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CENTRE
FOR HEALTH AND
POPULATION RESEARCH

Memorandum

22 February 2001

To : Mr. Ansaruzzaman
Laboratory Sciences Division

From: David A. Sack, M.D.
Chairman, Research Review Committee

Sub : Protocol # 2001-001

Thank you for your Protocol # 2001-001 entitled "Phenotypic and genotypic analysis of clinical and environmental *Vibrio cholerae* non 01 non 0139 to identify pathogenic clones and their pathogenic mechanism" which the RRC reviewed in its meeting held on 18th February 2001. After thorough discussion, the RRC made the following observations on the protocol:

(a) Title of the study should be the same throughout the protocol (face sheet and page 5).

(b) The hypothesis of the study should be re-written to make it more clear and specific.

(c) It would be better if the investigators could give some background information regarding isolation of *Vibrio cholerae* non O1 and non O139 from patients attending ICDDR,B treatment centre.

(d) Justification for the duration of the study should be provided.

(e) PCR assays for virulence genes should be included in the methodology section.

(f) The investigators should explain whether it a case control study or not.

(g) The investigators plan lot of animal testing (mice, rabbit). Further details of their plan should be provided.

(h) Sample size calculation: How the sample size calculation was done? With surveillance patients the total number of positive isolates would be 64 in two years assuming 2% isolation rate (not 128, page 12). Similarly 20 isolations from control children are confusing (assuming 0.1% isolation).

(i) Control: Healthy, matched human control: The idea was not favoured due to the fact that with a very low incidence of asymptomatic infection (among the controls) even a large number of controls may not help achieving statistical significance. Therefore, provision for control may be deleted.

(j) The upper row in both cases represents exposure among diarrhoeal patients, and the lower row exposure among apparently health controls. Please clarify this issue.

(k) Data analysis: Needs further elaboration (data entry and checking-single/double entry, analysis plan, description of study subjects by age, sex, results by different age sex groups, environmental isolations, comparison of rates, analysis of case control etc.)

(l) List of references should be checked. Some references have been mentioned in the text but not given in the reference list (e.g. Murry and Thompson). Similarly the publication year needs to be corrected (e.g. Booth et al, Ansaruzzaman et al, Ujiiye and Kobari etc.).

(m) The budget should be revised and to include cost for shipping the specimens to collaborating institutes.

You are therefore advised to revise the protocol incorporating the above observations of the committee and resubmit a modified copy of the protocol for approval of the Chair

Thank you.

cc: Acting Head
Laboratory Sciences Division

ICDDR,B: Centre for Health & Population Research **RRC APPLICATION FORM**

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RESEARCH PROTOCOL

Protocol No.: 2001-001

RRC Approval: Yes/ No Date:

ERC Approval: Yes/No Date:

AEEC Approval: Yes/No Date:

Project Title: Phenotypic and genotypic analysis of clinical and environmental *Vibrio cholerae* non O1 non O139 to identify pathogenic clones and their pathogenic mechanism

Theme: ***(Check all that apply)***

- ☐ Nutrition
- ☑ Emerging and Re-emerging Infectious Diseases
- ☐ Population Dynamics
- ☐ Reproductive Health
- ☐ Vaccine evaluation
- ☐ Environmental Health
- ☐ Health Services
- ☐ Child Health
- ☐ Clinical Case Management
- ☐ Social and Behavioural Sciences

Key words: : *V. cholerae* non O1 non O139, serogroup, diarrhoea

Principal Investigator: M. Ansaruzzaman **Division: LSD Phone:8811751-60,Extn.2406**
Address: Enteric Bacteriology Lab **Email: ansar@icddrb.org**

Co-Principal Investigator(s):

Co-Investigator(s): G.B. Nair, M.S. Islam, K.A. Talukdar, K. Alam, ASG Fruque, A.K. Siddique I. Kuhn & R.B. Sack

Student Investigator/Intern:

Collaborating Institute(s): NICED, Calcutta, India and MTC, Karolinska University, Sweden

Population: Inclusion of special groups (*Check all that apply*):

Gender
- ☑ Male
- ☑ Females

Age
- ☑ 0 – 5 years
- ☑ 5 – 9 years
- ☑ 10 – 19 years
- ☑ 20 +
- ☑ > 65

- ☐ Pregnant Women
- ☐ Fetuses
- ☐ Prisoners
- ☐ Destitutes
- ☐ Service providers
- ☐ Cognitively Impaired
- ☐ CSW
- ☐ Others (specify : North, Centre, South and Western part of Bangladesh_____)
- ☐ Animal

Project / study Site (*Check all the apply*):

- ☑ Dhaka Hospital
- ☐ Matlab Hospital
- ☐ Matlab DSS area
- ☐ Mirzapur
- ☐ Dhaka Community
- ☐ Chakaria
- ☐ Abhoynagar
- ☐ Mirsarai
- ☐ Patyia
- ☑ Other areas in Bangladesh , North, South & Western parts of Bangladesh
  tside Bangladesh
  name of country: _____
- ☐ Multi centre trial
  (Name other countries involved)

Revised on: 30 May 2000

Type of Study *(Check all that apply)*:

- ☑ Case Control study
- ☐ Community based trial / intervention
- ☐ Program Project (Umbrella)
- ☐ Secondary Data Analysis
- ☐ Clinical Trial (Hospital/Clinic)
- ☐ Family follow-up study
- ☐ Cross sectional survey
- ☐ Longitudinal Study (cohort or follow-up)
- ☐ Record Review
- ☐ Prophylactic trial
- ☑ Surveillance / monitoring
- ☐ Others

Targeted Population *(Check all that apply)*:

- ☑ No ethnic selection (Bangladeshi)
- ☐ Bangalee
- ☐ Tribal groups
- ☐ Expatriates
- ☐ Immigrants
- ☐ Refugee

Consent Process *(Check all that apply)*:

- ☐ Written
- ☐ Oral
- ☐ None
- ☐ Bengali language
- ☐ English language

Proposed Sample size:

Sub-group : 128 from Dhaka hospital surveillance
32 from field sentinel surveillance

☐ Total sample size: 492 (24th Month ☐
☐ 20 (appor.) from neighbourhood control ☐
☐ 312 from environmental sample ☐

Determination of Risk: Does the Research Involve *(Check all that apply)*:

- ☐ Human exposure to radioactive agents?
- ☐ Fetal tissue or abortus?
- ☐ Investigational new device?

(specify_____)

- ☐ Human exposure to infectious agents?
- ☐ Investigational new drug
- ☐ Existing data available via public archives/source
- ☑ Pathological or diagnostic clinical specimen only
- ☐ Existing data available from Co-investigator
- ☐ Observation of public behaviour
- ☐ New treatment regime

Yes/No

☐ ☐ Is the information recorded in such a manner that subjects can be identified from information provided directly or through identifiers linked to the subjects?

☐ ☐ Does the research deal with sensitive aspects of the subject's behaviour; sexual behaviour, alcohol use or illegal conduct such as drug use?

Could the information recorded about the individual if it became known outside of the research:

☐ ☐ a. place the subject at risk of criminal or civil liability?
☐ ☐ b. damage the subject's financial standing, reputation or employability; social rejection, lead to stigma, divorce etc.

Do you consider this research *(Check one)*:

- ☐ greater than minimal risk
- ☑ no risk
- ☐ no more than minimal risk
- ☐ only part of the diagnostic test

Minimal Risk is "a risk where the probability and magnitude of harm or discomfort anticipated in the proposed research are not greater in and of themselves than those ordinarily encountered in daily life or during the performance of routine physical, psychological examinations or tests. For example, the risk of drawing a small amount of blood from a healthy individual for research purposes is no greater than the risk of doing so as a part of routine physical examination".

