LSD 2002

International Centre for Diarrhoeal Disease Research, Bangladesh
CENTRE FOR HEALTH AND POPULATION RESEARCH
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Cable : Cholera Dhaka

MEMORANDUM

19February 2002

To : Dr. Shah. M. Faruque
Laboratory Sciences Division

From : Dr. David A. Sack
Chairman, Research Review Committee (RRC)

Sub : Protocol # 2002-002

Thank you for your memo of 19 February 2002 attaching the modified version of your protocol # 2002-002 entitled "**Genetic variants of *Vibrio cholerae* 0139 and development of a yaccine against 0139 cholerae**". The protocol is hereby approved upon your addressing the issues raised by the RRC in its meeting held on 14th February 2002.

Thank you.

cc: Associate Director
Laboratory Sciences Division

ACCOUNTING SHEET OF PHOTOCOPIER

Date	Opening meter	Details of copy(s)	Closing meter	Total # of copies	Budget code	Copy by whome
31.7.2001-4.9.2001	412.678	Epid course handouts, Committee, Staff Dev. papers —	415499	~~3~~ 2821 (35 days)		& Munir, Salam & Anjuman, Bejoy
5-10-2001	415499	Epid Course, ERC+RRC notice, minutes, review papers, approval letters, Modification letters etc	417,659	2,160 (38 days)		Salam, Bejoy, Munir & Anjuman
14-10-2001	~~417702~~ 417659	face sheets + others.	417702	43 pags		~~faceshets~~ Salam
15-10-2001	417702	face sheet (ERC), Staff Dev. papers	417762	60		Salam
21-10-2001	417762	Approval letters to PIs.	417,813	51		Salam
29-10-2001	417,813	ERC papers for chair —	418,300	487		Salam
5-11-2001	418,300	— do —	418,975	675		Salam

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Memorandum

DATE: February 19, 2002

TO: Chairman, Research Review Committee

FROM: Shah M Faruque Shah Faruque

SUB: Protocol # 2002-002

Thank you for your letter of 18th February and the comments and suggestions regarding our protocol entitled " Genetic variants of *Vibrio cholerae* O139 and development of a vaccine against O139 cholera". We have carefully considered the comments and made modifications in the protocol accordingly. Our specific response to the comments and suggestions are as follows.

a. We have considered the comments made by the RRC, and decided **not** to obtain samples from patient's contacts. It may be mentioned that the objectives of the protocol can be addressed without obtaining samples from contacts. We have accordingly made modification in specific aim # 1 of the protocol (page 4) and dropped the term "**contacts**".

b. As suggested by the RRC, we have now included a flow chart for implementation of the protocol (page 26 of the revised protocol). Kindly note that as shown in the flow chart timings of some of the activities will overlap. For example, as we perform genetic analysis of strains, collection of samples and isolation of more strains will continue during the first 2 years of the study.

c. As suggested by the RRC we have modified the face sheets and identified one PI who will be responsible for the ICDDR,B part of the protocol.

I am attaching with this letter a copy of the revised protocol for your consideration. May I hope that you will feel able to approve the revised protocol.

Thank you.

Enclo: A copy of the revised protocol.

APPROVED COPY

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

RESEARCH PROTOCOL

Protocol No. 2002-002

FOR OFFICE USE ONLY

RRC Approval: Yes/ No Date:

ERC Approval: Yes/No Date:

AEEC Approval: Yes/No Date:

Project Title:

Genetic variants of *Vibrio cholerae* O139 and development of a vaccine against O139 cholera

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: _____

Principal Investigator: **Dr. Shah M. Faruque**

Division: Laboratory Sciences Division

Address: Laboratory Sciences Division, ICDDR,B, GPO BOX-128, DHAKA-1000, BANGLADESH

Phone/Ext: 880 2 871751 Ext-2410

Co-Investigator(s): **G. Balakrish Nair, Qazi Shafi Ahmad, Dr. Andrej Weintraub, Dr. Firdausi Qadri**

Student Investigator/Intern:

Collaborating Institute(s): **Division of Clinical Bacteriology, Karolinska Instute, Huddinge, 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 _____)
- ☐ Animal

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

- ☑ Dhaka Hospital
- ☑ Matlab Hospital
- ☑ Matlab DSS area
- ☑ Matlab non-DSS area
- ☐ Mirzapur
- ☐ Dhaka Community
- ☐ Chakaria
- ☐ Abhoynagar
- ☐ Mirsarai
- ☐ Patyia
- ☐ Other areas in Bangladesh _____
- ☐ Outside Bangladesh
  name of country: _____
- ☐ Multi centre trial
  (Name other countries involved)
  _____

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:

Total sample size: _____ ☐

Sub-group _____ ☐ _____ ☐

_____ ☐ _____ ☐

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

- ☐ Human exposure to radioactive agents?
- ☐ Fetal tissue or abortus?
- ☐ Investigational new device?
  (specify_____)
- ☐ Existing data available from Co-investigator
- ☐ Human exposure to infectious agents?
- ☐ Investigational new drug
- ☐ Existing data available via public archives/source
- ☐ Pathological or diagnostic clinical specimen only
- ☐ 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".

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) _____

(2) _____

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

(Day, Month, Year - DD/MM/YY)

Beginning date _____

Duration 3 years _____

Cost Required for the Budget Period ($)

a.	*1st Year*	*2nd Year*	*3rd Year*	*Other years*
ICDDRB	65,424	66,570	68,423	
KI	16,983	16,847	17,346	
TOTAL	82,407	83,417	85,769	

b. Direct Cost : _____ *Total Cost :* 251,593

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

Dec 12, 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, 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

Signature of PI

DR. SHAH M. FARUQUE Shah Faruque 9/12/2001

Table of Contents

☐ Check here if appendix is included

Principal Investigators **DR. SHAH M. FARUQUE & DR. G. BALAKRISH NAIR**

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: Dr. Shah M. Faruque

Project Name:
Genetic variants of *Vibrio cholerae* O139 and development of a vaccine against O139 cholera

Total Budget US$ 251,593 Beginning Date: January, 2002 Ending Date: December, 2004

Vibrio cholerae O139 is the recently discovered second etiologic agent of epidemic cholera. Although there are striking similarities between *V. cholerae* O139 and the El Tor biotype of *V. cholerae* O1 (the traditional serogroup that causes cholera), the former differs from the latter in the possession of a polysaccharide capsule (CPS). Since the emergence of *V. cholerae* O139, the strain has been undergoing rapid genetic re-assortments and several phenotypic and genotypic changes have been noticed. We plan to continue to investigate genetic variation among O139 isolates, and also attempt to develop a vaccine against O139 cholera. We will investigate several possibilities for a new vaccine using different approaches. The vaccine candidates will be based on (a) the lipopolysachharide (LPS) since the O-antigen is structurally similar to the CPS; (b) from CPS generated oligosaccharides conjugated to proteins and (c) oligopeptides mimicking the immunogenic epitopes present in the CPS.

This study proposes environmental and clinical surveillance, and characterization of nontoxigenic progenitor strains as well as toxigenic strains of *V. cholerae*. This study is also designed to perform comparative molecular analysis of *V. cholerae* strains to determine the origination of new toxigenic strains, and their relation to epidemics of cholera. This year-round effort will (1) monitor the presence of toxigenic *V. cholerae* and nontoxigenic progenitor strains in environmental waters and patients during epidemic and interepidemic periods in Bangladesh, (2) perform genetic fingerprinting of *V. cholerae* strains isolated from cholera patients and from the environment during the study period, and compare with a collection of epidemic strains isolated from different geographic regions, (3) Catalogue different genotypes of epidemic strains and monitor their appearance or disappearance in environmental and clinical sources, and (4) determine the presence of various virulence genes and genes for antibiotic resistance in the different clonal groups. This will allow generating basic information on the movement of virulence, and other genes, of clinical significance among different clones of *V. cholerae*, and the emergence of new clones with epidemic potential.

The vaccine candidates will be based on either the LPS or the CPS present as unique antigens on the surface of the bacterium. The LPS molecule, although toxic due to the presence of lipid A, has a very good adjuvant activity as well as it possess the polysaccharide chain to which immunity may confer protection. The endotoxic activity may be abolished by mixing the LPS with synthetic anti-endotoxic peptides. The CPS is a large molecular weight polysaccharide not suitable for immunization because of its size and nature (polysaccharides are T-cell independent antigens). By depolymerising the CPS and conjugating the oligosaccharides to suitable protein carriers may convert the CPS to a T-cell dependent antigen. Depolymerisation will be done enzymatically. A third approach is to generate peptides that mimic the specific immunogenic epitopes of the CPS.

Principal Investigators **DR. SHAH M. FARUQUE & DR. G. BALAKRISH NAIR**

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

NameProfessional	Discipline/ Specialty	Role in the Project
1.DR. SHAH M. FARUQUE	Molecular Biologist	Principal Investigator
2. DR. G. BALAKRISH NAIR	Microbiologist	Co-Investigator
3.DR. ANDREJ WEINTRAUB	Chemist/Microbiologist	Co-Investigator
4.DR. FIRDAUSI QADRI	Immunologist	Co-Investigator
5.QAZI SHAFI AHMAD	Microbiologist	Co-investigator
6. DR. A.S.G. FARUQUE	Clinician/Epidemiologist	Co-investigator
7. DR. DAVID A. SACK	Clinician/Microbiologist	Co-investigator

DESCRIPTION OF THE RESEARCH PROJECT

Hypothesis to be tested:

Concisely list in order, 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.

We hypothesize that in the natural habitat, pathogenic strains of *V. cholerae* with epidemic potential originate from nonpathogenic environmental progenitor strains by progressive acquisition of virulence genes. The emergence and enrichment of toxigenic *V. cholerae* strains may thereafter lead to epidemic outbreaks of cholera. Thus, in areas where cholera exhibits a seasonal behavior characterized by fluctuations in incidence, surveillance for the presence of progenitor strains which are convertible to epidemic strains, may serve as a feasible means to monitor the emergence of new toxigenic *V. cholerae* strains as well as possible cholera outbreaks. Frequent origination of new clones by genetic reassortment possibly triggered by increasing immunity in the host population against the existing epidemic clones, new clones are enriched by a process of natural selection, and these clones causes subsequent outbreaks of cholera. In areas of endemic infection, molecular analysis of nontoxigenic and toxigenic strains of *V. cholerae* for the relevant virulence characteristics, and comparative genetic fingerprinting of the strains with previously isolated epidemic strains may serve as a means to monitor the emergence of new epidemic strains of *V. cholerae.* In addition, we will investigate several possibilities for a new vaccine against *V. cholerae* O139.

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 perform molecular analysis of clinical and environmental isolates of *V. cholerae* O1 and O139 in a cholera endemic region in order to study the genetic variations of *V. cholerae* O139, and catalogue different genotypes of toxigenic and non-toxigenic *V. cholerae* O139 strains, and to analyze the nontoxigenic strains for susceptibility and lysogenic conversion by CTXΦ.

