RESEARCH PROTOCOL

Protocol No. 2002-019

FOR OFFICE USE ONLY		
RRC Approval:	☐ Yes / ☐ No	Date:
ERC Approval:	☐ Yes / ☐ No	Date:
AEEC Approval:	☐ Yes / ☐ No	Date:

Project Title: Analysis and genetic modification of *Vibrio cholerae* strains carrying atypical combination of virulence genes and phenotypes to develop potential vaccine candidates.

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: _____ **Cholera, *Vibrio cholerae*, vaccine, virulence genes**

Relevance of the protocol:

Routine vaccination for cholera in areas where the disease is endemic and epidemic has not been implemented due to unavailability of a suitable vaccine with optimum efficacy in an at-risk population, at an affordable cost. Unfortunately, in the past the strategy to develop a cholera vaccine for use in an endemic or epidemic setting was unrealistically based on needs for developed countries (e.g. travelers). The need for efficient cholera vaccines as an additional public health tool for cholera prevention is an urgent requirement in endemic areas, and the standards for the development and deployment of a cholera vaccine need to stress convenience of vaccination and low cost. For example, developing and routine use of a cholera vaccine, which is even 60 to 70% effective is significant, judging by the sheer numbers that such a vaccine would protect in an endemic or epidemic region. Continued use of such a vaccine would ultimately bring the incidence of cholera to manageable levels. The present study is therefore designed to develop an inexpensive cholera vaccine using naturally occurring genetic variants of *Vibrio cholerae*.

Principal Investigator: **Dr. Shah M. Faruque** **Division:** **LSD** **Phone:** 2410

Address: **Laboratory Sciences Division, ICDDR,B** **Email: faruque@icddrb.org**

Co-Investigator(s): **Dr. G. B. Nair**
Dr. A. S. G. Faruque
Dr. David A. Sack

Student Investigator/Intern:

Collaborating Institute(s):

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	**Direct Cost Required for the Budget Period ($)**				
(Day, Month, Year - DD/MM/YY)	*a.*	*Ist Year*	*2nd Year*	*3rd Year*	*Other years*
Beginning date: When funds become available		**105,876**	**109,730**	**111,156**	**108,964**
Duration: 4 years	*b.*	*Direct Cost :*	**435,726**	*Total Cost :*	**544,658**

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

7th July, 2002
Date of Approval

Certification by the Principal Investigator

I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

Signature of PI Shah Faruque

Date: July 7, 2002

Name of Contact Person : Dr. Shah M. Faruque, LSD

Principal Investigators: Last, first, middle - **FARUQUE, SHAH M.**

International Centre for Diarrhoeal Disease Research, Bangladesh

RESEARCH PROTOCOL

FOR OFFICE USE ONLY
Protocol No: Date:
RRCApproval: Yes/ No Date:
ERC Approval: Yes/No Date:

1. Title of Project
Analysis and genetic modification of *Vibrio cholerae* strains carrying atypical combination of virulence genes and serotypes, to develop potential vaccine candidates.

2a. Name of the Principal Investigator(s) (Last, First, Middle).	**2b. Position / Title**	**2c. Qualifications**
FARUQUE, SHAH M.	SCIENTIST	M.Sc., Ph.D.

3. Name of the Division/ Branch / Programme of ICDDR,B under which the study will be carried out.
LABORATORY SCIENCES DIVISION

4. Contact Address of the Principal Investigator
4a. Office Location:
Molecular Genetics Laboratory,
Laboratory Sciences Division, ICDDR,B.
GPO BOX-128, DHAKA-1000, BANGLADESH

4b. Fax No: 880 2 872529 and 880 2 883116
4c. E-mail: **faruque**@icddrb.org
4d. Phone / Ext: 880 2 871751 to 880 2 871760 Ext- 2410

5. **Use of Human Subjects** Yes [ ] No [X]

5a. Use of Live Animal Yes [X] No [ ]

5b. If Yes, Specify Animal Species
Rabbits

6. **Dates of Proposed Period of Support**
(Day, Month, Year - DD/MM/YY)

7. Cost Required for the Budget Period
7a. Total Direct Cost ($) 435,726 **7b. Overhead:** 25%

8. 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
July 7, 2002 — Date of Approval

9. **Certification by the Principal Investigator**

I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

10. Signature of PI
Shah Faruque
Date: July 7, 2002

Table of Contents

☐ Check here if appendix is included

Principal Investigators: Last, first, middle - FARUQUE, SHAH M.

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:
Analysis and genetic modification of *Vibrio cholerae* strains carrying atypical combination of virulence genes and serotypes, to develop potential vaccine candidates.

Total Budget US$ 435,726 for 4 years Beginning Date: August, 2002 Duration: 4 years

SUMMARY:

Routine vaccination for cholera in areas where the disease is endemic and epidemic has not been implemented due to unavailability of a suitable vaccine with optimum efficacy in an at-risk population, at an affordable cost. Unfortunately, in the past the strategy to develop a cholera vaccine for use in an endemic or epidemic setting was unrealistically based on needs for developed countries (e.g. travelers). The need for efficient cholera vaccines as an additional public health tool for cholera prevention is an urgent requirement in endemic areas, and the standards for the development and deployment of a cholera vaccine need to stress convenience of vaccination and low cost. For example, developing and routine use of a cholera vaccine, which is even 60 to 70% effective is significant, judging by the sheer numbers that such a vaccine would protect in an endemic or epidemic region. Continued use of such a vaccine would ultimately bring the incidence of cholera to manageable levels. The present study is therefore designed to develop an inexpensive cholera vaccine using naturally occurring genetic variants of *Vibrio cholerae*. Recent studies have identified naturally occurring *V. cholerae* strains which appear to be genetic hybrids of more than one serotype or biotype and carry an unusual combination of virulence and serotype specific genes. These strains are expected to produce antigens which can be used to immunize against a variety of virulent *V. cholerae* strains. Because killed whole cell vaccine preparations are safe, and newer antigens can be added conveniently, identification, characterization and genetic modification of naturally occurring genetic hybrid strains or more than one strain of different serotypes may be useful in developing potential vaccine candidates. To begin to investigate this possibility, we plan to carry out studies to (a) Identify *V. cholerae* strains carrying atypical combination of virulence genes and serotypes, (b) Perform genetic modifications to construct nontoxigenic derivatives, or select spontaneous nontoxigenic mutants, and make these strains resistant to toxigenic conversion by CTXΦ (c) perform comparative molecular analysis of these strains with virulent strains isolated from cholera patients (d) Use the attenuated strains to immunize rabbits, and study the protective efficacy against challenge with virulent strains. The long term goal of this work is to explore the possibility of using these strains as inexpensive killed vaccines for use in areas of endemic and epidemic cholera.

Principal Investigators: Last, first, middle - **FARUQUE, SHAH M.**

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

Name	Professional 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. A. S. G. FARUQUE	Clinician	Co-investigator
4. QAZI SHAFI AHMAD	Microbiologist	Co-investigator
5. DR. DAVID A. SACK	Microbiologist	Co-Investigator

DESCRIPTION OF THE RESEARCH PROJECT

Hypothesis to be tested:

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

Although *V. cholerae* O1 and O139 responsible for epidemic outbreaks of cholera carry a set of defined virulence genes as well as genes encoding serotype specific antigens, there are also naturally occurring *V. cholerae* strains which appear to be genetic hybrids of different epidemic strains and carry atypical combinations of virulence genes and serotypes. If used as vaccines, these strains may provide protective immunity against diverse clones of toxigenic *V. cholerae*. Identification, and genetic modification of these strains may lead to the development of potential vaccine candidates. Such attenuated strains can be used to prepare a safe and inexpensive multivalent killed whole cell vaccine, and can be used routinely to immunize population in areas of endemic and epidemic cholera.

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).

(a) We will screen our *V. cholerae* collections to identify genetic hybrids carrying atypical combinations of virulence and serotype specific genes. The *V. cholerae* collection in our laboratory includes a large number of strains isolated from different cholera outbreaks as well as from environmental sources under different previous studies and cholera surveillance programs.

(b) Genetically modify the strains to construct nontoxigenic derivatives or select spontaneous nontoxigenic mutants.

(c) Perform comparative molecular analysis of the nontoxigenic strains with virulent strains isolated from cholera patients to study their clonal relationship

(d) Test the safety of the strains in adult rabbits using the RITARD model

(e) Use the attenuated strains to immunize rabbits, and study the protective efficacy against challenge with virulent strains.

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).**

Epidemiology of pathogenic *Vibrio cholerae*

Epidemics of cholera caused by toxigenic *Vibrio cholerae* belonging to the O1 or O139 serogroup are a major public health problem in developing countries (1). The disease is an acute dehydrating diarrhea caused principally by the potent enterotoxin cholera toxin produced by these organisms during pathogenesis. Cholera is endemic in Southern Asia and parts of Africa and Latin America, where outbreaks occur widely and are particularly associated with poverty and poor sanitation. In areas of endemic infection cholera outbreaks occur in a seasonal pattern. Cholera is a waterborne disease, and the importance of water ecology is suggested by the close association of *V. cholerae* with surface water and the population interacting with the water. Although *V. cholerae* is known to be a human pathogen the bacteria constitute part of the normal aquatic flora in estuarine and brakish waters and are able to persist in the absence of the human host (2, 3). *V. cholerae* is classified on the basis of its somatic antigens (O-antigens) into at least 200 serovars or serogroups, of which only sergroups O1 and O139 are known to cause epidemics of cholera. Non-O1 serogroups of *V. cholerae* had been mostly associated with sporadic cases of diarrhea and extraintestinal infections (4) until 1993, when a large cholera-like outbreak in Bangladesh and India was caused by a *V. cholerae* non-O1 strain (5, 6). This organism did not belong to any of the 138 O serogroups for *V. cholerae* described until then, but belonged to a new serogroup, which was later designated as O139 (6). Since then *V. cholerae* O139 has been persisting as a second etiologic agent of cholera. Hence, there are now two serogroups, O1 and O139, that have been associated with epidemic disease, but there are also strains of these serogroups which do not produce cholera toxin, and are not involved in epidemics. It is generally accepted that seven distinct pandemics of cholera have occurred since the onset of the first pandemic in 1817 (7). Except for the seventh pandemic which originated in Indonesia, the other six pandemics arose from the Indian subcontinent, usually from the Ganges delta and spread to reach other continents affecting many countries and extending over many years. Available information on these pandemics have been reviewed by Kaper *et.al.* (8).

