1. Gen. check

Date JULY 07, 1990

ET) ETHICAL REVIEW COMMITTEE, ICDDR,B.

Investigator DR. ASHFAQUE HOSSAIN Trainee Investigator (if any) 27

n No. PCC/04/90 Supporting Agency (if Non-ICDDR,B) _____

tudy A comparative study on -associated properties of cter jejuni strains obtained ents and healthy carriers

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

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

of Population: NA
ll subjects Yes No
on-ill subjects Yes No
inors or persons nder guardianship Yes No
he study involve: NA
hysical risks to the ibjects Yes No
ocial Risks Yes No
sychological risks o subjects Yes No
iscomfort to subjects Yes No
nvasion of privacy Yes No
isclosure of information damaging to subect or others Yes No
ie study involve:
se of records, (hospal, medical, death, rth or other) (Yes) No
e of fetal tissue or portus Yes No
se of organs or body uids Yes No
jects clearly informed about: NA
ture and purposes of tudy Yes No
rocedures to be llowed including ternatives used Yes No
ysical risks Yes No
nsitive questions Yes No
nefits to be derived Yes No
ght to refuse to rticipate or to withaw from study Yes No
nfidential handling data Yes No
mpensation &/or treatnt where there are risks privacy is involved in y particular procedure Yes No

5. Will signed consent form be required: NA
   (a) From subjects Yes No
   (b) From parent or guardian (if subjects are minors) Yes No
6. Will precautions be taken to protect anonymity of subjects NA Yes No
7. Check documents being submitted herewith to Committee:
   - ND 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.

obtain approval of the Ethical Review Committee for any changes he rights and welfare of subjects before making such change.

ipal Investigator

Trainee

APPLICATION FOR PROJECT GRANT

1. PRINCIPAL INVESTIGATOR : Dr. Ashfaque Hossain
   Department of Microbiology
   University of Dhaka

2. COINVESTIGATORS : Dr. Firdausi Qadri
   ICDDR,B

   Dr. S. M. Faruque
   ICDDR,B

   Dr. M. John Albert
   ICDDR,B

3. TITLE OF PROJECT : A comparative study on virulence-associated properties of *Campylobacter jejuni* strains obtained from patients and healthy carriers

4. STARTING DATE : When fund is available

5. COMPLETION DATE : One year from starting date

6. TOTAL BUDGET REQUIRED : US$ 7,577

7. HEADS OF PROGRAM :

Dr. Mozammel Hoq
Chairman
Department of Microbiology
University of Dhaka

Chairman,
Department of Microbiology
University of Dhaka

Dr. S. Tzipori
Associate Director
Laboratory Sciences Division
ICDDR,B

8. AIMS OF PROJECT

a) General aim

The present investigations aims to make a comparative study of the putative virulence factors of the *Campylobacter jejuni* strains isolated from patients with cholera-like secretory diarrhoea, dysentery-like bloody-mucoid diarrhoea and from healthy carriers. Qualitative and quantitative differences in production of enterotoxins and cytotoxin and invasiveness in HeLa cell model will be determined. Probing of the chromosomal DNA of *C. jejuni* strains will be carried out with *Escherichia coli* heat-labile toxin (LT) or cholera toxin (CT) gene probes to determine the nucleotide homology of the enterotoxin gene of *C. jejuni* with that of CT or LT. Similarly, probing with Shiga-like toxin 1 and 2 (SLT-1 and SLT-2) genes will be done to detect the similarity between cytotoxin of *C. jejuni* with SLT-1 and SLT-2 at the gene level. Gel diffusion experiments will be performed to determine the immunological similarity between toxins of *C. jejuni* with CT/LT and SLT-1 and SLT-2. In addition, antibiotic sensitivity, cell-surface hydrophobicity and serum susceptibility of the different groups of strains will be determined.

b) Specific aims

1) To determine the enterotoxigenicity of the *C. jejuni* strains by Y-1 mouse adrenal cell assay and by GM_1 ganglioside ELISA

2) To determine the cytotoxigenicity and invasiveness of the strains by HeLa cell assay

3) To determine serum susceptibility of the strains and compare their cell surface hydrophobicity and cell envelope protein profile