2. To study the immunogenicity and protective efficacy of different potential O139 vaccine candidates, namely LPS based, CPS based and peptides mimicking the specific antigens present in the CPS.

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).**

Molecular Epidemiology of Cholera: Epidemiological surveillance of cholera was limited before the seventies by the lack of suitable typing systems. However, recent developments in DNA analysis techniques have introduced several new typing methods and has enabled to study the epidemiology of *V. cholerae* on a larger global perspective (1-9). These techniques include the analysis of restriction fragment length polymorphisms (RFLPs) in different genes. The use of gene probes to study RFLPs in the *ctxAB* genes and their flanking DNA sequences which are part of a larger genetic element (CTX element) indicated that the U.S. Gulf coast isolates of toxigenic *V. cholerae* are clonal and that they are different from other seventh pandemic isolates (7). RFLPs in conserved rRNA genes have also been used to differentiate *V. cholerae* strains into different ribotypes. Analysis of isolates from the Latin American epidemic in 1991 showed that these were related to the seventh pandemic isolates from other parts of the world and that Latin American cholera epidemic was an extension of the seventh pandemic (6, 8, 9). Analysis of toxigenic El Tor strains by multilocus enzyme electrophoresis has also been used to group the El Tor strains into major clonal groups. The clones seem to reflect broad geographical and epidemiological associations. The clonal diversity and epidemiological associations of toxigenic *V. cholerae* have been reviewed by Wachsmuth and coworkers (10). Comparative molecular analysis of the El Tor strains of *V. cholerae* O1 and the epidemic O139 strains suggested that the O139 strains are related to El Tor strains and were derived from the El Tor strains by genetic changes in the serotype specific gene clusters (5, 10).

Molecular analysis of epidemic isolates of *V. cholerae* between 1961 and 1996 in Bangladesh revealed clonal diversity among strains isolated during different epidemics (1, 11, 12). These studies demonstrated the transient appearance and disappearance of more than six ribotypes among classical vibrios, at least 5 ribotypes of El Tor vibrios and 3 different ribotypes of *V. cholerae* O139. More recently genetic analysis of *V. cholerae* O139 strains isolated in Bangladesh and India since the first appearance of the serogroup in 1992 has revealed the presence of at least 6 different ribotypes within this serogroup (13, 14). Different ribotypes often showed different CTX genotypes resulting from differences in copy number of the CTX element and variations in the integration site of CTX element in the chromosome (11, 12). These studies indicated that there had been a continual emergence of new clones of toxigenic *V. cholerae* which replaced existing clones, possibly through natural selection involving unidentified environmental factors and immunity of the host population.

Virulence associated genes in *V. cholerae*: The pathogenesis of cholera is a complex process and involves a number of genes encoding virulence factors which aid the pathogen in its passage to reach the epithelium of the small intestine, colonize the epithelium, and produce cholera toxin (CT) that disrupts ion transport by intestinal epithelial cells. In *V. cholerae*, the major virulence genes required for pathogenesis exist in clusters. These include the CTX genetic element, which is the genome of a lysogenic bacteriophage designated CTXΦ (15) that carries the genes encoding CT, and the TCP

pathogenicity island which carries genes for a pilus colonization factor known as toxin coregulated pilus (TCP). The structural features of the TCP pathogenicity island which include the presence of groups of virulence genes, a regulator of virulence genes, a transposase gene, specific (*att*-like) attachment sites flanking each end of the island, and an integrase with homology to a phage integrase gene suggest that the TCP pathogenicity island also has been derived from a bacteriophage (16, 17). Thus the major virulence gene clusters in *V. cholerae,* appear to have phage origins, and this suggests that horizontal transfer of gene clusters may be a possible mechanism for the origination of new pathogenic clones of *V. cholerae*. Since colonization is a prerequisite to establishing a productive infection by *V. cholerae* the existence and role of possible other factors responsible for colonization has also been investigated. This includes the mannose-fucose-resistant cell associated hemagglutinin (MFRHA) the mannose sensitive hemagglutinin (MSHA), and some outer membrane proteins (OMPs) of *V. cholerae* (18-21). Although some of these factors including MFRHA, MSHA and OMPs are suspected to have a role in enhancing adhesion and colonization when tested in animal models, the exact role of these factors in the virulence of *V. cholerae* in humans is still uncertain. Studies to date have shown that the major virulence genes of *V. cholerae* required for pathogenesis are the genes involved in the production of TCP and CT (22).

The TCP Pathogenicity Island. Early works established that the genes encoding TCP were clustered, and were present in clinical isolates of O1 El Tor and classical vibrios but not in environmental isolates of *V. cholerae* O1, with the exception of a few strains from the Gulf Coast of the United States (23). Expression of CT and TCP are co-regulated by the ToxR regulatory system which includes the ToxT protein (24). The genes encoding ToxT and TCP are located in the same choromosomal region (25) together with other ToxR-regulated genes including those for a potential accessory colonization factor (ACF) (26). Molecular analysis has revealed that although the major subunit of TCP is encoded by the *tcpA* gene, the formation and function of the pilus assembly require the products of a number of other genes located on the chromosome adjacent to the *tcpA* gene, and these constitute the *tcp* gene cluster (27). At least 15 open reading frames are found in the *tcp* cluster which is located immediately downstream of the *tagD* gene. The *tcpH* and *tcpI* genes are two ToxR-regulated genes that influence TcpA synthesis. Inactivation of *tcpH* results in decreased pilin synthesis, whereas inactivation of *tcpI* leads to increased synthesis of TcpA. It has been suggested that regulators such as TcpI that acts downstream of ToxR and ToxT may function to fine tune the expression of the TCP virulence determinant throughout the pathogenic cycle of *V. cholerae* (28). Immediately adjacent and downstream to the *tcp* cluster the *acf* gene cluster is located. The exact nature of the colonization factor is not clear, but *acfD* one of the four open reading frames (*acfABCD*) encodes a lipoprotein. Further analysis revealed the presence of a putative integrase gene (*int*) and a putative *att*-like 20-bp attachment site adjacent to the TCP/ACF gene cluster (16). The entire region of nearly 40-kb flanked by the *att*-like sequences and including the TCP/ACF gene clusters, the integrase and a transposase genes appear to constitute a pathogenicity island. A recent report suggested that the TCP pathogenicity island is the genome of a bacteriophage (17).

The CTX genetic element. Toxigenic *Vibrio cholerae* strains carry one or more copies of CT genes (*ctxAB*). The A and B subunits of CT are encoded by two separate but overlapping open reading frames. *V. cholerae* also produces a putative toxin known as zonula occludens toxin (Zot) that increases the permeability of the small intestinal mucosa by affecting the structure of the intercellular tight junction, or zonula occludens (29). The *zot* gene consists of a 1.3 kb open reading frame, which could potentially encode a 44.8-kDa polypeptide, and is located immediately upstream of the *ctxA* gene (29). A third toxin that has been described is Accessory cholera enterotoxin (Ace) which is capable of inducing fluid accumulation in rabbit ligated ileal loops (30). The genes encoding

the toxins (*ace*, *zot* and *ctxAB*), a core-encoded pilin (*cep*), and an open reading frame of unknown function (*orfU*) are located on a 4.5 kb "core region", flanked by one or more copies of a repetitive sequence called RS1 (31). Together these DNA units comprise the CTX element, which had originally been perceived as a transposon-like genetic element. It has recently been discovered that the CTX genetic element is the genome of a lysogenic filamentous bacteriophage designated CTXΦ, and that genes in the core region of the CTX element particularly *zot* and *orfU* are crucial for the morphogenesis of the phage (15).

Cholera toxin converting bacteriophage (CTXΦ). It has been demonstrated that under appropriate conditions toxigenic *V. cholerae* strains can be induced to produce extracellular CTXΦ particles (15, 32). The phage can be propagated in recipient *V. cholerae* strains in which the CTXΦ genome either integrates chromosomally at a specific site forming stable lysogens or is maintained extrachromosomally as a replicative form (RF) of the phage DNA (15). Cultures of *V. cholerae* harboring the RF of CTXΦ produce high titers of the phage in their supernatants. Further studies have confirmed that some naturally occurring nontoxigenic strains of *V. cholerae* are infected by CTXΦ and converted to toxigenic strains with epidemic potential (33). The bacteriophage has been shown to use the TCP as a receptor, and hence expression of TCP by the bacterium is a prerequisite for its susceptibility to the phage. The CTXΦ genome has two regions, the "core" and the RS2 (34). Genes with related functions are clustered in the genome of CTXΦ in a way similar to those of other filamentous phages. Analysis of phage morphogenesis revealed that most of the genes of the "core region" are essential for the formation of the CTXΦ particles and hence for its propagation as an infectious phage. The open reading frames in RS2 were designated as *rstR, rstA2* and *rstB2*, and these were found to encode products required for the integration, replication and regulatory functions of CTXΦ (34). The deletion of a portion of the genes encoding CT by marker exchange, however, did not affect the morphogenesis of the bacteriophage. It appears that the *ctxAB* genes do not participate directly in the formation of phage particles, but these genes are important for the phage to provide a survival advantage to its host bacteria in the gastrointestinal environment (34).

Ecology of *V. cholerae*: *V. cholerae* has been regarded as a member of a group of organisms whose major habitats are aquatic ecosystems (35). Although, *V. cholerae* is part of the normal, free-living bacterial flora in riverine and estuarine areas, non-O1 non-O139 strains are more commonly isolated from the environment than are O1 or O139 strains. Moreover, outside of epidemic areas and away from areas that may have been contaminated by cholera patients, environmental isolates of *V. cholerae* O1 have been found to be mostly CT negative. The major pathogenic genes in *V. cholerae* are clustered in several regions of the *V. cholerae* chromosome and the structure of these pathogenic gene clusters indicates that these are capable of being propagated horizontally (15-17, 31). This is suggestive of the possibility that environmental strains of *V. cholerae* may develop the ability to adapt to the intestinal environment through acquisition of the virulence genes. In view of the available information on the epidemiology of cholera, the lysogenic conversion by a bacteriophage encoding cholera toxin, and the survival and enrichment of *V. cholerae* under in-vivo and in-vitro conditions, it is apparent that the ecosystem for *V. cholerae* should consists of a number of components. These include (a) the bacterium, (b) the aquatic environment, (c) CTXΦ and other unidentified genetic elements involved in the transfer of virulence genes, and (d) the intestinal environment of the host population. A summary of available information from studies regarding survival of toxigenic *V. cholerae* in the aquatic environment and in the host intestine, and the possible role of accessory genetic elements that mediate the horizontal transfer of virulence genes is as follows.

