RRC folder copy. Library.

ICDDR,B Library
Dhaka 1212

Attachment 1.

Date November 1, 1989

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr. Shah M. Faruque

Trainee Investigator (if any) ____

Application No. 89-012

Supporting Agency (if Non-ICDDR,B) ____

Title of Study Identification of enteric pathogens using specific DNA probes as an ..... epidemiological investigations

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

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

1. Source of Population:
   (a) Ill subjects — **Yes** No
   (b) Non-ill subjects — Yes **No**
   (c) Minors or persons under guardianship — **Yes** No
2. Does the study involve:
   (a) Physical risks to the subjects — Yes **No**
   (b) Social Risks — Yes **No**
   (c) Psychological risks to subjects — Yes **No**
   (d) Discomfort to subjects — Yes **No**
   (e) Invasion of privacy — Yes **No**
   (f) Disclosure of information damaging to subject or others — Yes **No**
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) — **Yes** No
   (b) Use of fetal tissue or abortus — Yes **No**
   (c) Use of organs or body fluids — **Yes** No
4. Are subjects clearly informed about:
   (a) Nature and purposes of study — **Yes** No
   (b) Procedures to be followed including alternatives used — **Yes** No
   (c) Physical risks — Yes No } NA
   (d) Sensitive questions — Yes No } NA
   (e) Benefits to be derived — Yes No } NA
   (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 NA
5. Will signed consent form be required:
   (a) From subjects — Yes **No**
   (b) From parent or guardian (if subjects are minors) — Yes **No**
6. Will precautions be taken to protect anonymity of subjects — **Yes** No
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ✓ Protocol (Required) ✓
   ✓ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

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

(PTO)

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

Shah M. Faruque
Principal Investigator

JAN - 1 1990

Trainee

A-033775

89-012

APPLICATION FOR PROJECT GRANT

1. Title of project : Identification of enteric pathogens using specific DNA probes as an aid to clinical and epidemiological investigations

2. Investigators : Dr. Shah M. Faruque
   Mr. Qazi Shafi Ahmad
   Mr. A.R.M. Abdul Alim

3. Consultant : Dr. Zia Uddin Ahmed

4. Project coordinator : Dr. S. Tzipori
   Associate Director
   Laboratory Sciences Division
   S. Tzipori

5. Starting date : January 01, 1990

6. Date of completion : December 31, 1992

7. Budget requested : US$ 263,546

8. Funding source :

9. Aims of the project :

9.1. General aim:

The objective of this protocol is to determine the frequency and clinical and epidemiological significance of pathogenic enteric bacteria and viruses, which will contribute to an understanding of the mode of transmission of these organisms and indicate possible prevention strategies.

9.2. Specific aim:

a) Establishment of appropriate techniques for use of DNA probes and training of staff.

b) Application of E. coli DNA probes to studies on persistent diarrhoea (in collaboration with scientists from CSD).

c) Application of DNA probes to identify specific virulence factors of diarrhoeagenic E. coli in strains which have been collected from previous studies and have not been analysed.

d) Application of new probes, as they become available for the detection of other enteric pathogens.

e) Application of non-radioactive probes for the detection of enteric pathogens

f) Application of DNA probes to major future epidemiological studies.

9.3. <u>Significance</u>

The utilization of specific DNA probes to identify enteric pathogens will help in the assessment of the relative importance of different enteric bacteria and virus-mediated diarrhoeal diseases in the population, and will aid in a better understanding of the mode of transmission and pathogenic mechanisms of these microorganisms. This will facilitate the development of possible prevention and treatment modalities and will also help to decide on research priorities to be addressed in future ICDDR,B investigations.

10. Background

Enteric bacterial pagthogens present one of the most challenging problems in microbial pathgenesis. These, together with some viruses and certain species of protozoa are responsible for most of the acute diarrhoeal diseases worldwide. In recent years, improved detection techniques have revealed that in addition to the previously identified enteric pathogens, such as strains of Shigella, Vibrio, Salmonella and Escherichia coli, a wide range of other bacterial species can also cause diarrhoeal diseases. These include pathogenic strains of Campylobacter, Yersinia, Aeromonas, Plesiomonas, Staphylococcus aureus, Bacillus cereus, Clostridia and Bacteroides (1,2). Diarrhoeagenic enteric viruses and certain protozoal parasites also contribute significantly to the occurrence of acute diarrhoeal diseases. Efforts are being made to improve the diagnosis and treatment of these diseases and devise strategies for their ultimate control and prevention. To achieve this aim, it is necessary to conduct studies on the relative importance and abundance of different enteric pathogens, and hence reliable and accurate identification techniques are essential.

Escherichia coli is a common inhabitant of the human (and animal) intestine, and based on routine laboratory tests the saprophytic strains cannot be effectively distinguished from the inherently pathogenic strains (3). Diarrhoeagenic strains of E. coli have been classified into four major categories: Enterotoxigenic

E. coli (ETEC), Enteroinvasive E. coli (EIEC), Enteropathogenic E. coli (EPEC), and Enterohemorrhagic E. coli (EHEC) (4). These diarrhoeagenic E. coli cause diarrhoea by at least one or more pathogenic mechanisms: enterotoxin production, enteroinvasion, enteroadherence, and cytotoxin production (4). The pathogenic strains of E. coli can be identified to some extent by biological assays (5,6) or immunoassays (7,8), but these assays are expensive and time consuming, or require specific antisera.

