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CENTRE
FOR HEALTH AND
POPULATION RESEARCH

Memorandum

23 September 1999

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

From : Professor V. I. Mathan
Chairman, Research Review Committee

Sub : Protocol # 99-032

I am pleased to inform you that your protocol # 99-032 entitled "Characterization of environmental and clinical strains of toxigenic and nontoxigenic *Vibrio cholerae* as an aid to predict the emergence of new epidemic strains" has been approved by the Research Review Committee in its meeting held on 20th September 1999 subject to the clearance of the Animal Experimentation Ethics Committee.

Thanking you and wishing your success in running he protocol.

copy:- Division Director
Laboratory Sciences Division

Attachment 1

Date: 6/9/99

(FACE SHEET) RESEARCH REVIEW COMMITTEE, ICDDR,B.

Principal Investigator: DR. SHAH M. FARUQUE

Application No. 99-032

Title of Study: "Characterization of environmental and clinical strains of toxigenic and non-toxigenic V.cholerae as an aid to predict the emergence of new epidemic strains".

Trainee Investigator (if any): _____

Supporting Agency (if Non-ICDDR,B) USAID

Project Status: _____

[✓] New Study

[ ] Continuation with change

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

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

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

2. Does the Study Involve:
   - (a) Physical risk to the subjects — Yes (No)
   - (b) Social risk — Yes (No)
   - (c) Psychological risks to subjects — Yes (No)
   - (d) Discomfort to subjects — Yes (No)
   - (e) Invasion of privacy — Yes (No)
   - (f) Disclosure of information damaging to subject or others — Yes (No)

3. Does the Study Involve:
   - (a) Use of records (hospital, medical, death or other) — (Yes) No
   - (b) Use of fetal tissue or abortus — Yes (No)
   - (c) Use of organs or body fluids — Yes (No)

4. Are Subjects Clearly Informed About:
   - (a) Nature and purposes of the study — Yes No NA
   - (b) Procedures to be followed including alternatives used — Yes No NA
   - (c) Physical risk — Yes No NA
   - (d) Sensitive questions — Yes No NA
   - (e) Benefits to be derived — Yes No NA
   - (f) Right to refuse to participate or to withdraw from study — Yes No NA
   - (g) Confidential handling of data — Yes No NA
   - (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure — Yes No NA

5. Will Signed Consent Form be Required:
   - (a) From subjects — NA Yes No
   - (b) From parents or guardian (if subjects are minor) — NA Yes No

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

7. Check documents being submitted herewith to Committee:
   - ____ Umbrella proposal - Initially submit an with overview (all other requirements will be submitted with individual studies
   - ✓ Protocol (Required)*
   - ✓ Abstract Summary (Required)
   - NA Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw) (Required
   - NA Informed consent form for subjects
   - NA Informed consent form for parent or guardian
   - NA Procedure for maintaining confidentiality
   - NA Questionnaire or interview schedule*

   * If the final instrument is not completed prior to review, the following information should be included in the abstract summary

   1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy
   2. Example of the type of specific questions to be asked in the sensitive areas
   3. An indication as to when the questionnaire will be presented to the Committee for review

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

Shah Md. Faruque

Principal Investigator

_____

Trainee

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

Characterization of environmental and clinical strains of toxigenic and nontoxigenic *Vibrio cholerae* as an aid to predict the emergence of new epidemic strains.

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
Mice

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

7. Cost Required for the Budget Period
7a. Ist Year ($): 43,340 *2nd Year ($):* 43,440

7b. Total Direct Cost ($) 86,780 **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.

Professor V. I. Mathan
Name of the Division Director

Signature

24/8/99
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 Md. Faruque

Date: 24/8/99

Table of Contents

☐ Check here if appendix is included

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

Principal Investigator	DR. SHAH M. FARUQUE
Project Name	Characterization of environmental and clinical strains of toxigenic and nontoxigenic *Vibrio cholerae* as an aid to predict the emergence of new epidemic strains.

Total Budget US$ 86,780 Beginning Date: January, 2000 Ending Date: December, 2001

Epidemics of cholera caused by toxigenic *Vibrio cholerae* belonging to the O1 or O139 serogroup is a major public health problem in developing countries of Asia, Africa and Latin America. Cholera causes worldwide deaths estimated at 120,000 and many more cases each year, of which the vast majority occur in children. In areas of endemic infection, epidemics of cholera occur in a seasonal pattern, and molecular epidemiological surveillance has revealed clonal diversity among epidemic *Vibrio cholerae* strains and a continual emergence of new epidemic clones.

Although during epidemic outbreaks toxigenic *V. cholerae* are readily isolated from surface waters, these organisms are rarely found during interepidemic periods. It has been discovered recently that cholera toxin, which is responsible for the profuse diarrhea characteristic of cholera is encoded by a lysogenic bacteriophage designated CTXΦ. Further investigations in our laboratory has confirmed that naturally occurring strains of toxigenic *V. cholerae* are inducible lysogens of CTXΦ, and that CTXΦ infects certain nontoxigenic *V. cholerae* strains and converts them to toxigenic strains with epidemic potential.

We hypothesized that in the natural habitat, toxigenic strains of *V. cholerae* emerge from nontoxigenic progenitor strains carrying certain genetic and phenotypic markers. The emergence and enrichment of toxigenic *V. cholerae* strains may thereafter lead to epidemic outbreaks of cholera. Thus in areas where cholera exhibits a seasonal behavior characterized by fluctuations in incidence, surveillance for the presence of progenitor strains which are convertible to epidemic strains, may serve as a feasible means to predict the emergence of new toxigenic *V. cholerae* strains as well as possible cholera outbreaks.

The present study proposes environmental and clinical surveillance, and characterization of nontoxigenic progenitor strains as well as toxigenic strains of *V. cholerae*. This study is also designed to perform comparative molecular analysis of *V. cholerae* strains to determine the origination of new toxigenic strains, and their relation to epidemics of cholera. This year-round effort will (1) monitor the presence of toxigenic *V. cholerae* and nontoxigenic progenitor strains in environmental waters and patients during epidemic and interepidemic periods in an area of endemic infection in Bangladesh, (2) perform genetic fingerprinting of *V. cholerae* strains isolated from cholera patients and from the environment during the study period, and compare with a collection of epidemic strains isolated from different geographic regions, (3) obtain data on the number of cholera cases from hospital records during the surveillance period, and examine the association between number of cholera cases and presence of toxigenic *V. cholerae* as well as their progenitor strains in the environment. The long term goal of this work is to understand the epidemiology and evolution of toxigenic *V. cholerae* and develop a feasible means to predict the emergence of new toxigenic *V. cholerae* strains with epidemic potential.

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. QAZI SHAFI AHMAD	Microbiologist	Co-investigator
4. DR. M. YUNUS	Epidemiologist	Co-investigator
5. DR. A. K. SIDDIQUE	Epidemiologist	Co-investigator
6. PROF. JOHN J. MEKALANOS	Molecular Geneticist	Collaborator

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.

Nontoxigenic *V. cholerae* strains carrying a set of virulence genes and a specific attachment sequence (*att*RS) for a filamentous bacteriophage which encodes cholera toxin (CTXΦ) are the progenitors of toxigenic *V. cholerae* strains with epidemic potential. CTXΦ infects susceptible *V. cholerae* cells by using the toxin coregulated pilus (TCP) as its receptor, and the phage genome integrates into the host bacterial genome at the *att*RS site causing the origination of new toxigenic strains. Subsequent enrichment of toxigenic *V. cholerae* strains aided by both host and enviornmental factors leads to cholera epidemics. In areas of endemic infection, molecular analysis of nontoxigenic and toxigenic strains of *V. cholerae* for the relevant virulence characteristics, and comparative genetic fingerprinting of the strains with previously isolated epidemic strains may serve as a means to predict the emergence of new epidemic strains of *V. cholerae*. Intermediate events can also be sought to determine if a chain of events lead to a higher probability of cholera outbreak. For example, the presence of nontoxigenic progenitor strains (which are convertible to toxigenic strains) may be followed by an increase in the concentration of toxigenic *V. cholerae* in the environment and subsequently an outbreak of 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).**

1. To isolate nontoxigenic and toxigenic *V. cholerae* strains from patients and environmental water samples in a cholera endemic region every two weeks during a period of one year.
2. To analyze the strains for the possession and expression of different virulence associated genes
3. To perform molecular epidemiological analysis of toxigenic and non-toxigenic *V. cholerae* strains isolated from the environment and cholera patients.
4. To analyze the nontoxigenic strains for susceptibility and lysogenic conversion by CTXΦ.
5. To perform comparative molecular analysis of the strains with a collection of epidemic strains isolated in different countries to study clonal relationships among the strains and detect the origination of new clones of toxigenic *V. cholerae*.
6. To examine the association between the presence of toxigenic as well as CTXΦ-susceptible nontoxigenic *V. cholerae* in the environment and the onset of cholera outbreaks.