Environmental survival and persistence of *V. cholerae*: The physicochemical conditions for the survival of *V. cholerae* O1 have been investigated and the possibility of survival of the organism in an estuarine environment and other brackish waters is widely accepted (35, 36). However, the nature of the survival and persistence of toxigenic *V. cholerae* O1 or O139 in aquatic ecosystem and the factors involved in the conservation of the CTX element (the lysogenic form of CTXΦ) and other pathogenic genes in the aquatic environment is not clear. The survival may be dependent on several factors, such as occurrence of particular physico-chemical conditions, specific association of the bacteria with aquatic plants or animals, and /or the existence of specific ecological association involving several components of the aquatic environment. It has been postulated that under stress conditions the vibrios are converted to a viable but non-culturable (VNC) form, that cannot be recovered by standard culture techniques, and that such VNC forms are able to produce infection and can revert into the culturable form (36). The public health and ecological importance of the possible survival forms such as VNC depends on whether these forms are re-convertible to live infectious bacteria. There is, however, very little evidence to conclusively establish that the possible non-culturable phenomenon is reversible. Hence there is considerable scope to further investigate the role of postulated VNC forms of *V. cholerae* through carefully controlled studies. It has also been suggested that during inter-epidemic periods toxigenic *V. cholerae* exists in an unexplained ecological association with aquatic organisms possibly in the VNC form until the next epidemic season, when environmental factors triggers the dormant bacteria to multiply and lead to cholera outbreaks (37, 38). However, differences in genetic or phenotypic properties have been often noticed among *V. cholerae* O1 and O139 strains isolated during different epidemics (39, 40). Analysis of rRNA gene restriction patterns of *V. cholerae* strains has also shown clonal diversity among epidemic strains (1, 11, 12, 41). These events have raised questions whether seasonal epidemics are caused by periodic appearance of the same strains of *V. cholerae* or due to a continual emergence of new toxigenic clones from nontoxigenic progenitors. Hence further studies are also required to understand more definitive roles of environmental factors in the emergence and reemergence of toxigenic *V. cholerae*.

Enrichment of toxigenic *V. cholerae* in the intestinal environment: Although there is considerable understanding of the mechanism how CT causes diarrhea, it is not clearly known why *V. cholerae* should infect and elaborate the lethal toxin in the host system. It seems worth wondering whether the role of the toxin is to simply cause diarrhea and thus disseminate the organism to its next victim or could the toxin be providing a more crucial function for the enrichment and continued existence of the bacteria. Studies directed towards the development of attenuated *V. cholerae* mutants altered in toxin production for use as live oral cholera vaccines provided a means to investigate the role of CT in the intestine. In 1971, Howard (42) reported the isolation of nontoxigenic mutants of the classical strain 569B by mutagenesis with nitrosoguanidine. The mutants were unable to induce a secretory response in the rabbit intestinal loop model, and did not survive or multiply in the intestinal environment. During 1974-75, Finkelstein et al (43) and Holmes et al (44) observed that the ability of various mutants to grow in the intestinal environment correlates with the ability of the mutants to induce a residual secretory response in the infant rabbit model. A variety of different toxin deficient mutants of *V. cholerae* tested in rabbit and infant mouse models also suggested that the mutants showed enhanced killing and mechanical clearance in the intestinal environment compared to the toxigenic parental strain (45, 46). It seems possible that many of these early mutants were altered in TCP as well as CT expression, perhaps by carrying mutations in regulatory genes involved in the expression of CT and TCP. The selection for their reversion in vivo may have, therefore, been driven by the need to up regulate the expression of TCP more than that of CT (22). The characterization in vivo of site-specific *ctxAB* mutants constructed by in vitro recombinant DNA methods provided the most convincing evidence that the toxin is beneficial to growth in the intestinal environment. It was

demonstrated that the *ctx* mutants colonized rabbit intestines about 10 to 100-fold less efficiently than the parental strain (47). The details of the mechanisms involved have been reviewed by Mekalanos (47). Studies so far suggest that causation of cholera in humans is also linked with a natural process of enrichment of toxigenic *V. cholerae*, and partly explains the benefit imparted to the pathogen during the disease in humans. However, in order to understand the general epidemiological behavior of *V. cholerae*, which includes seasonal pattern of epidemics, transient appearance and disappearance of different clones, and emergence of new epidemic clones, it is important to study the interactions among the bacteria, genetic elements mediating the transfer of virulence genes, the human host and possible environmental factors.

Emergence of novel strains of toxigenic *V. cholerae*: *V. cholerae* offers a genetic system to study the relationship between pathogenesis and the natural selection of pathogens so as to ensure their continued existence. It appears that acquisition of pathogenic gene clusters by *V. cholerae*, which is normally a marine or brakish water species, has allowed the bacterium to become adapted to the human intestinal environment (22). Although the donor for the TCP pathogenicity island has not yet been identified, the TCP island seems to have a bacteriophage origin, and it has been speculated that the TCP island can be transferred by transducing phages (16, 17). On the other hand the donor for the CTX genetic element has been confirmed to be a filamentous bacteriophage, which has been characterized to some details (15, 34). It has been postulated that the acquisition of the CTX element by *V. cholerae* provides a survival advantage to the bacterium, and leads to enrichment of toxigenic *V. cholerae* in the intestinal environment. Thus the CTX phage confers increased evolutionary fitness to its host and hence to its own nucleic acids. With growing immunity in the host population against certain toxigenic clones of *V. cholerae*, new toxigenic clones emerge and replace existing clones by a process of natural selection. Thus a continual emergence of new strains of toxigenic *V. cholerae* and their selective enrichment during cholera outbreaks constitute an essential component of the ecosystem for the survival and evolution of *V. cholerae* and the genetic elements that mediate the transfer of virulence genes (22). Recent studies have demonstrated that potential epidemic strains can originate from nonpathogenic progenitor strains and thus accounts for the observed clonal diversity among epidemic *V. cholerae* strains (33). However, many aspects regarding the epidemiology and the ecology of the pathogen remain unknown, particularly the mechanisms controlling the seasonal pattern of epidemics in areas of endemic infection. The present proposal aims at further understanding the mechanisms involved in the emergence of new pathogenic strains and its relation to epidemics of cholera.

Vaccine development

Different vaccine candidates are under development. These are based on either live attenuated strain (48) or on conjugate vaccines with the CPS (49, 50). There are several possibilities for a new vaccine using different approaches. The vaccine candidates may be based on: (i) the LPS since the O-antigen is structurally similar to the CPS; (ii) from CPS generated oligosaccharides conjugated to proteins and (iii) oligopeptides mimicking the immunogenic epitopes present in the CPS.

LPS-based vaccine

The LPS is not a suitable antigen for immunization since it has toxic properties. However, by mixing the LPS with synthetic anti-endotoxin peptides it is possible to abolish the toxic effects of the molecule.

The peptides are cyclic and are variants of polymyxin B (51-54). Since the LPS is a very good adjuvant and in addition, possesses the antigenic properties conferring the O139 specificity, we plan to generate different endotoxoids and evaluate their immunogenic and protective efficacy. Preliminary

results have shown that one endotoxoid preparation, when used as immunogen in mice, gave rise to an antibody response with high titer and specificity. This preparation is currently being investigated in a pilot experiment using the RITARD model. We plan to evaluate the results from the current pilot experiment and then further examine the endotoxoid using different routes of immunization.

CPS-based vaccine

The capsular polysaccharide in *V. cholerae* O139 is the major surface antigen. Because of its nature, high molecular weight polysaccharide, the molecule is not suitable for vaccination. The main reason is that polysaccharides are usually T-cell independent antigens, which do not confer immunological memory. In addition, the antibodies are usually of IgM class. One way to convert a polysaccharide antigen to a good immunogen is to conjugate oligosaccharides to protein carriers. The CPS of *V. cholerae* O139 is a large molecule and not suitable for conjugation. It is, however, possible to depolymerize the CPS and use oligosaccharides for conjugation. Chemical depolymerization of the CPS is not possible since the antigen possess both alkali-labile (uronic acid) and acid-labile (colitose) residues which upon treatment are either destroyed or lost. Another possibility to generate oligosaccharides preserving specificity is to use enzymatic methods. The bacteriophage JA1, specific for encapsulated *V. cholerae* O139 possess an enzyme with CPS degrading capability. We have investigated the JA1 phage and found that it possesses an enzyme that hydrolyzes the linkage between the N-acetyl glucosamine and the galacturonic acid. We have been able to isolate oligosaccharides corresponding to two repeating units of the CPS (55). In addition, we are in the process of synthesizing one complete repeating unit of the CPS. The generated oligosaccharides as well as the synthetic repeating unit will be covalently conjugated to carrier proteins such as tetanus toxoid, cholera toxin B-subunit, diphtheria toxoid or to bovine serum albumin. The generated glycoconjugates will be tested for their immunogenicity as well as for the protective efficacy.

Peptide-based vaccine

Another approach to generate vaccine candidates is to generate peptides or globular proteins mimicking the immunodominant carbohydrate epitopes present in the CPS. Three different techniques for generation of mimicking peptides or proteins will be employed. In all methods, mAbs (generated at Karolinska Institute) specific for the *V. cholerae* O139 will be used. The selection of the peptides will be performed by different groups involved in an EU consortium. The selcted peptides will be tested for immunogenicity and specificity at Karolinska Institute.

1. Production of small globular proteins against monoclonal antibodies. The mAbs will be used for biopanning experiments using phage display libraries. The specificity and affinity of small protein binders isolated from these libraries will be studied by ELISA and by real-time interaction analysis using a BIAcore instrument. Small recombinant globular proteins, constructed on a stable protein scaffold of bacteriophage origin and which mimic the bacterial capsule, should be immunogenic and should represent useful tools for the development of an acellular antibacterial vaccine.

2. The construction of a peptide library in *Streptococcus gordonii* will be carried out by exploiting an expression system in which heterologous DNA sequences are cloned in *E. coli* and then transformed on especially engineered recipient strains of *S. gordonii*. The heterologous peptide is
3. then produced as a fusion to the vector molecule (streptococcal M protein), which in turn targets the peptide to the cell surface.

4. Different phage-displayed random peptide libraries: peptide collections are constructed in phagemid systems (using pC89 and pIF4 vectors) as fusions to the major coat protein of filamentous phage. These libraries encompass $>10^7$ different peptide sequences each, and display linear peptide sequences having a length of 9aa, 12aa, 15aa and 28aa respectively. Additionally, we will also perform the screening of two different Cysteine- bridged constrained libraries, one composed of 9aa-long loops, and another having the Cysteine residues at variable lengths. Phage peptide libraries will be probed by several round of bio-panning with each available mAb. Selected phage clones will be further characterised by ELISA with the relevant antibodies and their specificity will be tested by competition using the natural antigen. The clones found to be most promising from the above analysis will be evaluated for their ability to elicit cross reactive antibody responses (as measured by ELISA) to the nominal antigens when used as immunogens in mice.

We have now generated peptides using the method described under 3 (see above) and five different peptides of various lengths have been generated. These have been synthesized as free peptides as well as BSA and KLH conjugates. All conjugates react with the mAb used for selection and the CPS inhibits the reactivity between peptide and the mAb. The BSA conjugates will used as immunogens in mice and the antibody response measured against the corresponding KLH conjugate as well as against the CPS. The selection according to method 1 is ongoing. The selection according to method 2 will start shortly.

