Principal Investigator: M. John Albert
Application No.: 9C-002

Title of Study: "The role and characteristics of diarreheogenic E. coli in clinical epidemiological investigations"

Project status:
- New Study [X]
- 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 [NA]
(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 [NA]
(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]
(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 [Yes, No]
(c) 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 Ctteee for review.

Principal Investigator: [Signature]
Date: 01/11/90
Trainee Investigator (if any):
Supporting Agency (if Non-ICDDR,B):
1c. INVESTIGATORS: Mr. H. Al-Kabir
Dr. K. Haider
Ms. S. Nahar
Mr. P. K. B. Neogi
Dr. M. J. Albert

1b. COLLABORATING INVESTIGATOR: Dr. D. Mahalanabis, CSO

2. TITLE OF PROJECT: The role and characteristics of diarrhoeagenic E. coli in clinical and epidemiological investigations

3. STARTING DATE: When money becomes available

4. DATE OF COMPLETION: 3 years from starting date

5. TOTAL BUDGET REQUESTED: US$ 230,556

6. FUNDING SOURCE:

7. HEAD OF PROGRAMME: Dr. S. Izzoori
Laboratory Sciences Division

8. AIMS OF PROJECT:

a) General aims

To quantify and characterize microfloras from the jejunal fluids of patients with persistent diarrhoea and to develop bioassays and serological tests required for the detection and characterization of diarrhoeagenic E. coli in Bangladesh.

b) Specific aims

i) To quantify aerobic, microaerophilic and anaerobic microfloras from jejunal fluid of patients with persistent diarrhoea and matched controls to study the aetiology and pathogenesis of persistent diarrhoeas.
iii. To develop and standardize bioassays and serological tests required for the detection and characterization of diarrhoeagenic *E. coli* (enteropathogenic, enterohaemorrhagic, enterotoxigenic, enteroinvasive and enteroaggregative) for diagnosis and epidemiological investigations. Strains to be investigated include:

a) Approximately 60,000 *E. coli* strains collected from cholera-vaccinees and non-vaccinees with diarrhoea during 4 years of passive surveillance (1985-1989) in rural Matlab.

b) Approximately 29,000 *E. coli* strains from surveillance patients with diarrhoea admitted to the Dhaka Treatment Centre (Urban) during a 3-year period (1989-1991).

c) Approximately 750 *E. coli* strains from *Entamoeba histolytica* dysentery patients and matched controls.

d) Approximately 2,000 *E. coli* strains from jejunal fluids and stools of patients with chronic diarrhoea and matched controls.
c) Significance

There are several categories of pathogenic *E. coli* which are known worldwide to be associated with diarrhoea. Appropriate assays need to be developed to assess their relative contribution to diarrhoea in urban and rural Bangladesh. Moreover, the serological assays we intend to develop will help laboratories with lesser capabilities to study the role of these organisms.

9. ETHICAL IMPLICATIONS:

Only stool specimens will be studied from patients enrolled in 4 projects. From patients enrolled in a fifth project, in addition to stool, jejunal juice will be studied. This is a collaborative study with the Clinical Sciences Division, and permission to obtain jejunal juice has already been given by the Ethical Review Committee through a separate grant application (see Research Plan later for specimens).

10. BACKGROUND, RESEARCH PLAN AND BIBLIOGRAPHY

10(α). BACKGROUND

The magnitude of diarrhoeal diseases problem in children under 5 years old in developing countries is well documented (1). Efforts are being made on a global scale to improve diagnosis and treatment of this condition, and also devise strategies for its ultimate control and prevention. To
achieve these aims, a knowledge of aetiological agents of all diarrhoeas is mandatory.

Improvements in detection techniques in recent years have identified a burgeoning list of bacteria, viruses and parasites as agents of diarrhoea. Among bacterial pathogens, the various categories of *E. coli* deserve special attention. They are enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohaemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAgaEC). The epidemiological importance of these pathogens in rural and urban Bangladesh needs to be studied.