Revised on: 30 May 2000

Principal Investigator: Ansaruzzaman, M. _____

Yes/No

☐ ☐ Is the proposal funded?

If yes, sponsor Name:

Yes/No

☐ ☐ Is the proposal being submitted for funding ?

If yes, name of funding agency: (1) USAID

(2) ICDDR,B internal funding source

Do any of the participating investigators and/or their immediate families have an equity relationship (e.g. stockholder) with the sponsor of the project or manufacturer and/or owner of the test product or device to be studied or serve as a consultant to any of the above?

IF YES, submit a written statement of disclosure to the Director.

Dates of Proposed Period of Support	**Cost Required for the Budget Period ($)**
(Day, Month, Year - DD/MM/YY)	*a. Ist Year* 29567 *2nd Year* 32853 *3rd Year* 24892 *Other years*
Beginning date: 01 03 2001 End date: Three years from starting date	*b. Direct Cost :87312 Total Cost : 109140*

Approval of the Project by the Division Director of the Applicant

The above-mentioned project has been discussed and reviewed at the Division level as well by the external reviewers. The protocol has been revised according to the reviewer's comments and is approved.

Dr. G. Balakrish Nair — Name of the Division Director

Signature

30/1/2001 — Date of Approval

Certification by the Principal Investigator

I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware

that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

Signature of PI

Date: 30/1/2001

Name of Contact Person (if applicable)

Table of Contents

☐ Check here if appendix is included

PROJECT SUMMARY: Describe in concise terms, the hypothesis, objectives, and the relevant background of the project. Describe concisely the experimental design and research methods for achieving the objectives. This description will serve as a succinct and precise and accurate description of the proposed research is required. This summary must be understandable and interpretable when removed from the main application. **(TYPE TEXT WITHIN THE SPACE PROVIDED).**

Principal Investigator: M. Ansaruzzaman

Project Name:

Phenotypic and genotypic analysis of clinical and environmental *Vibrio cholerae* non-O1 non-O139 to identify pathogenic clones and the underlying pathogenic mechanism

Total Budget US 109140 Beginning Date 1st March , 2001 Ending Date, February 2004

PROJECT SUMMARY:

Prior to 1992, all epidemics and pandemics of cholera were associated with *V.cholerae* strains belonging to the somatic O1 serogroup of either the classical or ElTor biotype. The non-O1 serogroups of *V.cholerae* are ubiquitous members of the heterotrophic microflora of aquatic environs and are recognized as a cause of sporadic diarrhoea and localized outbreaks of diarrhoeal illness. In October 1992, however a dramatic shift in the etiology of cholera was observed in the Indian subcontinent with strains belonging to a novel non-O1 serogroup, classified as O139 Bengal, was associated with large outbreaks of cholera. Multiple virulence factors with different combinations have been reported to play an important role in the disease process caused by *V.cholerae* non-O1. A disturbing trend recently observed in Calcutta and other cholera endemic areas in India and South America is the increasing incidence of *V. cholerae* non-O1 non-O139 infections. In some months, the incidence of these organisms is higher than the O1 and O139 serogroups. The highest reported isolation rate of *V. cholerae* non O1 non O139 is 16% among 134 children and adults in Cancun, Mexico. In this study, no non O1 non O139 strains were isolated from the 22 Healthy children. Curiously, these serogroups do not produce cholera toxin and do not have any of the conventional virulence traits of the epidemic causing O1 and O139 serogrops associated with the ability to cause diarrhoea. This study is designed to conduct an in-depth study to understand the mechanism by which these isolates cause the acute secretory diarrhoea and also to determine whether these strains comprise an as yet undiscovered subset of *V. cholerae* strains which have a pathogenic capability by performing a systematic study on *V. cholerae* non-O1 non-O139 in Bangladesh

KEY PERSONNEL (List names of all investigators including PI and their respective specialties)

	Professional Discipline/ Specialty	Role in the Project
M. Ansaruzzaman	Microbiology	Principal Investigator
Dr. G.B. Nair	Microbiology	Co -Investigator
Dr. M. Sirajul Islam	Microbiology	Co-Investigator
Dr. Kaisar Ali Talukdar	Molecular Biology	Co-Investigator
Mr. Khorsahed Alam	Microbiology	Co-Investigator
Dr. ASG Faruque	Epidemiology	Co-Investigator
A.k. Siddique	Epidemiology	Co-Investigator
Dr. Inger Kuhn	Microbiology	Co-Investigator
Prof. R. Bradley Sack	Geographical Medicine	Consultant

DESCRIPTION OF THE RESEARCH PROJECT

Hypothesis to be tested:

Concisely list in order, in the space provided, the hypothesis to be tested and the Specific Aims of the proposed study. Provide the scientific basis of the hypothesis, critically examining the observations leading to the formulation of the hypothesis.

Hypothesis:

There is increasing evidence that some strains of *V. cholerae* belonging to serogroups other than O1 and O139 have the capacity to cause diarrhoea in humans of severity very similar to that of cholera. Investigations elsewhere have shown that such serogroups do not possess any of the recognized virulence attributes of the toxigenic *V. cholerae* O1 and O139 and most if not all of them do not produce cholera toxin. These non-O1 non-O139 serogroups are therefore able to cause acute secretory diarrhoea especially in children but in whom the mechanism of pathogenesis remains unknown. Therefore, the non-O1 non-O139 strains of *V. cholerae* capable of causing diarrhoea which requires hospitalization cause the diarrhoea by a hitherto unknown mechanism. This study is, therefore, directed to understand and study such strains both at the phenotypic and genotypic level to find out what may be the attribute responsible to cause this kind of secretory diarrhoea or whether only certain genotypes may be responsible for clinical disease.

Specific Aims:

Describe the specific aims of the proposed study. State the specific parameters, biological functions/ rates/ processes that will be assessed by specific methods (TYPE WITHIN LIMITS).

1. To isolate *V. cholerae* belonging to serogroup other than O1 and O139 from clinical cases and from healthy controls and from aquatic environs in Bangladesh during a year-long survey.
2. To serotype the strain of *V. cholerae* non O1 non O139 using the international serotyping scheme for *V. cholerae* and also phenotypically fingerprint the strains by PhP typing system.
3. To examine these strains for all genes comprising the CTXϕ, VPIϕ, hemolysin, HA/P and Nag-ST.
4. To examine selected clinical strains devoid of previously identified virulence genes of toxigenic *V. cholerae* by RITARD and infant mouse cholera model.
5. To examine the same set of selected strains for adherence using Hep-2 and HeLa cells and for actin condensation.
6. Strains exhibiting good adherence will be examined further for invasive potential using polarised CaCo-2 epithelial cells.
7. Strains exhibiting adherence, invasive potential and ability to evoke a positive response in RITARD and a infant mouse model will be fingerprinted by DNA typing methods to examine clonality and relatedness to O1 and O139.

