Date 29.12.92

achment 1.
ACE SHEET) ETHICAL REVIEW COMMITTEE, ICDDR,B.

incipal Investigator DR. A.K. SIDDIQUE Trainee Investigator (if any) DR. MD. SIRAJUL ISLAM

plication No. 93-001 Supporting Agency (if Non-ICDDR,B) _____

tle of Study: Role of various aquatic flora, fauna and physico-chemical conditions of water in maintaining endemicity and season ~~ality~~ of cholera in Bangladesh

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

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

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

Does the study involve:
(a) Physical risks to the subjects Yes No NA
(b) Social Risks Yes No NA
(c) Psychological risks to subjects Yes No NA
(d) Discomfort to subjects Yes No NA
(e) Invasion of privacy Yes No NA
(f) Disclosure of information damaging to subject or others Yes No NA

Does the study involve:
(a) Use of records, (hospital, medical, death, birth or other) (Yes) No
(b) Use of fetal tissue or abortus Yes No NA
(c) Use of organs or body fluids Yes No NA

Are subjects clearly informed about:
(a) Nature and purposes of study Yes No NA
(b) Procedures to be followed including alternatives used Yes No NA
(c) Physical risks Yes No NA
(d) Sensitive questions Yes No NA
(e) Benefits to be derived Yes No
(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 Yes No NA
(b) From parent or guardian (if subjects are minors) Yes No NA
6. Will precautions be taken to protect anonymity of subjects Yes No NA
7. Check documents being submitted herewith to Committee:
___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
___ Protocol (Required)
___ Abstract Summary (Required)
___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
___ Informed consent form for subjects
___ Informed consent form for parent or guardian
___ Procedure for maintaining confidentiality
___ Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:
1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

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

M. S. Islam
Principal Investigator

Trainee

93-001
1.1.93

APPLICATION FOR PROJECT GRANT

1.	PRINCIPAL INVESTIGATORS	:	Dr. Md. Sirajul Islam Dr. A.K. Siddique
	COINVESTIGATORS	:	Dr. M.J. Albert Dr. A. Huq Mr. A. Felsenstein
	ADVISORS	: :	Professor R.B. Sack Professor R.R. Colwell
2.	TITLE OF PROJECT	:	Role of various aquatic flora, fauna and physicochemical conditions of water in maintaining endemicity and seasonality of cholera in Bangladesh
3.	STARTING DATE	:	When fund is available
4.	COMPLETION DATE	:	Three years after starting date
5.	TOTAL BUDGET REQUIRED	:	US$ 167,465
6.	FUNDING SOURCE	:	
7.	HEAD OF PROGRAMME	:	Prof. R. Bradley Sack

R. Bradley Sack

8. AIMS OF PROJECT

a) General aim

To investigate the role of various aquatic flora, fauna and physicochemical factors of water in maintaining endemicity and seasonality of cholera in Bangladesh. This will be done simultaneously with a clinical cholera surveillance system.

b) Specific aims

1) To find out the role of various aquatic flora, both macrophytes and phytoplanktons, in maintaining endemicity and seasonality of cholera in Bangladesh.

2) To find out the role of various aquatic fauna, e.g. oysters, crabs, shrimps and zooplankton in the maintainance of seasonality and endemicity of cholera.

3) To study the role of various physicochemical conditions of water (e.g. pH, temperature, DO_2, DCO_2, salinity, Na, K, etc.). which may provide favourable conditions for multiplication of vibrios in the aquatic environment triggering cholera epidemics.

c) **Hypothesis**

From the available data we hypothesize that during cholera season certain physicochemical factors of water, e.g. temperature, pH, salinity, DO_2, etc. become favourable for algal bloom formation in the aquatic environment. This algal bloom is followed by copepod bloom. These algae copepod bloom ultimately becomes responsible for causing vibrio bloom in the water. Widespread use of this contaminated water triggers epidemic.

d) **Significance**

This study will demonstrate the role of various aquatic flora and fauna as reservoirs of cholera. It will also demonstrate the role of different biophysicochemical conditions in triggering the multiplication of *V. cholerae* 01 in the aquatic environment during cholera seasons. This study will show the microhabitat of *V. cholerae* in the aquatic environment. This will not only have relevance to the maintenance of endemicity and seasonality of cholera in Bangladesh.but will also have significant implications

2) To find out the role of various aquatic fauna, e.g. oysters, crabs, shrimps and zooplankton in the maintainance of seasonality and endemicity of cholera.

3) To study the role of various physicochemical conditions of water (e.g. pH, temperature, DO_2, DCO_2, salinity, Na, K, etc.). which may provide favourable conditions for multiplication of vibrios in the aquatic environment triggering cholera epidemics.

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for our understanding of the transmission of the disease and may influence future cholera control programmes.

If the likelihood of cholera epidemic can be predicted by determining the characteristics of water, early warning of an impending epidemic could lead to better preparations.

The information gained about the microhabitats in the aquatic environment supporting *V. cholerae* 01 growth may also give us some idea of the feasibility of control strategies aimed at reduction of these microhabitats.

This study will also provide an important contribution to the overall epidemiological understanding of the disease.

9. ETHICAL IMPLICATION

There is no ethical implication in this protocol.

10. BACKGROUND, RESEARCH PLAN AND BIBLIOGRAPHY

a) Background

Introduction

Cholera is known as a killer disease from time immemorial. In endemic areas of Bangladesh, cholera epidemics occur twice a year with a regular seasonal pattern reaching their peaks during November, December and January with a second peak in April and May (Merson *et al.*, 1980; Glass *et al.*, 1982). During epidemics, *Vibrio cholerae* 01 is isolated from patients as well as from surface water but disappears from the environment during inter-epidemic seasons (Khan *et al.*, 1984). The reservoirs or sites of survival and

multiplication of *V. cholerae* 01 during inter epidemic periods are not known. There are no satisfactory explanations of how the seasonality and endemicity of cholera are maintained.

Suggested mechanisms

Miller *et al.* (1984) suggested four mechanisms. (a) Carrier status in animals, (b) Carrier status in humans, (c) continuous transmission in humans and (d) an environmental reservoir. The evidence available suggests that the first three mechanisms are less likely.