4) To probe the total DNA of *C. jejuni* with CT/LT and SLT-1/SLT-2 gene probes

c) Significance

This study is primarily directed at correlating the various virulence associated properties of *C. jejuni* with the clinical characteristics of the diarrhoea caused by them. So, it is anticipated that the results obtained will help us to identify the array of virulence factors which enable the pathogen to cause either cholera-like watery diarrhoea, or dysentery-like bloody-mucoid diarrhoea, or subclinical infection. In addition, genetic relatedness of *C. jejuni* enterotoxin and cytotoxin genes with CT/LT and SLT-1/SLT-2 genes will be established.

d) Rationale

Diarrhoeagenic bacteria usually cause disease primarily either by enterotoxin production (example: *V. cholerae*) leading to watery diarrhoea or by invasion of intestinal epithelial cell (example: *Shigella* spp.) leading to bloody, mucoid diarrhoea. But to what extent this generalization applies to *C. jejuni* is unknown as clinical features of *C. jejuni* enteritis indicates the possible involvement of both of these mechanisms. In addition, strains from healthy carriers are occasionally found to be toxigenic. So, it is necessary to make a comparative study of the virulence-associated properties with strains from cholera-like secretory diarrhoea cases, dysentery-like bloody-mucoid diarrhoea cases and from healthy carriers.

Also immunological similarity between *C. jejuni* enterotoxin and CT/LT and that of cytotoxin with SLT-1 has been demonstrated, but no nucleic acid homology could be detected. One probable reason could be the use of few strains which are repeatedly passaged in the laboratory. The proposed study intends to probe the chromosomal DNA of freshly isolated toxigenic bacterial strains which might generate a decisive information in this aspect.

9. ETHICAL IMPLICATIONS

Not applicable. Bacterial strains will be studied in a laboratory-based investigation.

10. BACKGROUND, RESEARCH PLAN and BIBLIOGRAPHY

BACKGROUND

C. jejuni is a major cause of gastroenteritis on a global scale (1). Epidemiological studies have shown that prevalence of *C. jejuni* diarrhoea in Bangladesh is higher than those caused by classical enteropathogens, such as *V. cholerae*, *Shigella* spp. or *Salmonella* spp. (2).

Studies carried out with *C. jejuni* strains obtained from clinical sources have identified several virulence-associated properties, such as production of enterotoxin, cytotoxin, invasion of intestinal epithelial cells and survival within macrophages (3-5). In addition, certain cell surface components, such as flagella and LPS have also been found to act as adhesins in *in vitro* tissue culture assays (6). Klipstein *et al.* (3) reported that enterotoxigenicity was associated with strains from watery diarrhoea and cytotoxigenicity and invasiveness were associated with strains from bloody-mucoid diarrhoea. But, the interplay of these virulence attributes leading to overt disease is far from clear.

Toxins from C. jejuni

a) Enterotoxin

The enterotoxin of *C. jejuni* has been reported to possess immunobiological similarity with those of CT and LT (4).

Various assays used for CT and LT have been adapted for detection and quantitation of *C. jejuni* enterotoxin (CJT). These include ELISA, tissue culture assays, such as CHO cell and Y-1 adrenal cell tests and accumulation of fluid in the ligated ileal loops of animals (3,4,7). Like CT and LT, CJT induced cytotonic response in CHO and Y-1 mouse adrenal cell was inhibited by preincubation with GM_1 ganglioside (7). The level of cAMP increased by CJT in CHO cells was comparable to those elevated by CT and LT as determined by radioimmunoassay (7).

Klipstein and Engert (8) observed lines of partial identity between the B subunit of *C. jejuni* enterotoxin and those of CT and LT. Neutralization of fluid accumulation in the ileal loops and development of various forms of ELISA also demonstrated immunological similarity of CJT with CT and LT. However, no nucleic acid homology was noted between the chromosomal DNA of *C. jejuni* and CT or LT gene probes by several investigators (4,9). Thus, it appears that similarity of CJT with CT and LT at the gene level is not clear and deserves investigation with more freshly isolated toxigenic strains of *C. jejuni*.

Cytotoxin

Certain *C. jejuni* strains are reported to produce a cytotoxin detectable in various cell lines, such as HeLa, Vero, MRC-5, HEp-2, etc. (10-12). Antisera to cytotoxins

from several enteropathogenic bacteria failed to neutralize the cytotoxic effects of the toxin. These include SLT-1 and SLT-2, and cytotoxins from *Clostridium difficile*, *Aeromonas* spp. and non-01 *V. cholerae* (11-13). Moor *et al.* (14), however, reported that some *C. jejuni* strains produced a cell-associated Shiga-like toxin at low level which was active against HeLa cells. The cytotoxic activity could be neutralized by monoclonal antibody to the B subunit of SLT-1 and rabbit anti-Shiga antitoxin. However, no hybridization was observed between a DNA fragment containing cloned SLT-1 gene from *E. coli* phage 933J and restriction enzyme digested total DNA from a *Campylobacter* strain that produced low levels of SLT-1. So, further studies are necessary to clarify the relationship at the DNA level between the Shiga-like toxin of *Campylobacter* and the SLT-1 and SLT-2.