Background of the Project including Preliminary Observations

Describe the relevant background of the proposed study. Discuss the previous related works on the subject by citing specific references. Describe logically how the present hypothesis is supported by the relevant background observations including any preliminary results that may be available. Critically analyze available knowledge in the field of the proposed study and discuss the questions and gaps in the knowledge that need to be fulfilled to achieve the proposed goals. Provide scientific validity of the hypothesis on the basis of background information. If there is no sufficient information on the subject, indicate the need to develop new knowledge. Also include the **significance and rationale** of the proposed work by specifically discussing how these accomplishments will bring benefit to human health in relation to biomedical, social, and environmental perspectives. **(DO NOT EXCEED 5 PAGES, USE CONTINUATION SHEETS).**

BACKGROUND

Role of *Vibrio cholerae* non O1 in diarrhoea

Previous epidemics and pandemics of cholera have been associated with *V.cholerae* strains belonging to the somatic O1 serogroup of either the classical or ElTor biotypes. The non-O1 serogroups of *V.cholerae* are ubiquitous members of the heterotrophic microflora of aquatic environs (Nair *et al.*, 1987; 1988; Ghosh *et al.*, 1994) and are recognized as a cause of sporadic diarrhoea and localized outbreaks of diarrhoeal illness (Aldova *et al.*, 1968). A case control study with a total of 611 adults and 1971 childrens presenting to selected health facilities for diarrhea in Somali regions of Mogadishu during 1983-1985 and recruited 984 children as clinic and community controls were screened for *V.cholerae* O1 and non O1 and other enteric pathogens. No infections with *V.cholerae* O1 were detected, but *V.cholerae* non- O1 was identified in 5.5% of persons with diarrhea(26 of 611 [4.3%] of the adults and 116 of 1971 [5.9%] of the children) and in 0.4%(4 of 984) of the control children(5.9% versus 0.4%; $p < 0.001$)[Anna Coppo et al. ,1995]. *V.cholerae* non O1 is also associated, though rarely, with extraintestinal infections like cellulitis (Gelbart *et al.* 1986), wound infections (Hughes *et al.* 1978) and septicemia (Safrin *et al.* 1988). In October 1992, however a dramatic shift in the etiology of cholera was observed in the Indian subcontinent with strains belonging to a novel serogroup, classified as O139 Bengal, being associated with large outbreaks of cholera (Ramamurthy *et al.* 1993; Albert *et al.* 1993). Multiple virulence factors with different combinations have been reported to play an important role in the disease process caused by *V.cholerae* non-O1 (Ramamurthy *et al.* 1993). Among others, the known virulence factor of V.cholerae includes mannose sensitive haemagglutinin, cholera toxin, heat stable enterotoxin (NAG-ST), toxin corregulated pili, haemolysin, cytotoxin, zonula occludens toxin, accessory cholera enterotoxin, lipopolysaccharide and major outer membrane proteins.

Putative virulence factors of *V.cholerae* non-O1

A variety of virulence traits of *Vibrio cholerae* non-O1 have been recognized and are known to be associated with diarrhoea among humans. These virulence factors include cholera toxin-like enterotoxin, heat-stable enterotoxin (NAG-ST), cytotoxin, zonula occludens toxin (ZOT), ElTor-like haemolysin, thermostable direct haemolysin (TDH)-like hemolysin, Shiga-like toxin, various cell associated hemagglutinins, other ADP ribosylating toxin(s), lipopolysaccharide (LPS) and major outer membrane protein(s). The role of some of these factors has not yet been clearly established.

Enterotoxins

V.cholerae non-O1 strains produce a variety of enterotoxins that have deleterious effect on eukaryotic cells. Among them cholera toxin (CT) is the most important enterotoxin but strains deleted of genes encoding CT can still cause mild to moderate diarrhoea (Levine *et al.*, 1988). This led to the search for newer enterotoxic factors responsible for the cause of residual diarrhoea after ingestion of strains which were incapable of producing CT (Kaper *et al.*, 1994). Zonula occludens toxin and accessory cholera enterotoxin were subsequently discovered although their role in human disease still remains to be elucidated.

Cholera toxin-like enterotoxin

Some strains of *V.cholerae* non-O1 non O139 produce a heat-labile toxin that is similar to cholera toxin of *V.cholerae* O1 (Craig *et al.*, 1981; Yamamoto *et al.*, 1982). This toxin is indistinguishable from CT produced by the classical prototype strain 569B and is biologically, immunologically and genetically identical. The non-O1 *V.cholerae* strains are also known to produce a CT-like enterotoxin which is similar to prototype CT but not identical (Ohashi *et al.*,1972; Yamamoto *et al.*,1982). The CT like enterotoxin from *V.cholerae* non-O1 strain was not completely neutralized by anti cholera enterotoxin from *V.cholerae* O1. CT consists of one molecule of A subunit and five molecules of B subunit (Gill, 1976). B subunit of non-O1 CT-like toxin did not migrate as one would expect of a pentamer and this may be due to the presence of larger aggregates of B subunit in the non O1 CT-like toxin (Yamamoto *et al.*, 1983). Genetic studies also suggest that the DNA sequence encoding enterotoxin of *V. cholerae* non O1 strain is not identical to the sequence encoding CT gene of *V.cholerae* O1 strains (Kaper *et al.* (1982; Yamamoto *et al.*, 1983).

Heat-stable enterotoxin (NAG-ST)

The heat-stable enterotoxin of *V.cholerae* non-O1 (NAG-ST) has recently been described as a virulence factor of *V.cholerae* non-O1. NAG-ST has been purified to homogeneity (Arita *et al.* 1986) and belongs to the methanol soluble infant mouse-active class of stable toxins currently designated as STa or ST1 (Burgess *et al.*, 1978). The biological and immunological properties and amino acid composition of NAG-ST (Arita *et al.*, 1986) are remarkably similar to those of the STas of enterotoxigenic *E. coli* (STh and STp), *Yersinia enterocolitica* (Y-ST) (Yoshimura *et al.*, 1986; Takao *et al.*, 1985) and *Citrobacter freundii*, *Klebsiella pneumoniae* (Guarino *et al.*, 1989a, 1989b). A recent human volunteer study has clearly shown that NAG-ST plays a role in the pathogenesis of non-O1 *V.cholerae* gastroenteritis (Morris *et al.*, 1990)

Zonula occludens toxin

V.cholerae O1, O139 and non-O1 non-O139 produce another enterotoxin, which causes a decrease in tissue resistance in Ussing chambers (Fasano et.*al.*1991). The toxin was discovered by examining *ctx*A deleted *V.cholerae* vaccine strains which caused residual diarrhoea in human volunteers (Kaper et. *al.* 1995). ZOT may cause secretory diarrhoea by increasing the permeability of the intestinal mucosa. Baudry *et al.* (1992) cloned and sequenced the gene encoding *zot* and observed that this gene is located immediately upstream of the *ctxA* gene. A synergistic role of ZOT and CT in the causation of acute dehydrating diarrhoea has been suggested (Johnson *et al.*, 1993; Karasawa et *al.*, 1993). *V. mimicus* and *V.cholerae* non O1 serogroup O139 has also been reported to possess the *zot* gene and since the ecology and environmental distribution of both these species are very similar, interspecies transmission of the *zot* gene in the environment has been postulated (Chowdhury *et al.*, 1994).

Accessory cholera enterotoxin

Another potential enterotoxin, accessory cholera enterotoxin of *V.cholerae* has been identified by Trucksis *et al.* (1993).They also reported that the gene product of an open reading frame located immediately upstream of *zot* can increase short circuit current in Ussing chambers. Like CT, and in contrast to ZOT, this new toxin increases potential differences (PD) rather than tissue conductivity.

Shiga-like toxin

V.cholerae O1 and *V.cholerae* non-O1 (O'Brien *et al.*, 1984) have been reported to produce a shiga-like toxin which was identified on the basis of cytotoxicity to HeLa cells. The above workers also reported that this toxin could be neutralized by antibody raised against Shiga toxin purified from *Shigella dysenteriae* 1.

Thermostable direct haemolysin (TDH)

Among others, *V.parahaemolyticus* is known to produce a hemolysin called the thermostable direct hemolysin (TDH), which is reportedly the cause of enterotoxin activity (Nishibuchi *et al.*, 1992). TDH has shown to cross-react with *V.cholerae* Non-O1 haemolysin (Yoh *et al.*, 1986). Honda *et al.* (1986) reported that genes encoding a TDH-like toxin have been found on a plasmid in some strains of *V.cholerae* non O1 .

