ıchment 1.
ACE SHEET)

Library

Date 3/12/96

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Cb

incipal Investigator DR. AKM SIDDIQUE

Trainee Investigator (if any)

plication No. 96-022

Supporting Agency (if Non-ICDDR,B) NIH - USA

tle of Study Epidemiology & Ecology of V Cholerae in Bangladesh

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

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

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

Does the study involve:
(a) Physical risks to the subjects Yes (No)
(b) Social Risks 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)

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)
(c) Use of organs or body fluids Yes (No)

Are subjects clearly informed about:
(a) Nature and purposes of study (Yes) No
(b) Procedures to be followed including alternatives used (Yes) No
(c) Physical risks Yes (No)
(d) Sensitive questions Yes (No)
(e) Benefits to be derived (Yes) No
(f) Right to refuse to participate or to withdraw from study (Yes) No
(g) Confidential handling of data (Yes) No
(h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes (No)

5. Will signed consent form be required:
(a) From subjects (Yes) No
(b) From parent or guardian (if subjects are minors) (Yes) No
6. Will precautions be taken to protect anonymity of subjects (Yes) No
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.

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

Principal Investigator

Trainee

ENTERED 2 1 JUN 1998

CHECK-LIST FOR SUBMISSION OF PROPOSALS TO THE RESEARCH REVIEW COMMITTEE (RRC)

[Please tick (✓) the appropriate box]

1. Has the proposal been reviewed, discussed and cleared at the Division level ?

   Yes [✓]

   No [ ]

   If 'No', please clarify the reasons: _____

2. Has the proposal been peer-reviewed externally ?

   Yes [✓]

   No [ ]

   If the answer is 'NO', please explain the reasons: _____

3. Has the proposal scope to address gender issues ?

   Yes [ ]

   Not applicable

   No [ ]

   If the answer is 'YES', have these been adequately incorporated in the proposal. Please indicate: _____

4. Has a funding source been identified ?

   Yes [✓]

   No [ ]

   If the answer is 'YES', please indicate the name of the donor: _____

   National Institute of Health (NIH), USA

5. Whether the proposal is a collaborative one ?

Yes [✓]

No [ ]

If the answer is 'YES', the type of collaboration, name and address of the institution and name of the collaborating investigator be indicated:

Prof. RB Sack, The Johns Hopkins University, Baltimore, USA

Prof. Rita Colwell, The University of Maryland, Baltimore, USA

6. Has the budget been cleared by Finance Division ?

Yes [✓]

No [ ]

If the answer is 'NO', reasons thereof be indicated: _____

7. Does the study involve any procedure employing hazardous materials, or equipments ?

Yes [ ]

No [✓]

If 'YES', fill the necessary form.

3/12/96
Date

Signature of the
Principal Investigator

APPLICATION FOR PROJECT GRANT

1.	Principal Investigator(s):	Dr. AKM Siddique Dr. M John Albert Dr. R Bradley Sack
	Co-investigator(s):	Dr. Md.Sirajul Islam Dr. Shah M Faruque Dr. Firdousi Qadri Dr. Md Yunus
	Consultant:	Dr. Rita Colwell Dr. J Glenn Morris
2.	Title of the Protocol:	Epidemiology & Ecology of Vibrio Cholerae in Bangladesh
3.	Starting date:	January 1, 1997
4.	Completion date:	December 31, 2001
5.	Total Direct Cost:	US$ 2,517,683
	Funding source:	National Institute of Health, USA
6.	Signature of Division Director	_____
	Date	3/12/96

