**ETHICAL REVIEW COMMITTEE, ICDDR,B.**

**Investigator** DR. ASHFAQU HOSSAIN

**Trainee Investigator (if any)**

**In No.** PCC/04/90

**Study** A comparative study on properties of enter jejuni strains obtained from patients and healthy carriers

**Supporting Agency (if Non-ICDDR,B)**

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

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

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<td>Right to refuse to participate or to withdraw from study</td>
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<td>Compensation &amp; or treatment where there are risks</td>
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<td>Privacy is involved in any particular procedure</td>
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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 NA

(a) Anonymity of subjects | Yes | No |

7. Check documents being submitted herewith to Committee:

<table>
<thead>
<tr>
<th>Document</th>
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<td>Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).</td>
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<td>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)</td>
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<td>Procedure for maintaining confidentiality</td>
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<td>Questionnaire or interview schedule</td>
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*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 Ctte. for review.

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Date JULY 07, 1990

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Principal 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 Hoque
   Chairman
   Department of Microbiology
   University of Dhaka

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

   Chairman, Department of Microbiology
   University of Dhaka
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 \textit{C. jejuni} strains by Y-1 mouse adrenal cell assay and by \textit{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 \textit{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 \textit{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 \textit{C. jejuni} enterotoxin and cytotoxin genes with CT/LT and SLT-1/SLT-2 genes will be established.
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 cytotoxic response in CHO and Y-1 mouse adrenal cell was inhibited by preincubation with GM1 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 entero-pathogenic 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 pathogens.

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

*GM1 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 GM1 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 x 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 $37^\circ\text{C}$, cell monolayers will be charged with serial dilutions of toxin preparation. After further incubation at $37^\circ\text{C}$ 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°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 K₂HPO₄, 53 mM KH₂PO₄, 30 mM urea and 0.8 mM MgSO₄), 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 x 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 with glass beads (1 hr at room temperature)
    - Spin to remove unlysed cells (7000 x g, 15 min)
    - Collect supernatants (envelope preparation) and save

Ref: Huyer et al. (26)
p) 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 HindIII and Southern-blotted to nitrocellulose membrane according to Southern (30). Then probing will be carried out according to standard procedure (29).


1. PUBLICATIONS OF PRINCIPAL INVESTIGATOR

Dr. Ashfaque Hossain


3. FLOW CHART

Scheme of work

Group A strains → Group B strains → Group C strains

Growth of Brucella broth; 24-36 hr, 42°C

Cell pellet → Culture supernatants

a) Cell-surface hydrophobicity
b) Serum susceptibility
c) Cell envelope protein profile
d) Invasiveness assay

Polymyxin B treatment and sonication → Toxin assay

Toxin assay:
a) GM₁ ELISA
b) Y-1 cell assay
c) HeLa cell assay
d) Immunodiffusion

Probing with SLT-1 and SLT-2 gene probe

Probing with CT/LT gene probe

Cytotoxin positive strains

Enterotoxin positive strains

**EY:**
- Group A - strains from cholera-like secretory diarrhoea
- Group B - strains from dysentery-like bloody-mucoid diarrhoea
- Group 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) GM₁ 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
16. BUDGET

a) Personnel services

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<td>@ 3000x12 Tk.36,000</td>
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<tr>
<td>Dr. Firdausi Qadri</td>
<td>5%</td>
<td>0</td>
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<tr>
<td>Dr. S. M. Faruque</td>
<td>10%</td>
<td>0</td>
</tr>
<tr>
<td>Dr. M. John Albert</td>
<td>5%</td>
<td>0</td>
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<tr>
<td>Research Fellow</td>
<td>100%</td>
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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.