Haemagglutinins

Early works involving attachment of *V.cholerae* to erythrocytes (RBCs) and rabbit intestine (Bales *et al.* 1961) suggested that *V.cholerae* haemagglutinins may serve as possible mediators of attachment to intestine epithelial cell surface receptors. A large variety of haemagglutinins have been described in *V.cholerae* O1 which is both soluble and cell-associated. Among them, cell associated haemagglutinins have been implicated as possible virulence factors for *V.cholerae* non-O1 (Booth *et al.*, 1986).

Other components that contribute to pathogenesis

The outer membrane of *V.cholerae* is likely to play a role in the pathogenesis as it is located in contact but just external to the membrane. It includes lipids extending beyond the outer membrane that are thought to be instrumental in the pathogenesis of infection. The involvement of TcpA of the *V.cholerae* O1 classical biotype strain, a subunit of a 20.5 KDa protein in intestinal colonization process has been well documented (Herrington *et. al.*, 1988). *V.cholerae* non-O1, on the other hand, usually does not express TCP as shown by TCP-specific DNA probe (Taylor *et. al.*(1988). Recently, Sengupta *et al.* (1992) demonstrated the expression of pilus structure in diarrhoeagenic non O1 *V.cholerae* pili and termed the pili as `non cholera vibrio pili' (NCVP) which were shown to be composed of 20 KDa subunit protein antigenically unrelated to TcpA (Sengupta *et al.*, 1993). *V.cholerae* serogroup O139 pili were composed of 20 KDa subunit protein which were antigenically related to 20 KDa TcpA pilus protein (Sengupta *et al.*, 1993). These pili may be involved in the intestinal colonization process and therefore may contribute to the virulence of the O139 epidemic isolates.

Pili or fimbrial structures:

Both the factors *i.e.*, colonization of small intestine by *V.cholerae* strains as well as expression of a potent enterotoxin are responsible for the cause of diarrhoea. The process of colonization appears to be essential for pathogenesis, as strains without colonization ability are unable to cause the disease (Levine *et al.*, 1983).

Animal model studies have implicated several components of *V.cholerae* surface in attachment and colonization process *viz.*, lipopolysaccharide (Chitinis *et al.*, 1982), the flagellum (Attridge *et al.*, 1983; Sinha *et al.*, 1993), a 25 KDa outer membrane protein (OMP) (Manning *et al.*, 1982), OMPS (Sears *et al.*, 1984) and haemagglutinins (Hanne *et al.*, 1982). Taylor *et al.* (1987) showed that *V.cholerae* Ogawa strain 395 expressed toxin co-regulated pilli (TcpA) which was required for colonization in a mouse model. Subsequently, Mekalanos *et al.* (1983), showed that an isogenic mutant of *V.cholerae* 395 N1 lacking the TcpA, designated TCP2 was unable to colonize volunteers. Expression of TcpA, cholera toxin and other proteins is coordinately regulated by ToxR. Other factors which may be involved in mucosal adhesion of *V.cholerae* are known as accessory colonization factors (acf) (Sun *et al.*, 1990 a,b).

Lipopolysaccharide (LPS):

The lipopolysaccharide of *V.cholerae* induces a strong antibacterial immunity in the host and anti-LPS antibodies inhibit the intestinal colonization of vibrios (Levine *et al.*, 1979). *V.cholerae* LPS resembles that of other Gram negative organisms in having a lipid A moiety and an O- antigenic polysaccharide. In most other Gram negative LPS, these components are linked by means of 2 Keto-3 deoxyoctonate (KDO) acid labile core sugar. *V.cholerae* LPS lacks this core sugar. Rather it possesses the acid labile sugar fructose, which may serve the same linkage function as 2-Keto-3 deoxyoctonate. Serum, intestinal or milk secretory immunoglobulin A (sIgA) antibody responses can be shown by enzyme linked immunosorbent assay (ELISA) against purified LPS antigen (Majumder *et al.*, 1981 a,b). Serum vibriocidal antibody level rose significantly in more than 90% of North American volunteers who participated in cholera challenges (Levine *et. al.*, 1983).

Outer membrane proteins:

The outer membrane of Gram-negative bacteria is a highly specialized structure that lies outside the cytoplasmic membrane and peptidoglycan layer forming the intersurface between the cell and its external environment (Osborn *et. al.*,1972). Outer membrane proteins of *V.cholerae* play an important role in the protective immunity in host through inhibition of intestinal colonization (Kabir, 1980; Sengupta *et al.*, 1992; Sears *et al.*, 1984; Richardson *et al.*, 1989). The outer membrane of *V.cholerae* is composed of limited number of protein moieties which are protective antigens because it presumably comes in contact with the small bowel mucosa (Eubanks *et al.*, 1977, Sears *et al.*, 1984). Kabir (1980) and Kelly (1981) observed that the major protein has a molecular weight ranging between 40 and 45 KDa and is common to both biotypes and serotypes of *V.cholerae*. The major outer membrane proteins (MOMPs) of *V.cholerae* on an individual basis are responsible for induction of intestinal colonization (Sengupta *et al.*, 1992). This would explain the observation where infection with one serotype confers solid immunity against later challenge with the heterologous serotype (Levine *et al.*, 1979, 1983). The expression of outer membrane protein is controlled by the ToxR gene product, ompU which encodes a 38 KDa outer membrane, which is positively regulated by ToxR while ompT encoding a 40 KDa outer membrane protein, is negatively regulated by ToxR (Taylor *et al.*, 1987; Miller and Mekalonos, 1988).

Research Design and Methods

Describe in detail the methods and procedures that will be used to accomplish the objectives and specific aims of the project. Discuss the alternative methods that are available and justify the use of the method proposed in the study. Justify the scientific validity of the methodological approach (biomedical, social, or environmental) as an investigation tool to achieve the specific aims. Discuss the limitations and difficulties of the proposed procedures and sufficiently justify the use of them. Discuss the ethical issues related to biomedical and social research for employing special procedures, such as invasive procedures in sick children, use of isotopes or any other hazardous materials, or social questionnaires relating to individual privacy. Point out safety procedures to be observed for protection of individuals during any situations or materials that may be injurious to human health. The methodology section should be sufficiently descriptive to allow the reviewers to make valid and unambiguous assessment of the project. **(DO NOT EXCEED TEN PAGES, USE CONTINUATION SHEETS).**

Research Design and Methods

WORK PLAN

Dhaka hospital surveillance study

ICDDR,B hospital situated in Dhaka treats approximately 100,000 patients a year for diarrhea. Of these, a 2% systematic sample (every 50th patient) is studied in-depth including detection for most of the diarrhoeal pathogens. The diarrhoeal pathogens studied include: *Salmonella* spp., *Shigella* spp., *V.cholerae*, *Campylobacter* spp., *Aeromonas* spp., *Plesiomonas shigelloides*, diarrhoeagenic *Escherichia coli* (enterotoxigenic, enteropathogenic, enteroinvasive, enterohaemorrhagic, enteroaggregative and diffuse adherent), rotavirus, *Cryptosporidium parvum*, *Giardia lamblia* and *Entamoeba histolytica*. Most of the surveillance patients are children below 5 years of age.

Sentinel surveillance

Four sentinel surveillance centres are set up in the north, centre, south and western parts of Bangladesh to study cholera. Each centre has a population of approximately 200,000, and is served by a primary health care clinic. Physicians will visit the surveillance centres every two weeks for three successive days and study all cases of diarrhoea. Rectal swabs are sampled in these studies and this is also an ongoing study. Plates from such samples will also be used in our study to isolate the non O1 non O139 *V. cholerae*.

Environmental surveillance

Surface water, sediment, zooplankton and phytoplankton are being collected and sampled every two weeks from two selected ponds and two river sites from each surveillance area in another protocol. These water sources are used by the population for washing, bathing and also for cooking. The TCBS plates from these ongoing studies will be used to pick up environmental strains of *V. cholerae* non o1 non O139.