ABSTRACT SUMMARY

Cholera, the most severe of all diarrheal diseases, caused by *Vibrio cholerae* O1 (and most recently also by serogroup O139) is one of the few pandemic infections of man. In the heavily endemic regions such as Bangladesh, cholera also exhibits a regular twice-yearly periodicity. Unfortunately, the mechanisms controlling the periodicity and pandemicity of cholera are not known. The primary objective of this proposal is to test the hypothesis that environmental factors involving surface waters are responsible for the observed epidemiology of cholera. We postulate that plankton living in surface waters are the reservoir for cholera vibrios, and that their growth and life cycle(s) control vibrio populations in surface waters, and thus determine how vribrios are spread and when outbreaks of disease will occur. This information could lead to an improved warning system for prediction of cholera outbreaks, both in endemic areas, and in areas into which a cholera pandemic is threatening, and possibly lead to new strategies for controlling the disease through environmental interventions directed toward growth of plankton. This project, involves establishing four sentinel surveillance sites in Bangladesh, three of which are known to exhibit cholera periodicity, and one that is usually free of cholera. A combined year-round effort will monitor (1) the clinical cases/infections with *V.cholerae*, (2) the occurrence and density of *V.cholerae* in the surface waters of these sites, including those attached to plankton, both as viable and as non-culturable forms, using sensitive sampling techniques (including PCR), (3) the growth of phytoplankton and zooplankton in these areas, using direct sampling and identification techniques (4) the genetic and phenotypic markers of isolates to determine whether *V. cholerae* isolated from surface water are identical with those isolated from patients with cholera in the same vicinity. Based on these data, a model of cholera epidemiology will be developed which may be useful in predicting outbreaks of cholera, thereby allowing early mobilization of preventive and treatment measures.

EPIDEMIOLOGIC AND ECOLOGIC STUDY OF V. CHOLERAE

1. HYPOTHESIS

Cholera exists both as an endemic and epidemic disease, and the etiologic agent, V. cholerae, is the only bacterial pathogen known to cause global pandemics of disease in modern times. We hypothesize that the various epidemiological features of the disease are primarily the result of environmental factors, particularly involving aquatic habitats **(plankton)** which can now be identified. This information will allow the prediction of cholera outbreaks, and thereby facilitate the early introduction of preventative and treatment measures.

SPECIFIC AIMS

The long term objective of this proposal is the decrease of cholera mortality and morbidity in Bangladesh and other areas of the world that have been threatened by this disease. The specific aims include the following:

1) To establish and maintain an efficient clinical surveillance system in four representative sites in Bangladesh (three in cholera-prone areas and one in a usually cholera-free area) to monitor rates and trends in the incidence of cholera.

2) To monitor the presence and concentration of *V. cholerae* (O1 and O139) in the water, sediment, and **aquatic fauna and flora** of these four clinical surveillance sites, using cultural, immunobiological, and molecular techniques.

3) To monitor both directly and by remote sensing, specific characteristics of the aquatic systems at these sites, including zooplankton and phytoplankton, and selected physicochemical parameters.

4) To develop a model of cholera epidemiology, based on the clinical and environmental surveillance data, which will predict the onset of epidemics, thereby allowing the development and application of preventive and treatment measures.

Though not a specific objective, an additional benefit of the project will be the characterization of two other cholera endemic areas in Bangladesh which can be considered for future field trials of cholera vaccines presently under development.

2. BACKGROUND AND SIGNIFICANCE

Cholera is one of man's oldest scourges, having been described since time immemorial from the area of the Ganges River delta, and V. cholerae remain the only bacteria which still cause global pandemics of disease. In the past approximately 180 years, there have been 7 global pandemics, the last of which is still "in progress," having reached South America just 3 years ago, almost 30 years after it first started to spread out of Indonesia. The last 3 pandemics are known to have been caused by V. cholerae serogroup O1, and the

atest by a new biotype (El tor) of that serogroup. Only in the latter part of 1992 did a new serogroup (O139) arise for the first time in history, which also has pandemic potential (4,54).

Cholera as a Public Health Problem

Cholera is a major public health problem wherever it occurs, either as an endemic disease (as in the "home" of cholera in the Ganges River delta) or as a spreading epidemic disease, despite the fact that it is a simple and inexpensive disease to treat, when adequate supplies and knowledgeable, committed health workers are available. When cholera spread to Latin America in 1991, it caused a large number of cases (nearly 400,000 estimated during the first year) but with a low mortality rate of about 1% (9). This was undoubtedly a result of rapid mobilization of trained personnel and provision of adequate treatment facilities throughout the region. In contrast, during the recent 1994 outbreak among Rwandan refugees in Zaire, mortality rates were high (as much as 50%) because of the severe shortage of supplies and trained personnel (11).