Carrier status in animals: No animals have so far been demonstrated as reservoirs of cholera. Sack (1973) investigated canines as reservoirs of cholera in Calcutta, India. Over 500 dogs were examined bacteriologically but no evidence of *V. cholerae* 01 reservoir was found although 14% of dogs harboured non-cholera vibrios in their intestines. These results indicate that non-human reservoirs exist for non-cholera vibrios in contrast to *V. cholerae* 01. However, toxigenic *V. cholerae* 01 have been isolated from domestic animals only in the locality of current cholera cases in man (Sanyal *et al.*, 1974). It is likely that these animals are probably picking up the vibrios from the environment at the time when cholera infections are occurring locally and can not act as reservoirs.

Carrier status in man: The evidence for chronic carriage in man is also weak. Only one chronic carrier of cholera has been recognized (Azurin *et al.*, 1967). Some convalescent cholera patients excrete cholera vibrios for a few months after infection, though these strains are normally rough (Pierce *et al.*, 1970) and are non-pathogenic.

Continuous transmission in man: The model of continuous transmission in man has gained some support because the organism frequently causes sub-clinical infections and continuous transmission could remain undetected. However, continuous transmission of the organism from man to man is unlikely to be a successful strategy because of the comparatively high infectious dose of the vibrio (Hornick *et al.*, 1971), the sensitivity of the vibrios to dehydration (Koch, 1884), the adaptation of the organism to waters of high salinity, its limited survival in potable water (Miller *et al.*, 1982), and the sensitivity of the organism to acidic conditions (Pollitzer, 1959).

Environmental reservoirs: An environmental reservoir for *V. cholerae* 01 was suggested by early bacteriologists (Koch, 1884) but was subsequently rejected, mainly because most survival studies have shown that the organisms have only a limited potential for environmental survival (Felsenfeld, 1974; Pollitzer, 1959). This kind of conclusion based on survival studies of *V. cholerae* 01 in water (excluding its different kinds of plants and animals) in the laboratory is of tenuous.

The first case in the USA during the 7th pandemic was detected in 1973 from the stool of a 51 year old man in Texas, USA. *V. cholerae* El Tor Inaba was isolated. This is the first reported case in USA since 1911 (Weissman *et al.*, 1974). After 5 years of this report in 1978, another case of a 44 year old man was detected in Lousiana. Afterwards, 10 more cases were detected in four additional clusters (Blake *et al.*, 1980).

An attempt was made to isolate *V. cholerae* from environmental samples. *V. cholerae* 01 was isolated from a boiled crab, from a shrimp caught in a canal near Pecan Island and from two Moore swabs from two canals. All isolates were *V. cholerae* 01, biotype El Tor, serotype Inaba.

The phage sensitivity of 38 Lousiana isolates and 1 Texas isolate was the same. This phage sensitivity was unique for these Texas and Lousiana strains in comparison with 1085 strains which were isolated all over the world during 1964 to 1979.

It has been hypothesized that *V. cholerae* had been able to survive along the Gulf Coast for many years. The strongest evidence is the fact that the isolates from Texas and all the isolates from Lousiana five years later were of a single phage type limited to the United States. Moreover the strong haemolytic properties of these isolates were also different from all other El Tor strains isolated worldwide in the past 15 years.

Finally it has been suggested that toxigenic *V. cholerae* 01 can survive and multiply in the environment and persist indefinitely without human faecal contamination. Field studies in Bangladesh have demonstrated that *V. cholerae* 01 remained attached to various components of aquatic environments (Tamplin *et al.*, 1990; Huq *et al.*, 1990; Amako *et al.*, 1992; Islam *et al.*, 1992).

Aquatic fauna and flora as reservoirs

Adhesion to surfaces: The availability of nutrients must be one of the most significant factors limiting the distribution of *V. cholerae*. This alone suggests that the organism will be found absorbed to surfaces where the concentration of nutrients will be higher (Heukelekian and Heller, 1940). In addition to this, some studies have suggested that two likely sources of nutrients for this organism are chitin (Kaper, 1979) and aquatic plants (Gessner, 1955).

In the late 1970s, Islam and Aziz isolated *Vibrio cholerae* from hydrophytic plants (Islam and Aziz 1978, 1981). Spira *et al.* (1981) observed the uptake of *V. cholerae*, biotype El Tor by water hyacinths.

Recently, it was observed that toxigenic *V. cholerae* 01 can survive longer with a duckweed *Lemna minor* and an alga *Rhizoclonium fontanum* than the water on which these plants were floating and in control water without plants (Islam *et al.*, 1989, 1990c). These findings suggested that plants may serve as an effective environmental reservoir for *V. cholerae* 01 either through a non-specific association or by interaction with *V. cholerae* in commensal relationship.

Most recently a laboratory-based study was carried out to investigate the role of an aquatic blue green alga *Anabaena variabilis* as a possible reservoir of toxigenic *V. cholerae* 01 (Islam *et al.*, 1990d). It was observed that *V. cholerae* survived longer in water with *Anabaena* and in control water (without *Anabaena*) than the *Anabaena* itself. This was a puzzling observation because in all previous studies, *V. cholerae* always survived longer on plant surfaces than water on which the plants were floating (Islam *et al.*, 1988, 1989, 1990c). However, *V. cholerae* secrets an enzyme, mucinase (Schneider and Parker, 1982) and with the help of this enzyme *V. cholerae* may enter the mucilaginous sheath of this alga. The infected *Anabaena variabilis* of this alga was observed under phase contrast microscope and it was found that *V. cholerae* had entered the mucilaginous sheath and were dividing and clustering around detached heterocytes of this alga (Islam *et al.* 1990d) but became non-culturable.