Laboratory methods

Identification of *V.cholerae* non O1 non O139

Oxidase positive colonies will be picked and identified as *V.cholerae* by standard methods (WHO 1989). The procedure includes biochemical reactions in motility-indole-urease medium and Kligler's iron agar and slide agglutination with polyvalent antisera of O1 and O139. *V. cholerae* which produces negative agglutination with *V.cholerae* polyvalent antisera will be included in this study as *V.cholerae* non O1, O139

and stored at –70°C in Luria broth (Gibco, NY, USA) with 25% glycerol until further study. The same procedure will be used to pick and identify *V. cholerae* non-O1 non-O139 from sentinel surveillance samples and from environmental samples.

Estimated number of isolates available for the study : We plan to collect *V.cholerae* nonO1, O139 isolates during a two years period from the ICDDR,B surveillance patient as well as neighborhood control children and one year samples from environmental sources.

Dhaka hospital surveillance: From an estimated 4000 patients, 3200 will be paediatric patients. At a 2% isolation rate, 3200 pediatric patients will yield 64 isolates. During two years period the total number will be 128 isolates.

Neighborhood control children : The isolation rate of *V.cholerae* non O1,O139 from age matched control children are less than 0. 1%(Recent case control study conducted at ICDDR,B for *Aeromonas Spp.*, Unpublished data). So during this two years period only a few isolates (Approx 20 strains)may be available from Approx. 1600 control samples.

Field sentinel surveillance patients:

At a 2% isolation rate, 1600 patients from all four surveillance sites will yield 32 isolates.

Environmental isolates:

At least one sample each of surface water, sediment, phytoplankton and zooplankton from each of 2 ponds and 2 rivers from 4 sentinel sites samples, 26 times a year, will yield 1248 samples. A previous study has shown that isolation of *V.cholerae* NonO1, O139 from environment is as high as 25% (Islam *et al.*, 1992). At this isolation rate, 1248 samples will yield 312 isolates. Since the number of environmental isolates is disproportionately higher compared to human isolates, all but every third isolate can be selected for specific molecular study. All the isolates will be stored in Luria broth with 15% glycerol at -70oC until studied.

Serogrouping: *V.cholerae* strains which do not agglutinate with O1 and O139 antisera will be assumed to belong to the group non O1 non O139. These strains will be further serogrouped by O-antigen serogrouping scheme at the National Institute of Cholera and Enteric Diseases where the entire serotyping antisera for non O1 and non O139 is available.

PhP typing: *V. cholerae* non O1 non O139 isolates stock will be subcultured onto Gelatin agar (GA), and colonies transferred into 10 ml protease peptone broth contained 0.1% bromothymol blue and the mixture is then dispensed into the automated PhP biochemical fingerprinting plates, which are read kinetically as described earlier (Mollby et al., 1993). According to the data obtained from the PhP system, the isolates will be subdivided into phenotypes, and isolates representing relevant phenotypes can be selected for further studies. The diversity of bacteria belonging to the relevant groups are calculated (Bianchi ., 1982; Atlas ., 1984; Khun., 1991) and compared between bacterial populations from environmental samples, healthy controls and patients with diarrhoea (Ansaruzzaman et al., 1996, 2000, Khun et al., 1997). A lower diversity among bacteria from patients with diarrhoea than among those isolated from other sources is a first indication of the predominance of certain clones, and isolates representing PhP types predominating among patients will be carefully studied. The method that gives maximum discrimination between strains will be chosen subsequently to subtype PhP types of interest.

Experimental cholera in infant mice

Infant mice can developed fetal infection by oral administration of Vibrios (Ujiiye A. et al. 1968) and this model can be used to demonstrate passive protection by specific antibodies (Ujiiye A et al. 1970). The experiment is carried out by separating young mice (5 to 6 days old) from their mothers and put in a boxes lined with tissue papers for 2-3 h before they were challenged; the challenge dose is given through a round tipped 22 gauze needle connected with a tuberculin syringe. The needle is inserted into the lower esophagus via the month. The mice were returned to the boxes after challenge. They are observed and sacrificed at various periods up to 48 hr after infection, unless they are dead before that time.

RITARD assay

The ability of bacteria to cause diarrhea will be tested in rabbits with removable intestinal ties (removable intestinal tie adult rabbit diarrhea (RITARD) model; rabbits used for this assay are referred to herein as RITARD rabbits) (Spira et al 1981). Abdominal surgery will be performed after rabbits are fasted for 24h, and a permanent tie is introduced in the caecum close to the ileocecal junction. A temporary tie is introduced in the terminal part of the small intestine above the mesoappendix. Aliquots (10 ml) of overnight shaker cultures of bacteria grown in TSBY at 37°C (each aliquot contained 10^{10} bacteria) or bacterium-free culture filtrates will be injected into the anterior part of the jejunum. The temporary tie is removed 2 h later. Control experiments are performed on four rabbits with *E. coli* K-12EC101 whole culture. Unless stated otherwise, the animals will be observed for 7 days for diarrhea, other symptoms, and death. Rectal swabs are taken daily and plated onto TTG agar to monitor shedding of the challenge bacteria. The animals are sacrificed at various periods after challenge when they develop diarrhea or hind limb paralysis. All animals, including control animals, that do not develop any symptom (see results below) are sacrificed at the end of day 7. At autopsy, the intestines are examined for fluid accumulation and other gross pathological changes. Intestinal sections are taken from the mid-jejunum, upper and loser ileum, caecum, proxlymph nodes. Sections are fixed in buffered formal saline for histology . Mucosal scrapings of the mid jejunum and upper and lower ileum are cultured quantitatively. Serial 10-fold dilutions of homogenized tissue will be prepared in sterile physiological saline, plated on TTGA agar, and incubated at 37oC for 24h.

Cell culture adherence

Adherence to HEp-2 cells will be tested by the method of (Cravioto et al. 1979) with some modifications. Stationary and shaker cultures (4 and 20 h) in Luria broth incubated at 37°C are used. Bacteria will be incubated with HEp-2 cell monolayers at 37°C for 3 h in the presence and absence of 0.5% D-mannose. After the monolayers will be washed to remove nonadherent bacteria, they are fixed in 70% methanol and stained with Giemsa stain. A localized adherence-positive serotype O127:H6 enteropathogenic E. coli (EPEC) strain will be included as a positive control in the adherence assays.

Fluorescent actin staining test for attachment-effacement lesions

The proxy test for attachment effacement lesions in the intestine will be performed with HEp-2 cell monolayers in 3 and 6h assays as described previously (Knutton et al 1991). The localized adherence-positive EPEC serotype 0127:H6 strain and the invasive S. flexneri 2a 611R strain will be included as controls.

Invasion of epithelial cells

The assay will be carried out using the human colonic epithelial cell line, Caco-2, in normal monolayer as well as in polarized monolayer in transwell filters for translocation. In the normal monolayer assay, a standard dose of bacteria will be incubated with the monolayer. After the infection period, the monolayer will be incubated in presence of gentamicin, cells will be fixed with glutaraldehyde in cacodylate buffer, stained with uranyl acetate, and examined by transmission electron microscope for intracellular bacteria. The effect of metabolic inhibitors on the invasion of Caco-2 cells by *V. cholerae* non O1,O139 will be examined as described previously in the quantitative cell culture assay containing gentamicin. The inhibitors that will be used are: cytochalasin D, chloroquine, ammonium chloride, dansylcadaverine, and colchicine.

Translocation of bacteria across polarized monolayer of Caco-2 cells will be carried out as described previously (Konkel et al 1992). Transwell filter units containing porous membranes will be seeded with Caco-2 cells and incubated in tissue culture medium for 10-14 days until a complete polarized monolayer is formed. Bacteria will be inoculated into apical layer. Translocation will be monitored by culturing the basolateral medium quantitatively periodically over a period.

Molecular Analysis

PCR assays for virulence genes.