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).**

Epidemics of cholera caused by toxigenic *Vibrio cholerae* belonging to the O1 or O139 serogroup are a major public health problem in developing countries. The disease is an acute dehydrating diarrhea caused principally by the potent enterotoxin cholera toxin produced by these organisms during pathogenesis. Epidemics of cholera cause worldwide deaths estimated at 120,000 and many more cases each year, of which the vast majority occur in children (1). Cholera has been categorized as one of the "emerging and re-emerging infections" threatening many developing countries (1). Several recent events that mark the epidemiological importance of the disease include (a) the reemergence of cholera in Latin America in 1991, (b) explosive outbreak of cholera among Rwandan refugees in Goma, Zaire, which resulted in about 70,000 cases and 12,000 deaths in 1994, and (c) the emergence of *V. cholerae* O139 in the Indian subcontinent during 1992-1993.

V. cholerae is a well-defined species on the basis of biochemical tests and DNA homology studies (2), but the species is not homogeneous with regard to pathogenic potential. Although, there are two serogroups, O1 and O139, that have been associated with epidemic disease, there are also strains of these serogroups which do not produce cholera toxin (CT), do not cause cholera, and are not involved in epidemics. Previous studies by us and others have identified certain genetic and phenotypic markers possessed by clones of toxigenic *V. cholerae* which are capable of causing widespread epidemics. Molecular analysis, has revealed that all strains capable of causing cholera invariably carry genes for cholera toxin (CT), a pilus colonization factor called toxin coregulated pilus (TCP) and ToxR, which coregulates the expression of both CT and TCP (3-6).

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. Molecular epidemiological surveillance has revealed clonal diversity among toxigenic *Vibrio cholerae* strains isolated from different epidemics and a continual emergence of new epidemic clones (3-5). Many aspects of the disease and the pathogen remain unknown, particularly the mechanism of emergence of new epidemic strains, and the ecology of toxigenic *V. cholerae* which maintains the seasonal pattern of outbreaks in areas of endemic infection.

EPIDEMIOLOGY OF CHOLERA

Cholera is a waterborne disease, and the importance of water ecology is suggested by the close association of *V. cholerae* with surface waters and the population interacting with the waters. Hallmarks of the epidemiology of cholera include (a) a high degree of clustering of cases by location and season, (b) highest rates of infection in children 1 to 5 years of age in endemic areas, (c) antibiotic resistance patterns that frequently change from year to year, (d) clonal diversity of epidemic strains, and (d) protection against the disease being afforded by improved sanitation/hygiene, and preexisting immunity (7).

Cholera pandemics: It is generally accepted that seven distinct pandemics of cholera have occurred since the onset of the first pandemic in 1817 (8). 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. The sixth pandemic and presumably the fifth pandemic were caused by *V. cholerae* O1 of the classical biotype. Available information on these and earlier pandemics have been reviewed by Kaper *et.al.* (9). The seventh pandemic is the most extensive of the pandemics in geographic spread and in duration, and the causative agent is *V. cholerae* O1 of the El Tor biotype. The pandemic which began in 1961 from the island of Sulawesi in Indonesia spread to other islands including Java, Sarawak, and Bourneo, and then to the Philippines, Sabah, and Taiwan thereby affecting the entire Southeast Asian archipelago by the end of 1962 (10). During 1963 to 1969, the pandemic spread to the Asian mainland and affected Malaysia, Thailand, Burma, Cambodia, Vietnam, India, Bangladesh and Pakistan. Soon after El Tor cholera reached Pakistan, Afghanistan, Iran, Iraq, and nearby republics within the Soviet Union experienced outbreak of cholera. By 1970, El Tor cholera invaded the Arabian Peninsula, Syria, and Jordan, and a limited outbreak occurred in Israel (11). The seventh pandemic reached the sub-Saharan West Africa in the early seventies and caused explosive outbreaks resulting in more than 400,000 cases with a high case fatality, due mainly to a lack of background immunity in population, and inadequacies in the health care infrastructure (12). In this epidemic, cholera spread along waterways along the coast and into the interior along rivers, and further disseminated into the interior of the Sahelian states by land travel fostered by nomadic tribes. Of the 36 countries that reported cholera in 1970, 28 were newly affected countries and 16 were in Africa (9).

The seventh pandemic reached South America in the form of an explosive epidemic that began in Peru in January 1991, and thus caused the return of cholera to the continent after more than a century (13-15). Subsequently epidemic cholera was reported in neighboring Equador, and then in Colombia. In each of these countries low socioeconomic populations lacking proper drinking water and sanitation facilities were most affected (14). By April 1991, a small outbreak occurred in Santiago the capital of Chile (13), and cholera began to travel along the Pacific coast of South America to progressively enter more countries in South and Central America. The Pan American Health Organization estimates that during 1991-1992 there were 750,000 cases of cholera in the Americas with 6,500 deaths (14). In recent times, one of the worst cholera outbreaks occurred in Goma, Eastern Zaire in July 1994 (16). Conflicts between tribes in neighboring Rwanda had displaced nearly a million people to Zaire and were sheltered in refugee camps. Outbreak of cholera in the poverty- stricken camps led to the death of an estimated 12,000 Rawandan refugees during a three week period (16). The seventh pandemic is ongoing and it continues to cause seasonal outbreaks in many developing countries, especially in Bangladesh and India.

In the late 1992 and in 1993 epidemic cholera was reported in different places in India and Bangladesh (17, 18). Although the clinical syndrome was typical of cholera, the causative agent was a *V. cholerae* non-O1 strain, later serogrouped as O139 and was recognized as the second causative agent of cholera after the O1 serogroup of *V. cholerae*. The O139 *V. cholerae* has since spread throughout India, and outbreaks or cases have been reported in Pakistan, Nepal, China, Thailand, Kazakhastan, Afghanistan, and Malaysia (19, 20). Imported cases have been reported from the United Kingdom and the United States (21, 22). If outbreaks of cholera due to this new serogroup continue to occur affecting more countries, this may represent the eighth pandemic (19).

Epidemiology of cholera in Bangladesh:

The present study will be carried out in Bangladesh, where cholera is an endemic disease, and substantial information is available on the incidence and seasonality of the disease in this country. Systematic surveillance of cholera has been carried out in Bangladesh by the International Centre for Diarrhoeal Disease Research (ICDDR,B) and the former Pakistan Seato Cholera Research Laboratory during a period of over 35 years (23-27). A number of studies have shown that in Bangladesh epidemic outbreaks usually occur twice during a year, with the highest number of cases just after the monsoon during September to December (23-27). A somewhat smaller peak of cholera cases is also observed during the spring between March and May. Until 1970, more than 90% of cholera in Bangladesh was caused by the classical Inaba serotype and by 1972, 85% of all cases were due to classical Ogawa serotype (28). The El Tor biotype of *V. cholerae* O1 appeared in Bangladesh in the middle of 1973, and since then this biotype had completely replaced the classical biotype. However, in 1982, the classical biotype reemerged in Bangladesh (28, 29) and coexisted with the El Tor vibrios until 1992. Data obtained from interventions of diarrhea epidemics in nearly 400 rural sub-districts by ICDDR,B medical teams between 1985 and 1991 showed that *V. cholerae* O1 was the most frequently (40%) isolated enteropathogens during the epidemics (27). The 1991 epidemic alone was estimated to have produced between 210,000 and 235,000 cases and over 8,000 deaths (27).

V. cholerae O139 emerged during 1992 and 1993 causing explosive epidemic throughout Bangladesh, India and neighboring countries (17-20). In the beginning the new strain totally displaced the existing *V. cholerae* O1 strains including both classical and El Tor biotypes which co-existed only in Bangladesh. However, during 1994 and till the middle of 1995, in most northern and central areas of Bangladesh, including the capital city Dhaka the O139 vibrios were replaced by a new clone of *V. cholerae* O1 of the El Tor biotype, whereas in the southern coastal regions the O139 vibrios continued to exist (4, 30). During the second half of 1995 and in 1996, nearly four years after the initial detection of O139 vibrios, cases due to both *V. cholerae* O1 and O139 were detected in various regions of Bangladesh. Recent surveillance conducted by the epidemic control preparedness programme of ICDDR,B revealed the coexistence of *V. cholerae* O1 and O139 in Dhaka as well as in many rural districts of the northern, central and southern regions of the country that were affected by outbreaks of cholera between June and December of 1996 (5). Cholera surveillance in Bangladesh has shown that until 1992 *V. cholerae* O1 belonging to both the biotypes caused regular epidemics and since then both *V. cholerae* O1 and O139 continue to be a significant cause of infection and morbidity, although the frequency of infection varies from year to year in different regions of the country (27, 31, 32).