Then attempts were made to detect *V. cholerae* by an indirect fluorescent antibody technique because this technique has been used to detect *V. cholerae*

from both clinical and environmental samples. Sack and Barua (1964) detected *V. cholerae* 01 from the rice water stool of cholera patients using this fluorescent antibody technique in early 1960s. Recently Colwell *et al.* (1985) also used the same technique to detect *V. cholerae* 01 from laboratory microcosms. By using this technique, *V. cholerae* 01 were detected inside the mucilaginous sheath of *A. variabilis* for 15 months. This is the first time it has been shown that *V. cholerae* 01 can persist such a long time inside a blue green alga. This finding demonstrated that blue green algae can act as a reservoir of cholera and provided a possible explanation for maintenance of the endemicity and seasonality of cholera in Bangladesh (Islam *et al.*, 1990d). However one major finding was that *V. cholerae* became non culturable 120 h after of inoculation into the experimental system (Islam, 1990d). Tamplin *et al.* (1990) studied the interactions between *V. cholerae* 01 and natural plankton populations in a cholera endemic area of Bangladesh. By using epifluorescent microscopes, they found that *V. cholerae* 01 could attach to phyto and zooplanktons.

The role of aquatic fauna such as oysters, crabs, prawns and zooplankton (Twedt *et al.*, 1981; Hood *et al.*, 1981, Huq *et al.*, 1983) has been much discussed.

Role of physicochemical factors on survival of V. cholerae

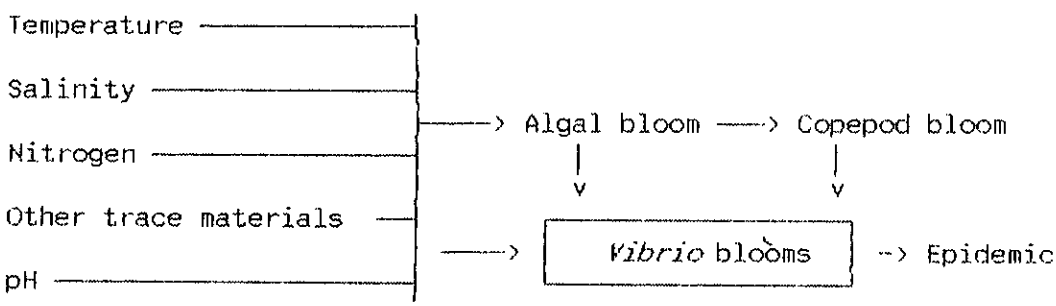
The physiological state of *V. cholerae* can be affected by various environmental conditions (e.g., sunlight, pH, temperature, salinity, competition with other bacteria for nutrients, etc.) during survival in the aquatic environment (Feachem *et al.*, 1981; Miller *et al.*, 1984; Siddique *et al.*, 1991; Singleton *et al.*, 1982). Recently it has been observed that the

production of toxin by the toxigenic *V. cholerae* 01 also depends on various physicochemical conditions in the microcosms (Islam, 1990a, 1990b, 1991). The conversion of *V. cholerae* from non-culturable to culturable forms may depend on various physicochemical factors in the aquatic environment. Recent studies have shown that *V. cholerae* cannot persist in water having pH below 7.0 and salinity of 0.005%. (Islam *et al.*, 1992). Therefore, water having acidic pH and lower salinity will not be a good habitat for longer survival of *V. cholerae*. Colwell *et al.* (1985) carried out a study on viable but non-culturable *V. cholerae* and related pathogens in the environment. They observed that over time *V. cholerae* enter a viable but non-culturable state in a series of microcosm studies. Direct viable counts by epifluorescent microscopy are consistently higher than corresponding plate counts. Furthermore, animal passage revealed that viability and pathogenicity persisted for such "non-culturable" cells. Therefore, indirect immunofluorescent microscopy offers a more sensitive detection system of *V. cholerae* from the environmental samples.

Oppenheimer *et al.* (1978) carried out a limnological study of three ponds in Dhaka, Bangladesh, for one year, from March 1975 to April 1976. They collected water samples once a month and determined physical and chemical parameters, including temperature, pH, turbidity, chloride, hardness, dissolved oxygen, carbon dioxide, phytoplankton, zooplankton, etc. They observed that the changes of pH of the surface water are due to algal photosynthesis. When the algae form a bloom, their high rates of photosynthesis take up the free CO_2 for carbohydrate synthesis and thus reduce the free carbon dioxide content, while the production of oxygen increased and the pH become more basic due to the reduction of carbonic acid in the surface

water. The algal bloom formation was mainly due to blue green algae. They also observed that the reasons of high pH coincided with the cholera seasons in the region.

All the previous literature reviews indicated that certain physicochemical factors may be responsible for producing algae/copepod blooms during cholera seasons in the environment. These algae/copepod bloom may ultimately be responsible for the formation of *Vibrio* blooms in the water. However, how this brings about *Vibrio* bloom remains unclear at the moment widespread use of heavily contaminated water may result in epidemics. From the foregoing data, the following model can be derived:



Using the above framework, we will investigate the interrelationship among aquatic fauna, flora various physiochemical parameters of surface water, the prevalence of culturable and non-culturable forms of *V. cholerae* 01 and the seasons.

Islam (1987) observed that stressed *V. cholerae* 01 could be recovered in non-selective media (trypticase soy agar) better than selective media (thiosulfate citrate bile-salt-sucrose agar) from artificial aquatic ecosystems. These results throw doubt on the efficacy of the alkaline peptone water and TCBS agar isolation technique which was originally developed for clinical specimens

but is currently employed for environmental samples. Moreover, as the number of *V. cholerae* non-01 and other vibrios are more than *V. cholerae* 01 in the environmental samples, frequently the non-01 overgrow the 01 serotypes which could then not be recovered following presently available cultural techniques. Therefore, the isolation of lower numbers of *V. cholerae* 01 from among other vibrios and the growth of stressed bacteria from the environmental samples pose problems. Therefore, attempts will be made to detect the stressed cells as well as the lower number of *V. cholerae* using the recently-developed very sensitive polymerase chain reaction technique (Shirai *et al.*, 1991).

b) Research plan

The search for *V. cholerae*, though involving water sampling, will be based also on an examination of various aquatic flora and fauna. Sediments from various aquatic ecosystems will also be examined. The sites investigated will be chosen on the basis that the composition of the water at a specific site falls within the range that allows moderate survival of *V. cholerae*. The likely survival habitat will be determined in light of information obtained from the previous studies (Islam *et al.*, 1988, 1989, 1990c, 1990d; Huq *et al.*, 1983).

