This is a pilot study

Attachment 1. CO

Date 13.5.92

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator S.Q. AKHTAR Trainee Investigator (if any) X

Application No. 92-022(P) Supporting Agency (if Non-ICDDR,B) X

Title of Study Studies on the incidence of Clostridium difficile in clinical and PMC.

Project status:
(✓) New Study Pilot
( ) 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: 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)
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.

We 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. Akhtar
Principal Investigator

Trainee

82-022(P)
17/5/82

SECTION I - RESEARCH PROTOCOL

1. Title : Studies on the incidence of Clostridium difficile in clinical and pseudomembranous colitis.

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

   Consultant : Dr. John G. Bartlett

   Co-Investigators : Drs. K.M.S. Aziz, M.M. Rahaman, P. Speelman, H. Ali

   Advisors : Dr. K.A. Monsur

3. Starting Date : May 16, 1982

4. Completion Date : November 15, 1982

5. Total Direct Cost : US$ 3,000.00

6. Scientific Programme Head :

   This protocol has been approved by the DTWG Working Group.

   Signature of the Scientific Programme Head : Hamadi

   Date : 12/5/1982

7. Abstract Summary :

Clostridium difficile is the established and most common cause of antibiotic-associated pseudomembranous enterocolitis in humans. At ICDDR,B we know uptil now approximately 80 per cent of the causes of diarrhoeal illness. The remaining 20 per cent or more is still unknown. Most of the bacterial pathogen responsible for diarrhoea

are either aerobic or facultative. Until date no significant study was done on the role of anaerobic organisms in diarrhoeal diseases at ICDDR,B.

Search would be made for Cl. difficile in diarrhoeal cases clinically diagnosed as pseudomembranous colitis (or suspected as PMC) having no established diarrhoeal pathogen. Isolation of this organism from stool samples would be attempted by using selective media. Cl. difficile would be identified following established standard identifying criteria. Toxin detection from the isolated/identified Cl. difficile strains would be performed using tissue culture system for cytotoxicity assay. Rapid direct detection of Cl. difficile toxin in stool would be done with stool extracts in tissue culture system.

8. Reviews:

a. Ethical Review Committee : _____

b. Research Review Committee : _____

c. Director : _____

d. BMRC : _____

e. Controller/Administrator : _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION:

1. Objectives:

The main objective of this study is to explore whether anaerobic bacteria specifically Cl. difficile is responsible for diarrhoeal illness in Bangladeshi population. Another aim is to set up anaerobic techniques in Microbiology Laboratory at ICDDR,B to facilitiate the isolation of anaerobic organisms.

2. Background:

Recent studies on the pathophysiology of antibiotic-associated pseudomembranons colitis have emphasized the importance of clostridia as human intestinal pathogen. Due to difficulty in culture of anaerobic organisms, literature survey shows that work on attempts on isolation of pathogenic anaerobic bacteria from diarrhoeal patients is rare. Currently a few laboratories are performing some work on isolation and characterization of toxigenic and anaerobic bacterial pathogens responsible for producing diarrhoea in man (Bartlett et al 1977, 1978, 1979, 1980, 1981, 1982; George et al 1978; Chang et al 1980; Batts et al 1980; Falson et al 1980; Larson et al 1978; Mullingan et al 1980). Recently Cl. difficile has been established as the most common cause of antibiotic-associated colitis in man (Fekety et al 1979;

George et al 1980). Using a special selective medium Falson et al (1980) have shown 3% isolation of Cl. difficile from diarrhoea patients. They also reported that any change of the normal bacterial fecal flora due to antimicrobial treatment or enteric infections like Salmonella increases the possibilities of isolating Cl. difficile. Larson et al (1978) and Bartlett et al (1978) reported that in many cases toxin-producing clostridia caused pseudomembranons enterocolitis in patients treated with antibiotics. Bartlett et al (1979) have been able to detect Cl. difficile toxin from stool samples of 98% patients with pseudomembranons enterocolitis and 15% of patients with antibiotic induced diarrhoea without signs of pseudomembranons enterocolitis. Until recently Cl. difficile was considered non-pathogenic for humans (Larson et al 1978; Bartlett et al 1979). Nord and Heimdahl (1979) reported isolation of Cl. difficile from 2% of healthy individuals.