A recent approach to identify diarrhoeagenic E. coli and other enteric pathogens using developments in molecular genetic technology is to identify nucleotide sequences of pathogenic determinants directly in specimens (9-11). Nucleotide probes have been especially useful in identifying pathogenic strains of E. coli, that are virtually indistinguishable phenotypically from non-pathogenic E. coli. The use of nucleotide probes to identify enteric pathogens is based on a combination of gene cloning and nucleic acid hybridization techniques. Gene cloning provides probes for the specific polynucleotide sequence of a pathogenic determinant, and nucleic acid hybridization with either radiolabelled or non-radiolabelled probes involves the use of these probes to search for homologous sequences in the total nucleic acid present in a specimen.

10.1. DNA probes against pathogenic E. coli

Nucleotide probes have been constructed to identify plasmid-encoded genes coding for heat-labile (LT) and heat-stable (ST)

enterotoxins (12), genes involved in enteroinvasion (13), genes coding for adherence to tissue culture and human intestinal cells (14,15), and bacteriophage-encoded genes for Shiga-like enterotoxins (SLT) (16). DNA probes refer to specific DNA fragments of plasmids containing cloned genes coding for enteropathogenic determinants or synthetic oligonucleotides that are constructed from the nucleotide sequences of these fragments.

The pathogenesis of enterotoxigenic E. coli (ETEC) has been frequently reviewed (17,18) and it has been suggested that ETEC requires two virulence factors, colonization adhesins and enterotoxin production, in order to cause diarrhoea. ETEC must first adhere to the proximal small bowel which is mediated by protein fimbrial adhesins termed colonization factor antigens (CFAs) or coli surface (CS) fimbriae. A number of adhesins have been recognized including CFA I (19), CS1, CS2, CS3, CS4, CS5 and CS6 (20-22). CS1, CS2 and CS3 in various combinations were first reported as a single factor CFA II (20,22), and CS4, CS5 and CS6 were reported as colonization factor PCF 8775 (21), now called CFA IV (23). Genes coding for CFA I and CFA II are plasmid-encoded and are closely associated with genes coding for heat-stable enterotoxins (24). Additional colonization factors have been described but it is not clear if these are distinct from previously reported adhesins (25). These include CFA III presumably CS6 (26,27) and CFA V presumably CS5 (28). Many ETEC strains have been isolated that do not have any of the CFAs so

far described, and hence additional CFAs of ETEC possibly exist and remain to be identified.

ETEC produce LT or ST or both of these toxins, and subclasses of LT and ST have also been identified. E. coli LT I and its subunits cross react serologically with the closely related enterotoxins of Vibrio cholerae (CT) (29) but LT II originally described in E. coli SA 53 is not neutralized by antibodies to CT, although LT I and LT II have resemblances with respect to several of their biological properties. DNA sequences coding for LT II do not hybridize with genes coding for LT I under hybridization conditions which allow for upto 20% base pair mismatch (30). Two different types of LT II have been identified serologically. These are LT IIa from E. coli SA 53 and LT IIb from E. coli 41 (31). Genes coding for LT I and ST are plasmid mediated while genes for LT II are chromosomally encoded (32). DNA probes have been developed for both LT I and LT II genes and consist of an 850 base pair fragment in pEWD299 and an 800 base pair fragment in pCP2725 respectively. Genes coding for the subclasses of ST (ST Ia, ST Ib, and ST II) have been identified. Genes for ST Ia are located on a transposon (33) while genes for ST Ib are often associated with plasmids coding for CFA I and CFA II (24). Genes coding for ST Ia and ST Ib have a sequence divergence of 31% (34) and are thus distinct when examined by DNA hybridization under stringent conditions. Neither the DNA nor the amino acid sequence of ST II has any resemblance to ST Ia or

ST Ib (35,36). Specific DNA probes have been developed to identify genes coding for ST Ia, ST Ib, and ST II.

Enteroinvasive E. coli and Shigella cause dysentery by invading epithelial cells of the colon (37,38). Both Shigella and EIEC possess plasmids of 120-140 Mdal that are necessary for virulence.

There is no specific biochemical test for differentiating between EIEC and non-pathogenic E. coli, but a 17 kilo base pair Eco RI digestion fragment of the 140 Mdal plasmid of S. flexneri 5 (M90T) has been found to be specific in identifying EIEC from non-pathogenic strains of E. coli (39). Additional probes for the differentiation between EIEC and Shigella have been derived from the other fragments of the 140 Mdal plasmid of Shigella (40).

Enteropathogenic E. coli (EPEC) demonstrate adherence to the small bowel mucosa in infants and animals, and close adherence has been suggeted as imported for the induction of diarrhoea (41,42) although cytotoxin production has also been identified in EPEC (43,44). HEp-2 cell adherence in tissue culture has been reported to be associated with 50-70 Mdal plasmids in EPEC (43) and with a 60 Mdal plasmid (pMAR-2) in EPEC 2348 (44). The genes for HEp-2 adherence have been cloned and used to identify E. coli that adhere to tissue culture cells and human intestinal enterocytes in a localized pattern (45,46). A DNA probe for EPEC

adherence factor (EAF) has also been constructed and is both sensitive and specific in identifying EPEC that adhere to HEp-2 cells (47). In addition to EAF, E. coli that adhere to HEp-2 and human intestinal cells in a localized pattern, E. coli also adhere in either an aggregative or diffuse adherence pattern (48). A DNA probe to identify diffusely adherent E. coli has been constructed. This probe is derived from a chromosomal gene encoding a protein associated with the expression of diffuse adherence by E. coli in the HEp-2 cell assay.