Cholera in Bangladesh has been studied in great detail by the International Centre for Diarrheal Disease, Bangladesh (ICDDR,B; established in 1979) and it's predecessors, the Pakistan-SEATO Cholera Research Laboratory, (1960 - 1971) and the Cholera Research Laboratory (1972-1979). In these facilities, which include an urban (Dhaka) and rural (Matlab) diarrheal disease hospital, and a rural surveillance population of 200,000 (Matlab), much of the epidemiology of the disease has been elucidated, six cholera vaccine field trials were carried out, and many of the early clinical studies of antibiotic therapy and oral rehydration therapy were successfully performed. The Johns Hopkins University, and the University of Maryland have had close links with the ICDDR,B and its predecessors for the past approximately 25 years. In 1972, an NIH-sponsored International Center for Medical Research and Training (ICMRT) project with Johns Hopkins University was undertaken over an 8 year period. Thereafter there has been a continuing exchange of faculty and students. One Director (Dr. W.B. Greenough, III) and two Associate Directors (Dr's D.A. Sack and R.B. Sack) of the ICDDR,B have been Johns Hopkins University faculty.

Cholera continues also to be a major public health problem in Bangladesh. In 1991, during a 12 week period there were approximately 220,000 cases of cholera with over 8,000 deaths, more than had occurred in all of Latin America that same year (63).

Emergence of <u>V. cholerae</u> O139

At the end of 1992, a new strain of enterotoxigenic <u>V. cholerae</u> (serogoup O139) was first identified in the southern coastal areas of Bangladesh (4). This strain was reported first in October 1992 from Madras, India, then a few months later from Calcutta (54). Since its appearances were in locations adjacent to the Bay of Bengal, it was tentatively given the name of O139, synonym Bengal. During the first four months of 1993 in Bangladesh, with the rapid spread of this new strain, there were an estimated 150,000 cases with 2,000 deaths. (See Figure 1 and Appendix 1,2,3) (10,61). The new strain of vibrios caused disease in adults at a high rate, as well as in children, indicating that prior exposure to <u>V. cholerae</u> O1 had not resulted in solid immunity (10). Within a few months of its appearance, this new vibrio had been studied in great detail by many laboratories around the world in collaboration with

ICDDR,B, resulting in numerous publications (1,39). The new vibrio appears to be closely related to the El Tor O1 serogroup, as evidenced by a number of genetic studies. Major differences have been identified in cell wall structures, e.g. O139 has an immunologically distinct LPS and also has a capsule, which differentiates it from O1 vibrios (46). V. cholerae O139 has now spread during the past two years to involve Nepal, Myanmar, Thailand, Malaysia, China, Pakistan, and imported cases have occurred in the United States, United Kingdom, Saudi Arabia and Japan.

During the past year, however, there seems to have been no further spread of this organism. It continues to be isolated in Bangladesh and India, but at much reduced levels, as compared to V. cholerae O1.

Cholera Epidemiology

Unfortunately, in spite of the many years of study, it is still not known how V. cholerae spreads around the world, what determines its regular seasonal peaks, and what are determinants of the evolution of the new O139 strain.

Hallmarks of the epidemiology of cholera (26,56) include I) a high degree of clustering of cases by location and season and sometimes long periods of time without apparent transmission, ii) highest rates of infection in children 1 to 5 years of age in endemic areas, iii) antibiotic resistance patterns that frequently change from year to year, and iv) protection against the disease being afforded by improved sanitation/hygiene, pre-existing immunity and breast-feeding.