Recently Bartlett (1981) demonstrated that toxin in stools of patients who had antibiotic-associated diarrhoea or colitis could be neutralized by Cl. sordellii antitoxin. Many investigators have shown a similar aetiologic mechansims in animal models in which there was a toxin in the stool that could be neutralized

with Cl. sordellii antitoxin (Silva 1979; Bartlett et al 1978; Fekety et al 1979) . Intracecal injection of either the organism or the partially purified toxin produces an analogous disease in experimental animal models (Bartlett 1977). Same toxin was found in the stool specimens from patients with antibiotic associated PMC. Report in the literature provide evidence for Cl. difficile being responsible for producing this toxin. Willey and Bartlett (1979) observed that stool cultures from these patients almost invariably yield this organism which produces a cytotoxin neutralizable by Cl. sordellii antitoxin. In vitro production of similar or identical toxin has also reported by Bartlett (1978). From Bartletts recent reviews it is apparent that 100% of the PMC patients showed the presence of toxin producing Cl. difficile and about 20% in patients with antibiotic-associated diarrhoea in which there are relatively mild symptoms and normal endoscopic picture.

3. Rationale:

Recent investigations have shown that the anaerobic bacteria specially Cl. difficile, Cl. perfringens are responsible for diarrhoeal illness including pseudomembranons and necrolizing

enterocolitis. In ICDDR,B no attempt has yet been made to isolate anaerobic bacterial pathogens from patients with these syndromes. This study is necessary to explore the role played by Cl. difficile in the aetiology of diarrhoeal illness in Bangladesh and to establish anaerobic abcteriology laboratory at ICDDR,B.

B. SPECIFIC AIMS:

1. The main aim of this study is to explore whether anaerobic bacteria like Cl. difficile is responsible for causing diarrhoeal illness in Bangladeshi population.

2. To set up a laboratory for anaerobic diagnostic work.

C. METHODS AND MATERIALS:

Patient Selection:

Approximately 100 patients would be included in this study irrespective of age and sex. Selected cases would be:

a) Suspected/clinically diagnosed as pseudomembranous colitis. Suspected cases should have persistent diarrhoea or dysentery (more than 3 days) and a history of antibiotic treatment.

b) Patients with persistent diarrhoeal or dysentery but not had any antibiotic therapy.

Patients selected should have no established diarrhoeal pathogens.

c) Patients under Dr. Speelman's colitis study. In this protocol pateints with different types of colitis are studied including colonoscopic investigation. Group 3 patients of "colitis study" who would have no established diarrhoeal pathogen would be of interest of the proposed study.

Collection of stool samples:

Two types of clinical specimens would be included in this study:

1) Stool samples - catheter stool samples would be collected under liquid parafin by the PI.

2) Colonic fluid - would be supplied by Dr. Speelman from group 3 patients under his colitis study.

Direct Toxin assay from stool:

Stool sample would be tested for the presence of cytopathic toxin that is neutralized by C. sordellii antitoxin (Bartlett 1981; Chang et al 1979).

a. Tissue culture:

All cell types eg. primary human amnion Wl-38, babt hamster kidney, HeLa, monkey kidney, mouse kidney, mouse fibroblast, human chorion and human brain cells are reported to be susceptible to clostridial toxin (Bartlett et al 1979). For this study we woild use HeLa cells which is widely used and readily available (Burdon 1981). We would look for rounding of cells and neutralization of this effect by C. sordellii antitoxin.

b. Test sample preparation:

The test specimens would consists of liquid stool or aquons extracts of solid stools prepared by adding an equal volume of phosphate buffer saline (PBS). The sample would be centrifuged at 2,000 g for 20 min. The supernate is removed for sterilization either by passing through a membrane filter of 0.45 µm average pore diameter or by treating with antimicrobial mixtures (penicillin, 100 µg/ml; streptomycin 50 µg/ml; polymyxin, 100 µg/ml; neomycin 100 µg/ml; amphotericin B, 25 µg/ml). As the sterilization by antibiotic treatment is easier we would treat the supernate with the above mentioned antimicrobial mixtures.

c. Assay:

Aliquots of 0.1 ml of the stool supernate diluted 1:50 in tissue culture maintenance medium would be inoculated into the tissue culture by replacing the miantenance fluid over the cell monolayer would be read at 24 hours. The criterion for a positive assay would be the demonstration of actinomorphic changes (rounded cells with radiating processes) that would be neutrilized by C. sordellii antitoxin.

d. Neutraliation:

Neutralization of the cytotoxin with antitoxin is considered necessary for test specificity. This is because stool from 20% healthy persons may contain cytopathic substances (Bartlett, 1979). Neutralization should be instantaneous at either room temperature or 37°C so that preincubation of the specimen with the antitoxin is unnecesssary. Samples showing positive results would be tetested by mixing with an equal volume of gas gangrene antitoxin diluted 1:10 or C. sordellii antitoxin. A known positive control (C. difficile broth culture) would also be tested with the antitoxin to ensure continued neutralizing activity and would be used with each run. High titre specimen, might fail to show neutralization due to the large amount of toxin and would require repeat testing with 1:100 or 1:1000 dilution. High titre

specimens should show cytopathic changes within 4 hours, low titre specimens would require 12-24 hours, rare specimens will require a 48 hours reading. Toxin neutralization may be difficult to interpret after 48 hours due to toxin antitoxin dissociation.