PROGRESS REPORT ON PREVIOUS PROJECTS:

This project is a continuation of previous proposals: **"Studies on the capsule of *Vibrio cholerae* O139 Bengal"** (period July 1996-December 1998) and **"Studies on virulence of *Vibrio cholerae* O139"** (period January 1999-December 2001) both funded by SIDA/SAREC. The PI at ICDDR,B for the above mentioned projects was Dr M. J. Albert. Since, Dr. Albert has left ICDDR,B and Dr G. B. Nair has come in the place of Dr. Albert, for the present proposal Dr. Nair will be the principal investigator.

Following is a summary of the progress on the two previous projects.

a) Studied the effect of capsular polysaccharide (CPS) on increased virulence of *V. cholerae* O139 such as serum resistance, resistance to phagocytosis, and invasion of epithelial cells. We have compared the phagocytic killing of *V. cholerae* O139 strains with a standard capsulated strain of *Klebsiella pneumoniae*. There was no killing of *K. pneumoniae* with 100% survival, whereas *V. cholerae* O139 isolates were partially resistant with approximately 40% survival (results have been published, ref 6 bellow).

b) Studied the protective efficacy of monoclonal antibodies (MAbs) to CPS in a suckling mouse cholera model. We have generated six MAbs to *V. cholerae* O139. All have been tested for their protective efficacy in a suckling mouse cholera model, and provided various levels of protection. The binding of these MAbs to CPS has been verified by immunoelectron microscopy. (These results will be published later).

c) Studied the interaction between phage specific for *V. cholerae* O139 and the CPS of *V. cholerae* O139 to generate oligosaccharides. We have isolated a phage (JA1) that

specifically lyse capsulated *V. cholerae* O139 bacteria. We have studied the interaction between the CPS and the phage and found that it hydrolyzes the CPS at the site between the glucosamine residue and the galacturonic acid residue and generates an oligosaccharide. The enzyme responsible for the hydrolysis is a lyase and will be used for generation of oligosaccharides (results have been published, ref 7).

d) Studies on the conjugation of oligosaccharides of CPS of *V. cholerae* O139 to carriers such as tetanus toxoid, B-subunit of cholera toxin or polymerized acrylamide to generate specific capsule-based antigen for immunodiagnostic tests. Since LPS and CPS in *V. cholerae* O139 are cross-reactive, preliminary studies were carried out with oligosaccharide from LPS. Oligosaccharide was conjugated to bovine serum albumin and used to raise antibodies in rabbits. The antibodies reacted with both LPS and CPS of *V. cholerae* O139. Due to technical problems, phage generated oligosaccharides have not been conjugated as yet.

e) Study of the structural basis of cross-reaction between the CPS of *V. cholerae* O139 and other cross-reacting bacteria. *V. cholerae* O139 cross-reacts with a number of bacteria including *V. cholerae* O155, O22 and some strains of *V. mimicus*. The structures of

f) O-specific polysaccharides of a strain of *V. cholerae* O155, O22 and *V. mimicus* N-1990 have been determined. For each of the strains it was possible to identify partial structures that could explain the cross-reactivity. The results have been published (ref 3-5)

g) Based on the unique sequences encoding the surface polysaccharides of *V. cholerae* O139, design primers and develop a polymerase chain reaction (PCR) assay for detection of *V. cholerae* O139 in clinical and environmental studies. The PCR assay developed with the primer pair produced an amplicon only from *V. cholerae* O139. The assay was evaluated for diagnosis of *V. cholerae* O139 directly from 180 watery stools. All the 67 *V. cholerae* O139 culture-positive stool specimens were positive by PCR, and the remaining specimens which had either other recognized pathogens or no pathogens, were all negative by PCR (Ref. 1).

h) The CAMP haemolytic activity has been used for presumptive diagnosis of *V. cholerae* O1 infection. El Tor biotype is always positive, classical biotype is negative, but some non-O1, non-O139 strains produce a weak atypical zone of haemolysis. *V. cholerae* O139 isolates were tested for CAMP haemolysis. All the 1993-94 isolates and the majority of 1995 isolates were CAMP positive, and TMP-SMX and vibriostatic compound O/129 resistant. However, the subsequent isolates exhibited opposite characteristics. The role of CAMP haemolysin in *V. cholerae* virulence has not been investigated. This can be done by studying the diarrhoeagenic potential of purified CAMP haemolysin, and by making CAMP haemolysin mutant and then comparing the virulence of wild type parent and mutant. The amino acid sequences of the CAMP factor gene of *Streptococcus uberis* and *Streptococcus agalactiae* were compared to find homologous areas. Three primers were selected and ambiguous bases were used where matching bases were not obtained. The primers where used in a seminested PCR that gave a product of 386 bp in the positive control *S. uberis*. This seminested PCR was used to investigate if an analogous CAMP factor gene was present in isolates of *V. cholerae* O139. One CAMP-test positive isolate of *V. cholerae* O139 from 1993, and one CAMP-test negative isolate of *V. cholerae* O139 from 1996, were tested together with *V. cholerae* O1 El Tor (CAMP-test positive) and *V. cholerae* O1 classical (CAMP-test negative). All of the tested isolates gave a single PCR fragment of expected length. In order to examine if the amplified fragment was the correct gene, the PCR products were cloned, sequenced and compared with

the published sequences of *S. uberis* and *S. agalactiae*. In spite of several attempts, no unique sequence for the CAMP-hemolysin in *V. cholerae* O139 could be identified. Therefore, this part of the project will not be continued.

i) Fingerprinting of a *Vibrio cholerae* O139 specific DNA region encoding the somatic antigen in strains isolated during 1993-1998. Previous studies indicated significant phenotypic and genotypic changes in O139 isolates over the years since its first appearance. This prompted us to study possible polymorphism in the 35-kb novel region encoding the O139 specificity. A total of 17 *V. cholerae* O139 isolates originating from different countries and years in South Asia and China, and a single unrelated *V. cholerae*

O139 isolate from Argentina were studied. The 35-kb chromosomal region was amplified as two fragments of 12- and 23-kb regions in an extended PCR from all isolates. These amplicons were then treated separately with seven different restriction enzymes and separated by agarose gel electrophoresis. The South Asian and Chinese isolates gave identical patterns for the same enzymes, but different patterns for different enzymes, thus exhibiting no polymorphism in the 35 –kb region. However, the Argentine isolate gave distinct patterns for most of the enzymes confirming its different origin. This data indicated that the portion of the chromosome encoding the O139 antigen specificity is highly conserved. As found in previous studies, the early O139 isolates were resistant to trimethoprim-sulfamethoxazole (TMP-SMX) and vibriostatic compound, O/129, and CAMP- haemolysin positive. The isolates of later years diverged exhibiting different patterns by pulsed-field gel electrophoresis (PFGE), and becoming susceptible to TMP-SMX and O/129, and CAMP-haemolysin negative (Ref. 8).

PUBLICATIONS:

The following publications have resulted from these projects.

1. Albert MJ, Islam D, Nahar S, Qadri F, Falklind S, Weintraub A. Rapid detection of *V. cholerae* O139 Bengal from stool specimens by PCR. J Clin Microbiol 1997; 35:1633-5.
2. Knirel YA, Widmalm G, Senchenkova SN, Jansson, PE, Weintraub A. Structural studies on the short-chain lipopolysaccharide of *Vibrio cholerae* O139 Bengal. Eur J Biochem 1997, 247:402-410
3. Landersjo C, Weintraub A, Ansaruzzaman M, Albert MJ, Widmalm G. 1998. Structural analysis of the O-antigenic polysaccharide from *Vibrio mimicus* N-1990. Eur J Biochem 1998; 251:986-90.
4. Senchenkova N, Zatonsky GV, Shashkov AS, Knirel YA, Jansson P-E, Weintraub A, Albert MJ. Structure of the O-antigen of *V. cholerae* O155 that shares a putative D-galactose-4, 6-cyclophosphate-associated epitope with *V. cholerae* O139 Bengal. Eur J Biochem 1998; 254:58-62.
5. Knirel YA, Senchenkova SN, Jansson, PE, Weintraub A. More on the structure of *Vibrio cholerae* O22 lipopolysaccharide. Carbohydr Res. 1998, 310:117-119
6. Albert MJ, Qadri F, Bhuiyan NA, Ahmad SM, Ansaruzzaman M, Weintraub A. Phagocytosis of *Vibrio cholerae* O139 Bengal by human polymorphonuclear leukocytes. Clin Diagn Lab Immunol 1999, 6:276-278

7. Linneborg M, Weintraub A, Albert MJ, Widmalm G. Depolymerization of the capsular polysaccharide from *Vibrio cholerae* O139. Carbohydr Res. 2001, 333:263-269.

8. Falklind-Jerkérus S, Albert MJ, Weintraub A. Fingerprinting of a *Vibrio cholerae* O139 specific DNA region encoding the somatic antigen in strains isolated during 1993-1998. FEMS Microbiol. Lett. 2001, Submitted for publication

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

Surveillance: Environmental water sources including rivers and ponds which are frequently used by the population, and sewage runoff will be sampled every two weeks in Dhaka, which is an area of endemic cholera, and where epidemics occur every year without exception. Stools of patients from population interacting with the same aquatic environment will be collected whenever a cholera case is reported, as well as during seasonal epidemics. The environmental samples and stools will be cultured for *V. cholerae*, and presence of genes for CT will be screened using specific probes or PCR assays. Both toxigenic and non-toxigenic *V. cholerae* strains isolated from the environmental samples as well as from patients during the same period will be analyzed by genetic fingerprinting to detect possible emergence and transmission of novel toxigenic strains, and the identity of their parental nontoxigenic strains. Nontoxigenic strains will be tested for the possession of virulence-associated genes and for susceptibility to the bacteriophage encoding cholera toxin using a genetically marked derivative of CTX phage. The fortnightly number of reported cholera cases in the surveillance area during the period of observation will be obtained from the hospital records. The study area for this project has been described elaborately in the method section. Computer assisted analysis of the data will be performed to study the correlation between the presence of toxigenic *V. cholerae* strains and progenitor strains in the environment and the probability of cholera outbreaks.

Study Area: The environmental sampling and surveillance of patients will be carried out in Matlab and Dhaka. Matlab is a rural area of Bangladesh, where the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR;B) has been maintaining a field research project since 1963. Matlab is a low lying riverine, cholera endemic area, located about 70 kilometers southeast of Dhaka, the capital of Bangladesh. One component of the field project is a longitudinal demographic surveillance system (DSS) which encompasses 200,000 people, resident in 149 villages. A central hospital, manned by physicians and laboratory technicians, located in Matlab, provides free treatment to all patients reporting to the hospital with diarrhea. Approximately 10,000 patients are treated per year in the Matlab Hospital. The ICDDR,B also maintains a diarrhea hospital in the capital Dhaka. Patients with diarrhea from who come to the Matlab or Dhaka hospital routinely have a stool specimen cultured for *V. cholerae*. For the purpose of the study 10 already established environmental surveillance sites in Dhaka and

Matlab will be used for obtaining environmental strains. Cholera patients reporting to the hospital will be recorded and *V. cholerae* strains isolated from patients will be obtained from the microbiology laboratory of the Dhaka and Matlab hospitals.