PCR assays will be performed to test for the presence of the species specific *toxR* gene and also to screen for other virulence genes of *V.cholerae*, using specific primers. Amplification will be carried out using 25 μl volumes containing 2.5 μl of 10X PCR amplification buffer (500 mM KCl; 100 mM Tris-HCl, pH 9.0; 0.1% triton-X, 150 mM $MgCl_2$), 2 μl of dNTP (2.5 mM each); 2 μl (10 pmole/μl) of each of the primers, 0.2 μl (5 U/ml) of rTaq DNA polymerase (Takara Shuzo, Tokyo, Japan) 11 μl of sterile triple distilled water and 5.3 μl of template DNA. Template DNA will be prepared by growing the bacteria in Luria broth (Difco, Detroit, USA) with 3% NaCl at 37 C overnight, centrifuging the culture, resuspending the pellet in 500 μl of sterile distilled water, and boiling for 10 min. The cycling conditions include a preincubation at 94°C for 5 min, followed by a middle step of 30 cycles at 94°C for 1 min 30 sec, 50°C for 1 min 30 sec, 72°C for 1 min 30 sec and a final extension of 72°C for 10 min using an automated thermal cycler (Biometra, Gottingen, Germany). Amplified products were electrophoresed in a 1% agarose gel (SRL, India), stained with ethidium bromide (Sigma, St. Louis, MO, USA), visualized and documented using a video documentation system (Pharmacia Biotech, Uppsala, Sweden).

Purification of genomic DNA

A modification of the method of Murray and Thompson (13) will be employed for the extraction of chromosomal DNA. Briefly, cells from 18-h culture in LB broth, Miller (Difco) with 3% NaCl will be harvested by centrifugation at 6000 x g for 5 min. The pelleted cells will be resuspended in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0), treated with 10% SDS and proteinase K and incubated for 1 h at 37°C. After incubation, CTAB/NaCl (10% cetyl trimethyl ammonium bromide in 0.7 M NaCl) will be added and incubated at 65°C for 10 min. The aqueous phase will be then treated with phenol-chloroform and the DNA pelleted and washed with 70% ethanol. Purified DNA will be suspended in TE buffer and treated with RNase at 37°C for 30 min.

Ribotyping

Restriction enzyme *BglI* (Boehringer Manheim Gmbh, Manheim, Germany) will be used for ribotyping of the *V. cholerae* strains. The genomic fragments will be electrophoresed on a 1% Seakem agarose gel (FMC Bioproducts, USA) using TAE (pH 8.0) buffer. For southern blotting, the gel will be treated successively in 0.25N HCl for 10 min to allow partial depurination and cleavage of large fragments, in denaturation solution composed of 0.5M NaOH, 1.5M NaCl for 30 min and in 0.5M Tris-HCl (pH 7.4) and 1.5M NaCl for 30 min. DNA will be then transferred to Hybond N^+ membrane (Amersham International PLC, Buckinghamshire, England), using 20X SSC by vacuum blotter (Pharmacia, Sweden). The membrane will be then washed with 20X SSC and dried at room temperature followed by fixation in UV cross-linker (Bio-Rad, USA). A 7.5 kb *Bam*HI fragment of the recombinant plasmid pKK3535 containing an rRNA operon of *Escherichia coli* (14) will be used as the *rrn* gene probe for ribotyping. Labeling of the probes, hybridization conditions, washing conditions of filters and detection of bands will be performed using ECL detection system (Amersham Life Science, UK).

DNA extraction and digestion for PFGE

The test strains grown on LB agar, Miller (Difco) with 3% NaCl will be transferred to 3 ml LB broth, Miller (Difco) with 3% NaCl and cultured overnight at 37°C with shaking at 100 rpm. 100 μl of the overnight culture will then be transferred to 8 ml of LB broth, and incubated at 37°C with shaking at 100 rpm till the culture attained an O.D. value of 0.9 at 600 nm. Bacterial cells will be harvested from 1 ml of the culture by centrifugation and resuspended in 0.5 ml cell lysis buffer (10 mM Tris-HCl [pH7.2], 20 mM NaCl, 50 mM EDTA). Agarose plugs will be prepared by mixing equal volumes of bacterial suspension with 2% low-melting agarose. The bacterial cells in the agarose plugs will be lysed by treatment with lysis solution (1 mg of lysozyme per ml, 0.4% N-sodium lauryl sarcosine, 0.2% Na-deoxycholate, 10 mM Tris [pH 7.2], 50 mM NaCl) at 37°C overnight and then treated with proteinase K at 50°C overnight. The plugs will then be washed with washing buffer containing 20 mM Tris HCl [pH 8.0] and 50 mM EDTA. Agarose plugs containing genomic DNA will be equilibrated in enzyme buffer for 1 h at room temperature and will then be cleaved in 600 μl of enzyme buffer H (500 mM Tris-HCl [pH7.5], 100 mM $MgCl_2$, 10 mM DTT, 100 mM NaCl) containing 50U of *Not*I enzyme at 37°C overnight.

PFGE

PFGE of *Not*I-digested inserts will be performed on 1% agarose (Biorad,USA, Richmond, California, USA) by the contour-clamped homogenous electric field method on a CHEF Mapper system (Biorad,USA) in 0.5X TBE buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1.0 mM EDTA, pH 8.0) for 40 h 34 min. A DNA size standard (Bacteriophage λ -ladder, Bio-Rad, USA) will be used as the molecular mass standard, and a minichiller (Bio-Rad, USA) was used to maintain the temperature of the buffer at 14 C. Run conditions will be generated by the auto algorithm mode of the CHEF Mapper PFGE system by using a size range of 20 to 300 kb. After electrophoresis, the gel will be stained in ethidium bromide (1 μg/ml) for 30 min and will be destained in water for 15 min twice. The DNA bands will be visualized and photographed using the Gel Doc 2000 (Biorad,USA) (8).

Facilities Available

Describe the availability of physical facilities at the place where the study will be carried out. For clinical and laboratory-based studies, indicate the provision of hospital and other types of patient's care facilities and adequate laboratory support. Point out the laboratory facilities and major equipments that will be required for the study. For field studies, describe the field area including its size, population, and means of communications. **(TYPE WITHIN THE PROVIDED SPACE).**

This is a laboratory based study which will be carried out with clinical and environmental isolates. The project will require tissue culture facilities, small laboratory animals' molecular biological facilities and biochemical fingerprinting. Most of them are available in ICDDR,B and other in Karolinska Institute.

Data Analysis

Describe plans for data analysis. Indicate whether data will be analyzed by the investigators themselves or by other professionals. Specify what statistical softwares packages will be used and if the study is blinded, when the code will be opened. For clinical trials, indicate if interim data analysis will be required to monitor further progress of the study. **(TYPE WITHIN THE PROVIDED SPACE).**

The data will be analyzed using conventional procedures. Where a test of significance is required, it will done using defined statistical programme. All data on virulence genes and on serogroups of the V. cholerae non O1 non O139 will be meticulously kept.

Ethical Assurance for Protection of Human Rights

Describe in the space provided the justifications for conducting this research in human subjects. If the study needs observations on sick individuals, provide sufficient reasons for using them. Indicate how subject's rights are protected cited and if there is any benefit or risk to each subject of the study.

Only stool samples from diarrhoeal patients will be required for this study so no direct involvement of human subject.

Use of Animals

Describe in the space provided the type and species of animal that will be used in the study. Justify with reasons use of particular animal species in the experiment and the compliance of the animal ethical guidelines for conducting the proposed procedures.

Standard animal assays such as adult rabbit RITARD model and suckling mouse assay will be used to investigate the diarrhoeagenic potential of bacterial isolates. However, standard animal ethical guidelines will be abided to perform these studies and it will be ensured that the animals will not be allowed to suffer.