Enterohemorrhagic E. coli (EHEC) produce either one or both of two antigenically distinct Shiga-like toxins, SLT I and SLT II, which are encloded by two different bacteriophages 933J and 933W in E. coli 933 (49). The genes for SLT I and SLT II have subsequently been cloned, sequenced and DNA probes specific for each have been constructed (50,51). SLT producing E. coli 0157:H7 also possesses plasmid-encoded fimbriae that promote attachment to intestinal epithelial cells in tissue culture (52). A DNA probe consisting of a 3.4 kilo base pair HindIII fragment of the plasmid pCVD 419 is referred to as the EHEC probe.

10.2. Current reports on the applicability of E. coli DNA probes

DNA probe technology has been rapidly advancing and has allowed the development of useful investigative tools. The applicability of examining clinical specimens using DNA probes for genes encoding enterotoxins has been tested in stool samples of

patients with diarrhoea in Thailand (12). The hybridization technique has been reported to be useful in accurately detecting the presence of ETEC in stool samples. The hybridization assay has also been used to examine water specimens in Thailand (11) and has been reported to be 10^4 times mor sensitive than testing random E. coli in the Y-1 adrenal and suckling mouse assays in identifying ETEC in water. Generally, DNA probes for toxins, enteroadherence, and enteroinvasiveness have been found to be comparable or superior to bioassays or immunoassays in detecting enteric pathogens (53,54). Enterotoxin gene probes for LT I, ST Ia, and ST Ib have been used to detect ETEC in isolates from Asia (55) and Africa (56). In two separate outbreaks of neonatal diarrhoea in Ohio and in Burma, DNA probes have been used successfully to identify EPEC serotypes 0114:NM and 0114:H2. In a community study of bloody diarrhoea, excellent correlation has been reported between culture results and hybridization analysis using the ipaC probe for enteroinvasiveness (57). With the availabiity of a growning number of new DNA probes, the analysis of large numbers of specimens and isolates is now extensively used in diagnostic and epidemiological investigations (53,58).

In a surveillance of diarrhoeal patients conducted at the ICDDR,B during 1979-80, pathogenic agents were identified in only 66% of patients (59) in spite of the application of all the techniques available at ICDDR,B during the period. However, the importance of various categories of diarrhoagenic E. coli, species of Aeromonas, Plesiomonas, Yersinia, Clostridia and Campylobacter,

various serotypes and serogroups of enteric viruses, and enteric protozoa could not be assessed due mainly to lack of appropriate techniques for detecting these organisms. The application of DNA probes at ICDDR,B will open up an opportunity to conduct the much warranted epidemiological studies to assess the relative contribution of all enteric pathogens to diarrhoea.

10.3. DNA probes available at the ICDDR,B

DNA probes presently available at ICDDR,B for the detection of enteric pathogens are summarized as follows:

Determinant	Recombinant plasmid	Probe DNA (base pairs)
SLT I	pJN37-19	1154
SLT II	pNN110-8	842
EIEC	pMR17	17000
EHEC	pCVD419	3400
DA	pSLM852	450
LA	pJPN16	1000
LT	pCVD403	1300
STh	pCVD402	216

10.4. Need for new DNA probes

The DNA probes presently available at this Centre are mainly useful for the identification of pathogenic E. coli strains. In order to study the importance of other enteric pathogens by the DNA hybridization technique, it is necessary to obtain specific DNA probes against them. The diarrhoeagenic role of species of Aeromonas spp., Plesiomonas, Yersinia, Campylobacter and above all Shigella spp. has to be investigated with a broad perspective.

Enterotoxin and cytotoxin production, and invasiveness have been demonstrated in most of these organisms and these properties can be exploited to identify the pathogenic genes. Detection of specific DNA sequences unique to each of these genes can lead to possible development of new DNA probes.

In addition to the development of new probes against enteric pathogens, such probes already developed or in the process of developmnt, can be obtained from other advanced laboratories and utilized for epidemiological studies. The use of oligonucleotide probes against the different serotypes of rotaviruses has just been introduced at ICDDR,B, and a DNA probe against Entamoeba histolytica will be available soon. Other probes which will be requested and tried at ICDDR,B include:

a) Cryptosporidium spirozoites (Flinders University, Adelaide, Australia)

b) Attachment-effacement (CVD, Maryland, USA)

c) Aggregatuve-adherence (CVD, Maryland, USA)

d) Salmonella (University of Zimbagwee)

e) Four CFA probes (University of Adelaide, Adelaide, Australia)

11. Outline of experimental methods

11.1. Preparation of probe DNA fragments

Recombinant clones carrying the probes of interest will be grown in nutrient broth containing the appropriate antibiotic. Plasmids will be isolated by modifications of the alkaline lysis method of Birnboim and Doly (61) and will be purified by using a commercially available column (NACS-52 PREPAC, BRL). The recombinant plasmids will be digested with the appropriate restriction enzymes to excise the inserts and the digests will be electrophoresed in agarose gels. The probe DNA fragments (inserts of recombinant plasmids) will be extracted from agarose gels by electroelution into dialysis bags as described by Maniatis et al. (62).