Collection of environmental water samples: Sampling for the study will be conducted every two weeks during a period of one year. All water samples will be collected in sterile containers from the village ponds and the adjacent river and from the sewage runoff. Temperature, and pH of the water will be measured. Initial processing and culture of the samples to detect the presence of *V. cholerae* will be done within 2 h of collection. Strains will be saved for further analysis in the Molecular Genetics Laboratory of ICDDR,B in Dhaka, as described later. In addition aliquots of the water samples will be stored for further analysis by PCR.

***V. cholerae* strains:** Strains from environmental samples will be isolated by enrichment in alkaline peptone water and culturing on selective media. Isolates from cholera patients will be obtained by culturing stools or rectal swabs by standard methods. The identity of all isolates will be confirmed biochemically and serologically by standard methods recommended by WHO (81). Presence of genes for cholera toxin, toxin coregulated pili, ToxT and ToxR will be determined by using DNA probes and PCR assays (4, 5). Number of colonies of toxigenic *V. cholerae* recovered per 100 ml of water will reflect a measure of the concentration of viable cells of toxigenic *V. cholerae* in the samples. Reference strains of *V. cholerae* and other control strains, as well as epidemic strains isolated in different geographical locations will be obtained from the culture collections of the Molecular Genetics laboratory of ICDDR,B.

Laboratory Methods

Probes and hybridization: The gene probes specific for the CTX element will consist of a probe for the *ctxA* gene or a defined portion of the *zot* gene. The *ctxA* probe will be a 0.5 kb *Eco*RI fragment of pCVD27 (56) which is a pBR325 derived plasmid containing an *Xba*I-*Cla*I fragment representing 94% of the gene encoding the A subunit of CT, cloned with *Eco*RI linkers. The *zot* gene probe will consist of an 840 bp region internal to the *zot* gene, and will be amplified by PCR from the recombinant plasmid pBB241 as described by us previously (57). The *toxR* probe will be a 2.4 kb *Bam*HI fragment of pVM7 (58) which is a pBR322 derived plasmid carrying the entire *toxR* sequence. The rRNA gene probe will be a 7.5-kb *Bam*HI fragment of pKK3535 (59). Preparation and use of these probes have been described by us previously (1, 11, 12). Colony blots or Southern blots will be prepared using nylon filters (Hybond, Amersham International plc. Ayelesbury, United Kingdom), and processed by standard methods (60). The polynucleotide probes will be labeled by random priming (61) using a random primers DNA labeling kit (Bethesda Research Laboratories, Gaithersburg, Md.) and [α-^{32}P]-deoxycytidine triphosphate (3,000 Ci/mmol, Amersham) and oligonucleotide probes will be labeled by 3'tailing using terminal deoxynucleotide transferase and [α-^{32}P]-dCTP. Southern blots and colony blots will be hybridized with the probes and autoradigraphed as described by us previously (1, 11, 12).

PCR Assays: Presence of the *tcpA* gene will be determined using a PCR assay as described previously (62). The *tcpI* gene will be detected by a PCR assay based on the published sequence of *tcpI* as described by us previously (11). The *toxT* and *acfB* genes will also be detected by PCR assays described previously. PCR assay to detect the presence of the CTX prophage in the environmental samples will be done using the two primers 5'GAACGCATAGCTAAGTAC, and 5'TCTATCTCTGTAGCCCCTATTACG to amplify a 1.6 kb region of the CTX genetic element encoding portions of the *zot* gene and the *ctxA* gene. A multiplex PCR assay for the

simultaneous detection of the *tcpA* gene and the *ctxA* gene will be used to discriminate between the presence of toxigenic *V. cholerae* and cell free CTXΦ particles in the environmental samples. All primers will be synthesized in the Molecular Genetics laboratory of ICDDR,B using a Beckman oligo-1000 DNA synthesizer and PCR reagents will be obtained from Perkin-Elmer Cetus (Norwalk, Conn.) Aliquots of the amplified products will be analyzed by agarose gel electrophoresis to ascertain the expected size of the amplified DNA segments. When required, the identity of the amplified DNA will be further confirmed by Southern blot hybridization using specific DNA probes.

Susceptibility of nontoxigenic *V. cholerae* strains to CTX-KmΦ: Susceptibility of *V. cholerae* strains to the genetically marked derivative of CTXΦ will be assayed under laboratory conditions and inside the intestines of infant mice by previously described methods (64, 65). The recipient strains will be grown in LB at 37°C; the cells will be precipitated by centrifugation and washed in fresh LB. The recipient cells and phage particles will be mixed in LB to a make an approximate final concentration of 10^6 bacterial cells and 10^6 phage particles per ml. For the in vitro assay the mixture will be incubated for 16 h at 30°C, and aliquots of the culture will be diluted and plated on LA plates containing kanamycin (50 μg/ml) to select for kanamycin resistant colonies and on plates devoid of kanamycin to determine the total number of colonies. For the in vivo assay, the same mixtures of phage and recipient cells will be used to gastrointestinally inoculate groups of 5-day old Swiss Albino mice obtained from the breeding facilities of the Animal Resources Branch of ICDDR,B as described previously (32). Animals will be sacrificed after 16 h and their intestines were removed and homogenized in 10 mM phosphate-buffered saline (PBS) pH 7.2. The homogenate will be centrifuged at low speed to precipitate debris, the supernatant will then be centrifuged to precipitate bacterial cells, and the pellet will be resuspended in PBS. The suspension will be screened for the presence of Km^R *V. cholerae* colonies. The ratio of Km^R colonies to total colonies will be calculated and expressed as the percentage of recipient cells carrying the phage genome.

Analysis of infected cells: Representative infected colonies will be grown overnight in LB containing kanamycin (50 μg/ml) and cells will be precipitated by centrifugation. The supernatant fluids of the cultures will be titrated for the presence of CTX-KmΦ particles using strain RV508 as the recipient as described previously (32). Total DNA or plasmids will be extracted from bacterial pellets by standard methods (60), and purified using microcentrifuge filter units (Ultrafree-Probind; Sigma). Integration of the phage genome into the chromosome of the recipient cells will be studied by comparative Southern blot analysis of total DNA and plasmid preparations from the phage-infected and the corresponding native strains using the *zot* probe (57).

Genetic fingerprinting of *V. cholerae* strains: High molecular weight chromosomal DNA will be isolated from strains grown overnight by standard methods. Approximately 5 μg of purified whole cell DNA will be digested with appropriate restriction endonucleases as instructed by the manufacturer (Bethesda Research Laboratories) using 5 units of enzyme per μg of DNA. The digested DNAs will be electrophoresed in 0.8% agarose gels, and transferred onto nylon membranes (Hybond-N, Amersham) by Southern blotting (63). Southern blots will be hybridized using appropriate probes and autoradiographed.
Computer assisted analysis and comparison of the pattern of bands produced by different strains will be done to find clonal relationships among strains (1).

O139 Vaccine Studies

Isolation and purification of *V. cholerae* O139 LPS and CPS: *V. cholerae* strain AI-1838 is grown in a 30 L fermentor (Belach AB, Stockholm, Sweden) in a rich tryptone-yeast extract (TY-medium) as described (64) to late logarithmic phase. The culture is checked for purity by Gram-stain and inoculation on appropriate agar plates at the end of the growth cycle and the bacteria killed with formaldehyde (1% wt/vol). After incubation at 4°C overnight, the bacteria are collected by centrifugation (8.000 X *g*, 4°C, 20 min) and washed with phosphate buffered saline (PBS, 0.01 M, pH 7.3). Pelleted bacteria are then suspended in water and extracted with hot phenol-water (65), followed by phenol- chloroform-petroleum ether (PCP) extraction (66, 67). Two fractions are obtained: one PCP-soluble and one PCP-insoluble. The PCP-soluble fraction (containing LPS) is further processed as follows. The chloroform and petroleum ether are removed by evaporation and the LPS precipitated from the phenol phase by 6 volumes of diethyl ether-acetone (1:5 v/v) (68). The PCP-insoluble fraction (containing the CPS) is air-dried, suspended in water and dialyzed against distilled water for three days at 4°C. The CPS is further purified by treatment with RNase and DNase as well as with proteinase K (Sigma Chemical Company, St. Louis, MO. USA) followed by repeated ultracentrifugations (2 x 100.000 x *g*, 16 h at 4°C). The CPS present in the supernatant after ultracentrifugations is lyophilized and used for further studies.

Generation of oligosaccharides and glycoconjugates: In order to generate oligosaccharides from *V. cholerae* O139 CPS, the previously described bacteriophages will be used (42). We have recently published about the generation and characterization of oligosaccharides using the bacteriophage JA1. The enzyme associated with the phage is a lyase that depolymerizes the CPS in a specific way without destroying the immunogenic epitopes (55). The purified oligosaccharides will then be conjugated to different carriers depending on the purpose of the conjugate i.e. (a) acrylamide-CPS conjugates will be used as antigens in enzyme immunoassay; (b) protein-CPS conjugates (bovine serum albumin, cholera toxin B-subunit or tetanus toxoid) will be used as immunogens for generation of specific antibodies. We have now generated small amounts of oligosaccharides by incubation of bacteriophage with purified CPS. After the incubation at 37°C, oligosaccharides were isolated by gel-permeation chromatography. Initial analyses of the oligosaccharides show that the major fraction corresponds to two repeating units. It is also evident that the enzyme present in the bacteriophage is specific for the linkage between the N-acetyl glucosamine and the galacturonic acid. Attempts to conjugate this oligosaccharide to protein carrier are in progress. The conjugates will be used to immunize rabbits and the antiserum will be tested for protective efficacy in infant mouse cholera model against challenge with virulent *V. cholerae* O139.

Evaluation of endotoxoids as vaccine candidate: The LPS, isolated and purified as described above will be used for the generation of LPS-based vaccine candidates i. e. endotoxoids. The principle is to use synthetic anti-endotoxic peptides (Polymyxin B analogues) in a mixture with the LPS. The peptide binds to the negatively charged epitopes in the lipid A part of the molecule and due to re-arrangement of the physical state of the LPS the molecule losses its biological activity. Several different endotoxoid preparations with different peptides and different proportions will be generated and tested for the biological activity in order to ensure their endotoxoid property. Preliminary results showed that some preparations were completely nontoxic and, when injected i. p. in mice, elicited high titer of specific anti-O139

antibodies. These preparations will be used to study the immunogenicity in rabbits (using the ELISA method with both the LPS and CPS as antigens) as well the protective efficacy using the passive protection assay in mice and the RITARD model.