Literature Cited

Identify all cited references to published literature in the text by number in parentheses. List all cited references sequentially as they appear in the text. For unpublished references, provide complete information in the text and do not include them in the list of Literature Cited. There is no page limit for this section, however exercise judgment in assessing the "standard" length.

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Dissemination and Use of Findings

Describe explicitly the plans for disseminating the accomplished results. Describe what type of publication is anticipated: working papers, internal (institutional) publication, international publications, international conferences and agencies, workshops etc. Mention if the project is linked to the Government of Bangladesh through a training programme.

The finding will be presented in Divisional Scientific forum and Annual Scientific conference of ICDDR,B and published in peer reviewed international journals.

Collaborative Arrangements

Describe briefly if this study involves any scientific, administrative, fiscal, or programmatic arrangements with other national or international organizations or individuals. Indicate the nature and extent of collaboration and include a letter of agreement between the applicant or his/her organization and the collaborating organization. (DO NOT EXCEEDONE PAGE)

The only collaboration which we will be having in this study will be with the National Institute of Cholera and Enteric Diseases, Calcutta, India who will perform the serotyping of our strains of V. cholerae non O1 non O139. The collaboration with MTC, karolinska Institute, Sweden will be involved only for data analysis and suppling of PhP typing plates.

Biography of the Investigators

Give biographical data in the following table for key personnel including the Principal Investigator. Use a photocopy of this page for each investigator.

Name M. Ansaruzzaman	Position:Asstt. Scientist	Date of Birth: 25/08/1952

Academic Qualifications (Begin with baccalaureate or other initial professional education)

Institution & Location	Degree	Year	Field of Study
Dhaka University	B.Sc (Hons)	1975	Biochemistry
Dhaka University	M.Sc	1981	Biochemistry

PUBLICATIONS

1. Hossain A, Haider K, Huq MI, **Ansaruzzaman M.** Studies on the factors affecting the hemolysin production of Vibrio mimicus isolated from clinical and environmental sources. Trans roy Soc Trop Med Hyg. 1988; 82:337-339.

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23. Albert MJ, Kabir I, Azim T, Hossain MA, **Ansaruzzaman M**, Unicomb L Diarrhoea associated with Cyclospora sp. in Bangladesh. Diagn Microbiol Infect Dis. 1994; 19:47-49.

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polysaccharide from Vibrio mimicus N-1990. Eur J Biochem 1997; 251:986-990.

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41. **Ansaruzzaman M**, Albert MJ, Nahar S, Byun R, Katouli M, Kuhn I, Mollby R. 2000. Clonal groups of enteropathogenic Escherichia coli isolated in case control studies in Bangladesh. J Med Microbiol. 49: 177-85.

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43. Ahmed F, **Ansaruzzaman M**, Haque E, Rao MR, Clemens JD. 2001. Epidemiology of post-Shigellosis persistent diarrhea in young children. Pediatric Infect Dis J. (in press)

Principal Investigator: Ansaruzzaman, M. _____

Research and Professional Experience

Concluding with the present position, list, in chronological order, previous positions held, experience, and honours. Indicate current membership on any professional societies or public committees. List, in, chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. **(DO NOT EXCEED TWO PAGES, USE CONTINUATION SHEETS).**

Assistant Scientist, Enteric Bacteriology Laboratory, Laboratory Sciences Division from April 1993 onward.

In June 1993, I was promoted to Assistant Scientist on the recommendation of the Scientific Ranking Committee and now I am working in Enteric Bacteriology Laboratory. Recently we have reported some unusual bacteria which produce diarrhoeal disease and characterizing its virulence factor and pathogenesis experiment and other cross-reacting antigen associated with diarrhoea. Set up a number of research technique in Enteric bacteriology lab.

1987 - June 1993: Section Chief, Research Microbiology, Dhaka. I looked after many research protocols and contributed in setting up a variety of new rapid diagnostic tests for various diarrhoeal pathogens.

1984 - 1987: Section Chief, Microbiology Laboratoy, Matlab. Supervised microbiological tests for the Oral Cholera Vaccine Trial. Developed new routine tests and set up the quality control for each of the microbiological tests. Supervised the production of high quality work for more than three hundred thousand (300,000) samples for Vaccine Trial.

1980 - 1984: Research Officer. Supervised and coordinated the environmental microbiology which was a collaborative study with Prof. Rita Colwell of University of Maryland.

Principal responsibilities

To develop and conduct research project on enteric pathogens and participated in other related research programme, working at bench level.

Principal Investigator: Ansaruzzaman, M. _____

Detailed Budget for New Proposal

Project Title: Phenotypic and genotypic analysis of clinical and environmental *Vibrio cholerae* non-O1 non-O139 to identify pathogenic clones and the underlying pathogenic mechanism

Name of PI: M Ansaruzzaman

Protocol Number: Name of Division: Laboratory Sciences Division

Funding Source: Amount Funded (direct): $87312 Total: $ 109140

Overhead (25%) $21828

Starting Date: March 2001 Closing Date: February 2004

Strategic Plan Priority Code(s):

Sl. No	Account Description	Salary Support			US $ Amount Requested		
	Personnel	Position/Grade	Effort%	Salary Rate	1st Yr	2nd Yr	3rd Yr
1.	M. Ansaruzzaman	NO-B	50%	1150	6900	7250	7612
2.	Dr. Kaisar Ali Talukdar	NO-B	5%	1063	638	702	772
3.	Mr. Nurul Amin bhuiyan	GS-V	25%	467	1401	1541	1695
4.	Rabindra Nath Bishwas	GS-I	25%	250	750	825	908
5.	Research technician (New)	GS-IV	100%	269	3228	3550	3905
6.	Research Assistant	GS-III	50%	225	1350	1485	-
	Sub Total				**14267**	**15353**	**14892**
	Local Travel				100	100	100
	International Travel					4000	
	Sub Total				**100**	**4100**	**100**
	Supplies and Materials (Description of Items)						
1.	Bacteriological media				2000	2000	2000
2.	Chemicals and PhP plate				4000	4000	2000
3.	Tissue culture media and plate				1000	1000	1000
4.	Molecular Biology reagent and enzyme				2000	3000	2000
5.	Stock items				800	1000	500
	Sub Total				**9800**	**11000**	**7500**
	Other Contractual Services						
1.	Repair and Maintenance				300	300	300
2.	Printing and Publication				500	500	500
	Sub Total				**800**	**800**	**800**
	Interdepartmental Services						
1.	Research Animals				1000	1000	1000
2.	Staff Clinic charges				600	600	600
3.	Capital expenditure: Computer and accessories				3000	-	-
	Sub Total				**4600**	**1600**	**1600**
	Total Operating Costs				**29567**	**32853**	**24892**
	Overhead (25%)				7392	8213	6223
	GRAND TOTAL				**36959**	**41066**	**31115**

TOTAL DIRECT COST $ 87312
Overhead (25%) $ 21828

S. Moin
30-1-2001
Shamima Moin
Controller, Budget & Costing

Principal Investigator: Ansaruzzaman, M. _____

Budget Justifications

Please provide one page statement justifying the budgeted amount for each major item. Justify use of man power, major equipment, and laboratory services.

1. Salary support is budgeted for personnel who are essential to carry out the work.

1. The materials include bacteriological media and reagents, tissue culture and molecular biology reagents which are essential for this work.

2. Capital expenditure for procurement of computers and accessories will be required for data analysis.

3. The budget for International Travel is needed to visit Karolinska Institute, Sweden for data analysis on biochemical fingerprinting. We need to visit NICED, Calcutta for serotyping *V.cholerae* non O1,O139

Other Support

Describe sources, amount, duration, and grant number of all other research funding currently granted to PI or under consideration. (DO NOT EXCEED ONE PAGE FOR EACH INVESTIGATOR)

2000-20001: Title of project: Characterisation of enterotoxigenic *E. coli* strains isolated from childhood diarrhea in Bangladesh. Funded by: USAID, Role in the project: Principal Investigator

1999-2001: Title: Studies on virulence of *V. cholerae* O139 Bengal. Funded by: SIDA/SAREC, Role in the project: Co-Investigator.