Molecular Epidemiology: 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, 9-17). 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 indicated that the U.S. Gulf coast isolates of toxigenic *V. cholerae* are clonal and that they are different from other seventh pandemic isolates (13). 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 (14, 15, 16). 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 (9). 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 changes in the serotype specific gene cluster (17). Molecular analysis of epidemic isolates of *V. cholerae* between 1961 and 1996 in Bangladesh revealed clonal diversity among strains isolated during different epidemics (17-20). 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. 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. These studies indicated that there had been frequent 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 (21) 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 (22, 23). Thus the major virulence gene clusters in *V. cholerae*, appear

to have the ability to propagate laterally and disperse among different strains. 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* (24, 25, 26). 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.

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 (27). Expression of CT and TCP are co-regulated by the ToxR regulatory system which includes the ToxT protein (28). The genes encoding ToxT and TCP are located in the same choromosomal region (29) together with other ToxR-regulated genes including those for a potential accessory colonization factor (ACF) (30). 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 (31). 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* (32). 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 (22). 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 termed VPIΦ (23), but this finding has not been confirmed in subsequent reexamination.

Cholera toxin converting bacteriophage (CTXΦ): 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 (33). A third toxin that has been described is Accessory cholera enterotoxin (Ace) which is capable of inducing fluid accumulation in rabbit ligated ileal loops (34).

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 (35). 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 (21).

It has been demonstrated that under appropriate conditions toxigenic *V. cholerae* strains can be induced to produce extracellular CTXΦ particles (21, 36). 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 (21). 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 (37). 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 (38). 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 (38).

Coordinate regulation of V. cholerae genes: There are multiple systems involved in the regulation of genes in *V. cholerae*. Expression of several critical virulence genes in *V. cholerae* is coordinately regulated so that multiple genes respond in a similar fashion to environmental conditions (28, 39). Co-ordinate expression of virulence genes results from the activity of a cascading system of regulatory factors. ToxR, a 32 kDa transmembrane protein is the master regulator which is itself regulated by environmental signals. The ToxR protein binds to a tandemly repeated 7-bp DNA sequence found upstream of the ctxAB structural gene and increases transcription of ctxAB resulting in higher levels of CT expression. ToxR regulates not only the expression of ctxAB, but at least 17 distinct genes, that constitute the ToxR regulon. These include the TCP colonization factor, the accessory colonization factor, outer membrane proteins OmpT and OmpU, and three other lipoproteins (40-42). Except for the ctxAB genes, other genes in the ToxR regulon are controlled through another regulatory factor called ToxT, a 32-kDa protein. ToxR controls the transcription of the toxT gene, which encodes a member of the AraC family of bacterial transcription activators. The resulting increased expression of the ToxT protein then leads to activation of other genes in the ToxR regulon. Thus ToxR is at the top of the regulatory cascade that controls the expression of a number of genes, while the expression of ToxR itself remains under the control of environmental factors (39, 43). The coordinate regulation of a

number of genes through the toxR regulon demonstrates that the organism has developed a mechanism of sampling and responding to its environment. Hence it seems obvious that *V. cholerae* is able to activate or inactivate a set of genes including those encoding colonization factors or toxins as an appropriate response to changing environmental conditions.

Genetic exchanges in Vibrio cholerae: . The species *V. cholerae* contains a wide variety of strains and biotypes, receiving and transferring genes for virulence associated factors as well as genes for other biochemical functions including antibiotic resistance, capsular polysaccharides and new surface antigens (1). Understanding the lateral or horizontal transfer of genes by phage, pathogenicity islands and other accessory genetic elements can provide insights into how this bacterial pathogen emerge and evolve to become new strains. *V. cholerae* is a bacterium autochthonous to riverine, coastal, and estuarine ecosystems, but at the same time, pathogenic for humans. Because of its coexistence with diverse microorganisms, and the ability to acquire genes from other strains in its natural habitat, there is frequent emergence of new V. cholerae strains with altered genetic and phenotypic properties. Recent studies have shown the presence of a versatile system in *V. cholerae* for acquisition of genes from other organisms. This consists of a distinctive class of integron which are gene expression elements that may capture open reading frames (ORF) and convert them to functional genes (44). The insertion of an ORF or a gene cassette into an integron takes place by site specific recombination between the circularized cassette and the recipient integron which carries an integrase gene and a specific attachment site. All integron-inserted gene cassettes have been found to possess an imperfect inverted repeat sequence located at the 3' end of the gene that functions as a recognition site for the site-specific integrase. The repeat sequence is referred to as the 59-base element, which is a diverse family of sequences that are recognized by the integrase. Until recently,,all known integrons were associated only with genes conferring antibiotic resistance (45, 46). Recently, a gene designated as *intI4* that encodes a previously unknown integrase have been identified in *V. cholerae* (44).

This integrase recognizes a family of *V. cholerae* repeated sequences (VCRs) and is associated with a "gene-VCR" organization, similar to that of the well characterized antibiotic resistance integrons (44, 46). The VCRs are a family of 123 to 126 base pair sequences that are highly repeated (60 to 100 copies) and situated in a region corresponding to about 10% of the *V. cholerae* genome (47). Two such sequences flank a heat-stable toxin gene in both O1 and non-O1 *V. cholerae* isolates (48), and nine other VCRs have been found in a 6-kb DNA fragment encoding the genes for MFRHA, a lipoprotein gene, and 8 other unidentified ORFs (49). The variation observed in codon usage of the gene-VCR cassettes as well as their GC content (between 33 and 45%, compared with 47% for the *V. cholerae* genome) suggested that the VCR associated genes were probably recruited by *V. cholerae* from other microbial sources (44). Thus the demonstration of the distinctive integron system in *V. cholerae* suggested that integrons may also play a part in the acquisition of pathogenic genes as well as genes for different biological functions by *V. cholerae*.

Vaccines against cholera

The need for a vaccine: Improved water quality, sanitation and personal hygiene significantly reduce the spread of many water-related diseases. For cholera, supplying populations with safe water and sanitation has worked to reduce the incidence, where trade barriers, quarantine, antibiotic prophylaxis, and ineffective vaccines have failed (50). The Global Water Supply and Sanitation Assessment 2000 estimates that 2.4 billion people world-wide still do not have any acceptable means of sanitation, while 1.1 billion people do not have an improved water supply (51). Provision of improved sources of water and improved sanitation services is a daunting tasks. It has been estimated that in order to half the proportion of the population without access to water supply, sanitation and hygiene by 2015 in Africa, Asia and Latin America alone, would mean providing water supply services to 300,000 more people and providing sanitation facilities to 400,000 more people every day for the next 15 years (52). For cholera, it is most cost effective from the point of view solely of protecting human health, to treat patients than to invest huge sums in cleaning up water or environmental pollution. Hence, the need for efficient cholera vaccines as an additional public health tool for cholera prevention is an urgent interim requirement.

Review of available vaccines: Early studies have shown that parenterally administered cellular vaccines, composed of inactivated *Vibrio cholerae* O1, elicit side reactions, limited immunity in adults and a lesser efficacy in children (53-55). The recognition that protection against *V. cholerae* infection is highly dependent on stimulation of a mucosal immune response has favored the concept of an oral vaccine. Since the withdrawal of the traditional injectable cholera vaccine in 1973, substantial progress has been made in the development of oral cholera vaccines. During the last two decades, an enormous amount of information has accumulated regarding the organism *V. cholerae*, its virulence factors, including cholera toxin, and the molecular basis of its pathogenicity (1). In recent years, with the advent of recombinant DNA technology and major breakthroughs in molecular biology and immunology, a new dimension has been given to the design of vaccine strains. Several strategies have been adopted to develop oral vaccines against cholera and some of these vaccines have undergone field trials. Significant attenuation has been achieved by deleting genes for various virulence determinants, biosynthetic genes, and regulatory genes. However, a suitable cholera vaccine with optimum efficacy in an at-risk population, especially children, is not yet available.

Currently, the two available types of cholera vaccines include (i) a killed-whole-cell formulation representing both biotypes and serotypes representative of serogroup O1 that is combined with the purified cholera toxin (CT-B) subunit and (ii) genetically engineered, live-attenuated *V. cholerae* vaccine (56).

The killed whole cell B subunit vaccine is a two-dose oral vaccine given one week apart in a buffer solution. Studies conducted in Sweden, Bangladesh and South America has proven its safety and high-grade (85%) protection against cholera caused by the classical and El Tor biotypes. The vaccine contains antigens representing both biotypes and both serotypes. Protection is obtained one week after the second immunization. After 6 months of follow-up the protection decreases in children less than 5 years of age. After 3 years of follow-up, the protection decreases but is still 70% in people more than 5 years of age (57, 58). The average protection after 3

years is around 50% in all age groups. This vaccine has been developed in Sweden and licensed in Sweden, Norway, Peru, Argentina, Guatemala, El Salvador, Honduras and Nicaragua.