Antibiotic resistance: In 1979, an outbreak of cholera due to multiple drug resistant *V. cholerae* O1 occurred in Matlab, a rural sub-district of Bangladesh (33, 34). Screening of isolates from the outbreak showed that 16.7% of the isolates were resistant to five antibiotics: tetracycline, ampicillin, kanamycin, trimethoprim-sulfamethoxazole, and streptomycin, and another 10% of the isolates were resistant to any four of these antibiotics including tetracycline. An antibiotic resistance plasmid was identified in these isolates and the plasmid was transferable to an *E. coli* K-12 recipient by conjugation. Epidemiological assessment of the outbreak suggested that the outbreak began from the introduction into the area of a single multiple drug resistant strain of *V. cholerae* O1 (34). By 1986, the drug resistance pattern changed, and screening of *V. cholerae* O1 isolated from cholera patients in January 1986 in Dhaka showed that none of these isolates was resistant to tetracycline, streptomycin, chloramphenicol, amoxicillin or nalidixic acid (35). However, during 1988 and 1989, nearly all classical *V. cholerae* isolated in Bangladesh were resistant to tetracycline, whereas strains

belonging to the El Tor biotype were sensitive to the drug (36). After almost a decade, reemergence of tetracycline resistant El Tor strains was observed during the 1991 epidemic in Bangladesh (37), and 70% of strains isolated were resistant to tetracycline, often in addition to other antibiotics. The O139 serogroup of *V. cholerae* which emerged during 1992-1993, were sensitive to tetracycline (17). Although this new serogroup of toxigenicv *V. cholerae* has shown a trend of increased resistance to trimethoprim-sulfamethoxazole, it has remained more susceptible to ampicillin and tetracycline than the O1 serogroup of *V. cholerae*. Waldor and coworkers (38) reported the presence of a 62-kb self-transmissible transposon-like element (SXT element) encoding resistance to sulfamethoxazole, trimethoprim and streptomycin in *V. cholerae* O139. However, recent isolates of *V. cholerae* O139 are mostly sensitive to SXT. Considering the rapidly changing pattern of antibiotic resistance observed among toxigenic *V. cholerae* isolated from different outbreaks, it appears that there is substantial mobility in genetic elements encoding antibiotic resistance in *V. cholerae*.

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 (3, 39-46). These techniques include the analysis of restriction fragment length polymorphisms (RFLPs) in different genes. The use of gene probes to study RFLPs in the *ctxAB* genes and their flanking DNA sequences which are part of a larger genetic element (CTX element) indicated that the U.S. Gulf coast isolates of toxigenic *V. cholerae* are clonal and that they are different from other seventh pandemic isolates (44). 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 (43 45, 46). 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 (47). Comparative molecular analysis of the El Tor strains of *V. cholerae* O1 and the epidemic O139 strains suggested that the O139 strains are related to El Tor strains and were derived from the El Tor strains by genetic changes in the serotype specific gene clusters (42, 47).

Molecular analysis of epidemic isolates of *V. cholerae* between 1961 and 1996 in Bangladesh revealed clonal diversity among strains isolated during different epidemics (3-5). 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 (4, 5). These studies indicated that there had been a continual emergence of new clones of toxigenic *V. cholerae* which replaced existing clones, possibly through natural selection involving unidentified environmental factors and immunity of the host population.

VIRULENCE ASSOCIATED GENES IN *V. CHOLERAE* :

The pathogenesis of cholera is a complex process and involves a number of genes encoding virulence factors which aid the pathogen in its passage to reach the epithelium of the small intestine, colonize the epithelium, and

produce cholera toxin (CT) that disrupts ion transport by intestinal epithelial cells. In *V. cholerae*, the major virulence genes required for pathogenesis exist in clusters. These include the CTX genetic element, which is the genome of a lysogenic bacteriophage designated CTXΦ (48) 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 (49, 50). Thus the major virulence gene clusters in *V. cholerae,* appear to have phage origins, and this suggests that horizontal transfer of gene clusters may be a possible mechanism for the origination of new pathogenic clones of *V. cholerae.*

Since colonization is a prerequisite to establishing a productive infection by *V. cholerae* the existence and role of possible other factors responsible for colonization has also been investigated. This includes the mannose-fucose-resistant cell associated hemagglutinin (MFRHA) the mannose sensitive hemagglutinin (MSHA), and some outer membrane proteins (OMPs) of *V. cholerae* (51-54). Although some of these factors including MFRHA, MSHA and OMPs are suspected to have a role in enhancing adhesion and colonization when tested in animal models, the exact role of these factors in the virulence of *V. cholerae* in humans is still uncertain. Studies to date have shown that the major virulence genes of *V. cholerae* required for pathogenesis are the genes involved in the production of TCP and CT (7).

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 (55). Expression of CT and TCP are co-regulated by the ToxR regulatory system which includes the ToxT protein (56). The genes encoding ToxT and TCP are located in the same choromosomal region (57) together with other ToxR-regulated genes including those for a potential accessory colonization factor (ACF) (58). 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 (59). 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* (60). 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 (49). 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 (50).

The CTX genetic element:

Toxigenic *Vibrio cholerae* strains carry one or more copies of CT genes (*ctxAB*). The A and B subunits of CT are encoded by two separate but overlapping open reading frames. *V. cholerae* also produces a putative toxin known as zonula occludens toxin (Zot) that increases the permeability of the small intestinal mucosa by affecting the structure of the intercellular tight junction, or zonula occludens (61). The *zot* gene consists of a 1.3 kb open reading frame, which could potentially encode a 44.8-kDa polypeptide, and is located immediately upstream of the *ctxA* gene (61). A third toxin that has been described is Accessory cholera enterotoxin (Ace) which is capable of inducing fluid accumulation in rabbit ligated ileal loops (62).

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 (63). 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 (48).

Cholera toxin converting bacteriophage (CTXΦ):

It has been demonstrated that under appropriate conditions toxigenic *V. cholerae* strains can be induced to produce extracellular CTXΦ particles (48, 64). 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 (48). 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 (65). 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 (66). 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 (66). The deletion of a portion of the genes encoding CT by marker exchange, however, did not affect the morphogenesis of the bacteriophage. It appears that the *ctxAB* genes do not participate directly in the formation of phage particles, but these genes are important for the phage to provide a survival advantage to its host bacteria in the gastrointestinal environment (66).

ECOLOGY OF *V. CHOLERAE*:

V. cholerae has been regarded as a member of a group of organisms whose major habitats are aquatic ecosystems (67). Although, *V. cholerae* is part of the normal, free-living bacterial flora in riverine and estuarine areas, non-O1 non-O139 strains are more commonly isolated from the environment than are O1 or

O139 strains. Moreover, outside of epidemic areas and away from areas that may have been contaminated by cholera patients, environmental isolates of *V. cholerae* O1 have been found to be mostly CT negative. The major pathogenic genes in *V. cholerae* are clustered in several regions of the *V. cholerae* chromosome and the structure of these pathogenic gene clusters indicates that these are capable of being propagated horizontally (48-50, 63). This is suggestive of the possibility that environmental strains of *V. cholerae* may develop the ability to adapt to the intestinal environment through acquisition of the virulence genes. In view of the available information on the epidemiology of cholera, the lysogenic conversion by a bacteriophage encoding cholera toxin, and the survival and enrichment of *V. cholerae* under in-vivo and in-vitro conditions, it is apparent that the ecosystem for *V. cholerae* should consists of a number of components. These include (a) the bacterium, (b) the aquatic environment, (c) CTXΦ and other unidentified genetic elements involved in the transfer of virulence genes, and (d) the intestinal environment of the host population. A summary of available information from studies regarding survival of toxigenic *V. cholerae* in the aquatic environment and in the host intestine, and the possible role of accessory genetic elements that mediate the horizontal transfer of virulence genes is as follows.

Environmental survival and persistence of *V. cholerae*:

The physicochemical conditions for the survival of *V. cholerae* O1 have been investigated and the possibility of survival of the organism in an estuarine environment and other brackish waters is widely accepted (67, 68). However, the nature of the survival and persistence of toxigenic *V. cholerae* O1 or O139 in aquatic ecosystem and the factors involved in the conservation of the CTX element (the lysogenic form of CTXΦ) and other pathogenic genes in the aquatic environment is not clear. The survival may be dependent on several factors, such as occurrence of particular physico-chemical conditions, specific association of the bacteria with aquatic plants or animals, and /or the existence of specific ecological association involving several components of the aquatic environment. It has been postulated that under stress conditions the vibrios are converted to a viable but non-culturable (VNC) form, that cannot be recovered by standard culture techniques, and that such VNC forms are able to produce infection and can revert into the culturable form (68). The public health and ecological importance of the possible survival forms such as VNC depends on whether these forms are re-convertible to live infectious bacteria. There is, however, very little evidence to conclusively establish that the possible non-culturable phenomenon is reversible. Hence there is considerable scope to further investigate the role of postulated VNC forms of *V. cholerae* through carefully controlled studies.