The aetiological mechanisms of chronic diarrhoeas are ill-understood. A concerted effort should be made to ascertain the role of known bacterial agents of acute diarrhoea in the initiation and perpetuation of lesions in chronic diarrhoea. Bacterial contamination of upper small intestine with Enterobacteriaceae is a feature of chronic diarrhoea, but the aetiology of this bacterial overgrowth is unknown (2). In order to elucidate their role, these bacteria need to be studied for virulence mechanisms such as epithelial cell adherence and toxin production (3) and their eradication by specific antimicrobial agents with the resultant amelioration of the condition.
ETEC
They cause disease worldwide, but are specially common in developing countries. In hospital-based studies, the percentage of cases in which ETEC have been identified ranged from 10 to 50%, with an average of about 20% in children under 5 years of age. In endemic areas, at least 2-3 infections appear to be asymptomatic. In Bangladesh, the highest infection rates occur in young children and decrease with increasing age (4). In community-based studies in rural Bangladesh, ETEC were identified in 27% of diarrhoeal cases and in 4.3% of children without diarrhoea (5).

They produce diarrhoea by attaching to the small intestine by colonization factor antigen (CFA) and producing enterotoxin of which a heat-labile variety (LT) and heat-stable variety (ST) are recognized. The genetic information for these properties is borne on transmissible plasmids. LT is structurally, antigenically and functionally related to cholera toxin (CT), and it effects fluid secretion in the small bowel by stimulating adenylate cyclase-cyclic AMP. LT-producing E. coli can be detected by rabbit ileal loop assay, tissue culture assays involving Chinese hamster ovary cells and adrenal tumor Y1 cells, ELISA and DNA probes (6,7,8).

ST occurs in two forms: STA and STb. STA has two subclasses: ST human (STh) and ST porcine (STp). STA
induces fluid secretion via guanylate cyclase-cyclic GMP. 
SLb is inactive in suckling mouse but active in weaned pigs, 
and its mechanism of action is unknown. SLb producing ETEC 
is very rare in human infections. STa producing ETEC can be 
diagnosed by suckling mouse assay, monoclonal antibody based 
ELISA and DNA probes (7,8).

At least 4, if not more, CFAs have been described in strains 
of ETEC: CEA/1, CFA/I1 and colonization factor antigens 
present in strains E 8775 and PCF 0159. Studies on 
screening of ETEC from Bangladesh for up to 3 CFAs have 
demonstrated that a significant number of isolates possess 
these antigens (9,10). However, ETEC need to be screened 
for the presence of all known CFAs, because this information 

is directly relevant to the polyvalency of anti-colonization 
factor vaccine against ETEC and to the breadth of protection 
that might be expected from such a vaccine. CFAs can be 
detected by different agglutination patterns of RBC from 
various animal species, slide agglutinations of isolates 
with specific antisera or DNA probes (P. Manning. personal 
communication).

EPEC

They have been historically associated with outbreaks of 
diarrhoea in nurseries. Their pathogenetic mechanism 
remained unknown until recently. However, in recent 
ultrastructural studies of small intestine of experimental
animals and infected humans, bacteria were seen closely adherent to the microvilli with the resultant destruction of microvilli and membrane cupping or partially enveloping the bacterium. For these reasons, the bacteria are called attaching-effacing bacteria (11). In HeLa or Hep-2 cells in tissue culture, the majority of serotypes exhibit characteristic localised adherence (LA) mediated by a EPEC adherence factor (EAF) which is encoded by a plasmid. These EPEC are called class I EPEC. The serotypes negative for EAF and not adherent to cells, but definitely diarrhoeagenic are called Class II EPEC.

There is a complete correlation between the presence of EAF, LA and disease-producing ability of these organisms. The role of LA EPEC in endemic diarrhoeal cases have been investigated recently in several studies. Even though, some asymptomatic controls also carried these organisms, the isolation rates in diarrhoeal patients were significantly higher in all these studies (12-14).

EPEC strains exhibiting diffused adherence (DA) in tissue culture cells have been noted. The pathogenic significance of such strains are uncertain. In some studies, they have been isolated with equal frequency from controls and cases (15).

Both Class I and Class II EPEC can be diagnosed by serotyping with commercial antisera. and Class I EPEC by its
characteristic adherence to HeLa or Hep 2 cells and hybridization with a DNA probe derived from EAF plasmid (16).