11.2. Preparation of specimens

Colony blots, dot blots, or stool blots will be prepared by standard techniques (62) using nylon membranes (HYBOND-N, AMERSHAM). For each set of specimens, a number of replica filters will be prepared for hybridization with different probes.

The colonies will be lysed and the DNA denatured by placing the filters on pieces of Whatman 3MM sheets soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl). The filters will then be neutralized by placing on Whatman 3MM sheets soaked in neutralizing solution (1 M Tris-Cl pH 8.0, 1.5 M NaCl). The DNA will be fixed to the nylon membranes by exposing the membranes to UV light for 2-5 minutes on a UV transilluminator.

For each set of specimens, a set of master filters carrying live bacterial colonies will be properly stored for possible future studies on the hybridization-positive colonies.

11.3. Radioactive labelling of DNA probes

The probe DNAs will be radioactively labelled by the method of Feinberg and Volgelstein (63,64) with a-^{32}P-dATP (10 uCi ul, 3000 Ci/mM, AMERSHAM) and oligonucleotide primers [P(dN)$_6$, PHARMACIA] using the Large Fragment of E. coli DNA polymerase I. In case of small DNA probes (less than 200 base pairs) the method of choice will be 5'-end labelling with -^{32}P-dATP using a 5' end-labelling kit (BRL). Radio-labelled probes will be denatured by boiling followed by quick chilling on ice, before using these for hybridization experiments.

11.4. Hybridization of DNA blots

Hybridization with labelled probes will be carried out as described by Maniatis et al. (62). The filters will be

prehybridized in the pesence of denatured salmon sperm DNA to block unspecific binding sites on the filters, and will then be hybridized with the denatured probe DNA for 12-16 hours at the appropriate temperature. After hybridization, the filters will be washed under conditions of increasing stringency.

11.5. Preparation of autoradiographs

The hybridized filters will be exposed to X-ray films in metal cassettes at -70°C for the appropriate time. In case of weak signals, intensifying screens will be used. The exposed X-ray films will be developed and fixed by standard procedure.

11.6. Non-radioactive labelling of probes

The use of DNA probes as a clinical tool has been hampered by the need to use radioactive isotopes for labelling DNA probes. Non-radioactive DNA-labelling procedures wich provide stable probes free from special handling precautions and regulations, will be tested. Biotinylated nucleotides will be used to label probes, and hybridization will be detected using a commercially available non-radioactive DNA hybridization kit (BRL). The sensitivity of non-radioactive methods will be compared with the radioactive methods of DNA hybridization. Other newly developed non-radioactive DNA labelling and detection systems will be similarly tested for their sensitivity and applicability in routine diagnosis of enteric pathogens.

12. Ethical implications:

Bacterial isolates obtained from stool specimens of diarrhoeal patients in a surveillance study and from another different project will be used in this study. In addition, only stool specimens and environmental samples will be involved in this investigation. No aspect of this project is in any way harmful to humans.

13. References:

1. Guerrant, R.L., Lohr, J.A. and Williams, E.K. (1986). Acute infectious diarrhoea. I. Epidemiology, aetiology and pathogenesis. Paediatr. Infect. Dis. 5:353-359.

2. Myers, L.L., Shoop, D.S., Stackhouse, L.L., Newman, F.S., Flaherty, R.J., Letson, G.W. and Sack, R.B. (1987). Isolation of enterotoxigenic Bacteroides fragilis from humans with diarrhoea. J. Clin. Microbiol. 25:2330-2333.

3. DuPont, H.L., Formal, S.B ., Hornick, R.B., Synder, M.J., Libonati, J.P., Sheahan, D.G., La Brec, E.H. and Kalas, J.P. (1971). Pathogenesis of Escherichia coli diarrhoea. N. Eng. J. Med. 285:1-9.

4. Levine, M. M. (1987). Escherichia coli that cause diarrhoea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. 155:377-389.

5. Dean, A.G., Ching, Y.C., Williams, R.G. and Harden, L.B. (1972). Test for Escherichia coli enterotoxin using infant mice: Application in a study of diarrhoea in children in Honolulu. J. Infect. Dis. 125:407-411.

6. Sack, D.A. and Sack, R.B. (1975). Test for enterotoxigenic Escherichia coli using Y-1 adrenal cells in miniculture. Infect. Immun. 11:334-336.

7. Handl, C., Ronnberg, B., Nilsson, B., Olsson, E., Jonsson, H. and Flock, J.I. (1988). Enzyme-linked immunosorbent assay for Escherichia coli heat-stable enterotoxin type II. J. Clin. Microbiol. 26:1555-1560.

8. Svennerholm, A. and Holmgren, J. (1978). Identification of Escherichia coli heat-labile enterotoxin by means of a ganglioside immunosorbent assay (GM-1 ELISA) procedure. Curr. Microbiol. 1:19-23.