It has also been suggested that during inter-epidemic periods toxigenic *V. cholerae* exists in an unexplained ecological association with aquatic organisms possibly in the VNC form until the next epidemic season, when environmental factors triggers the dormant bacteria to multiply and lead to cholera outbreaks (69, 70). However, differences in genetic or phenotypic properties have been often noticed among *V. cholerae* O1 and O139 strains isolated during different epidemics (71, 72). Analysis of rRNA gene restriction patterns of *V. cholerae* strains has also shown clonal diversity among epidemic strains (3-5, 73). These events have raised questions whether seasonal epidemics are caused by periodic appearance of the same strains of *V. cholerae* or due to a continual emergence of new toxigenic clones from nontoxigenic progenitors. Hence further studies are also required to understand more definitive roles of environmental factors in the emergence and reemergence of toxigenic *V. cholerae*.

Enrichment of toxigenic *V. cholerae* in the intestinal environment:

Although there is considerable understanding of the mechanism how CT causes diarrhea, it is not clearly known why *V. cholerae* should infect and elaborate the lethal toxin in the host system. It seems worth wondering whether the role of the toxin is to simply cause diarrhea and thus disseminate the organism to its next victim or could the toxin be providing a more crucial function for the enrichment and continued existence of the bacteria.

Studies directed towards the development of attenuated *V. cholerae* mutants altered in toxin production for use as live oral cholera vaccines provided a means to investigate the role of CT in the intestine. In 1971, Howard (74) reported the isolation of nontoxigenic mutants of the classical strain 569B by mutagenesis with nitrosoguanidine. The mutants were unable to induce a secretory response in the rabbit intestinal loop model, and did not survive or multiply in the intestinal environment. During 1974-75, Finkelstein et al (75) and Holmes et al (76) observed that the ability of various mutants to grow in the intestinal environment correlates with the ability of the mutants to induce a residual secretory response in the infant rabbit model. A variety of different toxin deficient mutants of *V. cholerae* tested in rabbit and infant mouse models also suggested that the mutants showed enhanced killing and mechanical clearance in the intestinal environment compared to the toxigenic parental strain (77, 78). It seems possible that many of these early mutants were altered in TCP as well as CT expression, perhaps by carrying mutations in regulatory genes involved in the expression of CT and TCP. The selection for their reversion in vivo may have, therefore, been driven by the need to up regulate the expression of TCP more than that of CT (7). The characterization in vivo of site-specific *ctxAB* mutants constructed by in vitro recombinant DNA methods provided the most convincing evidence that the toxin is beneficial to growth in the intestinal environment. It was demonstrated that the *ctx* mutants colonized rabbit intestines about 10 to 100-fold less efficiently than the parental strain (79). The details of the mechanisms involved have been reviewed by Mekalanos (79).

Studies so far suggest that causation of cholera in humans is also linked with a natural process of enrichment of toxigenic *V. cholerae*, and partly explains the benefit imparted to the pathogen during the disease in humans. However, in order to understand the general epidemiological behavior of *V. cholerae*, which includes seasonal pattern of epidemics, transient appearance and disappearance of different clones, and emergence of new epidemic clones, it is important to study the interactions among the bacteria, genetic elements mediating the transfer of virulence genes, the human host and possible environmental factors.

Emergence of novel strains of toxigenic *V. cholerae*:

V. cholerae offers a genetic system to study the relationship between pathogenesis and the natural selection of pathogens so as to ensure their continued existence. It appears that acquisition of pathogenic gene clusters by *V. cholerae*, which is normally a marine or brakish water species, has allowed the bacterium to become adapted to the human intestinal environment (7). Although the donor for the TCP pathogenicity island has not yet been identified, the TCP island seems to have a bacteriophage origin, and it has been speculated that the TCP island can be transferred by transducing phages (49, 50). On the other hand the donor for the CTX genetic element has been confirmed to be a filamentous bacteriophage, which has been characterized to some details (48, 66). It has been postulated that the acquisition of the CTX element by *V. cholerae* provides a survival advantage to the bacterium, and leads to enrichment of toxigenic *V. cholerae* in the intestinal environment. Thus the CTX phage confers increased evolutionary fitness to its host and hence to its own nucleic acids. With growing immunity in the host population against certain toxigenic clones of *V. cholerae*, new toxigenic clones

emerge and replace existing clones by a process of natural selection. Thus a continual emergence of new strains of toxigenic *V. cholerae* and their selective enrichment during cholera outbreaks constitute an essential component of the ecosystem for the survival and evolution of *V. cholerae* and the genetic elements that mediate the transfer of virulence genes (7). Recent studies have demonstrated that potential epidemic strains can originate from nonpathogenic progenitor strains and thus accounts for the observed clonal diversity among epidemic *V. cholerae* strains (65). However, many aspects regarding the epidemiology and the ecology of the pathogen remain unknown, particularly the mechanisms controlling the seasonal pattern of epidemics in areas of endemic infection. The present proposal aims at further understanding the mechanisms involved in the emergence of new pathogenic strains and its relation to epidemics of cholera.

SUMMARY OF PRELIMINARY STUDIES AND AVAILABLE DATA

1. **Surveillance of cholera**: There has been systematic surveillance of cholera in Bangladesh for over the last 35 years, and data are available on the occurrence and seasonality of cholera, as well as microbiological information regarding epidemic strains. Cholera surveillance in Matlab since 1966 shows that cholera outbreaks occur every year, without exception, although the yearly attack rates vary from 0.5-8.0 per 1000 population. There are two cholera epidemics each year; the spring peak occurs in April-May, and the fall peak in September-November. A geographic information system recently introduced in Matlab, has demonstrated a clustering of cases in certain villages and in relation to bodies of water (unpublished information).

2. **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. These studies have demonstrated a continual emergence of new epidemic strains (3-5). By ribotyping and CTX genotyping we have catalogued different toxigenic strains, and these are available in our collection to be used as reference strains in detecting the origination of new toxigenic strains.

3. **Isolation and characterization of nontoxigenic *V. cholerae* O1 and non-O1 strains from the environment:** We have isolated and characterized *V. cholerae* strains carrying different combinations of virulence associated genes from the environment. The techniques for isolation and identification of such strains are available in our laboratory (65).

4. **Identification and enumeration of CTXΦ:** We have developed and standardized assays for the identification of CTXΦ using specific probes, and for the quantitative determination of infectious phage particles. Assay for transduction of *V. cholerae* strains by CTXΦ in the intestines of suckling mice as well as under in vitro laboratory conditions has also been standardized (64).

5. **Lysogenic conversion of nontoxigenic strains to toxigenic strains:** Recent studies in our laboratory have shown that nontoxigenic *V. cholerae* carrying certain genetic markers can be infected and lysogenized with the CTX phage (65). This showed that environmental nonpathogenic *V. cholerae* strains may be progenitors of toxigenic *V. cholerae* which are involved in epidemic outbreaks.

6. **Seasonal change in toxigenicity of environmental vibrios** : Surveillance for toxigenicity of *V. cholerae* in a freshwater lake in Calcutta, showed that during an epidemic of cholera there was a 16-fold increase in the number of toxigenic strains among the resident non-O1 non-O139 serogroups of *V. cholerae* (80). We have also noticed a periodic increase in the isolation of toxigenic non-O1 non-O139 strains from the environment (unpublished data). Further studies are under way to determine whether such strains can act as reservoirs of the toxin converting bacteriophage, or serve as an indicator for the presence of the phage in the environment.

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

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

METHODS

Study Area:

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

from previous records. Cholera patients reporting to the hospital will be recorded and *V. cholerae* strains isolated from patients will be obtained from the microbiology laboratory of the Matlab hospital.

Collection of environmental water samples:

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

V. cholerae strains:

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

Laboratory Methods

Probes and hybridization:

The gene probes specific for the CTX element will consist of a probe for the *ctxA* gene or a defined portion of the *zot* gene. The *ctxA* probe will be a 0.5 kb *Eco*RI fragment of pCVD27 (82) which is a pBR325 derived plasmid containing an *Xba*I-*Cla*I fragment representing 94% of the gene encoding the A subunit of CT, cloned with *Eco*RI linkers. The *zot* gene probe will consist of an 840 bp region internal to the *zot* gene, and will be amplified by PCR from the recombinant plasmid pBB241 as described by us previously (83). The *toxR* probe will be a 2.4 kb *Bam*HI fragment of pVM7 (84) which is a pBR322 derived plasmid carrying the entire *toxR* sequence. The rRNA gene probe will be a 7.5-kb *Bam*HI fragment of pKK3535 (85). Preparation and use of these probes have been described by us previously (3-5).

Colony blots or Southern blots will be prepared using nylon filters (Hybond, Amersham International plc. Ayelesbury, United Kingdom), and processed by standard methods (86). The polynucleotide probes will be labeled by random priming (87) 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 (3-5).