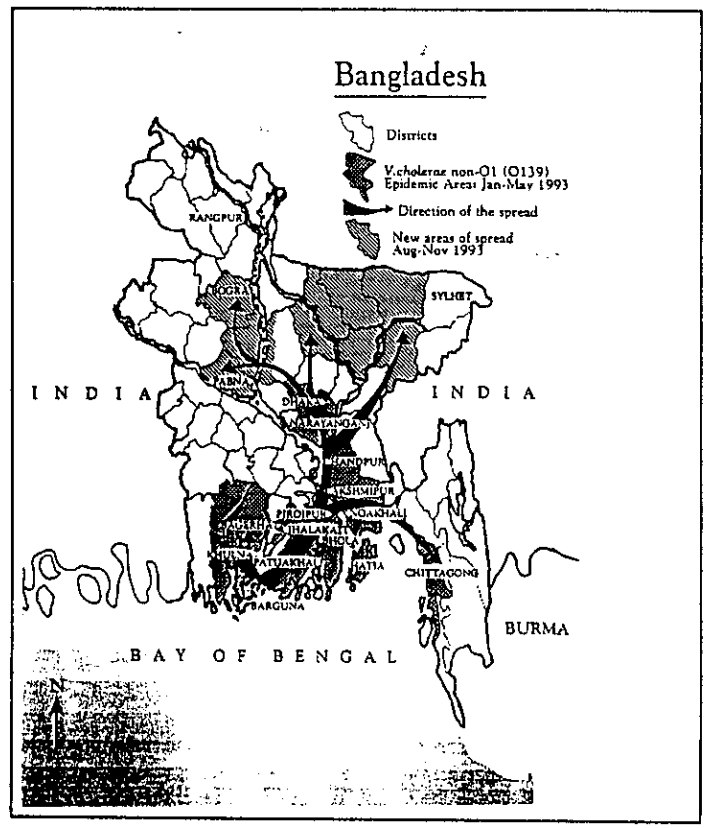

Figure 1 V. cholerae 0139 in Bangladesh

Cholera occurs in rural Bangladesh year-round, but with peaks of disease in Matlab, in the spring and fall (before and after the monsoons) (26,56). Little is known of the patterns of cholera in the rest of the country, because of lack of surveillance (61, 62).

Cholera is the classical "water-born" disease, and the importance of water ecology is suggested by the close association of V. cholerae with surface water (30,32,34,35,36). Because of its low-lying deltaic environment, the water volumes in ponds and rivers change; during the dry season they are usually low, but during the monsoons, they frequently overflow their banks with fresh water from the local rains as well as from rains and snow melt from the Himalayan and Tibetan plateau. Besides raising the river levels, the monsoons also wash the sewage, and fertilizers from fields and villages into the rivers. Though the rivers contain fresh water (except along the coast where it is brackish), tidal changes are evident as far

north as Dhaka. Having primarily a traditional and rural society, the people of Bangladesh are in direct contact with the surface water for drinking, bathing, cooking, and irrigation. Thus, if changes in water ecology result in increasing concentrations of pathogenic bacteria, the bacteria can be easily transmitted to the people.

Survival Forms of V. cholerae in the Environment

Survival of V. cholerae between epidemic seasons has long been a key question. Koch first postulated that cholera vibrios were associated with surface waters (41A), and Cockburn and Cassanos in 1960 (12A) were the first to study the association of V. cholerae with phytoplankon and the pH of water in ponds. More recently Colwell and her associates have demonstrated that many bacteria can enter into a nonculturable state (13,14) but remain viable and capable of producing disease (16,16A). More specifically, nonculturable V. cholerae strains have been found to become culturable again when passed through rabbit-ileal loops (14) or fed to human volunteers (16). A three year study conducted in Matlab, Bangladesh has demonstrated the presence of cells of V. cholerae O1 throughout the year, associated with zooplankton, as determined by fluorescent antibody techniques using a monoclonal antibody, when cultures were not always positive (30). The natural flora of zooplankton is predominantly vibrio species (33,64). Nonculturable forms of V. cholerae O1 and O139 have been reported recently in Bangladesh (33A). Nonculturable forms of V. cholerae have also been reported to survive in the mucilaginous sheath of blue green algae, Anabaena variabilis for up to 15 months (34). These algae form seasonal "blooms" which directly depend on the physico-chemical properties of water. Clearly the association of V. cholerae with plankton is well established (16B, 32,36). Hydrophytes (large water plants) such as Eichornia crassipes (water hyacinths) and Lemna minor (duckweed) may also have a role in the survival of the organisms in the aquatic environment (67A). However, at present, the strongest evidence, which is fully documented (33,64) suggests a close and important relationship between vibrio species and crustaceans.

It is known that zooplankton feed on phytoplankton and obviously a large number of zooplankton are expected to grow after a phytoplankton bloom, followed by a large number of vibrios (13). From the evidence we have to date the actual reservoir(s) of V. cholerae may be zooplankton and/or phytoplankton, the latter of which posses chitin exoskeletal structures to which vibrios attach (31,64). A hypothetical model was proposed by Huq et al (1987) and later modified by Colwell and Huq (13) which suggests the route of transmission of cholera and its survival as an autochthonous member of the aquatic environment.

A relatively new technology that should be useful in detecting phytoplankton and subsequent zooplankton blooms is that of remote sensing, or satellite imaging.(18,19,20). It is possible now to determine the extent of plant growth in surface waters of Bangladesh and to project from chlorophyll data the follow-on bloom of zooplankton that graze on the phytoplankton and carry the Vibrio species.