The sites for collection of environmental samples will be the same areas where a sentinel surveillance system for control of epidemics of cholera will be ongoing. The objective of the sentinel surveillance system is to implement a diarrhoeal surveillance system using selected sentinel areas in Bangladesh which will serve as early warning posts for impending epidemic outbreaks of diarrhoea particularly of cholera. The objectives of this protocol are: (a) the early recognition and documentation of diarrhoeal outbreaks, (b) the

detection of principal aetiological agents of diarrhoea outbreaks and any change of their characteristics, particularly antibiotic sensitivity. Five sentinel surveillance areas have been selected on the basis of:

a) representative geographical locations in the country,

b) risk of being affected by epidemic outbreaks as determined by historical information,

c) access both during the monsoon and dry months, and

d) the context of the administrative structure of the country.

The proposed surveillance areas are:

a) Gangachara (Rangpur)

b) Bhuapur (Tangail)

c) Sarail (Brahmanbaria)

d) Bakerganj (Barisal)

e) Morrelganj (Bagerhat)

Every month, the sentinel surveillance team will go and collect stool/RS samples from the human population, at the same time, the environmental microbiolgists will also go and collect the environmental samples.

Environmental sampling

Samples will be collected from the five sentinel diarrhoeal surveillance areas in the east, west, north and southern parts of Bangladesh. The water sampling from these sites will be determined on the basis of different physicochemical parameters of water e.g. temperature, pH, DO_2, suspended solids, salinity, and TCO_2.

Number of samples. Samples will be collected from 2 ponds and 2 river sites at each location. Samples will include oysters in addition to the plants, phytoplanktons, zooplanktons, soil, water and algae already mentioned. A total of 1680 samples (4 sites x 7 components x 5 districts x 12) per year will be collected.

Sampling procedure

Surface water (5 cm below the surface) will be collected in presterilized 1000 ml plastic bottles. Plant samples will be collected by hand using sterile plastic gloves and kept in sterile plastic bags. Sediment samples will be collected with the help of a core sampler and kept in 4 oz glass bottles. All the collected samples will be transported to the laboratory, inside an insulated foam box with ice bags so that the temperature can be maintained at approximately 4^{o}C. Samples will be processed within 4 hr after collection.

Plankton will be sampled by pulling a NO, 20 (77 µm mesh) plankton net through the water. A portion of each sample will be preserved in formaldehyde for identification.

Sampling time. Samples will be collected once in a month from each sampling site.

Treatment of samples. Plant samples will be blended for at least two minutes with an equal volume of phosphate buffered saline (PBS) to give a homogeneous mixture. Sediment samples will be shaken vigorously for 3 minutes before aliquots are taken for analysis. Plankton samples will be homogenized in a

Teflon-tipped tissue grinder (Wheaton Scientific, Millville, NJ) using a stedfast stirrer (Model 300, Fisher Scientific, USA).

A portion of the blended plants or homogenized plankton materials will be inoculated in enrichment media (alkaline bile peptone) and incubated for 6 hr, and subsequent plating will be done onto TCBS agar and TTGA medium. One gram of sediment will be taken and processed in the same way. One thousand ml of water will be filtered through a 0.45 µm membrane filter, and then the filter paper will be immersed in bile peptone, incubated for 6 hr at 37°C (in a shaker incubator) and subsequently plated onto TCBS agar and TTGA medium. Direct plating will also be done taking 0.1 ml from the blended or shaken samples onto TCBS and TTGA media.

Bacterial counts for vibrios

Water. One hundred ml of water will be filtered through a 0.45 µm membrane filter paper. Then the filter paper will be placed onto TCBS agar and incubated for 18-24 hr at 37°C. Vibrio-like organisms will be picked and streaked onto gelatin agar medium (Monsur, 1961) and incubated at 37°C to see the production of gelatinase and sensitivity to 0/129 vibriostatic compound; then they will be inoculated onto Kliglers Iron Agar (KIA) and Motility Indole Urea (MIU) agar. Presumptive vibrio isolates will be checked for lysine and ornithine decarboxylase, arginine dihydrolase and for fermentation of mannitol, sucrose, mannose and arabinose (Huq, 1979) and growth in 0, 3, 8 and 10% NaCl. Serology will be done if indicated. Faecal contamination of the water sources will also be determined following standard procedures of determining faecal coliform counts (APHA, 1985).

Colony hybridization. The isolated strains of *V. cholerae* 01 will be tested for toxigenicity by colony blot hybridization following procedures as described by Pal *et al.* (1992). In brief, the isolates will be inoculated on sterile nylon filters layered over nutrient agar plates and will be incubated at 37°C overnight. The membranes containing the freshly grown strains will be placed on the top of a denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 7 min, twice on a neutralization solution (0.5 M Tris-HCl [pH 7.2], 1.5 M NaCl) for 4 min each and finally on 2 x SSC. The membranes will be treated with proteinase K (1 mg/ml) for 30 min at room temperature, air dried and will be baked at 80°C for 2 h. The membranes will be hybridized with peroxidase labelled probe at 42°C overnight. After hybridization, the bound probe will be detected by enhanced chemoluminescence (ECL) gene detection system (Amersham International).

Fluorescent microscopy. All the samples will be tested by fluorescence microscopy following standard procedures (Islam *et al.*, 1990d). In brief, the planktons will be fixed on a slide and then *V. cholerae* 01 monoclonal antibody will be added and the slides will be incubated for 30 min in moist conditions. After washing, FITC anti-mouse globulin goat serum will be added and incubated for 30 min as before. Finally, after washing and drying, the slides will be mounted under a coverslip using buffered glycerol, pH 8.0-9.0 and examined under a fluorescent microscope.

Procedure for detection of *V. cholerae* 01 by PCR

All environmental samples will be centrifuged and the pellet will be used for DNA extraction.