Stool Culture:

For primary isolation, stool samples or colonic fluids would be inoculated in selective media (CCFA, George et al 1979; TCCFA, Wilson et al 1982) immediately after collection. For the better rates of isolation inoculation on frshly prepared plates is advised. Immediately after inoculation plates should be placed in an anaerobic jar and incubated for 48 hrs at 37°C. For subculturing freshly prepared thiaglycollate broth or blood agar plates would be used. Stocks would be maintained in cooked meat medium. Selective plates would be used to isolate Staph aureus and invasive E. coli from the same stool samples.

Setting up the Gaspak Jar:

1. Inoculated plates would be placed in the jar immediately after inoculation.
2. Anaerobic indicator should be used to ensure complete anaerobiosis.

3. Disposable hydrogen-carbon-dioxide generator would be opened, activated and placed upright in the jar.

4. The lid of the jar should be immediately secured and placed in the incubator.

After proper incubation the expected organisms would be identified following standard criteria.

Description of the Organisms:

C. difficile:

It is a long slender Gram-positive motile bacillus about 6-8 x 0.5 µm in size. It produces large, oval, subterminal spores which distent the bacillary body. C. difficile is most commonly encountered in the faeces of infants. It is a strict anaerobe. Colonies are 2-3 mm in diameter after 48 h incubation, slightly raised, white, apaque and circular, with an entire margin. Colonies of Cl. difficile growing on CCFA should have distinctive morphological and fluorescent properties which are sufficient for presumptive identification. Colonies of Cl. difficile growing on CFA, CCFA and blood agar would be examined under long-wavelength ultraviolet light (Mineralite UVSL-25; Ultraviolet Products, Inc., San Gabriel, Calif) for fluorescence. Whenever fluorescence, colonial morphology or gram stain morphology resembles that of Cl. difficile, the isolate would be identified followigng criteria outlined in the identification table.

Identification:

Cl. difficile

Motility	+
Haemolysis	-
Proteolysis	-
Gelatinase	-
Lecithinase	-
Cresoltolerance	+
Maltose	-
Lactose	-
Sucrose	-
Glucose	+
Indole	-
H_2S	-

Biochemically confirmed pure isolates of Cl. difficile would be subjected to tissue culture assay (as described earlier) for the detection of toxin.

D. SIGNIFICANCE

Recent reports in literature show the involvement of anaerobic bacterial pathogens for diarrhoeal illness. The significance of the study:

(1) Is the potential contribution to further understading of the unknown causes of diarrhoeal illness caused by anaerobic bacteria, particularly by C. difficile in Bangladesh.

(2) Additionally through this work we expect to set up anaerobic technology in our laboratory for continuing routine anaerobic diagnostic work which would also be significant for advancement of research in this area at ICDDR,B.

E. FACILITIES REQUIRED:

1. Office Space : Already provided.
2. Laboratory Spece : Already provided.
3. Hospital Resources : 100 patients.
4. Animal Resources : None.
5. Logistic Support : Yes.
6. Equipment : One anaerobic jar
7. Other Requirements : Chemical and Gas Pack.

F. COLLABORATIVE ARRANGEMENTS :

Dr. John G. Bartlett, Chief, Division of Infectious Diseases, The Johns Hopkins Hospital, Baltimore has consented to work as consultant and has agreed to the collaboration. Meanwhile Dr. Jesteenson, Department of Microbiology, University of Copenhagen has sent copies of literature and some reference strains.

REFERENCE

Anerobic Bacteriology, Clinical and Laboratory Practice. A Trevor Willis (3rd ed.). Bullerworths, London - Boston.

Bartlett JG.; Chang TW.; Gurwith M.; Gorbach SL and Onderdonk AB, 1978. Antibiotic-associated psendomembranons enterocolitis due to Toxin producing Clostridium. N. Engl. J. Med. 298:531-534.

Bartlett JG. et al. Antibiotic induced lethal enterocolitis in hamster: Studes with eleven agents and evidence to support the pathogenic role of toxin producin clostridia. Am. J. Vet. Res. 39:1525-1530, (1978b).

Bartlett JG. Et al. Clindamycin - Associated colitis due to toxin producing species of clostridium in hamsters. J. Intect. Dis. 136:701-705, 1977.

Bartlett JG.; Change, TW.; Taylor NS and Onderdonk AB, 1979. Colitis induced by C. difficiles. Rev. Infect. Dis. 1:370-378.

Bartlett, JG,; 1979. Antibiotic-associated colitis. Clinixs in Gastroenterol. 8:783-801.