EIEC
They cause invasive dysenteric form of illness. Like Shigella, their cardinal pathogenic feature is the capacity to invade and proliferate within colonic mucosal epithelial cells. Again as in Shigella, the invasive capacity of EIEC is dependent on a plasmid coding for the production of outer membrane proteins involved in invasiveness; the proteins are antigenically closely related in EIEC and in Shigella.

They have caused outbreaks of diarrhoea (17), traveller's diarrhoea (18) and were found in up to 7% of endemic diarrhoeal diseases (12). Asymptomatic infection with these organisms seems to be rare (14).

EIEC can be diagnosed by serotyping suspected E. coli colonies, Sereny's test, invasiveness in tissue culture, ELISA that detects the outer membrane proteins associated with invasiveness and DNA probe that detects genes for invasiveness (19,20).

EHEC
They are also called verocytotoxin producing E. coli (VTEC) because their toxins are cytotoxic for vero cells (or HeLa cells) in tissue culture. They are causative agents of haemorrhagic colitis and haemolytic uremic syndrome.
Although several serotypes are involved in the causation of these diseases, the predominant serotype recognized is 0157:H7. This serotype has caused several outbreaks of disease, and was also isolated as a significant pathogen from bloody diarrhoeal stools submitted for routine laboratory investigation of diarrhoea, in North America (21). However, they are also capable of causing non-bloody diarrhoea and asymptomatic infection.

The role of VTEC in developing countries is largely unknown. In the West, most of the disease due to VTEC is linked to animals and animal products, such as consumption of raw milk and undercooked hamburger meat (22). Since such dietary practices are not prevalent in developing countries, it is suspected that VTEC may be of lesser significance in these countries, but this hypothesis is yet to be tested (23). In studies conducted in Mexico, VTEC were found in higher proportion of diarrhoeal cases than controls up to one year of age, but beyond this age group, they were found in higher proportion of controls without diarrhoea than in diarrhoea cases (22). In a 13-month prospective study of children under 3 years of age, Bhan et al. (14) in North India did not detect VTEC in any case of diarrhoea, but detected in 1.0% of controls.

There is strong evidence to suggest that the pathogenesis of the disease is due to toxins. These \textit{E. coli} produce either or both of the immunologically distinct verocytotoxins: V1
and VT2, which are encoded by lysogenic phages. VT1 is almost identical to the cytotoxin produced by *Shigella dysenteriae* type 1. The majority of EHEC strains possess a plasmid encoded fimbrial antigen that mediates attachment to Henle 407 gut derived epithelial cells in tissue culture. EHEC can be diagnosed by testing for VT1 and VT2 in tissue culture cells. DNA probes based on the plasmid that encodes the production of fimbrial antigen or DNA probes based on gene sequences of phages coding for VT1 and VT2 (24,25).

**Enteroaggregative *E. coli* (EAggEC)**

*E. coli*, not belonging to classical serotypes, but giving either localized or diffuse adherence were observed in significantly higher proportion of travellers to Mexico with diarrhoea than controls without diarrhoea using a Hep-2 cell culture assay by Mathewson *et al.* (26). In this cell culture assay, the cells were washed after 1 hour incubation with bacteria, which was followed by a further 4 hour incubation and wash. before fixing and staining the cells. The adherent bacteria also produced diarrhoea when fed to human volunteers (27). Later, these *E. coli* were also found as a significant cause of diarrhoea in Mexican children (28).

These strains were later studied by Dr. M. Levine's group in Baltimore using a 3 hour adherence assay without a washing step. This time, they found that the same *E. coli* strains gave 'aggregative' or 'stacked brick' pattern of adherence.
Dr. Levine's group recommended that such *E. coli* should henceforth be called enteroaggregative *E. coli* (27). Thus, the pattern of adherence seems to be assay-dependent. The difference between the two assays is that in the former there is a wash-step after 1 hour and the total period of incubation is 5 hours, but in the latter there is no wash-step and the total period of incubation is 3 hours. It is important that divergent results obtained in the two laboratories in the US. have to be verified by other laboratories.