Evaluation of peptides mimicking the CPS as vaccine candidates

Different types of peptides mimicking the CPS of *V. cholerae* O139 will be generated as described above. The peptides, once identified and sequenced, will be synthesized and conjugated with both Bovine serum albumin (BSA) and Keyhole limpet hemocyanine (KLH). The BSA conjugate will be used to immunize mice (i.p using Alum as adjuvant) and KLH conjugate will be used to monitor the antibody response against the peptide. In addition, the LPS as well as the CPS will be used in order to investigate whether the peptide mimicking the epitope in the CPS will give rise to CPS and LPS specific antibodies. If the experiment become successful, the BSA-peptide conjugates will be used for immunization of rabbits in order to study the immunogenicity as well as the protective efficacy. The generation of the above-described peptides is a collaborative study supported by the European Union and will be done by other participants in the EU-consortium. Within this project, the peptides will be synthesized, conjugated and analyzed for immunogenicity and protective efficacy.

Facilities Available

The International Centre for Diarrhoeal Disease Research (ICDDRB) in Bangladesh has a long standing reputation of carrying out reasearch in diarrheal diseases with active collaboration with various national and international institutions. The Centre has the necessary infrastructure for both laboratory based and field or community based research. ICDDR,B has been maintaining a field station in Matlab, a rural cholera endemic area in Bangladesh, where routine surveillance of cholera has been carried out since 1966. In the present proposal the surveillance will be carried out in Bangladesh by investigators of the ICDDRB located in Dhaka, and through its rural substation in Matlab. Clinical samples will be obtained from patients who attend the treatment center of ICDDR,B located in Matlab and Dhaka. Environmental water samples will also be obtained from Matlab and Dhaka, and data on environmental parameters will be recorded. The laboratories of ICDDR,B will perform isolation and analysis of strains. The molecular genetics laboratory at the ICDDR,B has considerable expertise in processing clinical and environmental samples and performing the assays. This laboratory has the necessary equipment and setup for the synthesis of primers and probes, and carrying out PCR assays and relevant molecular characterization of *V. cholerae* strains. All necessary facilities for the part of the project performed at KI are also available.

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).

Molecular data generated by genetic finger printing of strains will be analyzed using computer programmes as described by us previously to calculate the similarity between each pair of isolates expressed as correlation coefficient and yield a similarity matrix consisting of Nx(N-1)/2 correlation coefficients, where N is the number of isolates. The similarity matrix will be clustered according to the unweighed-pair group method with arithmetic averages (UPGMA) to produce a dendrogram. To enable high discrimination levels clusters will be defined as groups of isolates with >98% similarity. Clusters were designated as ribotypes, and groups within a cluster with greater than 98% similarity but not identical will be further designated as subribotypes.

Strains will be catalogued as belonging to different ribotypes and possession of different virulence genes, and hence potential for causing disease. The goal of this study is to understand the diversity among epidemic strains of *V. cholerae* and the appearance of new pathogenic strains. Each study fortnight will be classified by the presence or absence of strains which are either pathogenic or has the potential to be converted into pathogenic strains. A fortnight will be considered positive if at least one of the sites are found positive for the presence of the strains. Data will be generated on the prevalence of different genotypes of strains isolated from environment and patients at different times of the surveillance period.

Strains of *V. cholerae* isolated from both clinical cases of cholera and from the environment will be compared in detail to determine whether the organisms are from the same clone, thereby strongly implicating a causal association. Comparison between toxigenic and nontoxigenic strains isolated from the environment by genetic fingerprinting will allow to detect the origination of new toxigenic strains.

The different vaccine candidates will be tested for immunogenicity and specificity in mice. The protective efficacy of the most suitable candidates will be tested in the passive protection assay in mice as well as in the RITARD model.

Ethical Assurance for Protection of Human Rights

Only stool samples from cholera patient will be required for the study. This project does not involve any study directly in humans. Stool culture is often routinely done for patients admitted with diarrhea in the ICDDR,B hospitals.

Use of Animals

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

Swiss Albino mice and New Zealand White rabbits will be used from the breeding facilities of ICDDR,B whenever required according to the ICDDR,B guidelines for use of animals in research. Any surgical procedure will be performed under anesthesia as appropriate to reduce suffering of the animals. The assays in mice will be required to determine the susceptibility of nontoxigenic *V. cholerae* strains to CTX phage. The assays in rabbits will be the RITARD model, which will be used to assess the immunogenicity of the various vaccine candidates and also to assess protection if the vaccine candidates are immunogenic.
The use of mice at Karolinska Institute will be to determine the specificity of the different vaccine candidates. The planned experiments are approved by the local ethical committee at KI.

Environmental considerations

Identify possible effects on the environment, both positive and negative, resulting from the planned project.

Microbiological analysis of environmental water samples under the current project will allow to evaluate microbial pollution of the environment. In future, this knowledge will help to develop policies on the use of environmental surface waters. The study will not have any adverse effect on the environment.

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.

DISSEMINATION OF RESEARCH FINDINGS

Research findings will be presented in International seminars and published in peer reviewed International Journals in order to make the results available to all researchers in the relevant field. The results will also be made available to government and non-government agencies, and are likely to influence policy decisions made by these organizations for mobilization of resources to control cholera epidemics.

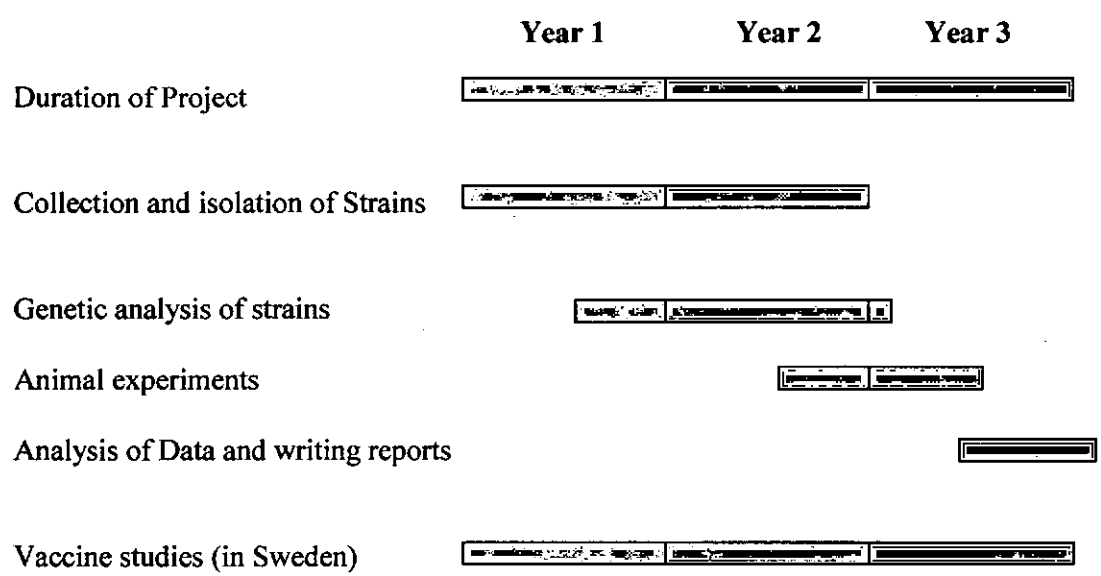
Collaborative Arrangements

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 EXCEED ONE PAGE)**

This study involves the participation of investigators and collaborators from laboratories in Sweden and Bangladesh, and reflects strengthening of scientific collaboration among laboratories of the developed and developing countries. In the ICDDR,B team, Dr. G. B. Nair is the principal investigator, Drs Shah M. Faruque, molecular microbiologists and F. Qadri, immunologist are the co-investigators, whereas Dr. A. S. G. Faruque is an epidemiologists with a background in clinical management of cholera. Dr. A.S.G. Faruque will provide strains collected under the hospital surveillance programs of ICDDR,B. Mr. Kazi Shafi Ahmad will be involved in the isolation and identification of strains from environmental samples.

Dr. Andrej Weintraub, is the Swedish co-investigator for different aspects of the proposed study. There is already existing collaborative linkages between the Karolinska Institute and ICDDR,B. The Karolinska Institute will also provide improved technologies and different candidates for vaccine evaluations to ICDDR,B to further build the capabilities of the ICDDR,B laboratories. Under this protocol, scientists from the collaborating institutions will share reagents and strains, exchange ideas, and visit each others laboratories to further build the technical capabilities and materialize the common research agenda described in this project.

Flow Chart for implementation of the project



Principal Investigators **DR. SHAH M. FARUQUE & DR. G. BALAKRISH NAIR**

Biography of the Principal Investigator (s)

A. Family name: Nair — Date of birth: January 5, 1954

First name(s): Gopinath Balakrish — Nationality: Indian

B. Education (subjects, university or school, degree, year)

B.Sc.	1973-75	Loyola College, Madras University, India	Zoology, Chemistry and Botany. Obtained **First Class**
M.Sc.	1975-77	Centre of Advanced Study in Marine Biology, Annamalai University, India	Marine Biology. Obtained **First Class** and ranked **Second** in the University. Recipient of the UGC National Merit
Ph.D	1978-82	Centre of Advanced Study in Marine Biology, Annamalai University, India	Marine Microbiology

C. Present and most recent positions held (type of posittion, institution/authority, dates)

Position	Dates	Institution	Responsibility
Research Microbiologist (P4, Step 1)	Apr. 8, 2000 - to date	International Centre for Diarrhoeal Diseases, Bangladesh (ICDDR,B)	Full time research
Deputy Director,	Dec. 26, 1996 – to date	National Institute of Cholera and Enteric Diseases (NICED) Calcutta, India	Full time research work on Microbiology of Diarrhoeal Etiologies
Sabbatical Leave	Sept. 1, 1994 – June 26, 1995	Department of Microbiology, Kyoto University, Kyoto, Japan	Molecular Microbiology
Assistant Director	July 1, 1993 - Dec. 25, 1996	NICED	Same as above
Senior Research Officer	July 1, 1988 - June 30, 1993	NICED	Same as above
Guest Research Fellow, Japan Health Sciences Foundation	March 1, 1987 – March 31, 1988	National Children's Medical Research Center, Tokyo, Japan	Research on Heat-stable enterotoxins of *Vibrio cholerae*
Research officer	Oct. 8, 1982 - June, 30, 1988	NICED	Same as above
Asst. Research Officer	Sept. 26, 1981 - Oct. 7, 1982	NICED	Same as above

D. Recent publications: list only the five most important and relevant publications over the last five years (papers in press or submitted for publication are also acceptable).
Please give full bibliographic references: author(s), title, journal, volume, page numbers, year

1. Basu, A., P. Garg, S. Datta, S. Chakraborty, T. Bhattacharya, A. Khan, T. Ramamurthy, S.K. Bhattacharya, S. Yamasaki, Y. Takeda and **G.B. Nair**. 2000. A seven-year analysis of the incidence, antibiogram and genotypes of *Vibrio cholerae* O139 in Calcutta, India and comparison of the Calcutta O139 genotypes with those isolated from other parts of India. **Emerging Infect. Dis.** 6 : 20-28.
2. Basu, A., A.K. Mukhopadhyay, P. Garg, S. Chakraborty, T. Ramamurthy, S. Yamasaki, Y. Takeda and **G.B. Nair** (2000). Diversity in the arrangement of CTX prophages in classical strains of *Vibrio cholerae* O1. **FEMS Microbiol. Lett.** 182: 35-40.
3. Faruque, S.M., M. N. Saha, Asadulghani, P. K. Bag, R. K. Bhadra, S.K. Bhattacharya, R.B. Sack, Y. Takeda, and **G.B. Nair** (2000). Genomic diversity among *Vibrio cholerae* O139 strains isolated in Bangladesh and India between 1992 and 1998. **FEMS Microbiol. Lett.** 15: 279-284.
4. Mukhopadhyay, A.K., D. Kersulyte, J.Y. Jeong, S. Datta, V.Ito, A. Chowdhury, S. Chowdhury, A. Santra, S.K. Bhattacharya, T. Asuma, **G.B. Nair** and D.E. Berg. 2000. Distinctiveness of genotypes of *Helicobacter pylori* in Calcutta, India. **J. Bacteriol.** 182: 3219-3227.
5. Mitra, R., P. Figueroa, A.K. Mukhopadhyay, T. Shimada, Y. Takeda, D. Berg and **G.B. Nair**. 2000. Cell vacuolation, a manifestation of the ElTor hemolysin of *Vibrio cholerae*. **Infect. Immun.** 68: 1928-1933.