An orally administered attenuated strain of *V. cholerae*, CVD103-HgR, is a vaccine strain constructed by deleting 94% of the gene encoding the enzymatically active A subunit of cholera toxin (*ctxA*) from classical Inaba *V. cholerae* 569B, leaving intact the gene encoding the immunogenic B (binding) subunit of cholera toxin (*ctxB*). A gene encoding resistance to Hg^{++} ions was inserted into the El Tor hemolysin gene to provide a marker to differentiate the vaccine strain from wild-type *V. cholerae* classical Inaba strains (59).

Several studies in a number of countries, among different age groups, have proven the safety, and immunogenicity of CVD 103-HgR against *V. cholerae* of either classical or El Tor biotype and either Inaba or Ogawa serotype, one week after administration of a single oral dose (60-62). The vaccine is packed in two sachets as a single dose presentation. The vaccine consists of one dose and should be administered with buffer solution and 100 ml of safe water. The onset of protection is after 8 days. A single dose of the live-attenuated vaccine used in clinical trials of North American adults provided 90% protection against the virulent homologous strain and 65 to 80% protection against virulent El Tor biotype, Inaba serotype strains. However, administration of the CVD 103-HgR vaccine in a large-scale field trial in an area of endemicity of Indonesia did not show any correlation between vaccination and protection (56).

It has been difficult to construct a safe El Tor vaccine strain. CVD 110 was constructed from wild-type strain El Tor Ogawa E7948. Although attenuated in much the same manner as CVD 103-HgR, CVD 110 caused mild to moderate diarrhea in 7 of 10 adult volunteers (63-66). Analysis of a series of other El Tor strains that were attenuated showed that there is a wide variability in the virulence of the strains. In addition to CT, *V. cholerae* produces other putative toxic factors such as Ace, Zot, hemolysin, hemagglutinin/protease (HA/P), and others. The roles of these factors in disease have not been determined. Another El Tor oral vaccine strain CVD 111, which is a live-attenuated El Tor biotype, Ogawa serotype strain, provided 80% protection in adult volunteers. CVD 111 was constructed by using a less virulent parent strain, El Tor Ogawa N16117 which was modified by deleting *ctx*, *zot*, *cep*, and *ace* and reintroducing *ctxB* to create CVD 111 (63, 65, 66) In a phase 1 study of CVD 111, 25 volunteers received $3x10^8$ CFU of freshly harvested CVD 111 with buffer. A mild diarrhea that was not accompanied by malaise, nausea, vom-iting or other notable adverse effects was observed in 12% of volunteers. The immune response was strong, and 92% of volunteers developed vibriocidal antibodies (65).

Unfortunately, most El Tor biotype vaccine strain candidates, even those lacking the whole core region of the CTX element (ΔAce, ΔZot, ΔCT) have been considered too reactogenic for wide-scale usage. Reactogenicity is manifested as mild to moderate diarrhea, abdominal cramps, malaise, vomiting, or low-grade fever. The cellular basis of these adverse reactions is not clear. Reactogenicity could be due to colonization of the intestinal mucosa

per se and/or synthesis by the vaccine strain candidate of unrecognized virulence factors. From the failure of each vaccine strain, new information had emerged and improved strategies were adopted. Recently, the nonmotile El Tor Inaba candidate vaccine strain Peru-15 was reported to be well tolerated and immunogenic in volunteers (67-69). The efficacy of this experimental vaccine remains to be confirmed by field trials in areas wherecholera is endemic, including epidemiological settings in which the Ogawa serotype is predominant. Experiments with Peru-15 have provided evidence that the capacity of motile vaccine strains to penetrate the mucous coat contributes to reactogenicity. Despite the potential of live vaccine strains, two problems are related to their use. First, the live-attenuated strains cause side effects such as mild diarrhea, abdominal cramps, and low fever. Second, because these live strains are attenuated by deleting the *ctx* genes that are carried on a bacteriophage, there is concern that infection of vaccine strains by *ctx*-carrying phage may lead to reversion to virulence if these vaccines are used in regions where cholera is endemic. In the case of Peru-15, specific deletions (ΔattRS1 and ΔrecA) have been introduced, rendering it incapable of performing homologous and site-specific recombination events that could lead to reacquisition of active cholera toxin genes. Mutations in recA might also limit the persistence of the live vaccine candidate in the environment.

Limitations of available cholera vaccines: Most of the recombinant vaccines tested so far produced adverse reactions in volunteers, although they provided varying degrees of protection upto about one year of surveillance. Moreover, as live vaccine candidates move to field trials, a variety of concerns needs to be addressed about the safety of these vaccines, not only for the vaccinee, but also for the community and the environment. Lastly, neither the protective moieties in the orally administered vaccines nor the host factors elicited by these products have been correlated with vaccine-induced protection. Though the efficacy of the live vaccine is yet to be supported by field trials, the inactivated oral vaccine has shown encouraging results in field trails on different population groups. The price of one-dose of the available killed vaccine is approximately 2-3 US $ (4-6 US$ per person vaccinated but is anticipated to be reduced in case of large production and multi-dose vial use (70). The price of one dose of CVD103HgR vaccine is 1.75 US $ if the range of order is between 50,000-1,50,000 doses and for orders in excess of 1,50,000 doses the price would be reduced to 1.50 US $. Since the role of cholera vaccines-including oral vaccines-as a public health tool in epidemic situations is debatable and cholera immunization for travellers will result in a high cost-benefit ratio, endemic cholera remains the main area of their application. The two oral cholera vaccines commercially available and licensed in several countries, even if available the high cost would limit its use in cholera-endemic countries. Moreover, none of these vaccines provide protection against *V. cholerae* 0139 Bengal. This shows the need to develop vaccines that would provide immunity not only against a particular serotype but across serotypes and biotypes. This is particularly important because of frequent genetic changes in *V. cholerae*. In summary, despite cholera being an old scourge, we are still challenged to develop a vaccine that is affordable for afflicted nations and that integrates long-lasting protection with clinical and environmental safety. A more effective, better tolerated vaccine that could be administered orally is therefore desirable. The accessibility of available cholera vaccines to the large majority of humans living in unsanitary conditions and who lack access to clean drinking water and for whom the risk of cholera is very real is limited by the cost of the vaccine. Therefore, development of cholera vaccines *per se* is not going to solve the problem; how inexpensive and how

available these vaccines are to populations that need them is another perspective that needs to be concurrently addressed.

Strategy for new vaccines: An ideal vaccine would provide a high level of long-term protection to even those at high risk for severe illness, e.g., individuals with blood group type O. An ideal oral cholera vaccine would provide protection against classical and El Tor biotypes and Inaba and Ogawa serotypes of *Vibrio cholerae* O1 as well as against V. cholerae O139. This protection should begin shortly after administration of a single oral dose. Because single-dose, oral vaccines are easy to administer and rapidly induce immunity, they may have considerable public health impact as a method to control epidemics (71).

The current WHO position on cholera vaccines is that i) cholera vaccine should be considered only for pre-emptive use, not reactively as a method of containing an outbreak once it has started ii) insufficient protection in children aged less than 2 years exclude these vaccines from use in national infant immunization programmes iii) vaccination to prevent cholera outbreaks should be undertaken only in concert with other prevention and control measures currently recommended by WHO and iv) in emergency situations high-risk populations such as refugees in primitive camps and urban slum residents should be immunized (56).

In a recent position paper on cholera vaccines, WHO has concluded that there is an urgent need for cholera vaccines that are efficient against the different epidemic types of *V. cholerae*, including the O139 serogroup, and that confer reliable and long-term protection in all age groups, also among children aged less than 5 years (56). Vietnam has taken a lead in this matter by demonstrating how an inexpensive, locally produced, and effective oral cholera vaccine may be within reach of the limited health-care budgets of poor countries with endemic cholera (72). The Vietnamese heat killed whole-cell vaccine modeled on the vaccines developed by Holmgren and colleagues at the University of Göteburg contains antigens representing both biotypes and both serotypes of *V. cholerae* (71). The Vietnamese whole-cell vaccine is inexpensive and said to be about US$0.20 per dose. It is adaptable to field conditions, it can be administered in a convenient two-dose regimen; it performed well without the use of strict cold chain for storage; and it appears not to require co administration with an oral buffer to retain its immunogenicity. The vaccine seemed to afford protection to young children, against cholera of life-threatening severity, and to members of households exposed to unclean water sources with potentially high loads of cholera vibrios (72).

Because killed vaccines are known to be safe, the oral killed cholera vaccine may be put into use very quickly. The advantage of killed cholera vaccine is that newer antigens, can be added on, especially in the scenario of emergence of new genetic variants of *V. cholerae* (18-20, 73). The O139 serogroup is already in wide circulation and hence the killed vaccine formulation should include both O1 and O139 strains. In view of rapid genetic changes in *V. cholerae*, a vaccine amenable to quick modifications to keep abreast of changes in traits would be extremely valuable.

Summary of Preliminary Studies

Molecular analysis of V. cholerae strains : We have analyzed toxigenic *V. cholerae* strains belonging to the O1 and O139 serogroups isolated during different epidemics of cholera in Bangladesh, and studied clonal diversity among the strains (12, 14, 17-20). These studies have demonstrated frequent emergence of new epidemic strains. By ribotyping and CTX genotyping we have catalogued different toxigenic strains, and these are available in our collection to be used as reference strains and as parent strains to construct attenuated mutants (73, 74).

Presence of virulence gene homologues in environmental strains: Although, genes encoding different virulence associated factors are supposed to be carried by pathogenic clinical isolates, recent studies have revealed that virulence genes or their homologues are also dispersed among environmental strains of *V. cholerae* belonging to diverse serogroups (75, 76). We examined the presence of five structural and regulatory genes involved in virulence, including *ctxAB, tcpA, toxR* and *toxT* in environmental strains of *V. cholerae* cultured from three different freshwater lakes and ponds in the eastern part of Calcutta, India. PCR analysis revealed the presence of these virulence genes or their homologues among diverse serotypes and ribotypes of environmental *V. cholerae*. This emphasized the potential importance of inter-strain gene exchange in the environment, leading to new genetic variants of *V. cholerae*.