Using Dr. Levine's assay, EAggEC were found in significantly more children with diarrhoea than age-matched controls in Chile (15). Furthermore, they were found more often in patients with acute and persistent diarrhoea than in age-matched controls in North India. Surprisingly, this category of *E. coli* were found significantly more often in patients with persistent diarrhoea than in those with acute diarrhoea in the North Indian study (14).

10(B). RESEARCH PLAN

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

About 78 children aged 3 months to 3 years with severe persistent diarrhoea will be studied. The controls for the study include 40 children with mild persistent diarrhoea and 40 children with acute diarrhoea. Of the 78 children with severe persistent diarrhoea, 26 will be treated with
co-trimoxazole and another 26 with coconut oil based chicken meat-diet to study the efficacy of these treatments for chronic diarrhoea. The remaining 26 will be treated with oral and I.V. fluid.

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

**Definition of severe persistent diarrhoea**

1) Diarrhoea duration of more than 14 days but less than 6 weeks

2) Requires prolonged I.V. maintenance (i.e. more than 48 hours)

3) Stool output more than 100 ml/kg/day during the initial 48 hours of observation

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

**Definition of mild persistent diarrhoea**

1) Duration of diarrhoea more than 14 days but less than 22 days

2) Does not require I.V. maintenance beyond first 24 hours
3) Diarrhoea does not last beyond 4 days after admission on supportive treatment and diet manipulation (but without antimicrobial therapy).

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

Justification of controls for persistent diarrhoea study

One problem is designing this study is the difficulty in obtaining data from suitable controls. We propose to include mild diarrhoeal patients as controls. We postulate that these patients are at the tail-end of normal distribution of patients with an acute attack of diarrhoea. In addition, for each case, an age-matched child admitted with acute watery diarrhoea will be identified concurrently and included in the acute diarrhoea controls.

b) Diagnostic service for persistent diarrhea patients

In the above study (a), the main objective is the investigation of the aetiology and pathogenesis of
persistent diarrhoea. In this study, if there is any suspicion of involvement of any pathogen(s) (particularly *E. coli*), the different treatment regimens are designed for comparison of their efficiencies in the eradication of suspected pathogens with the concomittant improvement in the condition of the patient. Based on our experience with this groups of study patients, we would like to offer additional diagnostic tests for diarrhoeagenic *E. coli*, in the routine management of persistent diarrhoea patients, who are not entered in the study (a). For this purpose, at least one stool specimen will be studied for all pathogens from approximately 300, 325 and 350 patients aged 3 months to 3 years, with persistent diarrhoea (with mild and severe forms) in the first, second and third year respectively seen at the Centre.

C) Diarrhoeagenic *E. coli* in ICDDR,B hospital surveillance study

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 belonging to all age groups will be tested for the bacterial pathogens mentioned. Approximately 3,000, 3,200 and 3,500 stool specimens in the first, second and third year respectively will be studied.
d) Epidemiology of diarrhoeagenic *E. coli* in *Entamoeba histolytica* dysentery study in collaboration with Clinical Sciences Division

Stool specimens from 50 patients with *Entamoeba histolytica* dysentery and samples from 200 controls, half of which is cases with *Shigella* dysentery and the other half, watery diarrhoea will be studied to study the prevalence of the pathogens in the 3 categories of patients. The cases and controls will be recruited within the same period and the ages of both groups will be more than one year.

e) *E. coli* diarrhoea in cholera vaccinees and controls

Children aged 2-15 years, vaccinated with oral cholera vaccine in 1985 in Matlab were kept under passive surveillance for diarrhoea for the past 4 years. Three *E. coli* colonies have been stored from each diarrhoeal stool culture of vaccinees and non-vaccinees. We plan to study 15,000 *E. coli* isolates per each year of 4 year surveillance to study the prevalence of various categories of *E. coli* in vaccinated versus non-vaccinated individuals. A total of 60,000 *E. coli* isolates will be studied during the 3 year project period.

Items (a) and (c) above suffer from a lack of appropriate controls. Since ICDDR,B is a Centre entirely devoted to the treatment of diarrhoea patients, it is impossible to recruit healthy controls. However, we hope to clarify aetiological relationship of various categories of *E. coli* by comparison of their isolation rates in acute diarrhoea, dysentery, and
chronic diarrhoea and in relation to the presence or absence of other well-established pathogens.