1998-2001 : Title: Nepal antimicrobial resistance surveillance: a technical cooperation project. Funded by: USAID, Co-investigator.

Ethical Assurance: Protection of Human Right:

APPENDIX

International Centre for Diarrhoeal Disease Research, Bangladesh
Voluntary Consent Form

Title of the Research Project: Phenotypic and genotypic analysis of clinical and environmental *Vibrio cholerae* non-O1 non-O139 to identify pathogenic clones and the underlying pathogenic mechanism

Principal Investigator: M Ansaruzzaman

Before recruiting into the study, the study subject must be informed about the objectives, procedures, and potential benefits and risks involved in the study. Details of all procedures must be provided including their risks, utility, duration, frequencies, and severity. All questions of the subject must be answered to his/ her satisfaction, indicating that the participation is purely voluntary. For children, consents must be obtained from their parents or legal guardians. The subject must indicate his/ her acceptance of participation by signing or thumb printing on this form.

Signature of Investigator/ or agents
Date:

Signature of Subject/ Guardian
Date:

Continuation Sheet (Number each sheet consecutively)

Principal Investigator: Ansaruzzaman, M. _____

Check List

After completing the protocol, please check that the following selected items have been included.

1. **Face Sheet Included** ☑
2. **Approval of the Division Director on Face Sheet** ☑
3. **Certification and Signature of PI on Face Sheet, #9 and #10** ☑
4. **Table on Contents** ☑
5. **Project Summary** ☑
6. **Literature Cited** ☑
7. **Biography of Investigators** ☑
8. **Ethical Assurance** NA
9. **Consent Forms** NA
10. **Detailed Budget** ☑

(FACE SHEET) **ETHICAL REVIEW COMMITTEE, ICDDR,B.**

Principal Investigator: M. Ansaruzzaman

Application No.

Title of Study: Phenotypic and genotypic analysis of clinical and environmental Vibrio cholerae non 01 non 0139 identify pathogenic clones and their pathogenic mechanism

Trainee Investigator (if any): _____

Supporting Agency (if Non-ICDDR,B) _____

Project Status: _____

[✓] New Study

[ ] Continuation with change

[ ] No change (do not fill out rest of the form)

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

1. Source of Population: NA
   - (a) Ill subjects — Yes No
   - (b) Non-ill subjects — Yes No
   - (c) Minor or persons under guardianship — Yes No

2. Does the Study Involve:
   - (a) Physical risk to the subjects — Yes (No)
   - (b) Social risk — 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 or other) — (Yes) No
   - (b) Use of fetal tissue or abortus — Yes (No)
   - (c) Use of organs or body fluids — Yes (No)

4. Are Subjects Clearly Informed About: NA
   - (a) Nature and purposes of the study — Yes No
   - (b) Procedures to be followed including alternatives used — Yes No
   - (c) Physical risk — 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: NA
   - (a) From subjects — Yes No
   - (b) From parents or guardian (if subjects are minor) — Yes No

6. Will precautions be taken to protect anonymity of subjects NA — Yes No

7. Check documents being submitted herewith to Committee:
   - ____ Umbrella proposal - Initially submit an with 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. Example 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 Committee for review

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

_____
Principal Investigator

_____
Trainee

Response to reviewer comments:

Reviewer 1

There is no specific suggestion or modification and recommended the project with high score.

Title : Phenotypic and genotypic analysis of clinical and environmental *Vibrio cholerae* non-O1 non-O139 to identify pathologic clones and their pathogenic mechanisms

	Rank Score		
	High	Medium	Low
• Quality of project	✓		
• Adequacy of project design	✓		
• Suitability of methadology	✓		
• Feasibility within time period	✓		
• Appropriateness of budget	✓		
• Potential value of field of knowledge	✓		

Conclusion

☑ I support the application :

- ☑ a) without qualification
- ☐ b) with qualification
  - on technical ground
  - on level of financial support

☐ I do not support the application

Name of Referee : Wanpen Chaicumpa

Signature : ..... Date Jan 6, 2001

Position : Professor of Microbiology and Immunology

Institution : Faculty of Tropical Medicine
Mahidol University
Bangkok 10400, Thailand

Title : Phenotypic and genotypic analysis of clinical and environmental *Vibrio cholerae* non-O1 non-O139 to identify pathologic clones and their pathogenic mechanisms

PI : Dr. M. Ansaruzzaman

Reviewer : The research proposal is scientifically original. It is a laboratory-based study which will be done by the most competent group of microbiologists and molecular biologists at the ICDDR-B, Bangladesh and Karolinska Institute, Sweden, where all needed facilities are available. The group has adequate experiences to master all methadologies so-proposed. The knowledge gained from the research should provide clear pictures on virulence factors and pathogenic mechanisms of the *Vibrio cholerae* non-O1 non-O139. The time requires and the budget so-requested are appropriate. The work should be supported without any qualification.

Wanpen Chaicumpa, D.V.M. (Hons.), Ph.D.
Professor of Microbiology and Immunology
Faculty of Tropical Medicine
Mahidol University, Bangkok,
Thailand

Response to reviewer comments:

Reviewer 2

1. All environmental isolates will be screened by cell culture adherence assays and specific PCR assays. Recommended the project without any other suggestion.

EVALUATION REPORT ON RESEARCH PROJECT

Title: Phenotypic and genotypic analysis of clinical and environmental strains of Vibrio cholerae non O1, non O139 to identify pathogenic clones and their pathogenic mechanisms.

Rank score: High ✓ Medium Low

Quality of the project: Excellent

Adequacy of Project Design: Adequate to meet the objectives of the project. (please see detailed comments)

Suitability of the methodology : The methodology proposed to be used are suitable to yield expected results

Feasibility within time period: Feasible

Appropriateness of budget: Appropriate

Potential value of field of knowledge: The knowledge to be gained from this project will be very useful both from the clinical point of view and from the environmental point of view. The study is expected to throw light on the ecology of diarrhegenic nonO1 non O139 strains of *V. cholerae* and the mechanism by which they cause disease..

Conclusions:

I support the application:

a) without qualification **Yes**

b) with qualification

-on technical grounds

-on level of financial support

I do not support the application

Name of Referee: Dr. I. Karunasagar

Signature  Date 4.1.2001

Position: Professor and Head, Department of Microbiology

University of Agricultural Sciences

College of Fisheries, Mangalore-575002, India

DETAILED COMMENTS:

The project aims at studying whether the non O1, non O139 strains of *Vibrio cholerae* involved in cases of diarrhoea are clonal and understanding the mechanism of their virulence. To achieve this, it is proposed to select clinical isolates that do not posses genes known to be associated with virulence in *V. cholerae*, and study their interaction with epithelial cells and their ability to cause diarrhea in infant mouse model and in removable intestinal tie adult rabbit diarrhea (RITARD) model. Selected environmental strains are also proposed to be studied by these methods and here the strategy proposed is to study every third isolate.

The project design with respect of clinical strains is very good. With respect of environmental strains, it is proposed that one third of the isolates will be screened. However, it is suggested that all isolates be screened by a simple cell culture adhrence assay and those showing adherence may be further tested in other systems such as invasion and fluorescent actin staining. Similarly, PCR can be done on pools of environmental isolates and when positive, individual cultures can be further examined to locate the isolate harbouring the gene of interest.

Overall, the project is excellent and this study is very essential to understand the hazard due to non O1, nonO139 strains of *Vibrio cholerae* which are commonly found in the aquatic environment. I recommend the project strongly.