Extraction of DNA. The pellet will be resuspended in 75 µl of 50 mM Tris-HCl buffer, pH 8.0, containing 20% (w/v) sucrose, 50 mM EDTA and 100 µg of lysozyme and the reactants will be incubated at 37°C for 30 min. Then 300 µl of 50 mM NaCl containing 1% (w/v) sodium dodecyl sulphate (SDS) and 800 µg of proteinase K will be added and the incubation continued for an additional 60 min. The extracted DNA will be precipitated by adding absolute ethanol, harvested by centrifugation, and then resuspended in 100-300 µl of 10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA. The extracted DNA will be stored in distilled H_2O at 4°C until needed. DNA will be extracted following the procedures described by Escheverria *et al.* (1992).

Amplication of DNA by PCR. 1-5 µl of the extracted DNA will be amplified by PCR using 10 µg of each of the primers following procedure described by Shirai *et al.* (1991). The sequence of primers are 5'-CTCAGACGGGATTTGTTAGGCACG-3' and 3'-GCATTATCCCCGATGTCTCTATCT-5'. PCR will be performed for 35 cycles of 1 min at 94°C (denaturation), 1.5 min at 60°C (annealing of primers to single stranded DNA) and 1.5 min at 72°C (DNA polymerase mediated primer extension). Then 10 µl of the amplified product will be analyzed by electrophoresis using 1.5% agarose gels. The identify of the amplified product will be further confirmed by a probe as described below.

Detection of PCR-amplified products with the alkalike phosphatase labeled oligonucleotide probe

The PCR-amplified products (as described above) will be anlalyzed by Southern hybridization with the *V. cholerae* CT DNA as a probe. The sequence of the CT probe is 5'-ACTATATTGTCTGGTCATTCTACT-3' (Shirai *et al.*, 1991). Before hybridization, the DNA will be incubated in 0.3 M NaOH for 5 min at 70°C and will be applied to a nylon membrane. Before hybridization the membrane will

be incubated in 5X SSPE, containing 5X Denhart's, 0.5% SDS and 0.1 mg/ml yeast tRNA for 15 min at 42^{o}C. Then 4 ng of the AP probe will be added and hybridization will be allowed to proceed for 15 min. Then the membrane will be washed four times at room temperature. 2X SSC, 05% SDS for 10 min; 1X SSC, 0.1% SDS for 10 min; 1X SSC, 0.5% Triton X-100 for 5 min, 1X SSC for 1 min. Then the membrane will be incubated with a substrate for the AP probe consisting of a mixture of nitro blue tetrazolium (NTB) and 3-bromo-4-chloro-3-indolyl phosphate (BCIP) for 4 hr at room temperature.

Detection of aquatic environmental parameters

Water temperature, salinity, pH, dissolved oxygen content of water will be determined using portable electronic meters.

Type of data

1) Isolation of *V. cholerae* 01 from various components of aquatic environments by using conventional cultural technique

2) Detection of non-cultureable but viable *V. cholerae* 01 from various aquatic components by using fluorescent microscopy

3) Detection of low numbers of *V. cholerae* 01 and non-culturable *V. cholerae* 01 using the polymerase chain reaction technique

4) Determination of various physicochemical parameters of water

Interpretation of data

If the *V. cholerae* 01 are found in a particular flora throughout the year and the number of both *V. cholerae* 01 and the associated hosts increase during

cholera seasons (clinical surveillance data), that will provide a clue about the reservoirs and role of thse reservoirs in maintaining endemicity and seasonality of cholera in various parts of Bangladesh.

If the multiplication of *V. cholerae* 01 and its associated host correlate with the changes of some particular physicochemical conditions of water, that will give us some idea about the role of various physicochemical factors in initiating the multiplication of vibrios in the environment.

Correlation of *V. cholerae* 01 recovery rate from different environmental samples will be analyzed with particular reference to seasons, cholera prevalence. The epidemiological data will be collected from the concurrent sentinel surveillance programme.

The data will be analysed in statistical analysis system (SAS). dBase will also be included. Data will be entered in SOS data entry package. The data will be processed by Mr. M.A. Malek in Archive Unit of LSD.

We can cluster the environmental factors in two categories, high and low. During cholera seasons, the high values should be observed in algal count, hours of sunshine, pH, salinity and dissolved oxygen content of water. The low values should be observed in rainfall, water level, water temperature and CO_2 content. The available data suggest a pattern of increasing or decreasing trends of various physico-chemical factors during epidemic and inter-epidemic periods. We will therefore be able to categorize some physico-chemical factors on the basis of increasing or decreasing trends.

c) Bibliography

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11. PUBLICATIONS OF PRINCIPAL INVESTIGATORS

Dr. Md. Sirajul Islam

1) Islam MS, Drasar BS, Bradley DJ. (1990) Long term persistence of toxigenic *Vibrio cholerae* O1 in the mucilaginous sheath of a blue green alga, *Anabaena variabilis*. J Trop Med Hyg 93:133-139.

2) Islam MS. (1991) Toxigenicity and toxin genes of *Vibrio cholerae* O1 isolated from an artificial aquatic environment. World J Microbiol Biotechnol 7:269-271.

3) Islam MS, Alam MJ, Tzipori S. (1992) Abundance of *Aeromonas* spp. in various components of pond environment in Dhaka, Bangladesh. Inter J Environ Stud 39:297-304.

4) Islam MS, Drasar BS, Bradley DJ. (1990) Survival of toxigenic *Vibrio cholerae* O1 with a common duckweed, *Lemna minor* in artificial aquatic ecosystems. Transac Roy Soc Trop Med Hyg 84:422-424.

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6) Islam MS. (1990) Increased toxin production by *Vibrio cholerae* 01 during survival with a green alga, *Rhizoclonium fontanum* in artificial aquatic environment. Microbiol Immunol 34:557-563.

7) Islam MS, Drasar BS, Bradley DJ. (1989) Attachment of toxigenic *Vibrio cholerae* 01 to various fresh water plants and survival with a filamentous green alga. *Rhizoclonium fontanum*. J Trop Med Hyg 92:396-401.