PCR Assays:

Presence of the *tcpA* gene will be determined using a PCR assay as described previously (88). The *tcpI* gene will be detected by a PCR assay based on the published sequence of *tcpI* as described by us previously (4). PCR assay to detect the presence of the CTX element in the environmental samples 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. A multiplex PCR assay for the simultaneous detection of the *tcpA* gene and the *ctxA* gene will be used to discriminate between the presence of toxigenic *V. cholerae* and cell free CTXΦ particles in the environmental samples. All primers will be synthesized in the Molecular Genetics laboratory of ICDDR,B using a Beckman oligo-1000 DNA synthesizer and PCR reagents will be obtained from Perkin-Elmer Cetus (Norwalk, Conn.) Aliquots of the amplified products will be analyzed by agarose gel electrophoresis to ascertain the expected size of the amplified DNA segments. When required, the identity of the amplified DNA will be further confirmed by Southern blot hybridization using specific DNA probes.

Susceptibility of nontoxigenic V. cholerae strains to CTX-KmΦ:

Susceptibility of *V. cholerae* strains to the genetically marked derivative of CTXΦ will be assayed under laboratory conditions and inside the intestines of infant mice by previously described methods (64, 65). The recipient strains will be grown in LB at 37°C; the cells will be precipitated by centrifugation and washed in fresh LB. The recipient cells and phage particles will be mixed in LB to a make an approximate final concentration of 10^6 bacterial cells and 10^6 phage particles per ml. For the in vitro assay the mixture will be incubated for 16 h at 30°C, and aliquots of the culture will be diluted and plated on LA plates containing kanamycin (50 µg/ml) to select for kanamycin resistant colonies and on plates devoid of kanamycin to determine the total number of colonies.

For the in vivo assay, the same mixtures of phage and recipient cells will be used to gastrointestinally inoculate groups of 5-day old Swiss Albino mice obtained from the breeding facilities of the Animal Resources Branch of ICDDR,B as described previously (64). Animals will be sacrificed after 16 h and their intestines were removed and homogenized in 10 mM phosphate-buffered saline (PBS) pH 7.2. The homogenate will be centrifuged at low speed to precipitate debris, the supernatant will then be centrifuged to precipitate bacterial cells, and the pellet will be resuspended in PBS. The suspension will be screened for the presence of Km^R *V. cholerae* colonies. The ratio of Km^R colonies to total colonies will be calculated and expressed as the percentage of recipient cells carrying the phage genome.

Analysis of infected cells: Representative infected colonies will be grown overnight in LB containing kanamycin (50 μg/ml) and cells will be precipitated by centrifugation. The supernatant fluids of the cultures will be titrated for the presence of CTX-KmΦ particles using strain RV508 as the recipient as described previously (64). Total DNA or plasmids will be extracted from bacterial pellets by standard methods (86), and purified using microcentrifuge filter units (Ultrafree-Probind; Sigma). Integration of the phage genome into the chromosome of the recipient cells will be studied by comparative Southern blot analysis of total DNA and plasmid preparations from the phage-infected and the corresponding native strains using the *zot* probe (65).

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 (89). 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 (3).

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

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 understand the origination of new toxigenic strains and possible epidemics of cholera in relation to the detection of toxigenic *V. cholerae* or its progenitor strains in the environment. Each study fortnight will be classified by the presence or absence of relevant strains in the environmental water samples. A fortnight will be considered positive if at least one of the sites are found positive for the presence of the strains. An outbreak of cholera will be defined as ten or more new cases during a period of two weeks.

Regression of cholera incidences against the presence of toxigenic *V. cholerae* or progenitor strains which are convertible to toxigenic strains in the environment will be done and correlation coefficients will be used to measure the association between fortnightly cholera cases and the presence of relevant *V. cholerae* strains in the aquatic environment. To measure the relationship between an outbreak of cholera in the surveillance area and the presence *V. cholerae* in the environmental waters, the odds ratio will be used. This ratio will measure the odds of having an outbreak of cholera when toxigenic *V. cholerae* or progenitor strains is found present in the water samples in the previous 4 weeks compared to when it is not found.

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

Intermediate events will also be sought to determine if a chain of events lead to a higher probability of cholera outbreak. For example, presence of nontoxigenic progenitor strains may be followed by an increase in the concentration of toxigenic *V. cholerae* and subsequently an outbreak of cholera. Approximate number of viable cells of toxigenic *V. cholerae* per 100 ml of water samples, determined by a colony count will provide a measure of the concentration of toxigenic *V. cholerae*. Appropriate analysis will be used to quantifying the effect of risk factors that operate along a single causal pathway (90). Thus the search for a predictive model will attempt to understand mechanisms as well as look for statistical associations.

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.

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

Use of Animals

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

Swiss Albino mice will be used from the breeding facilities of ICDDR,B whenever required according to the ICDDR,B guidelines for use of animals in research. Any surgical procedure will be performed under anesthesia as appropriate to reduce suffering of the animals. The assays in mice will be required to determine the susceptibility of nontoxigenic *V. cholerae* strains to CTX phage.

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. World Health Organization. Report of a meeting on "The potential role of new cholera vaccine in the prevention and control of cholera outbreak during acute emergencies" (CDR/ GPV/ 95.1). Geneva: World Health Organization, 1995.
2. Baumann, P., A. L. Furniss, and J. V. Lee. 1984. Vibrio, p. 518-538. In N. R. Kreig, and J. G. Holt, (ed.), Bergey's manual of systematic bacteriology, vol. 1, Baltimore, Willams and Wilkins.
3. 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.
4. 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.
5. 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.
6. Herrington, D.A., R.H. Hall, G. Losonsky, J.J. Mekalanos, R.K. Taylor, and M.M. Levine. 1988. Toxin, toxin-coregulated pili and ToxR regulon are essential for *Vibrio cholerae* pathogenesis in humans. J. Exp. Med. 168:1487-1492.
7. 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.
8. Pollitzer, R. 1959. History of the disease, p. 11-50. In R. Pollitzer (ed.), Cholera. World Health Organization, Geneva.
9. Kaper, J. B., J. G. Morris, and M. M. Levine. 1995. Cholera. Clin. Microbiol. Rev. 8:48-86.
10. Kamal, A. M. 1974. The seventh pandemic of cholera, p. 1-14. In D. Barua, and W. Burrows (ed.), Cholera. Saunders, Philadelphia.
11. Cohen, J., T. Schwartz, R. Klasmer, D. Pridan, H. Ghalayini, and A. M. Davies. 1971. Epidemiological aspects of cholera El Tor outbreak in a non-endemic area. Lancet. ii:86-89.
12. Goodgame, R. W., and W. B. Greenough III. 1975. Cholera in Africa: a message for the West. Ann. Intern. Med. 82:101-106.
13. Levine, M. M. 1991. South America: the return of cholera. Lancet. 338:45-46.
14. Pan American Health Organization. 1991. Cholera situation in the Americas. Epidemiol. Bull. 12:1-4.
15. Ries, A. A., D. J. Vugia, L. Beingolea, A. M. Palacios, E. Vasquez, J. G. Wells, N. G. Baca, D. L. Swerdlow, M. Pollack, N. H. Bean, L. Seminario, and R. V. Tauxe. 1992. Cholera in Piura, Peru: a modern urban epidemic. J. Infect. Dis. 166:1429-1433.
16. Siddique, A. K., A. Salam, M. S. Islam, K. Akram, R. N. Majumdar, K. Zaman, N. Fronczak, and S. Laston. 1995. Why treatment centres failed to prevent cholera deaths among Rwandan refugees in Goma, Zaire. Lancet. 345:359-361.
17. 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.
18. 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.
19. Swerdlow, D. L., and A. A. Ries. 1993. *Vibrio cholerae* non-O1 -the eighth pandemic? Lancet. 342:382-383.
20. Chongsa-nguan, M., W. Chaicumpa, P. Moolasart, P. Kandhasingha, T. Shimada, H. Kurazono, and Y. Takeda. 1993. *Vibrio cholerae* O139 Bengal in Bangkok. Lancet. 342:430-431.
21. Centers for Disease Control. 1993. Imported cholera associated with a newly described toxigenic *Vibrio cholerae* O139 strain-California, 1993. Morbid. Mortal. Weekly Rep. 42:501-503.

22. Public Health Laboratory Service. 1993. *Vibrio cholerae* O139 and epidemic cholera. Commun. Dis. Rep. Weekly. 3:173.

23. Martin, A. R., W. H. Mosley, B. B. Sau, S. Ahmed, and I. Huq. 1969. Epidemiologic analysis of endemic cholerain urban East Pakistan, 1964-1966. Am. J. Epidemiol. 89:572-582.

24. McCormack, W. M., W. H. Mosley, M. Fahimuddin, and A. S. Benenson. 1969. Endemic cholera in rural East Pakistan. Am. J. Epidemiol. 89:393-404.