9. Boileau, C.R., d'Hauteville, H.M. and Sansonetti, P.J. (1984). DNA hybridization techniques to detect Shigella species and enteroinvasive Escherichia coli. J. Clin. Microbiol. 20:959-961.

10. Chityothin, O., Sethabutr, O., Echeverria, P., Taylor, D.N., Vongsthongsri, V. and Tharavanij, S. (1987). Detection of heatstable enterotoxigenic Escherichia coli by hybridization with an RNA transcript probe. J. Clin. Microbiol. 25:1572-1573.

11. Echeverria, P., Seriwatana, J., Chityothin, C., Chaicumpa, W. and Tirapat, C. (1982). Detection of enterotoxigenic **Escherichia coli in water by filter hybridization with three** enterotoxin gene probes. J. Clin. Microbiol. 16:1086-1090.

12. Moseley, S.L., Echeverria, P., Seriwatana, J., Tirapat, C., Chaicumpa, W., Sakuldaipeara, T. and Falkow, S. (1982). Identification of enterotoxigenic Escherichia coli using three enterotoxin gene probes. J. Infect. Dis. 145:863-869.

13. Sethabutr, O., Echeverria, P., Taylor, D.N., Pal, T. and Rowe, B. (1985). DNA hybridization in the identification of enteroinvasive Escherichia coli and Shigella in children with dysentery. In Infectious Diarrhoea in the Young (S. Tzipori, ed.), pp. 350-356. Elsevier Science Publishers BV (Biomedical Division), Amsterdam.

14. Lanser, J.A. and Anargyros, P.A. (1985). Detection of Escherichia coli adhesins with DNA probes. J. Clin. Microbiol. 22:425-427.

15. Nataro, J.P., Baldini, M.M., Kaper, J.B., Black, R.E., Bravo, N. and Levine, M.M. (1985). Detection of an adherence factor of enteropathogenic Escherichia coli with a DNA probe. J. Infect. Dis. 152:560-565.

16. Seriwatana, J., Brown, J.E., Echeverria, P., Taylor, D.N., Suthienkul, O. and Newland, J. (1988). DNA probe to

identify Shiga-like toxin I and II producing enteric pathogens isolated from patients with diarrhoea in Thailand. J. Clin. Microbiol. 26:1614-1615.

17. Levine, M.M., Kaper, J.B., Black, R.E. and Clements, M.L. (1983). New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. Microbiol. Rev. 47:510-550.

18. Sack, R.B. (1975). Human diarrhoeal disease caused by enterotoxigenic Escherichia coli. Ann. Rev. Microbiol. 29:333-353.

19. Evans, D.G., Silver, R.D., Evans, D.J., Chase, D.G. and Gorbach, S.L. (1975). Plasmid-controlled colonization factor associated with virulence in Escherichia coli enterotoxigenic in humans. Infect. Immun. 12:656-667.

20. Cravioto, A., Scotland, S.M. and Rowe, B. (1982). Hemagglutination activity and colonization factor antigens I and II in enterotoxigenicc and non-enterotoxigenic strains of E. coli isolated from humans. Infect. Immun. 36:189-197.

21. McConnel, M.M., Thomas, L.V., Day, N.P. and Rowe, B. (1985). Enzyme-linked immunosorbent assays for the detection of adhesion factor antigens of E. coli. J. Infect. Dis. 152:1220-1227.

22. Smyth, C.J. (1982). Two mannose-resistant hemagglutinins on enterotoxigenic E. coli of serotype 06:K15 or H-isolated

from travellers' and infantile diarrhoea. J. Gen. Microbiol. 128:2081-2096.

23. McConnell, M.M., Thomas, L.V., Willshaw, G.A., Smith, H.B. and Rowe, B. (1988). Genetic control and properties of coli surface antigens or colonization factor antigen IV (PCF 8775) of enterotoxigenic E. coli. Infect. Immun. 56:1974-1980.

24. Echeverria, P., Seriwatana, J., Taylor, D.N., Changchawalit, S., Smyth, C.J., Twohig, J., and Rowe, B. (1986). Plasmids coding for colonization factor antigens I and II, LT and STA- 2 in Escherichia coli. Infect. Immun. 51:626-630.

25. Honda, T., Arita, M. and Miwatani, T. (1984). Characterization of new hydrophobic pili of human enterotoxigenic Escherichia coli: a possible new colonization factor. Infect. Immun. 43:959-965.

26. Darfeuille, A., Lafeuille, B., Joly, B. and Cluzel, R. (1983). A new colonization factor antigen (CFA/III) produced by enteropathogenic Escherichia coli 0128:B12. Ann. Microbiol. (Paris) 134A:53-64.

27. Darfeuille-Michaud, A., Forestier, C., Joly, B., and Cluzel, R. (1986). Identification of non-fimbrial adhesive factor of an enterotoxigenic Escherichia coli strain. Infect. Immun. 52:468-475.

28. Manning, P.A., Higgins, G.D., Lump, R. and Lanser, J.A. (1987). Colonization factor antigens and a new fimbrial type (CFA/V) on 0115:H4 and H-strains of enterotoxigenic Escherichia coli in Central Australia. J. Infect. Dis. 156:841-844.