8) Islam MS, Drasar BS. Bradley DJ. (1988) Survival and attachment of toxigenic *Vibrio cholerae* 01 in association with four marine algae. Bangladesh J Microbiol 5:41-48.

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12) Morshed MG, Aziz KMS, Islam MS, Khan MR. (1986) Abundance of coliform bacteria in the river sediment at three sampling stations on dthe Buriganga river. Bangladesh J Microbiol 3:5-10.

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18) Islam MS, Drasar BS. A review on aquatic flora as possible reservoirs of toxigenic *Vibrio cholerae* 01 in the aquatic environment. Trop Dis Bull (submitted).

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12. FLOW CHART

Activities of the laboratory : 3 years

First year : Standardization of techniques - 6 months
Field sampling - 6 months

Second year : Field sampling

Third year : Field sampling - 6 months
Data analysis and writing - 6 months

13. ITEMIZED SPECIFIC TASKS FOR EACH LISTED INVESTIGATORS

Sequence of tasks

Standardization of procedure	...	2 months
Testing of specimens	...	2 years 6 months
Analysis of data and writing reports		4 months

Task of investigators

M. S. Islam M.J. Abert A. Felsenstein A. Huq	Standardization of procedure
M. S. Islam A. K. M. Siddique	Field sampling
M. S. Islam A. K. M. Siddique A. Felsenstein	Data analysis
Professor R. B. Sack Professor R.R. Colwell	Overall supervision and interpretation of data

14. BUDGET

BUDGET SUMMARY

First year				
1.	Personnel	...	US$ 24,360	
2.	Operating cost	...	30,095	
3.	Capital equipment	...	5,000	
				US$ 59,455
Second year				
1.	Personnel	...	25,212	
2.	Operating cost	...	28,095	
				53,307
Third year				
1.	Personnel	...	26,608	
2.	Operating cost	...	28,095	
				54,703
	Total cost (for 3 years)			US$ 167,465

DETAILED BUDGET

First Year

A. Personnel

Designation	Level and step		Salary per annum	
1. Associate Scientist	NOC-4	(30%)	US$ 3,798	
2. Senior Research Officer	GS-VI-1	(100%)	6,036	
3. Research Officer	GS-V-1	(100%)	4,668	
4. Senior Laboratory Technician	GS-IV-1	(100%)	3,768	
5. Laboratory Technician	GS-III-1	(100%)	2,952	
6. Senior Laboratory Attendant	GS-II-1	(100%)	2,508	
7. Consultant	-		630	
				US$ 24,360

B. Operating costs

a)	Sampling and processing of phytoplankton - 240 @ 7.63	US$ 1,831	
b)	Zooplankton - 240 @ 7.63	1,831	
c)	Algae - 240 @ 7.63	1,831	
d)	Sediment - 240 @ 7.63	1,831	
e)	Plants - 240 @ 7.63	1,831	
f)	Water - 240 @ 7.63	1,831	
g)	Water for FC - 240 @ 7.41	1,778	
h)	Oysters - 240 @ 7.63	1,831	
i)	Antiserum and reagents -	3,000	
j)	Non-stock supplies - (sampling box, sampling bottles, chemicals)	2,000	
k)	Stationary, communication, Xerox, Medical Illustration -	1,500	
l)	Transport - DA and TA -	6,000	
m)	International travel	3,000	
			US$ 30,095

Total cost (for first year) : US$ 24,360 + 30,095 = 54,455

C. Capital equipment

1)	Homogenizer	US$ 2,000	
2)	Portable DO_2 meter, pH meter, water sampler	3,000	
			US$ 5,000

Total cost (for first year) = US$ 24,360 + 30,095 + 5,000 = 59,455

Second year

A. Personnel

Designation	Level and step	Salary per annum		
1. Associate Scientist	NOC-5 (30%)	US$ 3,906		
2. Senior Research Officer	GS-VI-2 (100%)	6,264		
3. Research Officer	GS-V-2 (100%)	4,848		
4. Senior Laboratory Technician	GS-IV-2 (100%)	3,912		
5. Laboratory Technician	GS-III-2 (100%)	3,060		
6. Senior Laboratory Attendant	GS-II-2 (100%)	2,592		
7. Consultant	-	630		
			US$	25,212

B. Operating costs

a)	Sampling and processing of phytoplankton - 240 @ 7.63	US$	1,831	
b)	Zooplankton - 240 @ 7.63		1,831	
c)	Algae - 240 @ 7.63		1,831	
d)	Sediment - 240 @ 7.63		1,831	
e)	Plants - 240 @ 7.63		1,831	
f)	Water - 240 @ 7.63		1,831	
g)	Water for FC - 240 @ 7.41		1,778	
h)	Oysters - 240 @ 7.63		1,831	
i)	Antiserum and reagents -		3,000	
j)	Stationary, communication, Xerox, Medical Illustration -		1,500	
k)	Transport - DA and TA		6,000	
l)	International travel -		3,000	
				US$ 28,095

Total cost (for second year) : US$ 25,212 + 28,095 = 53,307

Third year

A. Personnel

Designation	Level and step	Salary per annum	
1. Associate Scientist	NOC-6 (30%)	US$ 4,014	
2. Senior Research Officer	GS-VI-3 (100%)	6,720	
3. Research Officer	GS-V-3 (100%)	5,208	
4. Senior Laboratory Technician	GS-IV-3 (100%)	4,000	
5. Laboratory Technician	GS-III-3 (100%)	3,276	
6. Senior Laboratory Attendant	GS-II-3 (100%)	2,760	
7. Consultant	-	630	
		----------	US$ 26,608

B. Operating cost as previous

a)	Sampling and processing of phytoplankton - 240 @ 7.63	US$ 1,831	
b)	Zooplankton - 240 @ 7.63	1,831	
c)	Algae - 240 @ 7.63	1,831	
d)	Sediment - 240 @ 7.63	1,831	
e)	Plants - 240 @ 7.63	1,831	
f)	Water for vibrio - 240 @ 7.41	1,778	
g)	Water for FC - 240 @ 7.63	1,831	
h)	Oysters - 240 @ 7.63	1,831	
i)	Antiserum and reagents -	3,000	
j)	Stationary, communication, Xerox, Medical Illustration -	1,500	
k)	Transport - DA and TA -	6,000	
l)	International travel -	3,000	
		----------	US$ 28,095

Total cost (for third year) : US$ 26,608 + 28,095 = 54,703

MSI:mh/S10:AFLORA.PRT

Reviewer NO.1.

