation of persons	-	-
8.	Transportation of Things	-	-
9.	Rent, Communication and Utilities	-	-
10.	Information Services (Library and Publication)	-	-
11.	Printing and Reproduction	-	-
12.	Other contractual services	-	-
13.	Construction, Renovation and Alterations	-	-
	Total	Tk.66,000.00	$ 2,250.00

= $2,750.00

Grand total $ 5,000.00