Invasiveness of C. jejuni

Clinical features of diarrhoea caused by certain *C. jejuni* strains resemble those of other invasive enteropathogens, such as *Shigella* spp. and Enteroinvasive *E. coli*. These include abdominal pain with bloody-mucoid diarrhoea and presence of leucocytes in stool (4). These findings led researchers to investigate the invasive nature of *C. jejuni*. Examination of colonic biopsy specimens of patients with colitis showed direct invasion of colonic mucosa by *C. jejuni* (3,4). Clinical strains, especially those obtained from bloody-mucoid diarrhoea, were found to be capable of invading HeLa cells which is often used as

an *in vitro* test for determining invasiveness of pathogenic microorganisms (3,15,16). However, the contributory role of the invasive capability in the pathogenesis of *C. jejuni* diarrhoea is not clear at present.

Cell-envelope proteins

Cell-envelope proteins are speculated to contribute to pathogenesis and immunity in many pathogenic microorganisms. Recent studies with *Yersinia* spp. demonstrated that a cell-surface protein can act as adhesin in cultured epithelial cell model (15). The virulence plasmid-associated outer membrane proteins of *Shigella* spp. are implicated to play an important role in the disease process (16).

Comparative cell-envelope protein analysis of virulent *C. jejuni* strains with spontaneous laboratory derived avirulent strains showed that transition to avirulent phase from virulent phase was associated with loss of a 42-Kilodalton (KDa) cell-surface protein (5). It would be interesting to see whether this protein band is absent in strains obtained from healthy carriers.

Cell-surface hydrophobicity and serum susceptibility

Cell-surface hydrophobicity is currently regarded as an important factor in mediating bacterial adherence to eucaryotic cells and has been correlated with virulence in several pathogenic microorganisms (17,18). Qadri *et al.* (18) reported that cell-surface hydrophobicity correlated closely with the relative virulence of the different species of *Shigella*. The

gradual reduction in cell-surface hydrophobicity in the order of *S. dysenteriae*, *S. sonnei* and *S. boydii* was in general agreement with the virulence of *Shigella* spp. and the severity of disease caused by these pathogen.

Cell-surface hydrophobicity of *C. jejuni* was found to be comparable with that of *Shigella* spp. Comparative assessment of cell-surface hydrophobicity of clinical *C. jejuni* isolates and carrier strains will provide information as to whether this property is associated with virulence. For the same purpose, serum susceptibility of clinical and carrier strains will be determined as capacity to survive in serum is considered a virulence-associated property of pathogens (19).

RESEARCH PLAN

Bacterial strains

Fresh clinical strains will be isolated from stools of patients attending the Clinical Research Centre of ICDDR,B. A collection of recently isolated strains will also be included. Stools of healthy individuals will be screened for isolation of carrier strains. At least 25 carrier strains and 50 clinical strains (25 from cholera-like and 25 from dysentery-like cases) will be included in this study.

Isolation and identification of C. jejuni

Stool samples will be plated on Brucella blood agar plates containing *Campylobacter* selective supplement (vancomycin 1.0 mg,

polymyxin B 250 IU, trimethoprim 0.5 mg, amphotericin B 0.2 mg and cephalothin 1.5 mg per 100 ml). *C. jejuni* strains will be identified and biotyped according to the new, extended biotyping scheme of Lior (20).

Growth of organisms

C. jejuni strains will be grown in Brucella broth for 24-36 hr at 42°C under microaerophilic atmosphere.

Assay of toxins

a) Enterotoxin assay

GM_1 ganglioside ELISA: The method of Sack *et al.* (21) will be used for detection and quantitation of cholera toxin (CT) equivalent of *C. jejuni* enterotoxin (CJT). Optimal concentration of GM_1 ganglioside, CT antiserum and alkaline phosphate labelled anti-antiserum to be used will be determined by checkerboard titration. Colour will be developed using the substrate p-nitrophenyl phosphate and will be read at 405 nm with an ELISA reader. A standard curve of purified CT (0-10,000 ng/ml) will be prepared and the amount of CT equivalent of CJT will be estimated from the standard curve.