Laboratory procedures

Jejunal juice will be cultured for aerobic, micro-aerophilic and anaerobic bacteria and stool for pathogenic bacteria by standard methods. For the study of jejunal juice, briefly 10-fold serial dilutions will be made in sterile heart-infusion broth, 0.1 ml of undiluted juice and dilutions from $10^{-1}$ to $10^{-5}$ will be plated onto plain blood agar and incubated anaerobically. The same dilutions will be plated onto blood agar, MacConkey’s agar and Sabouraud’s dextrose agar and incubated aerobically. The dilutions will be inoculated onto Rogosa SL agar and incubated micro-aerophilically for lactobacilli. All aerobic, microaerophilic and anaerobic floras will be identified by standard techniques (30). [The role of *Helicobacter pylori* will be studied through another protocol entitled "The role of anaerobic and microaerophilic bacteria in diarrheal illness in Bangladeshi population."] In addition, the identities of anaerobic bacteria will be confirmed by analysis of their metabolic end products by GLC.

Whenever jejunal biopsy specimens are available, their flora will be studied. For this purpose, homogenate of biopsy in sterile heart-infusion broth (HI) will be serially diluted in HI and dilutions cultured for aerobic, microphilic and anaerobic microfloras as above.
*E. coli* isolated from above studies (a to d)

From MacConkey plate, 3 individual *E. coli* colonies will be inoculated into 20% glycerol broth and stored at -70°C until studied. *E. coli* strains isolated in cholera vaccine surveillance (study no. e) have already been collected and stored on nutrient agar slants.

1) **Tissue culture assays**

*EPEC* and *EAaggEC* will be diagnosed by their characteristic adherence to cultured HeLa cells (27). *EHEC* will be detected by the toxicity of culture supernatant to the cells. VT1 and VT2 will be differentiated by neutralization of toxin with specific antisera. This assay system is well-established in the laboratory.

2) **Enzyme-linked immunosorbent assay**

Both LT and ST are currently detected in the laboratory by GM1 ganglioside based ELISAs (7). The anti-LT monoclonal antibody for LT-ELISA; and ST-C1B conjugate and monoclonal antibody to ST-BSA for ST-ELISA are supplied by Dr. Ann-Mari Svennerholm, University of Goteborg, Goteborg, Sweden.

3) **Animal models**

In suspected cases, confirmation of virulence properties will be carried out by using animal models such as guinea pig (Sereny's test) and reversible ileal tie in adult rabbit (RITARD model) (31,32).
4) Detection of CFAs on ETEC
The four types of CFAs described in the text will be detected by slide agglutination test with specific antisera which will be supplied by Dr. Ann-Mari Svennerholm. University of Goteborg, Goteborg. Sweden.
All diarrhoeagenic *E. coli* will be serotyped based on their O and H antigen by Dr. K. A. Bettelheim. Infectious Disease Hospital, Fairfield, Victoria, Australia.

5) Development of serological tests
At present, the laboratory has serological assay (ELISA) only for ST and LT. Pal *et al.* (19) have demonstrated that specific antiserum to surface virulent marker antigen of EIEC can be produced and used in an ELISA to detect these organisms. In fact, we have just succeeded in developing an ELISA for EIEC by following the procedure of Pal *et al.* An attempt will be made to develop both ELISAs and agglutination tests (latex agglutination or Staphylococcal coagglutination) by developing specific antisera to surface virulence antigens of EPEC, EAgg EC and EIEC. Since virulence properties of all these *E. coli* are determined by plasmids and DNA probes derived from these plasmids are used for diagnosis, it is our belief that surface virulence markers will be expressed by these plasmids. And if so, antibodies to the virulence
antigens can be made and used in ELISAs as in the case of EIEC.

6) Plasmid analysis

Attempts will be made to study the role of plasmids on virulence of *E. coli*. Wild type organisms and plasmidless derivatives will be compared. These organisms will also be used for production of reagents for developing serological assays (19).