Associate Director
Laboratory Sciences and Community Health Division
The International Center for Diarrheal Disease Research,
Bangladesh
P.O. Box 128
Dhaka, Bangladesh

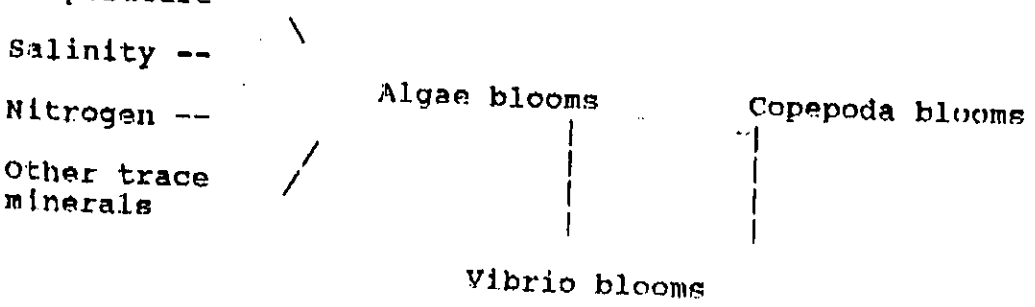
Dear Brad:

Thank you for sending the protocol of Dr. Sirajul Islam entitled, "Role of various aquatic flora, fauna, and physicochemical conditions of water in maintaining endemicity and seasonality of cholera in Bangladesh." Overall I am impressed with the proposal and feel it will complement nicely the planned surveillance protocol which is about to begin in Bangladesh. There are however a few aspects which I hope the investigators will consider.

1. Firstly, I find an overall framework or model lacking from the protocol, other than the general notion that the environmental reservoir is important in the maintenance of the reservoir. This no longer seems to be a controversial area, and by itself, is not worthy of such a large study. What does seem important is the linkage of the findings from this environmental study with the clinical surveillance, such that epidemics can be correlated with the environmental findings. Hopefully an environmental explanation can be found which will explain the timing of epidemics. Seasonal changes in surface waters should relate to seasonal epidemics; the purpose of this protocol should be to describe physicochemical changes in water, and the epidemics which result from these changes.

Previously we hypothesized that physicochemical factors (light, salt, nitrogen, temperature, etc) lead to blooms of algae which were followed by blooms in copepoda, and that these were followed by epidemics of cholera since the Vibrio could better survive in the copepodae/algae rich waters. This model suggests at least two intervention strategies. Firstly, if the likelihood of cholera epidemic can be predicted by determining the characteristics of water, early warning of an

impending epidemic could lead to better preparations. Secondly, some aspects of water may be amenable to intervention, especially nitrogen content. The nitrogen could come from feces run-off, but could also result from agriculture fertilizer run-off. I suspect Dr. Islam is aware of this model and may have a different model of his own. Statement of the model system with which he is working will allow him to better state more specific hypotheses. A drawing might assist him in conceptualizing his model.



The relationship between water ecology and Vibrio growth is obviously complex, and sorting out the important variables among an ever changing background of ecological factors will be very difficult, making the project extremely difficult to analyze unless the framework is well developed from the start.

2. Specific hypotheses. Related to the first point, specific hypotheses are needed which a) are based on the extensive data collection from ICDDR,B regarding Vibrio ecology, and b) would present the best "guess" as to what factors are most important in predicting an epidemic. A hypothesis might be stated: epidemic cholera is most likely to occur when salinity of surface waters rises above a certain level, algae are blooming, and the mean temperature exceeds 28 C. Obviously other hypotheses could also be proposed; the point is to predict which of the factors are thought to be important in the cholera epidemic.

3. Data analysis. Before initiating the project, the method of analysis must be determined. There will be a tremendously large number of data points and unless careful attention is given to data management and analysis, the data will not be analyzed.

The data base should include both environmental data as well as some epidemiologic data from the case detection; at least to know when an epidemic is occurring.

4. Definitions of environmental data clusters. Environmental changes are extremely complex with electrolyte concentrations changing, temperatures changing, nitrogen concentration changing, plankton concentrations changing, etc. Is there a way that to categorize these changes. Are there patterns? Is "summer" or "monsoon" characterized by a certain pattern. (Don't limit the data clusters to obvious seasons; however, since, your data may discover

more subtle patterns which are not obvious.) If data clusters can be defined, analysis would be greatly assisted.

4. Lab details. We have recently learned a few things in our lab about enrichment media which might be helpful. Firstly, aeration of the enrichment broth is extremely helpful in growing vibrios in bile peptone water. I believe this should be incorporated into the methods. Also, one should compare bile peptone with alkaline peptone. In our hands, bile peptone was better than alkaline peptone at the same pH.

Also since you are planning gene probe studies, I was surprised not to see a description of doing probes on colony blots of the primary plates to give a quantitative number of toxigenic V. cholerae.

5. "Viable but not cultured bacteria" is a fascinating field but not related to the epidemiologic study being undertaken. I would recommend not including the rabbit loop studies since I don't see the connection to the epidemiology.

The immunofluorescent tests will likely be a convenient way to detect V. cholerae so should be included.

In summary, I believe this is an important study, but I trust that the questions to be answered can become more focused, can be more related to the epidemiologic studies which will be ongoing, and especially will attempt to answer what environmental changes are responsible for the epidemics. One would hope that the combination of the epidemiologic surveillance plus this environmental study will not only describe the seasonal and geographic patterns of cholera in Bangladesh, but also explain the environmental causes as to why the patterns occur. Most optimistically, the studies could even suggest an environmental intervention or at least an early warning system.