Detection of hybrid strains: The causative agent of the sixth pandemic of cholera, the classical biotype of *Vibrio cholerae* O1 is currently believed to be extinct and has not been isolated for the past several years even in southern Bangladesh, the last of the niches where this biotype prevailed. We identified for the first time new varieties of *V. cholerae* O1, which are hybrids of the classical and El Tor biotypes, from hospitalized cases of acute diarrhoea in Bangladesh. Twenty-four strains of *V. cholerae* O1 isolated between 1991 and 1994 from hospitalized cases of acute diarrhoea in Bangladesh were examined for their response to phenotypic and genotypic traits that distinguish between the two biotypes of *V. cholerae* O1. Standard reference strains of *V. cholerae* O1 belonging to the classical and El Tor biotypes were used as controls in all the tests. The commonly used phenotypic traits to distinguish between El Tor and classical biotypes differentiated the 24 strains of toxigenic *V. cholerae* O1 into three types designated as the "Matlab" types. Type I strains appeared to be more like the classical biotype while type II and III strains appeared to be more like the El Tor biotype. However, the ribotypes of strains belonging to type I, II and III were like the El Tor biotype. Therefore, overall these strains were of the El Tor biotype displaying some traits of the classical biotype. Thus athough the classical and El Tor biotypes have different lineages, hybrids between the classical and El Tor biotype resulting from genetic exchange between different bacterial lineages exist in nature and can cause cholera. It is expected that these strains produce antigens which can be used to immunize against a variety of virulent *V. cholerae* strains. Identification, characterization and genetic modification of naturally occurring genetic hybrid strains may be useful in developing potential vaccine candidates.

Table 1. Phenotypic traits of the three types (Matlab I, Matlab II and Matlab III) of toxigenic *Vibrio cholerae* O1 isolated from patients hospitalized with acute secretory diarrhoea in Bangladesh

Type	Number of strains	Serotype		Voges Proskauer test	Polymyxin B sensitivity (50 U)	Chicken cell agglutination	Sensitivity to Phages	
		Inaba	Ogawa				Group IV (El Tor specific)	Group V (classical specific)
Matlab I	2	2	0	-	R	-	R	R
Matlab II	1	0	1	-	S	-	S	R
MatlabIII	21	0	21	-	R	-	S	R
El Tor	MAK757	0	1	+	R	+	S	R
Classical	154	0	1	-	S	-	R	S

Abbreviations: R, resistant; S, sensitive

Selection of nontoxigenic variants: We standardized techniques for induction of CTXΦ and investigated the production of extracellular phage particles by CTXΦ lysogens. CTX phage is believed to be maintained in V. cholerae cells by continuous reinfection. The receptor for CTX phage is the toxin coregulated pilus (TCP) which expresses in vitro only under defined conditions of temperature and pH. Hence if cells are induced to excise the prophage, while maintaining under a TCP non-permissive conditions, the reinfection can be stopped. Under such a condition a low proportion of cells become nontoxigenic. The nontoxigenic cells can be detected using CTX phage specific probes.

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

V. cholerae strains from our culture collection will be analyzed for different virulence and house keeping genes, and for phenotypic properties. Strains with atypical combination of virulence genes, serotype specific genes, and other marker genes will be selected. Genetic hybrids of different serogroups or biotypes will also be identified using probes, PCR assays, serological and and biochemical assays.These strains will also be compared with representative

strains of different biotypes and serogroups isolated from cholera patients, by genetic fingerprinting techniques. For development of vaccine candidates possible hybrid strains carrying traits of more than one types will be slected. The selected strains will be genetically manipulated to produce nontoxigenic derivatives, or spontaneous toxin deleted derivatives will be screened and selected. It is believed that CTX phage is maintained in toxigenic V. cholerae strains by a process of continuous reinfection. By chosing conditions that induce the phage but inhibits reinfection, it is possible to produce derivatives which have lost the phage. Such nontoxigenic derivatives can be identified using DNA probes. We plan to use this strategy for generating nontoxigenic derivatives. An alternative strategy would be to use a truncated CTX phage or an RS1 phage to replace the CTX phage by homologous recombination. We have already developed such inactivated CTX phages in our laboratory. The necessary probes and PCR assays, and the genetic manipulation techniques are already available in our laboratory.

The safety, immunogenicity and protective efficacy of these attenuated strains will be tested in adult rabbit models , using already established methods. To be able to formuate a multivalent vaccine reperesenting all the epidemic biotypes and serotypes, we plan to do genetic attenuation of strains belonging to at least three categories or their possible genetic hybrids. These are *V. cholerae* O1 El Tor Inaba, ElTor Ogawa and O139 Bengal strains.

Methods

Serology: The identity of all isolates will be confirmed biochemically and serologically by standard methods recommended by WHO (77). The identity of *V.cholerae* will be confirmed by agglutination with the somatic O antigen serogrouping scheme for *V.cholerae* developed at the National Institute of Infectious Diseases, Tokyo, Japan.

PCR Analysis: A series of PCR assays for different virulence and house keeping genes will be used. Presence of the *tcpA, tcpI,* and *acfB* genes representing the TCP pathogenicity island will be detected by PCR assays described previously (19, 20, 78). PCR assay to detect the presence of the CTX element will be performed 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. 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. Other PCR assays will detect *V. cholerae* regulatory genes, toxR and toxT. These PCR assays have been described previously by us and are available in our laboratory.

Probes and hybridization: Presence of different virulence genes or their homologues including *extAB, tcpA, tcpI, acfB, ToxR, hlyA, mshA* will also be determined by DNA probe hybridization of colony blots and Southern blots as appropriate. 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*R1 fragment of pCVD27 (79) 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*R1 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 (80). The *toxR* probe will be a 2.4 kb *Bam*HI fragment of pVM7 (81) which is a pBR322 derived plasmid carrying the entire *toxR* sequence. The rRNA gene probe will be a 7.5-kb *Bam*H II fragment of pKK3535 (82). Preparation and use of these probes have been described by us previously (36, 37). Colony blots or Southern blots will be prepared using nylon filters (Hybond, Amersham International plc. Ayelesbury, United Kingdom), and processed by standard methods (83). The polynucleotide probes will be labeled by random priming (84) 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 (18, 36, 37).

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 (85). 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.

Development of nontoxigenic mutants: Two different strategies will be used to construct nontoxigenic derivatives. This will include (a) detection of spontaneous mutants induced by CTX phage induction, and (b) mutations by homologous recombination. We have standardized both these methods. The mutants will be extensively tested to confirm the absence of genes encoding CT and compare with the parent strains by genetic and phenotypic means to rule out any other possible mutation.

Assay for CT production: Production of CT by *V. cholerae* strains and their nontoxigenic derivatives will be determined by the G_{M1}-ganglioside dependent enzyme linked immunosorbent assay (G_{M1}-ELISA) and the rabbit ileal loop assay as described previously (86). For each round of CT assay, 5 ml of AKI medium (1.5% Bacto Peptone, 0.4% yeast extract, 0.5% NaCl, 0.3% NaHCO3 [pH 7.4]) was inoculated with approximately 1 x 10^3 bacterial cells. All cultures will be grown for 16 h at 30°C with shaking. The cultures will be centrifuged at 4000xg for 5 min, and the supernatant will be collected and filtered through 0.22µm-pore size Millipore filters. Aliquots of the undiluted supernatant, 10-fold and 100-fold dilutions of the supernatant, and dilutions of purified CT (Sigma) will be used for the toxin assay. Quantification of CT production will be done using a standard curve prepared for each batch of assay.

Ileal loop assay: Culture filtrates prepared for the ELISA will also be tested in ileal loops of adult New Zealand White rabbits. A maximum of six ileal loops of approximately 10 cm in length will be made in each rabbit which had previously been fasted for 48 h, and 1 ml of the filtrate was inoculated into each loop as described previously (87). After 18 h rabbits will be sacrificed and the loops were examined for fluid accumulation.

Oral Immunization of rabbits: Locally bred New Zealand white rabbits will be used and immunized as described previously (87, 88). To prepare each inoculum, frozen stock of the bacterium will be thawed, inoculated into 1% peptone water (pH 7.4), and cultured overnight at 37°C. A 50-ml Erlenmeyer flask containing 10 ml of Casamino yeast extract medium will be inoculated with 0.05 ml of the overnight culture and shaken at 180 cycles per min for 4 h in a 37°C water bath. Bacteria will be then centrifuged and suspended in 0.01 M phosphate-buffered saline, pH 7.4. Viable bacterial counts will be determined on 3% gelatin agar by the drop-plate method. Inocula will be prepared by diluting the bacterial suspension in fresh Casamino yeast extract medium. Rabbits will be fasted overnight before inoculation. The oral inoculum will be given through a stomach tube in the following manner. At time zero, the rabbits will be given 50 mg of cimetidine into an ear vein. At 15 min, a stomach tube will be placed per os, and 15 ml of a 5% solution of sodium bicarbonate will be given. At 30 min, the stomach tube will be again placed. and another 15 ml of 5% sodium bicarbonate solution will be given, followed immediately by the bacterial inoculum suspended in 10 ml of PBS. At 60 min, 2 ml of tincture of opium will be given intraperitoneally, and the rabbits will be returned to their cages.The rabbits will be orally inoculated as described above on days 0, 7, and , 14. Blood samples will be obtained from the rabbits periodically to assay for serum vibriocidal antibodies. On day 33 (19 days after the last oral inoculation) the animals will be challenged by the RITARD procedure described bellow. A similar number of control unimmunized rabbits will be challenged on the same day as the immunized rabbits.