7) Analysis of diarrhoeagenic *E. coli* data from cholera vaccine trial samples

Demographic and clinical data of patients and controls who participated in the cholera vaccine trial are stored in a master computer. This information will be matched with that on diarrhoeagenic *E. coli* for analysis.
10. BIBLIOGRAPHY


4. Development of vaccines against cholera and diarrhoea due to enterotoxigenic E. coli. WHO/CDD/IMV/89.2.


11. PUBLICATIONS OF PRINCIPAL INVESTIGATORS

PUBLICATIONS OF K. HAIDER


PUBLICATIONS OF PKB NEOGI


**PUBLICATIONS OF M.J. ALBERT**


FLOW CHART 1

Activities of the Laboratory, 1-3 years

First year

Testing *E. coli* for enteropathogenicity and quantitative culture of jejunal fluid for aerobic, microaerophilic and anaerobic bacteria.

Hospital surveillance (*E. coli*) ... 3,000 stool

Persistent diarrhoea (Research - *E. coli*) ... 512 stool

Persistent diarrhoea (Research - *E. coli* and quantitative bacteriology) ... 276 jejunal juice

Persistent diarrhoea (Diagnostic - *E. coli*) ... 300 stool

Testing of *E. coli* isolates including those from Cholera Vaccine Trial (CVT) for enteropathogenicity ... 31,570

Second year

Hospital surveillance (*E. coli*) ... 3,200 stool

Persistent diarrhoea (Diagnostic - *E. coli*) ... 350 stool

Testing of *E. coli* isolates including those from CVT for enteropathogenicity ... 31,570

Third year

Hospital surveillance (*E. coli*) ... 3,500 stool

Persistent diarrhoea (Diagnostic - *E. coli*) ... 375 stool

Testing of *E. coli* isolates including those from CVT for enteropathogenicity ... 31,570
FLOW CHART 2

Working plan for diarrhoeagenic *E. coli*

Stool specimens
↓
Culture for *E. coli*
↓
Test for LT, ST, VT

LT, ST, VT Negative
↓
Adherence assay

LT & ST Positive
↓
Test for VI

LT or ST Negative
↓
Test for VI

Serotype, if positive
↓
Adherence positive
↓
Serotype
↓
Positive for invasiveness
↓
Serotype

Adherence negative
↓
Serotype
↓
Test for invasiveness

Negative for invasiveness
↓
RITARD assay

Serotype, if positive

*For isolates from jejunal fluids of persistent diarrhoea patients only (see later)*

**NOTE:** Isolates found positive for various categories of diarrhoeagenic *E. coli* by the above assays will be verified by specific DNA probes by Dr. S.M. Faruque, in the Molecular Biology Laboratory of our Division.
II. ITEMIZED SPECIFIC TASKS FOR EACH LISTED INVESTIGATOR

SEQUENCE OF TASKS

- Standardization of procedure  |  6 months
- Testing of specimens  |  24 months
- Analyses of data and writing reports  |  6 months

TASK OF INVESTIGATORS

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Task</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. Haider</td>
<td></td>
</tr>
<tr>
<td>S. Nahar</td>
<td>Bio-assay development and screening</td>
</tr>
<tr>
<td>P.K.B. Neogi</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>P.K.B. Neogi</td>
<td>Serological assay development and screening</td>
</tr>
<tr>
<td>M.J. Albert</td>
<td></td>
</tr>
<tr>
<td>H. Ali Kabir</td>
<td></td>
</tr>
<tr>
<td>M.J. Abert</td>
<td>Coordination</td>
</tr>
</tbody>
</table>
## BUDGET

### BUDGET SUMMARY

#### First Year

<table>
<thead>
<tr>
<th>Item</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capital equipment</td>
<td>US$ 9,800</td>
</tr>
<tr>
<td>Personnel</td>
<td>27,232</td>
</tr>
<tr>
<td>Operating cost</td>
<td>48,432</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>US$ 85,465</strong></td>
</tr>
</tbody>
</table>

#### Second Year

<table>
<thead>
<tr>
<th>Item</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Personnel</td>
<td>29,955</td>
</tr>
<tr>
<td>Operating cost (E. coli screening)</td>
<td>39,137</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>69,092</strong></td>
</tr>
</tbody>
</table>