Bartlett JG. Antimicorbial agents implicated in C. difficile toxin-associated diarrhoea or colitis. John Hopkins Med J. 149: 6-9, 1981.

Batts DH et al., 1980. Treatment of antibiotic-associated Clostridium difficile diarrhoea with oral vaneomycin. J. Pediatr. 97(1): 151-153.

Chang, T.W., Lin, P.S., Gorbach, S.L and Bartlett, JG. 1979. Infect Immun. 23:795-798.

Chang T.W.; Lauremann, M and Bartlett, JG, 1979. Cytotoxicity assay in Antibiotic associated colitis. J. Infect. Dis. 140: 765-770.

Chang Te-Wen.; Shorwood L,; Gorbach JG.; Bartlett and Raphael S, 1980. Gastroenterology, 78:1584-1586.

Falsen E,; Kaijder B,; Nehls L,; Nygreu B and Svedhem A, 1980. Clostridium difficile in realation to enteric bacterial pathogens. J. Clin. Microbiol. 12(3): 297-300.

Fekety R, et al. Antibiotic-associated colitis_ Effects of antibiotics on C. difficile and the disease in hamster. Rev. Intect. Dis. 1: 368-397, 1979.

George, W.L., R. Rolfe, and S. Finegold. 1980. Treatment and presentation of antimicrobial agent induced colitis and diarrhea. Gastroenterology, 79:366-372.

George RH et al. Identification of C. difficile as a cause of PMC. Br. Med. J. 1: 695, 1978.

George WL.; Sutter VL.; Citrol D and Finegold SM, 1979. Selective differential medium for isolation of C. difficile. J. Clin. Microbiol. 9:214-219.

Hafiz S and Oakley CL, 1976. C. difficile: Isolation and characteristic, J. Med Microbiol. 9:129.

Larson HE,; Price AB,: Honour P and Borriello SP, 1978. Clostridium difficile and the aetiology of psendomembranons colitis, Lancet 1: 1063-1066, 1.

Mullingan ME, et al. Epidemiological aspects of Clostridium difficile induced diarrhoea and colitis, Amer.J. Clin. Nutr. 1980, 3: (11 suppl.) 2533-2538.

Silva J,; Jr: Animal models of antibiotic induced colitis. In Microbiology 1979. Schlessinger D. ED. Washington DC: ASM. PP. 258-263, 1979.

Wilson, K.H. Kennedy M.J., and Fekety, F.R. 1982. Use of Sodium Taurocholate to enhance spore recovery on a medium selective for C. difficile. J. Clin. Microbiol, 15:443-446.

Willey S and Bartlett JG. Cultures of C. difficile in stools containing a cytotoxin neutralixed by C. sordellii antitoxin. J. Clin. Microbiol. 10: 880-884, 1979.

ABSTRACT SUMMARY

Clostridium difficile is the established and most common cause of antibiotic-associated pseudomembranous enterocolitis in humans. At ICDDR,B we know uptil now approximately 80 per cent of the causes of diarrhoeal illness. The remaining 20 per cent or more is still unknown. Most of the bacterial pathogen responsible for diarrhoea are either aerobic or facultative. Until date no significant study was done on the role of anaerobic organisms in diarrhoeal diseases at ICDDR,B.

Search would be made for *Cl. difficile* in diarrhoeal cases clinically diagnosed as pseudomembranous colitis (or suspected as PMC) having no established diarrhoeal pathogen. Isolation of this organism from stool samples would be attempted by using selective media. *Cl. difficile* would be identified following established standard identifying criteria. Toxin detection from the isolated/identified *Cl. difficile* strains would be performed using tissue culture system for cytotoxicity assay. Rapid direct detection of *Cl. difficile* toxin in stool would be done with stool extracts in tissue culture system.

SECTION III - BUDGET

A. DEATAILED BUDGET

1. PERSONNEL SERVICES

Name	Position	Efforts %	Annual Salary	Project Requirements Taka	Dollar
Dr. S.Q. Akhtar	Principle Investigator	35%		25,000	
Mr. Kaisar	Res. Officer	20%		4,000	

2. SUPPLIES AND MATERIALS

Gas Pack	$ 300
Chemicals	$ 850
HeLa Cell	$ 200

3. EQUIPMENTS

Anaerobic Jar	$ 200/Jar	$ 200

4. HOSPITALIZATION

Nil

5. OUTPATEINT

Nil

6. TRANSPORT

Nil

7. TRAVEL

Nil

8. TRANSPORTATION OF THINGS

Nil

9. RENT AND COMMUNICATION

Nil

10. PRINTING AND REPRODUCTION

Nil

11. CONTRACTUAL SERVICE

Nil

12. CONSTRACTION

Nil

13. ANIMAL REQUIREMENT

Nil