29. Gill, D.M. and Richardson, S.H. (1980). Adenosine diphosphateribosylation of adenylate cyclase catalyzed by heat-labile enterotoxin of Escherichia coli: comparison with cholera toxin. J. Infect. Dis. 141:64-70.

30. Pickett, C.L., Twiddy, E.M., Belisle, B.W. and Holmes, R.K. (1986). Coloning of genes that encode a new heat-labile enterotoxin of Escherichia coli. J. Bacteriol. 165:348-352.

31. Holmes, R.K., Twiddy, E.M. and Bramucci, M.G. (1983). Antigenic heterogeneity among heat-labile toxins from Escherichia coli. In advances in research on cholera and related diarrhoeas, Vol.1 (S. Kuwahara, N.F. Price, ed.), pp. 293-300. Martinus Nihoff Publishers, Boston.

32. Green, B.A., Neill, R.J., Ruyechan, W.T. and Holmes, R.K. (1983). Evidence that a new enterotoxin which activates adenyl cyclase in eucaryotic target cells is not plasmid mediated. Infect. Immun. 41:383-390.

33. So, M., Heffron, F. and McCarthy, B.S. (1979). The E. coli gene encoding heat-stable toxin is a bacterial transposon flanked by inverted repeats of ISI. Nature 277:453-856.

34. Moseley, S.L., Hardy, J.N., Huq, M.I., Echeverria, P. and Falkow, S. (1983). Isolation and nucleotide sequence determinations of a gene encoding a heat-stable enterotoxin of Escherichia coli. Infect. Immun. 39:1167-1174.

35. Lee, C.H., Mosley, S.L., Moon, H.W., Whipp, S.C., Gyles, C.L. and So, M. (1983). Characterization of the gene encoding heat-stable toxih II and preliminary molecular and epidemiological studies of enterotoxigenic Escherichia coli heat-stable toxin II. Infect. Immun. 42:264-268.

36. Picken, R.N., Mazaistis, A.J., Maas, W.K., Rey, M. and Heyneker, H. (1983). Nucleotide sequence of the gene for heat-stable enterotoxin II of Escherichia coli. Infect. Immun. 42:269-275.

37. LaBrec, E.H., Schneider, H., Magnani, T.J. and Formal, S.B. (1964). Epithelial cell penetration is an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. 88:1503-1518.

38. Formal, S.B., and Hornick, R.B. (1987). Invasive Escherichia coli. J. Infect. Dis. 137:641-644.

39. Sethabutr, O., Hanchalay, S., Echeverria, P., Taylor, D.N. and Leksomboon, U. (1985). A non-radioactive DNA probe to identify Shigella and enteroinvasive Escherichia coli in stools of children with diarrhoea. Lancet ii: 1095-1097.

40. Small, P.L.C. and Falkow, S. (1986). Development of a DNA probe for the virulence plasmid of Shigella spp. and enteroinvasive Escherichia coli. Microbiology. 1986. pp. 121-124. American Society of Microbiology, Washington, D.C.

41. Clausen, C.R. and Christie, P.L. (1982). Chronic diarrhoea in infants caused by adherent enteropathogenic Escherichia coli. J. Pediatr. 100:358-361.

42. Ulsen, M.H. and Rollo, J.L. (1980). Pathogenesis of Escherichia coli gastroenteritis in man: another mechanism. N. Engl. J. Med. 302:99-101.

43. Karch, H., Hessemann, J. and Lauf, R. (1987). Phage associated cytotoxin production and enteroadhesiveness by enteropathogenic Escherichia coli isolated from infants with diarrhoea in West Germany. J. Infect. Dis. 155:707-715.

44. Scotland, S.M., Day, N.P. and Rowe, B. (1980). Production of a cytotoxin affecting vero cells by strains of E. coli belonging to traditional enteropathogenic serotypes. FEMS Microbiol. Lett. 7:15-17.

45. Knutton, S., Lloyd, B.R. and McNeish, A.S. (1987). Adhesion

of enteropathogenic Escherichia coli to human intestinal enterocytes and cultured human intestinal mucosa. Infect. Immun. 55:69-77.

46. Kutton, S., Baldini, M.M., Kaper, J.B. and McNeish, A.S. (1987). Role of plasmid-encoded adherence factors in adhesion of enteropathogenic Escherichia coli to HEp-2 cells. Infect. Immun. 55:78-85.

47. Nataro, J.P., Scaletsky, I.C.A., Kaper, J.B., Levine, M.M. and Trabulsi, L.R. (1985). Plasmid-mediated factors conferring diffuse and localized adherence of enteropathogenic Escherichia coli. Infect. Immun. 48:378-383.

48. Scaletsky, I.C.A., Silva, M.L.M. and Trabulsi, L.R. (1984). Distinctive patterns of adherence of enteropathogenic Escherichia coli to HeLa cells. Infect. Immun. 45:534-536.

49. Newland, J.W., Strockbine, N.A., Miller, C.F., O'Brien, A.D. and Holmes, R.K. (1985). Cloning of Shiga-like toxin structural genes from a toxin converting phage of Escherichia coli. Science 230:179-181.

50. Newland, J.W., Strokbine, N.A. and Neill, R.J. (1987). Cloning of genes for production of Escherichia coli Shiga-like toxin type II. Infect. Immun. 55:2675-2680.