Reviewer No. 2.

Title: "Role of various aquatic flora, fauna and physicochemical conditions of water in maintaining endemicity and seasonality of cholera in Bangladesh".

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

	Rank Score		
	High	Medium	Low
Quality of Project			
Adequacy of Project Design	✓		
Suitability of Methodology	✓		
Feasibility within time period	✓		
Appropriateness of budget	✓		
Potential value of field of knowledge	✓		

CONCLUSIONS

I support the application:

a) without qualification ☐

b) with qualification ☑

- on technical grounds ☑ see review
- on level of financial support ☐

I do not support the application ☐

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 thay are justified.

(Use additional pages if necessary)

Title:

PI:

Reviewer: .....

ICDDR.B REVIEW

"Role of various aquatic flora, fauna, and physicochemical conditions of water in maintaining endemicity and seasonality of cholera in Bangladesh"

Dr. Md. Sirajul Islam and Dr. A.K. Siddique

This proposed research project should provide new and useful knowledge about the ecology of the etiologic agent of cholera. The project seems feasible and the budget appropriate. This study represents an excellent opportunity to define both the niche and the pathogen and to eliminate confusion resulting from earlier studies.

I strongly urge investigators to use a strict definition for toxigenic *Vibrio cholerae* O1 as the cause of cholera and to rigorously apply it throughout their work and analysis of the data:

There will undoubtedly be fluorescent antibody and PCR results in the absence of culturable organisms. A positive O1 reaction and toxin gene amplification are convincing evidence, but I suggest these data be analyzed separately or qualified when integrated with culture data.

Determination of toxigenicity is also important in face of nontoxigenic *V. cholerae* O1 strains which seem only distantly related to the toxigenic O1 strains. Volunteer studies indicate that these strains are not pathogenic for humans.

Investigators state in the introductory remarks on page 9 (Islam, 1987) that environmental isolates do not grow well on selective media. However, TCBS and TTGA are indicated in most of the proposed culturing procedures (pages 12-14).

It would be interesting to determine specifically where the toxigenic *V. cholerae* O1 strains exist/replicate on or in the various plants and animals. Investigators may be proposing this on page 13, but it was not entirely clear.

If it is possible to amplify DNA from nonculturable forms of *V. cholerae*, I would suggest using PCR more frequently during sampling. It can also be used as an assay for toxin genes in isolates; PCR is much faster and probably more accurate than most toxin assays.

I suggest that investigators do some pilot studies with the PCR for *ctxA* and am attaching a protocol and recent reprint describing a slightly different *ctxA* PCR. The reprint describes a more extensive look at control cultures than the report by Shirai, 1991. Initial amplification conditions did amplify LT genes of ETEC; the amplicons were visibly different in size. Since the number of ETEC examined by both PCR approaches is small, I recommend that the investigators assay some ETEC isolated recently in Bangladesh.

Given enough background data from the pilot study, it might be possible to simply run gels without hybridization. Investigators might also find that the probe could be designed to distinguish between LT and CT. In any event, initial studies should warrant some cultural follow up.

I believe that the project is well worth supporting and that the data may be helpful in determining both reservoirs and the best technical approaches to assay them. The information and the methodology might be broadly applicable in assessing risk and proposing control strategies in settings within and outside of Bangladesh.

Response to comments raised by Reviewer No. 1

1) As the reviewer suggested, a model has been incorporated in the revised protocol. P10.

2) As suggested by the reviewer, specific hypothesis is given which has been incorporated in the revised manuscript. P2, P3.

3) The surveillance team will provide data of the sources of infection of the index case and then that data will be correlated with the environmental data.

The data will be analysed in statistical analysis system (SAS). dBase will also be included. Data will be entered in SOS data entry package. The data will be processed by Mr. M.A. Malek in Archive Unit of LSD. This information has been incorporated in revised manuscript. P18, P4.

4) We can cluster the environmental factors in two categories, high and low. During cholera seasons, the high values should be observed in algal count, hours of sunshine, pH, salinity and dissolved oxygen content of water. The low values should be observed in rainfall, water level, water temperature and CO_2 content. The available data suggest a pattern of increasing or decreasing trends of various physico-chemical factors during epidemic and inter-epidemic periods. We will therefore be able to categorize some

periods. We will therefore be able to categorize some physico-chemical factors on the basis of increasing or decreasing trends. It has been incorporated in interpretation of data section. P18, P5.

5) As the reviewer suggested we will use bile peptone as enrichment media and *Vibrios* will be grown by agitation. This has been incorporated in the revised manuscript, P14, P2, L2.

Detection of toxigenic *V. cholerae* 01 will be carried out following colony blot hybridization as described by Pal *et al.* (1992). As suggested by the reviewer, this methodology has been incorporated in the revised manuscript, P15, P1.

6) As suggested by the reviewer, the RIL test has been omitted.

Response to comments raised by Reviewer No. 2

As the reviewer suggested, a strict definition for toxigenic *V. cholerae* 01 has been rigorously applied throughout the work and will be used to analyse the data.

As the reviewer suggested, the fluorescent antibody and PCR results will be analyzed separately or qualified when integrated with culture data.

All the isolated *V. cholerae* 01 strains will be tested for its toxigenicity by colony blot hybridization technique using CT probe which has been described in the revised protocol, P15, P1.

Though the laboratory based study showed better recovery of stressed *V. cholerae* 01 in non-selective media than selective media, but in case of environmental samples, it will not be possible to use non-selective media because of presence of various other bacterial flora. However, fluorescent antibody test and PCR technique will help to overcome the detection of stressed *V. cholerae* 01 from the environmental samples.

To determine where the toxigenic *V. cholerae* 01 strains exist/replicate on or in the various plants and animals, fluorescent microscopy will be done as described previously by Islam *et al.* (1990).

As the reviewer suggested, and we found that PCR can pick-up non-culturable cells, this tests will be carried out to amplify DNA

from both non-culturable and culturable cells and as the *ctx* gene will be used as target sequence, it will only detect the toxigenic *V. cholerae* 01.

MSI:mh/S7A:RESPN2.REV