25. Samadi, A. R., M. K. Chowdhury, M. I. Huq, and M. U. Khan. 1983. Seasonality of classical and El Tor cholera in Dhaka, Bangladesh: 17 year trends. Trans. R. Soc. Trop. Med. Hyg. 77:853-856.

26. Siddique, A. K., A. H. Baqui, A. Eusof, K. Haider, M. A. Hossain, I. Bashir, and K. Zaman. 1991. Survival of classical cholera in Bangladesh. Lancet. 337:1125-1127

27. Siddique, A. K., K. Zaman, A. H. Baqui, K. Akram, P. Mutsuddy, A. Eusof, K. Haider, S. Islam, and R. B. Sack. 1992. Cholera epidemics in Bangladesh: 1985-1991. J. Diarrhoeal Dis. Res. 10:79-86.

28. Khan, M. U., A. R. Samadi, M. I. Huq, and W. B. Greenough. 1986. Reappearance of classical *Vibrio cholerae* in Bangladesh, p. 3-12. In Advances in Research on Cholera and Related Diarrheas, 3, KTK Scientific Publishers, Tokyo.

29. Samadi A. R., M. I. Huq, N. Shahid, M. U. Khan, A. Eusof, A. S. M. M. Rahman, M. M. Yunus, and A. G. S. Faruque. 1983. Classical *Vibrio cholerae* biotype displaces El Tor in Bangladesh. Lancet. i:805-807.

30. Siddique, A.K., K. Akram, K. Zaman, P. Mutsuddy, A. Eusof, and R.B. Sack. 1996. *Vibrio cholerae* O139: How great is the threat of a pandemic? Trop. Med. Int. Health. 1:393-398.

31. Baqui, A. H., M. D. Yunus, K. Zaman, A. K. Mitra, and K. M. B. Hossain. 1991. Surveillance of patients attending a rural diarrhoea treatment centre in Bangladesh. Trop. Geog. Med. 43:17-22.

32. Stoll, B. J., R. I. Glass, M. I. Huq, M. U. Khan, J. E. Holt, and H. Banu. 1982. Surveillance of patients attending a diarrhoeal disease hospital in Bangladesh. Br. Med. J. 285:1185-1188.

33. Glass, R. I., I. Huq, A. R. M. A. Alim, and M. Yunus. 1980. Emergence of multiple antibiotic-resistant *Vibrio cholerae* in Bangladesh. J. Infect Dis. 142:939-942.

34. Glass, R. I., M. I. Huq, J. V. Lee, E. J. Threlfall, M. R. Khan, A. R. M. A. Alim, B. Rowe, and R. J. Gross. 1983. Plasmid-borne multiple drug resistance in *Vibrio cholerae* serogroup O1, biotype El Tor: evidence of a point-source outbreak in Bangladesh. J. Infect. Dis. 147:204-209.

35. Nakasone, N., M. Iwanaga, and R. Eeckels. 1987. Characterization of *Vibrio cholerae* O1 recently isolated in Bangladesh. Trans. Roy. Soc. Trop. Med. Hyg. 81:876-878.

36. Siddique, A. K., K. Zaman, and Y. Mazumder. 1989. Simultaneous outbreaks of contrasting drug resistant classic and El Tor *Vibrio cholerae* O1 in Bangladesh. Lancet i:396.

37. Siddique, A. K., K. Zaman, A. H. Baqui, K. Akram, P. Mutsuddy, A. Eusof, K. Haider, S. Islam, and R. B. Sack. 1992. Cholera epidemics in Bangladesh: 1985-1991. J. Diarrhoeal Dis. Res. 10:79-86.

38. Waldor, M.K., H. Tschape, and J.J. Mekalanos. 1996. A new type of conjugative transposon encodes resistance to sulfamethoxazole, trimethoprim, and streptomycin in *Vibrio cholerae* O139. J. Bacteriol. 178:4157-4165.

39. 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.

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

41. 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.

42. 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.

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

44. 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.\

45. 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.

46. 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.

47. 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.

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

49. 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.

50. 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.

51. 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.

52. 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.

53. Goldberg, M. B., V. J. DiRita, and S. B. Calderwood. 1990. Identification of an iron-regulated virulence determinant in *Vibrio cholerae*, using Tn*phoA* mutagenesis. Infect. Immun. 58:55-60.

54. 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.

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

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

57. 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.

58. 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.

59. 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.

60. 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.

61. 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.

62. 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.

63. 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.

64. 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.

65. 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.

66. 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.

67. 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.

68. 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.

69. Islam M. S., B. S. Drasar, and R. B. Sack. 1994. The aquatic environment as reservoir of *Vibrio cholerae*: A review. J. Diarrhoeal Dis. Res. 11:197-206.

70. Islam M. S., B. S. Drasar, and R. B. Sack. 1994. The aquatic flora and fauna as reservoirs of *Vibrio cholerae*: A review. J. Diarrhoeal Dis. Res. 12:87-96.

71. Mukhopadhyay, A. K., A. Basu, P. Garg, P. K. Bag, A. Ghosh, S. K. Bhattacharya, Y. Takeda, and G. B. Nair. 1998. Molecular epidemiology of reemergent *Vibrio cholerae* O139 Bengal in India. J. Clin. Microbiol. 36:2149-2152.

72. Nakasone, N., M. Iwanaga, and R. Eeckels. 1987. Characterization of *Vibrio cholerae* O1 recently isolated in Bangladesh. Trans. Roy. Soc. Trop. Med. Hyg. 81:876-878.

73. Sharma, C., G. B. Nair, A. K. Mukhopadhyay, S. K. Bhattacharya, R. K. Ghosh, and A. Ghosh. 1997. Molecular characterization of *Vibrio cholerae* O1 biotype El tor strains isolated between 1992 and 1995 in Calcutta, India: evidence for the emergence of a new clone of the El Tor biotype. J. Infect. Dis. 175: 1134-1141.

74. Howard, B. D. 1971. A prototype live oral cholera vaccine. Nature. 230:97-99.

75. Finkelstein, R. A., M. L. Vasil, and R. K. Holmes. 1974. Studies on toxinogenesis in *Vibrio cholerae*. I. Isolation of mutants with altered toxinogenicity. J. Infect. Dis. 129:117-123.

76. Holmes, R. K., M. L. Vasil, and R. A. Finkelstein. 1975. Studies on toxino-genesis in *Vibrio cholerae*. III. Characterization of nontoxigenic mutants in vitro and in experimental animals. J. Clin. Invest. 55:551-556.

77. Baselski, V. S., R. A. Medina, and C. D. Parker. 1978. Survival and multiplication of *Vibrio cholerae* in the upper bowel of infant mice. Infect. Immun. 22:435-440.

78. Baselski, V. S., R. A. Medina, and C. D. Parker. 1979. In vivo and in vitro characterization of virulence-deficient mutants of *Vibrio cholerae*. Infect. Immun. 24:111-116.

79. Mekalanos, J. J. 1985. Cholera toxin: genetic analysis, regulation and role in pathogenesis. Curr. Top. Microbiol. Immunol. 118:97-118.

80. Ghosh, A. R., H. Koley, D. De, S. Garg, M. K. Bhattacharya, S. K. Bhattacharya, B. Manna, G. B. Nair, T. Shimada, T. Takeda, and Y. Takeda. 1994. Incidence and toxigenicity of *Vibrio cholerae* in a freshwater lake during the epidemic of cholera caused by serogroup O139 Bengal in Calcutta, India. FEMS Microbiol. Ecol. 14: 285-291.

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

82. 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.

83. 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.

84. 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

85. 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.

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

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

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

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

90. Koopman JS. Epigenesis theory: A mathematical model relating causal concepts of pathogenesis in individuals to disease patterns in populations. Am J Epidemiol 1990; 132:366-390

Dissemination and Use of Findings

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

DISSEMINATION OF RESEARCH FINDINGS

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

Collaborative Arrangements

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

This study involves the participation of investigators and collaborators from several laboratories in the United States and Bangladesh, and reflects strengthening of scientific collaboration among laboratories of the developed and developing countries. In the ICDDR,B team, Dr. Shah M. Faruque, a molecular biologist is the principal investigator, whereas Dr. M. Yunus, and Dr. A. K. Siddique are epidemiologists with a background in clinical management of cholera. Dr. Yunus will provide information and *V. cholerae* strains isolated from patient admitted in the Matlab Hospital. Dr. Siddique will provide strains for comparison, collected under the epidemic control programs of ICDDR,B. Mr. Kazi Shafi Ahmad will be involved in the isolation and identification of strains from environmental samples.

Professor John Mekalanos, of the Department of Microbiology and Molecular Genetics at the Harvard Medical School is the collaborator for the molecular genetic aspects of the proposed study. There is already existing collaborative linkages between the Harvard Medical School and ICDDR,B. The school will also provide improved technologies and genetically manipulated strains to ICDDR,B to further build the capabilities of the ICDDR,B laboratories.