Assay for protection of immunized rabbits: Protection of immunized rabbits against challenge with wild type *V. cholerae* strain will be assayed using the removable intestinal tie-adult rabbit diarrhea (RITARD) model (90). The reference virulent El Tor strain N16961 available in our laboratory will be used as the challenge strain. Adult New Zealand white rabbits weighing 1.5-2.7 kg will be used to prepare the RITARD model. Rabbits will be starved for the previous 24 h, and surgery will be done under a local anaesthetic. The cecum of each animal will be ligated to prevent it from retaining fluid secreted by the small intestine, and a temporary removable tie of the small bowl will be introduced at the time of challenge. The challenge strain will be grown in Casamino acid-yeast extract broth as described previously, cells will be precipitated by centrifugation and resuspended in 10mM phosphate buffered saline, pH 7.4 at a concentration of approximately 1 $x10^9$ cells per ml. One ml of the suspension will be injected into the lumen of the anterior jejunum. The removable tie in the intestine will be removed 2 h after inoculation. Each strain will be inoculated in at least 5 different rabbits. Rabbits will be observed for overt diarrhea, and for death, and stools or rectal swabs will be cultured on gelatin agar plates to monitor shedding of the challenge organisms. Observations will be made at 6 h intervals during the following 7 days of inoculation, number of rabbits developing moderate to severe diarrhea will be arbitrarily scored and the

number of deaths will be recorded. Rabbits that die with or without diarrhea will be subjected to post mortem examinations to check the presence of fluid in the intestine.

Expected Results:

The studies described in this protocol are expected to generate the following results.

1. Identify strains of *V. cholerae* that are CT negative and are possible genetic hybrids showing different combination of classical and El Tor or O139 specific properties.
2. Information regarding genetic characteristics of these strains, and their clonal relationships with other pathogenic or non-pathogenic strains in our collection.
3. Data on the safety of these strains in the adult rabbit diarrhea model
4. Data regarding the induction of protective immunity by these strains in rabbits.
5. Information regarding culture conditions and media requirements for these strains, and their growth curves under different culture conditions.

Facilities Available

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

The Centre has the necessary infrastructure for both laboratory based research and research in animals. Vaccine strains will be developed in the molecular genetics laboratory. The molecular genetics laboratory at the ICDDR,B has considerable expertise in conducting genetic analysis of strains as well as doing animal experiments. This laboratory has the necessary equipment and setup for the synthesis of primers and probes, and carrying out multiplex PCR assays and relevant molecular characterization of *V. cholerae* strains.

Data Analysis

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

The goal of this study is to identify and analyze *V. cholerae* strains carrying atypical combination of virulence genes and test their immunogeneicity in animal models. Data generated in animal studies will be analyzed using appropriate statistical packages to determine the protective efficacy of the potential vaccine strains. Bacterial mean counts will be compared by using the Student t test; attack rates after challenge will be compared by using the Fisher exact test. Protection will be calculated by dividing the attack rate in immunized rabbits by the attack rate in controls and subtracting from 100 as described previously (87, 88).

Principal Investigators: Last, first, middle - FARUQUE, SHAH M.

Ethical Assurance for Protection of Human Rights

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

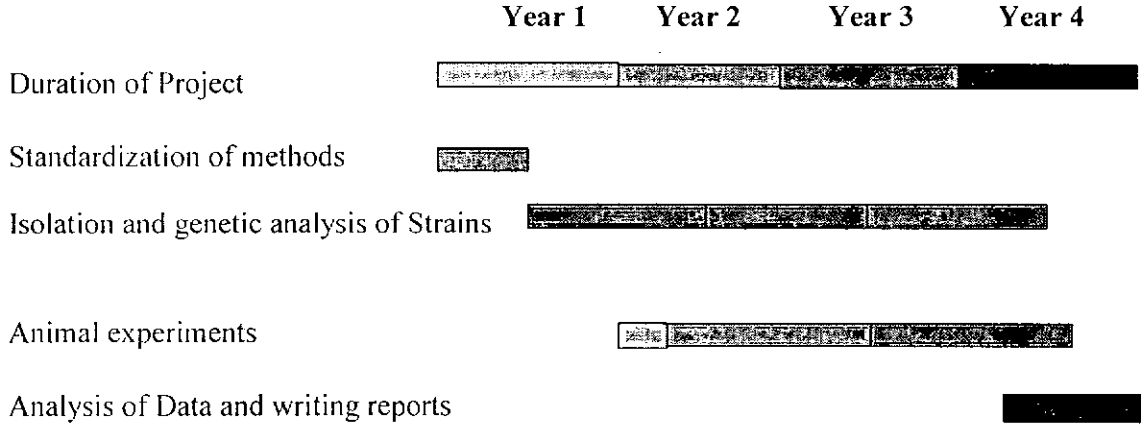
This project does not involve any study directly in humans.

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.

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 rabbits will be the RITARD model, which will be used to assess the diarrheagenic potential of different strains.

Flow Chart for implementation of the project



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.

1. Faruque S.M., M. J. Albert, and J. J. Mekalanos. 1998. Epidemiology, genetics and ecology of toxigenic *Vibrio cholerae* Microbiol. Mol. Biol. Rev. 62: 1301-1314.

2. Colwell, R. R., and W. M. Spira. 1992. The ecology of *Vibrio cholerae*. p. 107-127. In D. Barua, and W. B. Greenough III (ed.), Cholera. Plenum Medical Book Co., New York.

3. Colwell, R. R., and A. Huq. 1994. Vibrios in the environment: viable but non-culturable *Vibrio cholerae*. p. 117-133. In I. K. Wachsmuth, P. A. Blake, and O. Olsvik (ed.), *Vibrio cholerae* and cholera: molecular to global perspectives. ASM Press, Washington D.C.

4. West, P. A. 1989. The human pathogenic vibrios-A public health update with environmental perspectives. Epidemiol. Inf. 103:1-34.

5. Ramamurthy, T., S. Garg, R. Sharma, S. K. Bhattacharya, G. B. Nair, T. Shimada, T. Takeda, T. Karasawa, H. Kurazano, A. Pal, and Y. Takeda. 1993. Emergence of a novel strain of *Vibrio cholerae* with epidemic potential in Southern and Eastern India. Lancet. 341:703-704.

6. Cholera Working group, International center for diarrhoeal disease research, Bangladesh. 1993. Large epidemic of cholera like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. Lancet. 342:387-390.

7. Pollitzer, R. 1959. History of the disease, p. 11-50. In R. Pollitzer (ed.), Cholera. World Health Organization, Geneva.

8. Kaper, J. B., J. G. Morris, and M. M. Levine. 1995. Cholera. Clin. Microbiol. Rev. 8:48-86.

9. Wachsmuth, I. K., O. Olsvik, G. M. Evins, and T. Popovic. 1994. Molecular epidemiology of cholera. p. 357-370. In K. Wachsmuth, P. A. Blake, and O. Olsvik (ed.), *Vibrio cholerae* and cholera: Molecular to Global Perspectives. American Society of Microbiology, Washington DC.

10. Chen, F., G. M. Evins, W. L. Cook, R. Almeida, H. N. Bean, and I. K. Wachsmuth. 1991. Genetic diversity among toxigenic and non-toxigenic *Vibrio cholerae* O1 isolated from the Western Hemisphere. Epidemiol. Infect. 107:225-233.

11. Cook, W. L., K. Wachsmuth, J. Feeley, and I. Huq. 1983. The question of classical cholera. Lancet. i:879-880.

12. Faruque, S. M., A. R. M. A. Alim, M. M. Rahman, A. K. Siddique, R. B. Sack, and M. J. Albert. 1993. Clonal relationships among classical *Vibrio cholerae* O1 strains isolated between 1961 and 1992 in Bangladesh. J. Clin. Microbiol. 31:2513-2516.

13. Kaper, J. B., H. B. Bradford, N. C. Roberts, and S. Falkow. 1982. Molecular epidemiology of *Vibrio cholerae* in the U.S. Gulf Coast. J. Clin. Microbiol. 16:129-134.

14. Faruque, S. M., and M. J. Albert. 1992. Genetic relation between *Vibrio cholerae* O1 strains in Ecuador and Bangladesh. Lancet. 339:740-741.

15. Wachsmuth, I. K., C. A. Bopp, P. I. Fields, and C. Carrilo. 1991. Difference between toxigenic *Vibrio cholerae* O1 from South America and US gulf coast. Lancet. 337:1097-1098.

16. Wachsmuth, I. K., G. M. Evins, P. I. Fields, O. Olsvic, T. Popovic, C. A. Bopp, J. G. Wells, C. Crrillo, and P. A. Blake. 1993. The molecular epidemiology of cholera in Latin America. J. Infect. Dis. 167:621-626.

17. Faruque, S. M., A. R. M. A. Alim, S. K. Roy, F. Khan, G. B. Nair, R. B. Sack, and M. J. Albert. 1994. Molecular analysis of rRNA and cholera toxin genes carried by the new epidemic strain of toxigenic *Vibrio cholerae* O139 synonym Bengal. J. Clin. Microbiol. 32:1050-1053.

18. Faruque, S. M., S. K. Roy, A. R. M. A. Alim, A. K. Siddique, and M. J. Albert. 1995. Molecular epidemiology of toxigenic *V. cholerae* in Bangladesh studied by numerical analysis of rRNA gene restriction patterns. J. Clin. Microbiol. 33: 2833-2838.

19. Faruque, S. M., K. M. Ahmed, A. R. M. A. Alim, F. Qadri, A. K. Siddique, and M. J. Albert. 1997. Emergence of a new clone of toxigenic *Vibrio cholerae* biotype El Tor displacing *V. cholerae* O139 Bengal in Bangladesh. J. Clin. Microbiol. 35:624-630.