Y-1 mouse adrenal cell assay: The bioassay of enterotoxin will be carried out using Y-1 cell assay according to Donta and Smith (22). The wells of 96-well tissue culture plates will be seeded with 1×10^5 cell/well and will be grown to

near confluence. Dilutions of enterotoxin preparation will be added and after 20 hr the cell monolayers will be observed for morphological changes. The highest dilution of toxin causing rounding of 50% of the cells counted will be considered its titre.

b) Cytotoxin assay

Preparation of cytotoxin: *C. jejuni* strains will be grown for 48 hr as described before for optimal production of cytotoxin. Cell-free culture filtrates will be used for assay. In parallel assays, cells extracted with polymyxin B and sonicated cell suspensions will be tested for determining the proportion of cell-associated cytotoxin.

Assay procedure: HeLa cell line will be used for quantitation of cytotoxin according to Gurerrant *et al.* (13). The wells of 96-well flat-bottomed microtitre plates will be seeded with 10^4 cells. After 24 hr at $37^{o}C$, cell monolayers will be charged with serial dilutions of toxin preparation. After further incubation at $37^{o}C$ for 18 hr, the cell monolayers will be observed for cytotoxic effect (loss of typical cell morphology and detachment from plastic surface). The highest dilution of toxin exerting cytotoxic effect on 50% of the cell counted will be considered as its titre.

Invasion assay

C. jejuni strains will be assessed for their ability to invade HeLa cells according to the procedure described by Isberg *et al.* (15). HeLa cell monolayers will be inoculated with *C. jejuni* cell suspension at a multiplicity (number of bacteria per HeLa cell) of 100:1. After 2 hr incubation at 37^{o}C, the extracellular bacteria will be killed by the antibiotic gentamycin which has very limited capability to enter eucaryotic cells. The HeLa cells then will be lysed by sodium deoxycholate to release internalized bacteria and viable counts will be determined. The proportion of inoculated bacteria internalized will indicate the invasion potential of the strain.

Determination of cell-surface hydrophobicity

Bacterial adherence to hydrocarbon (BATH) test (23) and salt aggregation (SA) test (24) will be employed for determining the cell-surface hydrophobicity of the *C. jejuni* strains.

BATH test

This test measures the relative distribution of bacterial cells between an aqueous phase and a hydrocarbon phase. To 1.5 ml of washed bacterial cell suspension in phosphate-urea-magnesium (PUM) buffer (pH 7.1; 97 mM K_2HPO_4, 53 mM KH_2PO_4, 30 mM urea and 0.8 mM $MgSO_4$), 0.5 ml of n-octane will be added and mixed. After separation of the two phases, the aqueous phase will be removed and its OD will be read in a spectrophotometer at

550 nm. The decrease in OD in comparison to buffer treated control will indicate relative cell-surface hydrophobicity.

SA test

This test is based on the capacity of bacterial cells to agglutinate in presence of ammonium sulphate solution. Bacterial cell suspension will be mixed with an equal volume of salt solutions of different concentration (0.5 to 4.0 M differing by 0.5 M per dilution) on a glass slide. A visible aggregation of cells will indicate a positive reaction. The lowest concentration of ammonium sulphate solution causing positive reaction will indicate the SA test value of the test strain.

Determination of serum susceptibility

Serum susceptibility of the *C. jejuni* strains will be determined according to Blasser *et al.* (25). Bacterial cell suspension (1.0×10^8 cells/ml) will be incubated with equal volume of 50% normal human serum suspension for 1 hr. Serum bactericidal effect will be assessed by viable plate counts. Serum samples will be pretreated with EGTA or heated at 50°C for 30 min to selectively eliminate the classical and alternate complement fixation pathway, respectively.

Cell envelope protein profile analysis

a) Isolation of cell envelope proteins

Cell envelope proteins will be isolated according to Huyer *et al.* (26) (Flow diagram).

FLOW DIAGRAM

Isolation of *C. jejuni* cell envelope proteins

C. jejuni grown in
Brucella broth (200 ml)

↓

Harvest cells
(7000 X *g*, 20 min)

↓

Suspend in 10 ml of Tris-HCl buffer,
pH 7.4, containing 2.0 mM EDTA

↓

Add lysozyme 10 mg/ml,
incubate at room temperature
for 30 min

↓

Shake well glass beads
(1 hr at room temperature)

↓

Spin to remove unlysed cells
(7000 X *g*, 15 min)

↓

Collect supernatants
(envelope preparation)
and save

↓

SDS-PAGE

ef: Huyer *et al.* (26)

b) SDS-PAGE

SDS-PAGE of protein samples will be carried out essentially as described by Laemmli (27). The separating (lower) and stacking (upper) gels will be made up of 12.5 and 4.5% acrylamide, respectively. The gels will be stained with Coomassie blue. The molecular weights of the cell envelope proteins will be determined by comparing with migration of standard proteins.