Dr. G. B. Nair of the Department of Microbiology of the National Institute of Cholera and Enteric Diseases, Calcutta, will collaborate in providing reference *V. cholerae* strains, and participate in the characterization of environmental strains. Under this protocol, scientists from the collaborating institutions will share reagents and strains, exchange ideas, and visit each others laboratories to further build the technical capabilities and materialize the common research agenda described in this project.

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

Biography of the Principal Investigator

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

Name	Position	Date of Birth
Dr. Shah M. Faruque	Scientist ICDDR,B,; Dhaka, Bangladesh	November 11, 1956

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

Institution and Location	Degree	Year	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	Ph.D.	1988	Molecular Biology

Research and Professional Experience

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

1981-1984	Research Fellow, Department of Biochemistry, University of Dhaka
1984-1985 :	Lecturer, Department of Biochemistry, University of Dhaka.
1988-1989 :	Assistant Professor, Department of Biochemistry, University of Dhaka.
1989-1993:	Assistant Scientist, International Center for Diarrhoeal Disease Research.
1993-1996:	Associate Scientist, International Center for Diarrhoeal Disease Research.
1997-	Scientist & Head, Molecular Genetics Laboratory, International Center for Diarrhoeal Disease Research.

Bibliography (Selected publications)

1. Faruque, S. M., M. J. Albert, and J. J. Mekalanos. 1998. Epidemiology, genetics and Ecology of Toxigenic *Vibrio cholerae* (Review). Microbiol. Mol. Biol. Rev. 62:1301-1314
2. 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 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. 66: 5819-5825

3. 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.

4. Faruque SM, Rahman, MM, Asadulghani, Islam KMN, and Mekalanos JJ. Lysogenic conversion of environmental *V. mimicus* strains by CTXΦ. Infect. Immun. 1999; (in press).

5. Faruque, S.M., A. K. Siddique, M. N. Saha, Asadulghani, M. M. Rahman, K. Zaman, M. J. Albert, D. A. Sack, and R. B. Sack. 1999. Molecular characterization of a new ribotype of *Vibrio cholerae* O139 Bengal associated with an outbreak of cholera in Bangladesh. J. Clin. Microbiol. 37: 1313-1318.

6. 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.

7. 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.

8. 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.

9. 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.

10. 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.

11. 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.

12. 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.

13. 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.

14. 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.

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

16. Faruque SM, Haider K, Rahman MM, Alim ARMA, Baqui AH, Ahmad QS, Hossaim KMB, Albert MJ. Evaluation of a DNA probe to identify enteroaggregative *Escherichia coli* from children with diarrhoea in Bangladesh. J Diar Dis Res 1992; 10:31-34.

17. Faruque SM, Skidmore CJ. Transcription unit of the mouse α_1-casein gene. Biochem Soc Trans 1988; 16:1064.

18. Faruque SM, Antoniou M, Skidmore CJ. The fine structure of the mouse α_1-casein gene. Biochem Soc Trans 1987; 16:624.

19. Faruque SM, Kabir Y, Bashar SAMK. Effect of vitamin A supplementation on the serum levels of some immunoglobulins in vitamin A deficient children. Peadiat Indonesiana 1984; 24:249-253.

20. Faruque SM, Sharmeen L, Bashar SAMK. Study of some immune responses in postnatally malnourished rats. J Pak Med Assoc 1984; 34:270-271.

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

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

23. Albert MJ, Faruque SM, Faruque ASG, Neogi PKB, Ansaruzzaman M, Bhuyan NA, Alam K, Akbar MS. A controlled study of Escherichia coli diarrheal infections in Bangladeshi children. 1995; J. Clin. Microbiol. 33:973-977.

24. Alim ARMA, Faruque SM, Ahmad QS, Hossain KMB, Mahalanabis D, Albert MJ. Evaluation of a non-radioactive chemiluminescent method for using oligonucleotide and polynucleotide probes to identify enterotoxigenic *Escherichia coli*. J Diarrhoeal Dis Res 1994; 12:113-116.

25. Haider K, Faruque SM, Albert MJ, Nahar S, Neogi PKB, Hossain A. Comparison of a modified adherence assay with existing assay methods for identification of enteroaggregative *Escherichia cli*. J Clin Microbiol 1992; 30:1614-1616.

26. Haider K, Faruque SM, Nahar S, Alam AN, Albert MJ, Tzipori S. Enteroaggregative *Escherichia coli* infections in Bangladeshi diarrhoeal children: clinical and microbiological features. J Diarrhoeal Dis Res 1991; 9:318-322.

27. Albert MJ, Faruque SM, Ansaruzzaman M, Islam MM, Haider K, Alam K, Kabir I, Robins-Browne R. Sharing of virulence associated properties at the phenotypic and genetic levels between enteropathogenic *Escherichia coli* and *Hafnia alvei*. J Med Microbiol 1992; 37:310-314.

28. Strockbine NA, Faruque SM, Kay BA, Haider K, Alam AN, Tzipori S and Wachsmuth K. DNA probe analysis of diarrhoeagenic *Escherichia coli*: detection of EAF-positive isolates of traditional enteropathogenic *Escherichia coli* serotypes among Bangladeshi paediatric diarrheal patients. Mol Cell Prob 1992; 6:93-99.

29. Qadri F, Haque A, Faruque SM, Bettelheim KA, Robins-Browne R, Albert MJ. Hemagglutination properties of Enteroaggregative *Escherichia coli*. J Clin Microbiol 1994; 32:510-514.

30. Albert MJ, Ansaruzzaman M, Faruque SM, Neogi PKB, Haider K, Tzipori S. An enzyme-linked immunosorbent assay (ELISA) for the detection of localized adherent enteropathogenic *Escherichia coli* (EPEC). J Infect Dis 1991; 164:986-989.

31. Albert MJ, Siddique AK, Islam MS, Faruque ASG, Ansaruzzaman M, Faruque SM, Sack RB. Large outbreak of clinical cholera due to *Vibrio cholerae* non-01 in Bangladesh. Lancet 1993; 341:704.

32. Albert MJ, Ansaruzzaman M, Bardhan PK, Faruque ASG, Faruque SM, Islam MS, Mahalanabis D, Sack RB, Salam MA, Siddique AK, Yunus M, Zaman K. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. Lancet 1993; 342:387-390.

33. Qadri F, Azim T, Hossain A, Islam D, Mondal G, Faruque SM, Albert MJ. A monoclonal antibody to *Shigella dysenteriae* serotype 13 cross-reacting with Shiga toxin. FEMS Microbiol Lett 1993; 107:343-348.

34. Albert MJ, Ansaruzzaman M, Qadri F, Hossain A, Kibriya AKMG, Haider K, Nahar S, Faruque SM, Alam AN. Characterization of *Plesiomonas shigelloides* strains that share type-specific antigen with *Shigella flexneri* 6 and common group 1 antigen with *Shigella flexneri* spp. and *Shigella dysenteriae* 1. J Med Microbiol 1993; 39: 211-217.

35. Albert MJ, Ansaruzzaman M, Faruque SM, Haider K, Qadri F, Islam MM, Kibriya AKMG, Tzipori S. An out break of keratoconjunctivitis due to *Salmonella weltevreden* in a guinea pig colony. J Clin Microbiol 1991; 29:2002-2006.

36. Mahalanabis D, Faruque ASG, Hoque SS, Faruque SM. Hypotonic oral rehydration solution in acute diarrhoea: a controlled clinical trial. Acta Paediatr 1995; 84:289-293.

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

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

39. Albert MJ, Alam K, Ansaruzzaman M, Montanaro J, Islam M, Faruque SM, Haider K, Bettelheim KA, Tzipori S. Localized adherence and attaching-effacing properties of non-enteropathogenic serotypes of *Escherichia coli*. Infect Immun 1991; 59:1864-1868.

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

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

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

Detailed Budget for New Proposal

Project Title: Characterization of environmental and clinical strains of toxigenic and nontoxigenic *Vibrio cholerae* as an aid to predict the emergence of new epidemic strains.