20. Faruque, S. M., K. M. Ahmed, A. K. Siddique, K. Zaman, A. R. M. A. Alim, and M. J. Albert. 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. 35:2299-2306.

21. Waldor M. K., and J. J. Mekalanos. 1996. Lysogenic conversion by a filamentous bacteriophage encoding cholera toxin. Science. 272:1910-1914.

22. Kovach, M. E., M. D. Shaffer, and K. M. Peterson. 1996. A putative integrase gene defines the distal end of a large cluster of ToxR-regulated colonization genes in *Vibrio cholerae*. Microbiology. 142:2165-2174.

23. Karaolis, D. K., S. Somara, D. R. Maneval Jr, J. A. Johnson, and J. B. Kaper. 1999. A bacteriophage encoding a pathogenicity island, a type-IV pilus and a phage receptor in cholera bacteria. Nature 399:375-379.

24. Franzon, V. F., A. Baker, and P. A. Manning. 1993. Nucleotide sequence and construction of a mutant in the mannose-fucose-resistant hemagglutinin (MFRHA) of *Vibrio cholerae* O1. Infect. Immun. 61:3032-3037.

25. Jonson, G., M. Lebens, and J. Holmgren. 1994. Cloning and sequencing of *Vibrio cholerae* mannose-sensitive hemagglutinin pilin gene: localization of mshA within a cluster of type 4 pilin genes. Mol. Microbiol. 13:109-108.

26. Sengupta, D. K., T. K. Sengupta, and A. C. Ghose. 1992. Major outer membrane proteins of *Vibrio cholerae* and their role in induction of protective immunity through inhibition of intestinal colonization. Infect. Immun. 60:4848-4855.

27. Taylor, R., C. Shaw, K. Peterson, P. Spears, and J. Mekalanos. 1988. Safe, live *Vibrio cholerae* vaccines ?. Vaccine 6:151-154.

28. DiRita, V. J. 1992. Co-ordinate expression of virulence genes by ToxR in *Vibrio cholerae*. Mol. Microbiol. 6:451-458.

29. Brown, R. C., and R. K. Taylor. 1995. Organization of *tcp, acf*, and *toxT* genes within a ToxT-dependent operon. Mol. Microbiol. 16:425-439.

30. Everiss, K. D., K. J. Hughes, and K. M. Peterson. 1994. The accessory colonization factor and toxin-coregulated pilus gene clusters are physically linked on the *Vibrio cholerae* O395 chromosome. DNA Seq. 5:51-55.

31. Ogierman, M. A., S. Zabihi, L. Mourtzios, and P. A. Manning. 1993. Genetic organization and sequence of the promoter-distal region of the *tcp* gene cluster of *Vibrio cholerae*. Gene. 126:51-60.

32. Harkey, C. W., K. D. Everiss, and K. M. Peterson. 1994. The *Vibrio cholerae* toxin-coregulated pilus gene *tcpI* encodes a homolog of methyl-accepting chemotaxis proteins. Infect. Immun. 62:2669-2678.

33. Baudry B., A. Fasano, J. Ketley, and J. B. Kaper. 1992. Cloning of a gene (*zot*) encoding a new toxin produced by *Vibrio cholerae*. Infect. Immun. 60:428-34.

34. Trucksis, M., J. E. Galen, J. Michalski, A. Fasano, and J. B. Kaper. 1993. Accessory cholera enterotoxin (Ace), the third toxin of a *Vibrio cholerae* virulence cassette. Proc. Natl. Acad. Sci. USA. 90:5267-5271.

35. Pearson, G. D. N., A. Woods, S. L. Chiang, and J. J. Mekalanos. 1993. CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor. Proc. Natl. Acad. Sci. USA. 90:3750-3754.

36. Faruque S. M., Asadulghani, A. R. M. A. Alim, M. J. Albert, K. M. N. Islam, and J. J. Mekalanos. 1998. Induction of the lysogenic phage encoding cholera toxin in naturally occurring strains of toxigenic *V. cholerae* O1 and O139. Infect. Immun. 66:3752-3757.

37. Faruque S. M., Asadulghani, M. N. Saha, A. R. M. A. Alim, M. J. Albert, K. M. N. Islam, and J. J. Mekalanos. 1998. Analysis of clinical and environmental strains of nontoxigenic *Vibrio cholerae* for susceptibility to CTXΦ: molecular basis for the origination of new strains with epidemic potential. Infect. Immun. 66:5819-5825.

38. Waldor, M. K., E. J. Rubin, G. D. N. Pearson, H. Kimsey, and J. J. Mekalanos. 1997. Regulation, replication, and integration functions of the *Vibrio cholerae* CTX are encoded by regions RS2. Molecular. Microbiol. 24: 917-926.

39. Skorupski, K., and R. K. Taylor. 1997. Control of the ToxR virulence regulon in *Vibrio cholerae* by environmental stimuli. Mol. Microbiol. 25:1003-1009.

40. Hughes, K. J., K. D. Everiss, C. W. Harkey, and K. M. Peterson. 1994. Identification of a *Vibrio cholerae* ToxR-activated gene (*tagD*) that is physically linked to the toxin coregulated pilus (*tcp*) gene cluster. Gene 148:97-100.

41. Parsot, C., E. Taxman, and J. J. Mekalanos. 1991. ToxR regulates the production of lipoproteins and the expression of serum resistance in *Vibrio cholerae*. proc Natl Acad Sci USA. 88:1641-1645.

42. Peterson, K. M., and J. J. Mekalanos. 1988. Characterization of the *Vibrio cholerae* ToxR regulon: identification of novel genes involved in intestinal colonization. Infect. Immun. 56:2822-2829.

43. Parsot, C., and J. J. Mekalanos. 1990. Expression of ToxR, the transcriptional activator of the virulence factors in *Vibrio cholerae*, is modified by the heat shock response. Proc. Natl. Acad. Sci. USA. 87:9898-9902.

44. Mazel, D., B. Dychinco, V. A. Webb, and J. Davies. 1998. A distinctive class of integron in the *Vibrio cholerae* genome. Science. 280:605-608.

45. Recchia, G. D., and M. R. Hall. 1995. Gene cassettes: a new class of mobile element. Microbiology. 141:3015-3027.

46. Recchia, G. D., and M. R. Hall. 1997. Origins of the mobile gene cassettes found in integrons. Trends. Microbiol. 5:389-394.

47. Baker, A., and P. A. Manning.1997. VlpA of *Vibrio cholerae* O1: the first bacterial member of the alpha 2-microglobulin lipocalin superfamily. Microbiology 143:1805-1813.

48. Ogawa, A., and T. Takeda. 1993. The gene encoding the heat-stable enterotoxin of *Vibrio cholerae* is flanked by 123-base pair direct repeats. Microbiol. Immunol. 37:607-616.

49. Baker, A., C. A. Clark, and P. A. Manning. 1994. Identification of VCR, a repeated sequence associated with a locus encoding a hemagglutinin in *Vibrio cholerae* O1. J. Bacteriol. 176:5450-5458.

50. Barua D. Merson MH. 1992. Prevention and control of cholera. In: Barua D and Greenough III WB (eds) Cholera. Plenum, New York, USA

51. IRC. 2001. World water day: focus on water for health. Source Bull. No. 14:3-4 (http://www.wsscc.org/source).

52. IRC. 2000. Global water supply and sanitation assessment 2000. Source Bull. No. 14:3-4 (http://www.wsscc.org/source).

53. Benenson, A. S., W. H. Mosley, M. Fahimuddin, and R. O. Oseasohn. 1968. Cholera vaccine field trials in East Pakistan. Bull. W. H. O. 38:359–372.

54. Finkelstein, R. A. 1984. Cholera, p. 107–136. *In* R. Germanier (ed.), Bacterial vaccines. Academic Press, Inc., New York, N.Y.

55. Mosley, W. H., W. E. Woodward, K. M. A. Aziz, A. S. M. M. Rahman, A.K.M.A. Chowdhury, A. Ahmed, and J. C. Feeley. 1970. The 1968–1969 cholera-vaccine field trial in rural East Pakistan. Effectiveness of monovalent Ogawa and Inaba vaccines and a purified Inaba antigen, with comparative results of serological and animal protection tests. J. Infect. Dis. 121:S1–S9.

56. World Health Organization. 2001. Cholera vaccines. WHO position paper. Weekly Epidemiol. Rev. 76:117-124

57. Clemens JD, Sack D, Harris JR, Van Loon F, Chakraborty J, Ahmed F, Rao MR, Khan MR, Yunus M, Huda N, Stanton BF, Kay BA, Walter S, Eeckels R, Svennerholm AM, Holmgren T.1990. Field trial of oral cholera vaccines in Bangladesh: results of 3-year follow-up. Lancet. 335:270-73.

58. Clemens JD, Harris J, Sack D, Chakraborty J, Ahmed F, Stanton BF, Khan MU, Kay BA, Hunda N, Khan MR, Yunus M, Rao MR, Svennerholm AM, Holmgren J. 1988. Field trial of oral cholera vaccines in Bangladesh: results of one year of follow-up. J. Infect Dis. 158:60-69.

59. Levine, M. M., J. B. Kaper, D. Herrington, J. Ketley, G. Losonsky, C. O. Tacket, B. Tall, and S. Cryz. 1988. Safety, immunogenicity, and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103-HgR. Lancet ii:467–470.

60. Su-Arehawaratana, P., P. Singharaj, D. N. Taylor, C. Hoge, A. Trofa, K. Kuvanont, S. Migasena, P. Pitisuttitham, Y. L. Lim, G. Losonsky, J. B. Kaper, S. S. Wasserman, S. Cryz, P. Echeverria, and M. M. Levine. 1992. Safety and immunogenicity of different immunization regimens of CVD103-HgR live oral cholera vaccine in soldiers and civilians in Thailand. J. Infect. Dis. 165:1042–1048.