Immunodiffusion

Double-diffusion assays will be carried out according to Ouchterlony (32) to determine immunological similarity between *C. jejuni* enterotoxin (CJT) and cholera toxin (CT). Agarose (1% w/v) in PBS (pH 7.3) will be used to prepare the gel. Concentrated culture filtrate, sonicated and Polymyxin B treated cell extracts will be used against antiserum to purified CT raised in rabbit.

Gene probing

Initially, all the strains included in this study will be screened for nucleotide homology with CT/LT and SLT-1/SLT-2 gene probes by colony hybridization technique. To determine the location and size of gene, total DNA of the strains showing positive hybridization will be isolated according to Maniatis (29), digested with restriction enzyme *Hind*III and Southern-blotted to nitrocellulose membrane according to Southern (30). Then probing will be carried out according to standard procedure (29).

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3) Klipstein, F.A., Engert, R.F., Short, H. and Shenk, E.A. (1985) Pathogenic properties of *Campylobacter jejuni*: assay and correlation with clinical manifestations. Infect. Immun. 50:43-49.

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1. PUBLICATIONS OF PRINCIPAL INVESTIGATOR

)r. Ashfaque Hossain

) Haider, K., Huq, M.I., Hossain, A., Shahid, N. and Holmes, I.H. (1985) Electropherotypes of ds-RNA of rotavirus in infants and young children with gastroenteritis in Bangladesh. JDDR 3:219-222.

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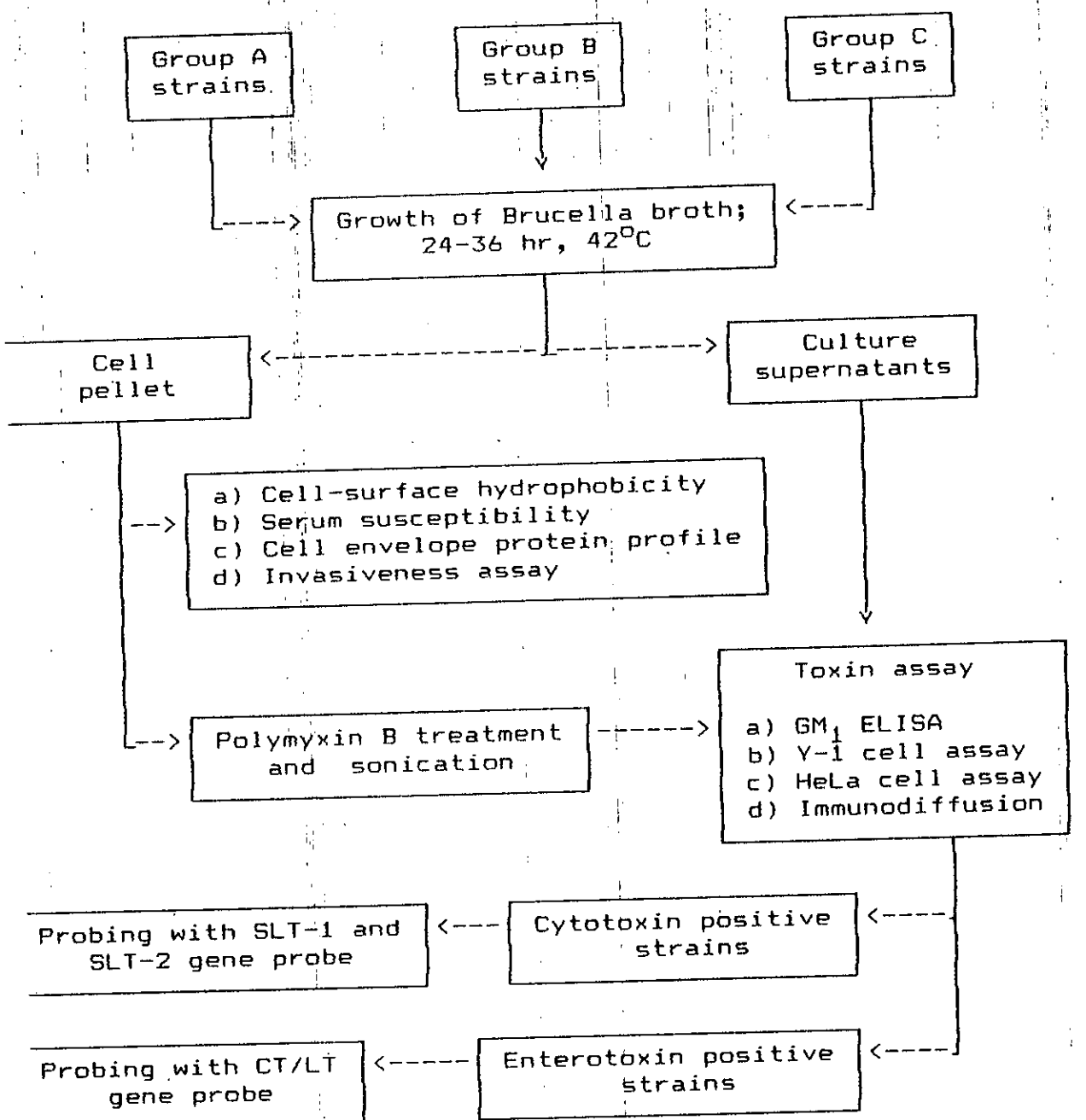
3. FLOW CHART