Name of PI: DR. SHAH M. FARUQUE

Protocol Number: Name of Division: Laboratory Sciences Division

Funding Source: USAID/T Amount to be Funded (direct): US $ 99,828
Overhead (%): 25%

Starting Date: July, 1999 Closing Date: 2 years from starting date

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

SL. NO	ACCOUNT DESCRIPTION	SALARY SUPPORT			US $ AMOUNT REQUESTED		
	Personnel	Position	Effort%	Salary	1st Yr	2nd Yr	TOTAL
	Dr. Shah M. Faruque	Scientist	50%	19,500	9,750	9,750	19,500
	Qazi Shafi Ahmad	Senior Scientific Off	20%	14,100	2,820	2,820	5,640
	Dr. M. Yunus	Scientist	10%	19,800	1,980	1,980	3,960
	To be named	Research Officer	100%	3,000	3,000	3,000	6,000
	M. H. Tarik	Laboratory Attend	20%	2,950	590	590	1,180
	Sub Total				18,140	18,140	36,280
							TOTAL
	Local Travel				1,500	200	1,700
	International Travel	Travel to calcutta, and Boston				5,000	5,000
	Sub Total				1,500	5,200	6,700
	Supplies and Materials (Description of Items)						
							TOTAL
	Molecular biology grade Reagents, enzymes etc.				9,000	9,000	18,000
	Culture media. Plastics, and supplies				6,000	6,000	12,000
	Office supplies				1,000	1,000	2,000
	Sub Total				16,000	16,000	32,000

	OTHER CONTRACTUAL SERVICES	1ST YR	2ND YR	TOTAL
	Repair and Maintenance	500	500	1,000
	Rent, Communications, Utilities	500	500	1,000
	Training Workshop, Seminars			
	Printing and Publication	1,000	1,000	2,000
	Sub Total	2,000	2,000	4,000

	INTERDEPARTMENTAL SERVICES	1ST YR	2ND YR	TOTAL
	Computer Charges	500	500	1,000
	Microbiological tests			
	Research Animals	1,000	1,000	2,400
	Transport			
	Xerox, Mimeographs etc.	200	200	400
	Sub Total	1,700	2,100	3,800
	Other Operating Costs			
	Capital Expenditure: Refrigerators, computer accessories, etc.	4,000		4,000
	TOTAL OPERATIONAL COST	43,340	43,440	86,780

TOTAL DIRECT COST: US$ 86,780

OVERHEAD COST (25%):

Budget Justifications

1. The recruitment of a research officer is essential for conducting the environmental sampling and for participation in the laboratory based characterization of strains. Samples from patients, and clinical and epidemiological information will be collected by scientists who are partly supported by other ongoing projects, and hence only part of their salaries have been budgeted under this project.

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

3. Budget for supplies have been kept at a realistic level considering the expensive reagents and enzymes used for molecular analysis of strains. Capital expenditure for procurement of refrigerators will be required for storing of large number of samples. Computer accessories will be required for maintenance and analysis of data.

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

6/9/99

Controller, Budget and Costing
ICDDR,B

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

Other Support

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

1996-2001 Title of project: Epidemiology and ecology of *Vibrio cholerae* infection in Bangladesh.
Donor: Unnited States National Institutes of Health (NIH).
Role in the project: Co-PI ; Budget: US$ 350,000 over 5 years

1997-1999 Title of project: Development and application of multiplex diagnostic PCR assays as an aid to clinical and environmental studies.
Donor: USAID/T
Role in the project: PI Budget: US$ 136,33 over 2 years

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

Check List

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

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

Reviewer # 1

ICDDR(B) Research proposal: Characterisation of environmental non toxigenic V. cholerae

Review by referee: Dr Paul Shears, Liverpool UK

The proposed research study relates to an important area of public health and its scientific understanding. The principal investigator is already acknowledged, through his research record and publications, to be at the forefront of scientific work on the molecular epidemiology of V. cholerae. I have had the privilege of visiting the Molecular Genetics Laboratory at ICDDRB, and, at an earlier time, the Matlab Field Station, and would recommend that the research team have the ability and facilities to carry out the proposed research.

The project is intended to develop, through an epidemiological and molecular study, the initial laboratory studies of the P.1. on the isolation and role of the cholera toxin bacteriophage, and its ability to infect non-toxigenic v. cholerae strains.

Previous studies on the ecology of V. cholerae in inter-epidemic periods have focused on the occurrence and viability of v. cholerae in aquatic habitats. This study is original in that it goes beyond the "whole bacteria" approach to investigate the role of the cholera toxin bacteriophage in the environment in the development of pathogenic strains, and subsequent epidemics. Given the past experience of the investigators, and the laboratory facilities at ICDDR(B), the intended study is feasible and should be effectively completed.

The public health and economic impact of cholera outbreaks is immense. Most cholera epidemics are characterised by high initial morbidity and some (or major, as in Zaire 1994) mortality, with mortality and morbidity being reduced once control and effective case management are implemented. The present study has the potential to contribute to the understanding of the inter-epidemic period and the "triggers" that initiate epidemics. Such findings may help in outbreak and epidemic prediction, ultimately contributing to reduce cholera morbidity and mortality.

The proposed budget and financial support requested are appropriate for the planned study.

As an external referee, I find the study worthy of support.

The following minor points may be considered:

1. Specific aims, 5, states: "To perform comparative molecular analysis of the strains with a collection of epidemic strains isolated in different countries....." There does not appear to be in "Methods", any mention of isolates from other countries.

2. Specific Aims, 6, states "To examine the concentration of toxigenic as well as CTX-susceptible ....." It is not explained clearly in the "data Analysis" section what is meant by the "concentration of toxigenic strains in the environment" and how the association between this and the onset of cholera outbreaks will be measured.

3. In Methods, the study area states Matlab, with no mention of Dhaka. In "Facilities Available" it states "clinical samples.... and environmental samples... will be collected from Matlab and Dhaka. The emphasis in methods and data analysis implies investigating associations between the environment and the well defined population in the Matlab area. It is not clear how such associations could be made in Dhaka, as presumably patients in Dhaka will cover different geographical areas. There would seem to be good reasons to restrict the environment/patient study to Matlab.

Reviewer #1

Title:

Summary of Referee's Opinion: 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	Y		
Adequacy of Project Design	Y		
Suitability of Methodology	Y		
Feasibility within time period	Y		
Appropriateness of budget	Y		
Potential value of field of knowledge	Y		

CONCLUSIONS

I support the application:

a) Without qualification Y ☐

b) With qualification ☐

- on technical groups ☐

- on level of financial support ☐

I do not support the application ☐

Name of Referee: Dr Paul Shears

Signature _____ Date 3 Sep 1999

Position:
Consultant Microbiologist/Director
Centre For Tropical Medical Microbiology
Liverpool School of Tropical Medcine UK

Institution:

Reviewer # 1

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)

See attached document
Title:

PI:

Reviewer: _____

Reviewer #2

Date: Thu, 26 Aug 1999 09:13:08 +0700
From: PETER ECHEVERRIA <pechev@mozart.inet.co.th>
To: mathan@icddrb.org
Subject: test and review

Dear Dr Mathan; lETS see if we conect. I give Dr Faraque's proposal scores of high scores in Quality of Project, Adequacy of Project Design, Suitability of Methodology, medium in feasibility within time period and high in budget and field of knowledge. conclusions: I support the application without qualificatioins. I sign it as a enteric consultant of enteric disease(formerely Dept of Bacti and molecular Genetics, Afrims, Bangkok, Thailand. Peter Echeverria 10/25/1999

Reviewer #2

Title:

Summary of Referee's Opinion: 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	X		
Adequacy of Project Design	X		
Suitability of Methodology	X		
Feasibility within time period		X	
Appropriateness of budget	X		
Potential value of field of knowledge	X		

CONCLUSIONS

I support the application:

a) Without qualifications X

b) With qualification ☐

- on technical groups ☐

- on level of financial support ☐

I do not support the application ☐

Name of Referee:

Signature Peter Echeverria Date 10/25/1999

Position: consultant in enteric disease at Afrims

Institution:Afrims

Reviewer #2

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: Characterization of environmental and clinical strains of toxigenic and non-toxigenic V. cholerae as a s an aid to predict the emergence of new epidemic strains.....

PI: Dr SM Faraque

Reviewer : Peter Echeverria MD

INVESTIGATORS' RESPONSE TO REVIEWERS' COMMENTS

Name of the Proposal: Characterization of environmental and clinical strains of toxigenic and nontoxigenic *Vibrio cholerae* as an aid to predict the emergence of new epidemic strains.

Name of Principal Investigator: Dr. Shah M. Faruque

General response: The reviewers have generally agreed with the objective, appropriateness and design of the project, and appear to be supportive of the study. One of the reviewers has provided some minor but constructive suggestions to further clarify some of the technical aspects. We have carefully considered the comments and suggestions made by the reviewers, and have incorporated appropriate modifications in the relevant sections of the proposal. Our specific response to the reviewers' comments are as follows:

Specific response:

Reviewer # 1:

1. As suggested by the reviewer, we have now clarified the use of epidemic strains isolated in other countries for comparison in the method section. We already have a collection of strains from different countries (pages 15 and 17).
2. We have made corrections in specific aim 6. We have also briefly described how concentration of *V. cholerae* in the environmental samples will be determined both in the methods and data analysis sections.
3. The study will be restricted in Matlab, as suggested by the reviewer. However, we will obtain epidemic strains for genetic comparison from other areas of Bangladesh through the Epidemic Control Preparedness program (ECPP).

Reviewer # 2:

The second reviewer did not make any adverse comments, and hence no response seems to be necessary.