61. Gotuzzo, E., B. Butron, C. Seas, M. Penny, R. Ruiz, G. Losonsky, C. F. Lanata, S. S. Wasserman, E. Salazar, J. B. Kaper, S. Cryz, and M. M. Levine. 1993. Safety, immunogenicity, and excretion pattern of single-dose live oral cholera vaccine CVD 103-HgR in Peruvian adults of high and low socioeconomic levels. Infect. Immun. 64:3994–3997.

62. Kotloff, K. L., S. S. Wasserman, S. O'Donnell, G. A. Losonsky, S. J. Cryz, and M. M. Levine. 1992. Safety and immunogenicity in North Americans of a single dose of live oral cholera vaccine CVD 103-HgR: results of a ran-domized, placebo-controlled, double-blind crossover trial. Infect. Immun. 60:4430–4432.

63. Michalski, J., J. E. Galen, A. Fasano, and J. B. Kaper. 1993. CVD 110, an attenuated Vibrio cholerae O1 El Tor live oral vaccine strain. Infect. Immun. 61:4462–4468.

64. Tacket, C. O., G. Losonsky, J. P. Nataro, S. J. Cryz, R. Edelman, A. Fasano, J. Michalski, J. B. Kaper, and M. M. Levine. 1993. Safety, immunogenicity, and transmissibility of live oral cholera vaccine candidate CVD 110, Δctx, Δzot, Δace derivative of El Tor Ogawa *Vibrio cholerae*. J. Infect. Dis. 168: 1536–1540.

65. Tacket, C. O., K. L. Kotloff, G. Losonsky, J. P. Nataro, J. Michalski, J. B. Kaper, R. Edelman, and M. M. Levine. 1997. Volunteer studies investigating the safety and efficacy of live oral El Tor Vibrio cholerae O1 vaccine strain CVD 111. Am. J. Trop. Med. Hyg. 56:533–537.21.

66. Taylor, D. N., C. O. Tacket, G. Losonsky, O. Castro, J. Gutierrez, R. Meza, J. P. Nataro, J. B. Kaper, S. S. Wasserman, R. Edelman, M. M. Levine, and S. J. Cryz. 1997. Evaluation of a bivalent (CVD 103-HgR/CVD 111) live oral cholera vaccine in adult volunteers from the United States and Peru. Infect. Immun. 65:3852–3856.

67. Kenner, J. R., T. S. Coster, D. N. Taylor, A. F. Trofa, M. Barrera-Oro, T. Hyman, J. M. Adams, D. T. Beattie, K. P. Killeen, D. R. Spriggs, J. J. Mekalanos, and J. C. Sadoff. 1995. Peru-15, an improved live attenuated oral vaccine candidate for *Vibrio cholerae* O1. J. Infect. Dis. 172:1126–1129.

68. Sack D. A., Sack R. B., Shimko J., Gomes G, O'Sullivan D., Metcalfe K, and Spriggs D. 1997. Evaluation of Peru-15, a new live oral vaccine for cholera, in volunteers. J Infect Dis. 76:201-5.

69. Sack D. A., Shimko J., Sack R. B., Gomes G. J., MacLeod K, O'Sullivan D., and Spriggs D. 1997. Comparison of alternative buffers for use with a new live oral cholera vaccine, Peru-15, in outpatient volunteers. Infect. Immun. 65:2107-2111.

70. World Health Organization.1999. Potential use of oral cholera vaccines in emergency situations. Report of a WHO meeting. (http://www.who.int/emc).

71. Trach DD. 1990. The problem of cholera vaccination in Vietnam. In: Sack D, Freij L, eds. Prospects for public health benefits in developing countries from new vaccines against enteric infections. Stockholm, SAREC Documentation, Conference Report 2, 217-218.

72. Trach DD, Clemens JD, Ke NT, Thuy HT, Son ND, Canh DG, Hang PVD, Rao MR. 1997. Field trial of a locally produced, killed, oral cholera vaccine in Vietnam. Lancet 349:231-235.

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

74. 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.

75. 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.

76. Mukhopadhay, A. K., S. Chakraborty, Y. Takeda, G. B. Nair, and D. E. Berg. 2001. Characterization of VPI pathogenicity island and CTXΦ prophage in environmental strains of *Vibrio cholerae*. J. Bacteriol. 183:4737-4746.

77. World Health Organization 1974. World Health Organization Guidelines for the laboratory diagnosis of cholera. Bacterial Disease Unit, World Health Organization, Geneva, Switzerland.

78. Keasler, S.P., and R.H. Hall. 1993. Detection and biotyping *Vibrio cholerae* O1 with multiplex polymerase chain reaction. Lancet. 341:1661.

79. Kaper, J.B., J.G. Morris Jr., and M. Nishibuchi. 1988. DNA probes for pathogenic Vibrio species, p. 65-77. *In* F. C. Tenover (ed.), DNA probes for infectious disease. CRC press, Inc., Boca, Raton, Fla.

80. Faruque, S.M., L. Comstock, J.B. Kaper, and M.J. Albert. 1994. Distribution of zonula occludens toxin (zot) gene among clinical isolates of *Vibrio cholerae* O1 from Bangladesh and Africa. J. Diarrhoeal Dis. Res. 12:222-224.

81. Miller, V.L., and J.J. Mekalanos. 1984. Synthesis of cholera toxin is positively regulated at the transcriptional level by toxR. Proc. Natl. Acad. Sci. USA. 81:3471-3475

82. Brosius, J., A. Ullrich, M.A. Raker, A. Gray, T.J. Dull, R.R. Gutell, and H.F. Noller. 1981. Construction and fine mapping of recombinant plasmids containing the rRNB ribosomal RNA operon of *E. coli*. Plasmid. 6:112-118.

83. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

84. Feinberg, A., and B. Volgelstein. 1984. A technique for radio labelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 137:266-267.

85. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

86. Sack, D.A., S. Huda, P.K.B. Neogi, R.R. Daniel, and W.M. Spira. 1980. Microtitre ganglioside enzyme linked immunosorbent assay for *Vibrio* and *Escherichia coli* heat labile enterotoxins and antitoxins. J. Clin. Microbiol. 1:35-40.

87. De, S. N., and D. N. Chatterje. 1953. An experimental study of the mechanisms of action of *Vibrio cholerae* on the intestinal mucous membrane. J. Pathol. Bacteriol. 46:559-562.

88. Cray, W.C., Tokuanga, E., and Pierce, N. F. 1983. Successful colonization and immunization of adult rabbits by oral inoculation with *Vibrio cholerae* O1. Infect. Immun. 41: 735-741.

89. Sack, R. B., Kline, R. L., and Spira, W. M. 1988. Oral immunization of rabbits with enterotoxigenic *Escherichia coli* protects against intraintestinal challenge. Infect. Immun. 56:387-394.

90. Spira, W. M., R. B. Sack, and J. L. Froehlich. 1981. Simple adult rabbit model for *Vibrio cholerae* and enterotoxigenic *E. coli* Diarrhea. Infect. Immun. 32:739-747.

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.

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.

Principal Investigators: Last, first, middle - FARUQUE, SHAH M.

Detailed Budget for New Proposal

Project Title: **Analysis and genetic modification of *Vibrio cholerae* strains carrying atypical combination of virulence genes and serotypes, to develop potential vaccine candidates.**

Name of PI: DR. SHAH M. FARUQUE Name of Division: Laboratory Sciences Division

Protocol Number:
Funding Source: Amount to be Funded (direct): US $ 435,726 Overhead (%): 25%

Starting date: When funds become available Duration: 4 years

Account Description	Salary Support		US $ Amount Requested				
			1st Yr	2nd Yr	3rd Yr	4th Yr	TOTAL
Personnel	**Position**	Effort%					
Dr. Shah M. Faruque	Scientist	50%	52,109	54,714	57,450	60,323	224,596
Q. S. Ahmad	Asst. Scientist	10%	1,547	1,624	1,706	1,791	6,668
M. Kamruzzaman	Research Officer	100%	4,176	4,385	4,604	4,834	17,999
Rasel Khan	Research Officer	100%	4,800	5,040	5,292	5,557	20,689
Afjal Hossain	Laboratory Asst.	100%	3,144	3,301	3,466	3,640	13,551
MH Tarik	Laboratory Attend.	100%	3,300	3,465	3,638	3,820	14,223
Subtotal			69,076	72,530	76,156	79,964	297,726
Local Travel			1,000	1,000	2,000	1,500	5,500
International Travel	conference, workshop,		3,000	4,000	4,000	3,500	14,500
Subtotal			4,000	5,000	6,000	5,000	20,000
Supplies and Materials (Description of items)							
	Molecular biology grade Reagents, enzymes etc.		18,000	18,000	15,000	11,000	62,000
	Culture media. Plastics, and supplies		6,500	6,000	4,600	6,000	23,100
	Office supplies		300	500	200	500	1,500
Sub Total			24,800	24,500	19,800	17,500	86,600

Principal Investigators: Last, first, middle - **FARUQUE, SHAH M.**

Other Contractual Services						
	Rent, communication, utilities	1,000	1,000	1,000	1,000	4,000
	print and publication	500	1,000	1,500	500	3,500
	Repair and maintenance	2,000	1,000	2,000	1,000	6,000
Sub Total		3,500	3,000	4,500	2,500	13,500
Interdepartmental Services						
	Computer charges	500	500	700	500	2,200
	Research animals	3,000	2,700	3,000	2,000	10,700
	Transport	500	500	500	500	2,000
	Telephone, fax, communication	500	1,000	500	1,000	3,000
Sub Total		4,500	4,700	4,700	4,000	17,900
Total direct cost		**105,876**	**109,730**	**111,156**	**108,964**	**435,726**
Overhead (25%)		26,469	27,432	27,789	27,241	108,932
Total Operationg Cost		**132,345**	**137,162**	**138,945**	**136,205**	**544,658**

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

Controller, Budget and Costing

ICDDR,B

11/7/2002

Principal Investigators: Last, first, middle - FARUQUE, SHAH M.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
DR. SHAH M. FARUQUE	SCIENTIST

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Dhaka, Dhaka, Bangladesh	B.Sc. (Honours)	1978	Biochemistry
University of Dhaka, Dhaka, Bangladesh	M.Sc.	1979	Biochemistry
University of Reading Reading, England, U.K	Ph.D.	1988	Molecular Biology

NOTE: The Biographical Sketch may not exceed four pages. Items A and B (together) may not exceed two of the four-page limit. Follow the formats and instructions on the attached sample.