DR. SHAH M. FARUQUE
LABORATORY SCIENCES DIVISION
INTERNATIONAL CENTER FOR DIARRHOEAL DISEASE RESEARCH
GPO BOX 128, DHAKA-1000, BANGLADESH

EDUCATION AND TRAINING:

Ph.D., (Molecular Biology) University of Reading, Reading, England, 1988
*Received training in recombinant DNA techniques including construction and screening of genomic libraries, cloning and sequencing of DNA and analysis of gene sequence.

M.Sc., (Biochemistry) University of Dhaka, Dhaka, Bangladesh, 1979: First Class
*M.Sc. thesis on interactions of vitamin A deficiency and immune responses in malnourished children
*Major courses: Biochemistry, Microbiology, Molecular biology, Immunology.

B.Sc. (Honours, Biochemistry) , University of Dhaka, Bangladesh, 1978: First Class
Major courses: Biochemistry, Microbiology, Organic and physical chemistry, Physiology Nutrition.

TRAINING: Center for Disease Control, Atlanta, Georgia, USA, 1989
*Received training on the identification and characterization of enteric pathogens using molecular techniques.

AWARDS AND HONOURS

1985-1988 Commonwealth Scholar
Commonwealth Scholarship Commission, U.K.

1997-present: Senior Associate
Department of International Health,
Johns Hopkins University, Baltimore, Maryland, USA

ACADEMIC AND RESEARCH APPOINTMENTS:

1981-1984 Research Fellow, Department of Biochemistry, University of Dhaka

1984-1985 : Lecturer, Department of Biochemistry, University of Dhaka.

1988-1989 : Assistant Professor, Department of Biochemistry, University of Dhaka.

1989-1993: Assistant Scientist, International Center for Diarrhoeal Disease Research.

1993-1996: Associate Scientist, International Center for Diarrhoeal Disease Research.

1997- present: Scientist & Head of Molecular Biology, International Center for Diarrhoeal Disease Research.

SELECTED PUBLICATIONS (Last 5 years):

1. **Faruque SM**, Rahman MM, Hasan AK, Nair GB, Mekalanos JJ, Sack DA. Diminished Diarrheal Response to *Vibrio cholerae* Strains Carrying the Replicative Form of the CTXΦ Genome instead of CTXΦ Lysogens in Adult Rabbits. Infect Immun. 2001; 69:6084-90.

2. **Faruque SM**, Saha MN, Asadulghani, Sack DA, Sack RB, Takeda Y, Nair GB. The O139 serogroup of *Vibrio cholerae* comprises diverse clones of epidemic and nonepidemic strains derived from multiple *V. cholerae* O1 and non-O1 progenitors. J. Infect. Dis. 2000. 182:1161-1168.

3. **Faruque SM**, Asadulghani, Rahman MM, Waldor MK, Sack DA. Sunlight-induced propagation of the lysogenic phage encoding cholera toxin. Infect. Immun. 2000; 68: 4795-4801.

4. **Faruque SM**, Rahman MM, Asadulghani, Islam KMN, Mekalanos JJ. Lysogenic conversion of environmental Vibrio mimicus strains by CTXΦ. In fect Immun 1999; 67:5723-5729.

5. **Faruque SM**, Siddique AK, Saha MN, Asadulghani, Rahman MM, Zaman K, Albert MJ, Sack DA, Sack RB. Molecular characterization of a new ribotype of Vibrio cholerae O139 Bengal associated with an outbreak of cholera in Bangladesh. J Clin Microbiol 1999; 37: 1313-1318.

6. **Faruque SM**, Albert MJ, Mekalanos JJ. Epidemiology, genetics and Ecology of Toxigenic Vibrio cholerae (Review). Microbiol. Mol. Biol. Rev. 1998; 62:1301-1314

7. **Faruque SM**, Asadulghani, Saha MN, Alim ARMA, Albert MJ, Islam KMN, Mekalanos JJ. Analysis of environmental and clinical strains of nontoxigenic Vibrio cholerae for susceptibility to CTXΦ: molecular basis for the origination of new strains with epidemic potential. Infect. Immun. 1998; 66: 5819-5825

8. **Faruque SM**, Asadulghani, Alim ARMA, Islam KMN, Albert MJ, Mekalanos JJ. Induction of the lysogenic phage encoding cholera toxin in naturally occurring strains of toxigenic Vibrio cholerae O1 and O139. Infect Immun 1998; 66:3752-3757.

9. **Faruque SM**, Saha MN, Asadulghani, Bag PK, Bhadra RK, Bhattacharya SK, Sack RB, Takeda Y, Nair GB. Genomic diversity among Vibrio cholerae O139 strains isolated in Bangladesh and India between 1992 and 1998. FEMS Microbiol Lett. 2000; 184:279-284.

10. **Faruque SM**, Ahmed KM, Siddique AK, Zaman K, Alim ARMA, Albert MJ. 1997. Molecular analysis of toxigenic Vibrio cholerae O139 Bengal isolated in Bangladesh between 1993 and 1996: evidence for the emergence of a new clone of the Bengal vibrios. J. Clin Microbiol. 1997; 35:2299-2306.

11. **Faruque SM**, Ahmed KM, Alim ARMA, Qadri F, Siddique AK, Albert MJ. Emergence of a new clone of toxigenic Vibrio cholerae biotype El Tor displacing V. cholerae O139 Bengal in Bangladesh. J Clin Microbiol 1997; 35:624-630.

12. Casswall, TH, Sarker SA, **Faruque SM**, Weintraub A, Albert MJ, Fuchs GJ, Alam NH, Dahlstrom AK, Link H, Brussow H, and Hammarstrom L. Treatment of enterotoxigenic and enteropathogenic Escherichia coli-induced diarrhoea in children with bovine immunoglobulin milk concentrate from hyperimmunized cows: A double-blind, placebo-controlled trial. Scand J Gastroenterol 2000; 35:711-718.

13. Bardhan PK, Albert MJ, Alam NH, **Faruque SM**, Neogi PKB, Mahalanabis D. Small bowel and fecal microbiology in children suffering from persistent diarrhea in Bangladesh. J Pediatr Gastroenterol Nutr 1998; 26: 9-15.

14. Chakraborty S., Mukhopadhyay AK, Bhadra RK, Ghosh AN, Mitra R, Shimada T., Yamasaki S, **Faruque SM**, Takeda Y, Colwell RR, and Nair GB. Virulence genes in environmental strains of Vibrio cholerae. App Env Microbiol 2000; 66:4022-4028.

15. Albert MJ, Faruque ASG, **Faruque SM**, Sack RB, Mahalanabis D. Case-control study of enteropathogens associated with childhood diarrhea in Dhaka, Bangladesh. J Clin Microbiol. 1999: 37:3458-3464.

16. Albert MJ, Bhuyan NA, Talukder KA, Faruque ASG, Nahar S, **Faruque SM**, Ansaruzzaman M Rahman M. Phenotypic and genotypic changes in Vibrio cholerae O139 Bengal. J Clin Microbiol 1997; 35:2588-2592.

17. Faruque ASG, Salam MA, **Faruque SM**, Fuchs GJ. Etiological, clinical, and epidemiological characteristics of a seasonal peak of diarrhoea in Dhaka, Bangladesh. Scan J Infect Dis 1998; 30:393-396

18. Albert MJ, **Faruque SM**, Faruque ASG, Bettelheim KA, Neogi PKB, Bhuiyan NA, Kaper JB. Controlled study of cytolethal distending toxin-producing *Escherichia coli* infections in Bangladeshi children. J Clin Microbiol 1996; 34:717-719.

19. Unicomb LE, **Faruque SM**, Malek MA, Faruque ASG, Albert MJ. Demonstration of a lack of synergistic effect of rotavirus with other diarrheal pathogen on severity of diarrhea in children. J Clin Microbiol. 1996; 34:1340-1342

20. Ansaruzzaman M, Albert MJ, Kuhn I, **Faruque SM**, Siddique AK, Molby R. 1996. Differentiation of Vibrio cholerae O1 isolates with biochemical fingerprinting and comparison with ribotyping. J. Diarrheal Dis Res. 14:248-254.

21. Qadri F, Mohi MG, Azim T, **Faruque SM**, Kabir AKMI, Albert MJ. Production, characterization and immunodiagnostic application of a monoclonal antibody to shiga toxin. J Diarrhoeal Dis Res 1996; 14:95-100.

22. Azim T., Islam LN, Sarker MS, Ahmad SM, Hamdani JD, **Faruque SM**, Salam MA. 2000. Immune response of Bangladeshi children with acute diarrhea who subsequently have persistent diarrhea. J. Pediatr. Gastroenterol. Nutr. 31:528-535.

23. Chowdhury HR, Yunus M, Zaman K, Rahman A, **Faruque SM**, Lescano AG, Sack RB. 2001. The efficacy of bismuth subsalicylate in the treatment of acute diarrhoea and the prevention of persistent diarrhoea. Acta Paediatr. 90:605-10.

24. Ryan ET, Bridges EA, Crean TI, Gausia K, Hamadani JD, Aziz A, Hawkes S, Begum M,

Bogaerts J, **Faruque SM**, Salam MA, Fuchs GJ, Calderwood SB. 2001. Local production of anti-*Vibrio cholerae* mucosal antibody in reproductive tract tissues after cholera. J. Infect. Dis.184:643-7.