#### Third Year

<table>
<thead>
<tr>
<th>Item</th>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Personnel</td>
<td>32,950</td>
</tr>
<tr>
<td>Operating cost (E. coli screening)</td>
<td>43,050</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>76,000</strong></td>
</tr>
</tbody>
</table>

**TOTAL COST (for 3 years):**

<table>
<thead>
<tr>
<th>Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>US$ 230,556</strong></td>
</tr>
</tbody>
</table>
DETAILED BUDGET

First year

A. Personnel

<table>
<thead>
<tr>
<th>Name</th>
<th>Level &amp; step</th>
<th>Salaries per annum</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. Al-Kabir</td>
<td>GS3 4</td>
<td>US$ 3,132</td>
</tr>
<tr>
<td>K. Haider</td>
<td>NDB 4</td>
<td>10,000</td>
</tr>
<tr>
<td>S. Nahar</td>
<td>GS5 2</td>
<td>4,176</td>
</tr>
<tr>
<td>P.K.B. Neogi</td>
<td>GS6 21</td>
<td>9,924</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US$ 27,232</td>
</tr>
</tbody>
</table>

B. Operating costs

Bacteriology of jejunal fluid
(quantitative culture and identification)
@ US$5.5 x 276 = US$ 1,518

Storage of E. coli colonies from all studies except cholera vaccine surveillance
@ US$ 0.05 x 34711 = 1,735

For the costing of pathogenicity tests of E. coli, we have assumed that 30% will be positive for LT/ST, 5% for VT, 20% for adherence and 5% for enteroinvasiveness. For the persistent diarrhoea study, we have assumed that 60% of patients with severe persistent diarrhoea, 40% of controls with mild persistent diarrhoea and 20% of controls with acute diarrhoea respectively will have coliform overgrowth in the small intestine. The coliform isolates from the small bowel of these patients only which are negative for known pathogenic properties will be tested in the RITARD model.
LT. SI testing of 31,570 colonies
\[ @ \text{US$} \ 0.4 \times 31,570 \ = \ 12,628 \]

VI testing of 70% of 31,570 colonies (22,099)
\[ @ \text{US$} \ 0.33 \times 22,099 \ = \ 7,293 \]

Adherence assay of 65% of 31,570 colonies (20,520)
\[ @ \text{US$} \ 0.59 \times 20,520 \ = \ 12,106 \]

Enteroinvasiveness assay of 45% of 31,570 colonies (14,206)
\[ @ \text{US$} \ 0.25 \times 14,206 \ = \ 3,552 \]

RITARD assay of 160 colonies (40% of 400) from jejunal juice in 160 rabbits
\[ @ \text{US$} \ 22.5 \times 160 \ = \ 3,600 \]

\[ \text{US$} \ 42,432 \]

The cost for pathogenicity tests includes items such as media, centrifuge tubes, pipettes, tips, cover-slips, microtitre plates, monoclonal antibodies, ganglioside, enzyme-conjugates, substrate, etc.

Development of ELISA for EPEC, EHEC, EAggEC and EIEC

Cost of 16 rabbits for antisera production
\[ \text{US$} \ 20 \times 16 \ = \ 320 \]

Feeding cost
\[ @ \text{US$} \ 0.5 \text{ a day} \times 35 \text{ days} \times 16 \text{ rabbits} \ = \ 280 \]

Cost of ELISA plate, enzyme conjugate, substrate, etc
\[ \text{US$} \ 400 \]

\[ \text{US$} \ 1,000 \]

\[ \text{TOTAL} \]
\[ \text{US$} \ 43,432 \]

32
Capital equipment

Refrigerator (Westinghouse) US$ 6,000
Revo ultra-low temperature freezer, 17.2 cft (Fisher) 6,000
Eppendorf microfuge (IEC) 2,000
Electrophoresis chamber (BRL) 1,000

US$ 9,800

Travel 3,000
Computing 2,000

Justification of budget

Capital equipments are very essential for the project. We expect to collect a large number of samples and since there is no room in the refrigerators and freezers currently in the laboratory, we need to buy them for the project. Eppendorf centrifuge and electrophoresis chamber are needed to characterize plasmids. Major expenditure is for reagents which are essential to carry out the project.