A. Positions and Honors. List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

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

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

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

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

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

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

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

B. Selected peer-reviewed publications (in chronological order). Do not include publications submitted or in preparation.
(Publications selected from 57 peer reviewed publications)

1. **Faruque SM,** Asadulghani, Kamruzzaman M., Nandi RK, Ghosh AN, Nair GB, Mekalanos JJ, Sack DA. 2002. RS1 element of *Vibrio cholerae* can propagate horizontally as a filamentous phage exploiting the morphogenesis genes of CTXΦ. Infect Immun. 70:163-170.

2. **Faruque SM,** and Nair GB. 2002. Molecular ecology of toxigenic Vibrio cholerae (Review). Microbiol. Immunol. 46:59-66.

3. **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.

4. **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.

5. **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.

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

7. **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.

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

9. **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

10. **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.

11. **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.

12. **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.

13. **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.

14. **Faruque SM**, Roy SK, Alim ARMA, Alber MJ. Molecular epidemiology of toxigenic *Vibrio cholerae* in Bangladesh studied by numerical analysis of rRNA gene restriction patterns. J Clin Microbiol 1995; 33: 2833-2838.

15. **Faruque SM**, Alim ARMA, Roy SK, Khan F, Nair GB, Sack RB, Albert MJ. Molecular analysis of rRNA and cholera toxin genes carried by the new epidemic strain of toxigenic *Vibrio cholerae* O139 synonym Bengal. J Clin Microbiol 1994; 32:1050-1053.

16. **Faruque SM**, Comstock L, Kaper JB, Albert MJ. Distribution of Zonula Occludens toxin (*zot*) gene among clinical isolates of *Vibrio cholerae* O1 in Bangladesh and Africa. J Diarrhoeal Dis Res 1994;12:222-224.

17. **Faruque SM**, Alim ARMA, Rahman MM, Siddique AK, Sack RB, Albert MJ. Clonal relationships among classical *Vibrio cholerae* 01 strains isolated between 1961 and 1992 in Bangladesh. J Clin Microbiol 1993; 31:2513-2516.

18. **Faruque SM**, Rahman MM, Alim ARMA, Hoq MM, Albert MJ. Antibiotic resistance pattern of heat-labile enterotoxin (LT) producing *Escherichia coli* isolated from children with diarrhoea in Bangladesh: Clonal relationships among isolates with different resistance phenotypes. J Diarrhoeal Dis Res 1993: 11:143-147.

19. **Faruque SM**, Haider K, Rahman MM, Alim ARMA, Ahmad QS, Albert MJ, Sack RB. Differentiatin of *Shigella flexneri* strains by rRNA gene restriction patterns. J Clin Microbiol 1992; 30:2996-2999.

20. **Faruque SM**, Haider K, Albert MJ, Ahmad QS, Nahar S, Tzipori S. A comparative study of specific gene probes and standard bioassays to identify diarrhoeagenic *Escherichia coli* in paediatric patients with diarrhoea in Bangladesh. J Med Microbiol 1992; 36:37-40.

21. **Faruque SM**, Albert MJ. Genetic relation between *Vibrio cholerae* 01 strains in Ecuador and Bangladesh. Lancet 1992; 339:740-741.

Title: Analysis and genetic modification of *Vibrio cholerae* strains carrying atypical combination of virulence genes and serotypes, to develop potential vaccine candidates.

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 qualification

b) with qualification

- on technical grounds

- on level of financial support

I do not support the application

Name of Referee: PROFESSOR YOSHIFUMI TAKEDA

Signature: .....

Date: JUNE 28, 2002

Position: .....Professor

Institution: Jissen Women's University

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.

(Use additional pages if necessary)

Title:

PI:

Reviewer:

The project intends to use understand the distribution of a newly identified set of hybrids carrying atypical combination of virulence and specific genes. The investigators also plan to do extensive molecular analysis of these strains and determine the ability of these strains to cause diarrhoea in the adult rabbits using the RITARD model. The design, the presentation and the feasibility of execution of the project are excellent. The project should be support financially.

Title: Analysis and genetic modification of *Vibrio cholerae* strains carrying atypical combination of virulence genes and serotypes, to develop potential vaccine candidates.

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 qualification

b with qualification

- on technical grounds

- on level of financial support

I do not support the application

Name of Referee: R. Bradley Sack, MD

Signature: R. Bradley Sack

Date: June 26, 02

Position: Professor Peter

Institution: Johns Hopkins Univ

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.

(Use additional pages if necessary)

Title: Analysis and Genetic modification of V. bivio cholerae

PI: Faruque, SM.

Reviewer: RBlack

See attached page

June 26, 2002

Review of Protocol of Dr. Shah Faruque, "Analysis and Genetic modification of Vibrio cholerae ....

The protocol is intended to develop a killed oral cholera vaccine that will incorporate all of the known antigens of V. cholerae 01 and 0139. This will be accomplished by genetically engineering strains that do not produce cholera toxin, or other possible diarrheagenic virulence factors, but do produce the appropriate antigens that will provide protection against clinical cholera. The vaccine will be designed to be inexpensive so that it may be used as a public health tool in the control of cholera.

The protocol is very well put together; background material has been adequately reviewed, and the hypothesis well developed.

As written the protocol is very open-ended, i.e. strains will be developed and tested as they prove to be useful. All of the methods described are certainly adequate and can be carried out by the investigator. I would suggest listing potentially useful strains that are in hand.

Review of the protocol "Analysis and genetic modification of Vibrio cholerae strains carrying atypical combination of virulence genes and phenotypes to develop potential vaccine candidate"
(protocol # 2002-019)
PI: Dr. Shah M. Faruque

The goal of the project is to develop an inexpensive cholera vaccine using naturally occurring genetic variants of *Vibrio cholerae*. A safe and effective cholera vaccine is urgently needed for prevention of cholera in endemic and epidemic areas. As such this important area of research should be given high priority. The protocol is well written and the background materials have been extensively reviewed. The investigators have long continued experience in genetic analysis of *V. cholerae* strains.

However, I have the following few comments:

x 1. Background - The previous B subunit killed whole cell vaccine was given ~~one month~~ two weeks apart (page 10).
2. Ref # 89 (Sack et al, 1988) has not been mentioned in the text. also correction of Ref. list should be
3. Face sheet- Funding status should be mentioned.
4. One of the names of the coinvestigators mentioned on page 4 should also be listed on face sheet.
5. What may be the next steps if the investigators are able to formulate an inexpensive multivalent vaccine representing all the epidemic biotypes and serotypes?

Thanks

O139
Combination of all strains

* some one from ARB should included in study.
RRC application should prop[illegible] method

Cham

Dr. Kfaruque

Review of the protocol "Analysis and genetic modification of Vibrio cholerae strains carrying atypical combination of virulence genes and phenotypes to develop potential vaccine candidate"
(protocol # 2002-019)
PI: Dr. Shah M. Faruque

The goal of the project is to develop an inexpensive cholera vaccine using naturally occurring genetic variants of *Vibrio cholerae*. A safe and effective cholera vaccine is urgently needed for prevention of cholera in endemic and epidemic areas. As such this important area of research should be given high priority. The protocol is well written and the background materials have been extensively reviewed. The investigators have long continued experience in genetic analysis of *V. cholerae* strains.

However, I have the following few comments:

1. Background - The previous B subunit killed whole cell vaccine was given one month apart (page 10).
2. Ref # 89 (Sack et al, 1988) has not been mentioned in the text.
3. Face sheet- Funding status should be mentioned.
4. One of the names of the coinvestigators mentioned on page 4 should also be listed on face sheet.
5. What may be the next steps if the investigators are able to formulate an inexpensive multivalent vaccine representing all the epidemic biotypes and serotypes?

Thanks

Dr. K. Zaman

Review of the protocol "Analysis and genetic modification of Vibrio cholerae strains carrying atypical combination of virulence genes and phenotypes to develop potential vaccine candidate"
(protocol # 2002-019)
PI: Dr. Shah M. Faruque

The goal of the project is to develop an inexpensive cholera vaccine using naturally occurring genetic variants of *Vibrio cholerae*. A safe and effective cholera vaccine is urgently needed for prevention of cholera in endemic and epidemic areas. As such this important area of research should be given high priority. The protocol is well written and the background materials have been extensively reviewed. The investigators have long continued experience in genetic analysis of *V. cholerae* strains.

However, I have the following few comments:

1. Background - The previous B subunit killed whole cell vaccine was given one month apart (page 10).
2. Ref # 89 (Sack et al, 1988) has not been mentioned in the text.
3. Face sheet- Funding status should be mentioned.
4. One of the names of the coinvestigators mentioned on page 4 should also be listed on face sheet.
5. What may be the next steps if the investigators are able to formulate an inexpensive multivalent vaccine representing all the epidemic biotypes and serotypes?

Thanks