51. Newland, J.W. and Neill, R.J. (1988). DNA probes for Shiga-like toxins I and II and for toxin converting bacteriophages. J. Clin. Microbiol. 26:1292-1297.

52. Karch, H., Hessemann, J., Lauf, R., O'Brien, A.D., Tucker, C.O. and Levine, M.M. (1987). A plasmid of enterohemorrhagic Escherichia coli 0157:H7 is required for expression of a new fimbrial antigen adhesins to epithelial cells. Infect. Immun. 55:455-461.

53. Morgan, D.R., DuPont, H.L., Wood, L.V. and Eriscon, C.D. (1983). Comparison of methods to detect Escherichia coli heat-labile enterotoxin in stool and cell free culturue supernatant. J. Clin. Microbiol. 18:798-802.

54. Echeverria, P., Taylor, D.N., Seriwatana, J., Chatkaeomorakot, A., Khungvalert, V., Sakuldaipeara, T. and Smith, R.A. (1986). A comparative study of enterotoxin gene probes and tests for toxin production to detect ETEC. J. Infect. Dis. 153:255-260.

55. Seriwatana, J., Echeverria, P., Escamilla, J., Glass, R., Huq, I., Rockhill, R. and Stoll, B.L. (1983). Identification of enterotoxigenic Escherichia coli in patients with diarrhoea in Asia with three enterotoxin gene probes. Infect. Immun. 42:152-154.

56. Georges, M.C., Wachsmuth, I.K. and Birkness, K.A. (1983). Genetic probes for enterotoxigenic Escherichia coli isolated

from childhood diarrhoea in the Central African Republic. J. Clin. Microbiol. 18:199-202.

57. Strockbine, N.A., Puhr, N., Venkatesan, M., shaffer, N., Tauxe, R., Wells, J. and Wachsmuth, I.K. (1988). Survey of patients with bloody diarrhoea on the Navajo Indian Reservation using culture techniques and DNA probes for detecting bacterial pathogens. Ann. Meet. Am. Soc. Microbiol., Miami. Beach, FL.

58. Echeverria, P., Seriwatana, J., Taylor, D.N., Tirapat, C., Chaicumpa, W. and Rowe, B. (1985). Identification by DNA hybridization of enterotoxigenic Escherichia coli in a longitudinal study of villages in Thailand. J. Infect. Dis. 151:124-130.

59. Stoll, B.J., Glass, R.I., Huq, M.I., Khan, M.U., Holt, J.E. and Banu, H. (1982). Surveillance of patients attending a diarrhoeal disease hospital in Bangladesh. Brit. Med. J. 285:1185-1188.

60. O'Brien, A.D. and Holmes, R.K. (9187). Shiga and Shiga-like toxins. Microbiol. Rev. 51:206-220.

61. Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513.

62. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular Cloning. A laboratory manual. Cold Spring Harbor Laboratory, New York.

63. Feinberg, A. and Volgelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.

64. Feinberg, A. and Volgelstein, B. (19844). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 137:266-267.

SOURCE OF SPECIMENS

The specimens and bacterial isolates to be used in this investigation will be either obtained from diarrhoeal patients in collaboration with the Clinical Sciences Division or from the Vaccine Trial project which has just been concluded and a large number of bacterial isolates have been collected and stored.

1) <u>ICDDR,B Hospital Surveillance Study</u>

A surveillance system has been set up at the Centre 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 these patients will be tested for the presence of bacterial pathogens.

2) <u>Aetiology of persistent diarrhoea in collaboration with Clinical Sciences Division</u>

About 160 children with persistent diarrhoea and 40 control children with acute diarrhoea will be studied per year. From each child jejunal juice will be collected twice, first at the time of enrollment and then at the end of the treatment or after recovery. Also three stool samples collected at different times at the time of enrollment and an additional sample collected at the end of the treatment or after recovery will be studied for bacterial pathogens from all patients.

3) Diagnostic service for persistent diarrhoea patients

At least one stool specimen will be studied for all bacterial pathogens from approximately 300 children with persistent diarrhoea seen at the Centre every year who are not entered into study 2.

4) Aetiology of non-Shigella dysenteriae in collaboration with Clinical Sciences Division

Stool specimens from 100 patients with non-Shigella dysenteriae and samples from 200 controls, half of which are cases with Shigella dysenteriae and the other half watery diarrhoea will be studied.

5) E. coli diarrhoea in cholera vaccine trial (CVT) study

Approximately 200,000 people vaccinated with oral cholera vaccine or given placebo in 1985 in Matlab were followed up for development of diarrhoea. From those individuals who developed diarrhoea, 3 separate E. coli colonies and a pool of 5 have been stored (8235 patients). E. coli colonies have also been stored from the stools of mothers of these children (2304 mothers). We plan to test these E. coli for all categories of diarrhoeagenic E. coli to find out their incidence in this population. This study will complement other studies which had been generated as a consequence of the CVT, and will utilize all the data with reference to age-distribution, seasonality, geographical location, association with breastfeeding. The study will have a bias toward severe cases of diarrhoea.