cheme of work



EY:

oup A - strains from cholera-like secretory diarrhoea
oup B - strains from dysentery-like bloody-mucoid diarrhoea
oup C - strains from healthy individuals

Sequence of work

Month 1-3

a) Collection and identification of strains

b) Antibiotic sensitivity determination

c) Standardization of assays

Month 4-6

a) GM_1 ganglioside ELISA

b) Bioassay of toxin

c) Immunodiffusion

d) Invasiveness assay

Month 7-9

a) Gene probing

Month 10-11

a) Cell envelope protein profile determination

b) Cell-surface hydrophobicity and serum susceptibility determination

Month 12

a) Analysis of data

b) Report preparation

14. ITEMIZED INVOLVEMENT OF RESEARCH INSTITUTES

a) Department of Microbiology, University of Dhaka

1) Culture of clinical samples
2) Determination of antibiotic resistance pattern
3) Cell envelope protein profile determination
4) Determination of cell-surface hydrophobicity
5) Determination of serum resistance
6) Immunodiffusion

b) ICDDR,B

1) Collection of samples
2) Tissue culture assays and gene probing

15. ITEMIZED SPECIFIC TASKS FOR EACH LISTED INVESTIGATORS

Dr. Ashfaque Hossain Dr. M. John Albert	Collection of strains, correlation with clinical symptoms, ELISA, bioassay of toxins and invasiveness assay
Dr. Ashfaque Hossain Dr. Firdausi Qadri	Cell-envelope protein preparation, SDS-PAGE
Dr. S. M. Faruque Dr. Ashfaque Hossain	Gene probing
Dr. Ashfaque Hossain	a) Identification of strains b) Antibiotic sensitivity assay c) Determination of cell-surface hydrophobicity d) Determination of serum susceptibility e) Immunodiffusion

A research fellow (to be appointed) will provide technical assistance

P2:CJEJUNI.PRT

16. BUDGET

a) Personnel services

Name	Time	Honorarium/salary p.a.		
Dr. Ashfaque Hossain	40%	@ 3000x12	Tk.36,000	
Dr. Firdausi Qadri	5%		0	
Dr. S. M. Faruque	10%		0	
Dr. M. John Albert	5%		0	
Research Fellow (to be recruited)	100%	4000x12	48,000	
				Tk. 84,000

b) Supplies

1) Bacteriological media, petridishes, slides and glasswares	32,000	
2) Chemical and reagents, tissue culture media, serum	70,000	
		102,000
c) Probing expenses		20,000
d) Photocopying, publication, printing		5,000
e) Overhead, contingency, stationary		5,000
f) Electrophoresis unit, including power pack		53,000
TOTAL PROJECT COST		Tk.269,000
		= $ 7,577

STIFICATION FOR PURCHASE OF ELECTROPHORESIS UNIT

present there is no electrophoresis unit in the Department of crobiology, University of Dhaka. Purchase of an unit is cessary for carrying out cell-envelope protein analysis of the oposed research protocol. The electrophoresis unit will also of great help in future research activities of the Department.