Principal Investigators **DR. SHAH M. FARUQUE & DR. G. BALAKRISH NAIR**

Detailed Budget

Project Title: Genetic variants of *Vibrio cholerae* O139 and development of a vaccine against O139 cholera
Name of PIs: Dr. Shah M. Faruque & Dr. G. Balakrish Nair

Protocol Number: Name of Division: Laboratory Sciences Division
Funding Source: SIDA/SAREC Amount to be Funded (direct cost): US $ 251,593

Starting Date: January, 2002 Closing Date: December 2004

Strategic Plan Priority Code(s): Research issue 11 (100%); Discipline 92 (50%), 93 (25%), 94 (25%)

Detailed Budget (Budget for ICDDR,B)

ACCOUNT DESCRIPTION	Salary support	(% Effort)	1st yr		2nd yr		3rd yr		TOTAL	
Personnel	Position/Level		US $	SEK	US $	SEK	US $	SEK	US $	SEK
Dr. Shah M. Faruque	Scientist (P-4)	15%	15078	155605	15832	163385	16623	171554	47533	490545
Dr. Firdausi Qadri	Scientist (P-5)	5%	5848	60351	6140	63369	6447	66537	18436	190258
MR. Q. S. Ahmad	Asst. Scientist	10%	1417	14623	1488	15355	1562	16122	4467	46100
RO (molecular genetics)	RO/GS-V	100%	3996	41239	4196	43301	4406	45466	12597	130005
SUB TOTAL			**26339**	**271818**	**27656**	**285409**	**29039**	**299680**	**83034**	**856908**
Supplies/operating cost (immunology)										
Reagents/plastics			3500	36120	3000	30960	3000	30960	9500	98040
Res Animals			1000	10320	1000	10320	1000	10320	3000	30960
SUBTOTAL			**4500**	**46440**	**4000**	**41280**	**4000**	**41280**	**12500**	**129000**
Supplies/operating cost (Molecular Genetics)										
Reagents, enzymes			9000	92880	9000	92880	9000	92880	27000	278640
Culture media, plastics			3000	30960	3000	30960	3000	30960	9000	92880
DNA labeling kits, cloning kits			3500	36120	3500	36120	3500	36120	10500	108360
Interdepartmental,	repair, maintenance etc.		200	2064	300	3096	200	2064	700	7224
Print, fax, publications,			300	3096	300	3096	500	5160	1100	11352
SUBTOTAL			**16000**	**165120**	**16100**	**166152**	**16200**	**167184**	**48300**	**498456**
Travel/ Consultancy	(Molecular Genetics)									
Local Travel			500	5160	500	5160	500	5160	1500	15480
International Travel			5000	51600	5000	51600	5000	51600	15000	154800
SUBTOTAL			**5500**	**56760**	**5500**	**56760**	**5500**	**56760**	**16500**	**170280**
TOTAL DIRECT COST			**52339**	**540138**	**53256**	**549601**	**54739**	**564904**	**160334**	**1654644**
INDIRECT COST (25%)			13085	135035	13314	137400	13685	141226	40083	413661
TOTAL PROJECT COST (ICDDR,B)			**65424**	**675173**	**66570**	**687002**	**68423**	**706130**	**200417**	**2068305**

Principal Investigators DR. SHAH M. FARUQUE & DR. G. BALAKRISH NAIR

Budget for Division of Clinical Bacteriology, Karolinska Instute, Huddinge, Sweden

ACCOUNT DESCRIPTION	Salary support	(% Effort)	1st yr		2nd yr		3rd yr		TOTAL	
Personnel	Position/Level		US $	SEK	US $	SEK	US $	SEK	US $	SEK
To be named	Technician	10%	3850	39732	4235	43705	4658	48071	12743	131508
									0	0
SUB TOTAL			**3850**	**39732**	**4235**	**43705**	**4658**	**48071**	**12743**	**131508**
Travel International			3000	30960	3000	30960	3000	30960	9000	92880
SUBTOTAL			**3000**	**30960**	**3000**	**30960**	**3000**	**30960**	**9000**	**92880**
Supplies and Materials (Description of Items)										
Chemicals and reagents			4000	41280	3500	36120	4000	41280	11500	118680
Plastics and supplies			2000	20640	2500	25800	2000	20640	6500	67080
Animals			2000	20640	1500	15480	1500	15480	5000	51600
SUBTOTAL			**8000**	**82560**	**7500**	**77400**	**7500**	**77400**	**23000**	**237360**
Overhead (except travel) (18%)			2133	22013	2112	21796	2188	22580	6433	66389
SUBTOTAL			**2133**	**22013**	**2112**	**21796**	**2188**	**22580**	**6433**	**66389**
TOTAL PROJECT COST (Karolinska Institute)			**16983**	**175265**	**16847**	**173861**	**17346**	**179011**	**51176**	**528136**
TOTAL PROJECT COST (ICDDR,B)			**65424**	675176	**66570**	687002	**68423**	706125	200417	2068303
TOTAL COST (ICDDR,B + KI)			**82407**	**850440**	**83417**	**860863**	**85769**	**885136**	**251593**	**2596440**

**5% of Dr. G. B. Nair's salary for the first 4 months of the project only, has been included in Dr. Shah Faruque's salary budget.

Total cost for three years (ICDDR,B + KI): US$ 251,593 SEK 2,596,440

Budget Justifications

1. The recruitment of a research officer is essential for conducting the environmental sampling and for participation in the laboratory based characterization of strains.

2. Funds for local travel will be required for sampling in areas away from Dhaka. Funds for International travel will be used for the travel of collaborators and the investigators to perform collaborative works in characterization of strains and data analysis.

3. Budget for supplies have been kept at a realistic level considering the expensive reagents and enzymes used for molecular analysis of strains.

The budget has been reviewed by the budget office of ICDDR,B and found to be appropriate

Controller, Budget and Costing
ICDDR,B

1. **Title:** Genetic variants of *Vibrio cholerae* O139 and development of a vaccine against O139 cholera.

Summary of Referee's Opinions: Please see the following table to evaluate the various aspects of the proposal by checking the appropriate boxes. Your detailed comments are sought on a separate, attached page.

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

Conclusions

I support the application:

a) without the qualification

b) with qualification

- on technical grounds

- on level of financial support

I do not support the application.

Name of Referee : WANPEN CHAICUMPA

Signature: ..... **Date:** 23 November 2001

Position: Professor

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

Detailed Comments

Please briefly provide your opinions of this proposal, giving special attention to the originality and feasibility of the project, its potential for providing new knowledge and the justification of financial support sought; include suggestions for modifications (scientific or financial) where you feel they are justified.

The investigators have provided an excellent and concise review on molecular biology of *Vibrio cholerae*. They have raised important research questions which answers will have great impact on improving public health of mankind. The proposed methodology and techniques are appropriate, especially the phage display system for production of vaccine mimotope which is a new vaccine strategy. The budget and the time required for the research are appropriate.

I agree completely with the proposal and give full moral supports to the investigators. Special admiration on the preparation of the proposal which they have done so well; they must be congratulated.

Title: Genetic variants of *Vibrio cholerae* O139 and development of a vaccine against O139 cholera

PI: G. Balakrish Nair and Shah M. Faruque

Reviewer: Professor Dr. WANPEN CHAICUMPA

COMMENTS

The project entitled "Genetic variants of *Vibrio cholerae* O139 and development of a vaccine against O139 cholera" by G.B.Nair, S.M.Faruque and others aims at the isolation of *V.cholerae* O139 strains from the environment as well as from patients and their molecular characterization to determine the possibility whether the toxigenic strains with epidemic potential are evolved from the nontoxigenic progenitor strains that are present in the environment. To test this hypothesis , the investigators plan to undertake a prospective study in two areas of Bangladesh which are known to be endemic for cholera over the years. As a continuation of the earlier projects, the investigators ,in collaboration with other scientists , also plan to explore the possibility of development of potential O139 vaccines using detoxified LPS preparations, CPS based glycoconjugates and peptide mimics of O139 CPS. The project is well written with adequate background information and defined objectives. The investigators are apparently quite familiar with the methodologies to be adopted and possess necessary expertise in their respective area related to the project. On completion, the project is likely to generate new information on the persistence and evolution of *V.cholerae* O139 strains in the cholera endemic area as well as on the possibility of the development of O139 polysaccharide based vaccines. I recommend the project for approval and funding with appropriate budgetary provision.

There are some minor issues which the investigators may take into consideration while designing their experimental protocol:

(1) The investigators hypothesize that the emergence of new clones is "possibly triggered by the increasing immunity in the host population against the existing epidemic clones". While such a possibility may explain the evolution of pathogenic *V.cholerae* strain(clone) of one serogroup from a different one (for example O139 from a O1 strain) in a population having immunity towards the progenitor (O1) serogroup, evolution of another O139 strain (clone) in an area already known to be endemic for O139 cholera and its replacement of the earlier strain(clone) in the population would require certain selective advantage of the newly evolved strain (clone)over the existing ones. This may be achieved as a result of(a) subtle variation in the antigenic properties between these O139 strains , (b) increased colonizing property of the newly evolved strain (clone) and/or, (c) increased capability of the new clone to persist in the environment. The investigators may like to address some of these issues through the design of appropriate experiments.

(2) Serum vibriocidal antibody titter is believed to be a surrogate marker of the protective immunity in cholera. Vibriocidal activity is primarily directed towards the polysaccharide determinant of the LPS. Although the exact relevance of the serum vibriocidal antibody in intestinal immunity is not clear, anti-LPS or anti-CPS antibodies in serum have been shown to mediate passive protection against *V.cholerae* challenge in experimental animal models through inhibition of intestinal colonization of vibrios. The investigators may evaluate the antisera raised against oligosaccharides/ glycoconjugates/ endotoxoids to this effect.

(3)"Presence of genes for CT, toxin coregulated pili and ToxR will be determined by using DNA probes and PCR assays"- Investigators may include ToxT in these experiments.

(4) "Both toxigenic and non-toxigenic *V.cholerae* strains isolated from environmental samples as well as from patients during the same period will be analyzed by genetic fingerprinting to detect possible emergence and transmission of novel toxigenic strains"(Research design: surveillance) ----- The serogroup of the *V.cholerae* strains to be included in this study need to be specified .

INVESTIGATORS' RESPONSE TO REVIEWERS' COMMENTS

Both reviewers have supported the objective and design of the project. While the first reviewer has no additional suggestion for modification, the second reviewer has given a few specific suggestion to further improve the project. We have carefully considered the reviewer's suggestions and made modifications accordingly. Our specific response to the 2nd reviewer's comments is as follows.

1. The reviewer has suggested to design new experiments to understand the factors involved in the emergence of new epidemic clones replacing old clones of the same serogroup. We agree with the reviewer that there is a need to study this aspect. But since this will involve many new investigations and experiments, considering the budget constraints of the present project, we have planned to investigate these aspects of *V. cholerae* evolution under a different project on the role of genetic and environmental factors influencing the emergence of new epidemic clones.

2. We agree with the reviewer on the relevance of different antibodies in mediating passive protection against *V.cholerae* challenge, and our Swedish collaborators have designed experiments to evaluate the antisera raised against oligosaccharides, glycoconjugates, and endotoxoids (page 18 of the project).

3. We have accepted the reviewer's suggestion and have included assay for toxT in the project (page 15 of the project)

4. We have accepted the reviewer's suggestion, and will serotype all *V. cholerae* strains.