PLAN OF LABORATORY WORKS: 1-3 YEARS

First year:

a) Hybridization of colony blots with DNA probes for SLT I, SLT II, EHEC, EIEC, ST, LT, DA and LA. The bacterial isolates consist of bacteria isolated and stored from two previous studies on diarrhoeal patients. In addition, bacterial isolates from stool specimens and jejunal fluid of diarrhoeal patients under an on-going protocol will be used.

- Hospital surveillance: 3 E. coli isolates from each of 400 patients
- Persistent diarrhoea (research): 800 stool specimens
- Vaccine Trial project: 15,000 bacterial isolates

b) Every 10th colony found positive by using DNA probes will be tested by possible biological assays.

Second year:

a) Continuation of colony hybridization:

- Hospital surveillance: 9,700 stool specimens
- Persistent diarrhoea (research): 400 jejunal fluid
- Vaccine Trial project: 15,000 bacterial isolates

Third year

a) Continuation of colony hybridization:

- Vaccine Trial project: 15,000 bacterial isolates
- Persistent diarrhoea (diagnostic): Possible application of new probes to examine 350 stool specimens

b) Comparison of colony hybridization results with results obtained from biological assays conducted on the same strains under a different protocol.

c) Testing the applicability of non-radioactive hybridization techniques for routine diagnosis of enteric pathogens.

d) Summarizing the results obtained in three years and identifying research priorities to be addressed in future ICDDR,B investigations.

TIME ALLOTTED FOR EACH INVESTIGATIONS

Standardization of procedure	...	6 months
Testing of specimens	...	24 months
Analysis of data and writing reports		6 months

TASK of INVESTIGATORS

Investigator	Task
S.M. Faruque Q.S. Ahmad	Standardization of hybridization procedures and DNA probes
S.M. Faruque Q.S. Ahmad A.R.M.A. Alim Lab technicians Research Officer	Preparation of colony blots and performing routine hybridizations for detecting enteric pathogens in clinical and epidemiological specimens
S.M. Faruque Research Officer	Introduction and testing of non-radioactive hybridization methods
S.M. Faruque Q.S. Ahmad Lab technicians Research Officers	Testing the specificity of new DNA probes against enteric pathogens obtained from other laboratories
Dr. Zia Uddin Ahmed	Academic and strategic feedback

BUDGET FOR THREE YEARS

Description			Yearly budget (in US$)			
			1st year	2nd year	3rd year	Total
1. Personnel						
No.	Level & step					
1	NOC	5 (100%)	15,948	16,488	17,028	49,464
1	NOB	10 (100%)	14,796	15,228	15,660	45,676
1	NOB	9 (50%)	7,824	8,040	8,256	24,120
1	NOA	9 (10%)	1,192	1,230	1,264	3,686
2	GSV	1 (100%)	5,440	5,616	5,832	16,848
2	GSIII	7 (100%)	4,440	4,584	4,728	13,752
Total personnel cost			49,600	51,178	52,768	153,546
2. Capital equipment						
	Gel electrophoresis apparatus (2 sets)		2,000	-	-	2,000
	Sequencing gel system and high voltage power supply (1 set)		3,000	-	-	3,000
	Darkroom set-up		2,000	-	-	2,000
	Shaking water bath		1,500	-	-	1,500
	Radioactivity monitor		2,500	-	-	2,500
Total equipment cost			11,000	0	0	11,000

128. Ward, H.D., Lev, B.I., Kane, A.V., Keusch, G.T., Pereira, M.E.A. Identification and characterization of taglin: a mannose-6-phosphate binding, trypsin activated lectin from Giardia lamblia. Biochemistry (in press).

129. Ward, H.D., Alroy, J., Lev, B.I., Keusch, G.T., Pereira, M.E.A. Analysis of surface carbohydrates of Giardia lamblia: Detection of N-acetyl-D-glucosamine as the only saccharide moiety and identification of two distinct subsets of trophozoites by lectin binding. J. Exp. Med. (in press)

130. Mobassaleh, M., Donohue-Rolfe, A., Jacewicz, M., Grand, R.J., Keusch, G.T. Pathogenesis of shigella diarrhea. XIII. Evidence for a developmentally regulated glycolipid receptor for shigella toxin involved in the fluid secretory response of rabbit small intestine. J. Infect. Dis. (in press).

131. Harari, I., Donohue-Rolfe, A., Keusch, G., Arnon, R. Synthetic peptides of Shiga toxin B-subunit induce antibodies which neutralize the biological activities of the toxin. Infect. Immun. (submitted).

****INTERDEPARTMENTAL SERVICES-1ST/2ND/3RD YEAR**

A/C code	Service Area	$ Amount
4801	Computer	
4802	Transport Dhaka	50
4803	Transport Matlab	
4804	Water transport-Matlab	
4805	Transport Teknaf	
4806	Xerox	50
4807	Pathology	
4808	Microbiology tests	
4809	Biochemistry	
4810	X-Ray	
4811	I.V. Fluid	
4812	Media	
4813	Patient hospitalisation study	
4814	Animal research	
4815	Medical illustration	
4817	Telex	
4818	Out patient care	
4819	Maintenance charges	
4820	Vehicle maintenance charges	
4821	Library service charges	150
4830	Transport subsidy	
	TOTAL	* 250

** Please contact Cost Office on Ext. 281. for rates.

*AGREES WITH PAGE 1 A/C 4800

Budget86.20
Aziz-13.