Another "survival form" of V. cholerae has also been recently rediscovered, that of the rugose form, which appears to be resistant to chlorination (55). Rugose colonies have an unusual colonial morphology on nonselective agar and appear similar to rough strains. They do however possess an LPS and are fully virulent in both animal models and human volunteers.

Microbiologic Diagnosis and Molecular Epidemiology

Standard bacteriologic methods for detection and characterization of V. cholerae involve the use of selective media (TTGA [tellurite, taurocholate, gelatin agar] and/or TCBS [thiosulfate-citrate-bile salts-sucrose agar) and enrichment in alkaline peptone water. Serotyping and biotyping are done by standard methods(72). Additional rapid techniques have been devised which detect specific vibrio antigens by immunomagnetic capture (49) fluorescent antibodies (27,30), modified Elisa assay, (29) co-agglutination (15,51,53), and vibrio genes by DNA probes (40,73) and PCR (25, 28, 42).

The DNA probe techniques are particularly valuable because they permit direct and quantitative identification of V. cholerae strains of interest (i.e. strains which carry the cholera toxin gene, or genes associated with specific O groups). Furthermore, by combining molecular and immunologic methods with classical cultural methods, it is also possible to detect cholera vibrios associated with specific components of plankton.

Once isolated, V. cholerae can be further characterized by a variety of molecular techniques, including plasmid analysis, ribotyping, pulsed field gel electrophoresis, and multifocus enzyme electrophoresis. These techniques are finding increasing usage in epidemiologic studies of cholera (40,70), and provide a means by which it will be possible to obtain a "molecular fingerprint" of isolates of interest.

Using these methods selectively should allow us to I) demonstrate a link between the environmental and the clinical isolates, ii) quantitate numbers of an individual Vibrio species in environmental samples (using gene probes with colony blots) when the concentrations of culturable bacteria are relatively high, and detect vibrios (using monoclonal antibody florescent microscopy and PCR techniques) when they are present in low concentrations or in a non-culturable form.

Rationale

It may be possible to predict the timing and severity of epidemics if one is able to correlate specific ecological factors with concentrations of V. cholerae in the environment and with incidence of disease. This information would provide a warning to the population and to the medical services. It should be possible to develop environmental interventions, based on knowledge of critical risk factors associated with survival and transmission of the cholera vibrio, as well as to intervene with cholera vaccine(s).

3. PRELIMINARY DATA

Epidemiologic Studies: The ICDDR,B and its predecessors have continuous demographic data for the past 26 years on a rural surveillance population of approximately 200,000 persons living in Matlab, which is approximately 45 km south of Dhaka. Established originally for vaccine field testing, the population is now being used for evaluation of various primary health care activities. All persons living in this surveillance population (as well as any others from adjacent areas) receive free treatment for diarrheal diseases at the 70 bed ICDDR,B Diarrhea Treatment Center; all patients coming from the surveillance area are routinely cultured for diarrheal pathogens. Approximately 10,000-15,000 persons per year are treated at the Diarrhea Treatment Center. In Dhaka, the ICDDR,B maintains a larger 200 bed diarrhea hospital, which provides free treatment to residents of Dhaka and vicinity. A regular 4% sample of hospital attendees (every 25th patient) is routinely cultured for enteric pathogens (67). Approximately 90,000-100,000 persons are treated annually.

Cholera surveillance from Matlab since 1966 shows: 1) there is cholera every year, without exception, although the yearly attack rates vary from 0.5 - 8.0 per 1000 population. In addition there is seen an 8- year cycle of peak and valley attack rates. (Figure 2) 2) Age-related attack rates show the highest rates in children between the ages of 1-5 years (5-7 cholera hospitalizations per 1000 population), with lower rates in adults (1-1.5 cholera hospitalizations per 1000 population), suggesting age-acquired immunity, and 3) there are two cholera "epidemics" yearly superimposed on an endemic baseline; the spring peak occurs in April-May (pre-monsoon) and the fall peak in September-November (post-monsoon).(See Figure 3) 4) A geographic information system (GIS) recently introduced in Matlab, has demonstrated a clustering of cholera cases in certain villages and in relation to bodies of water (unpublished information.)

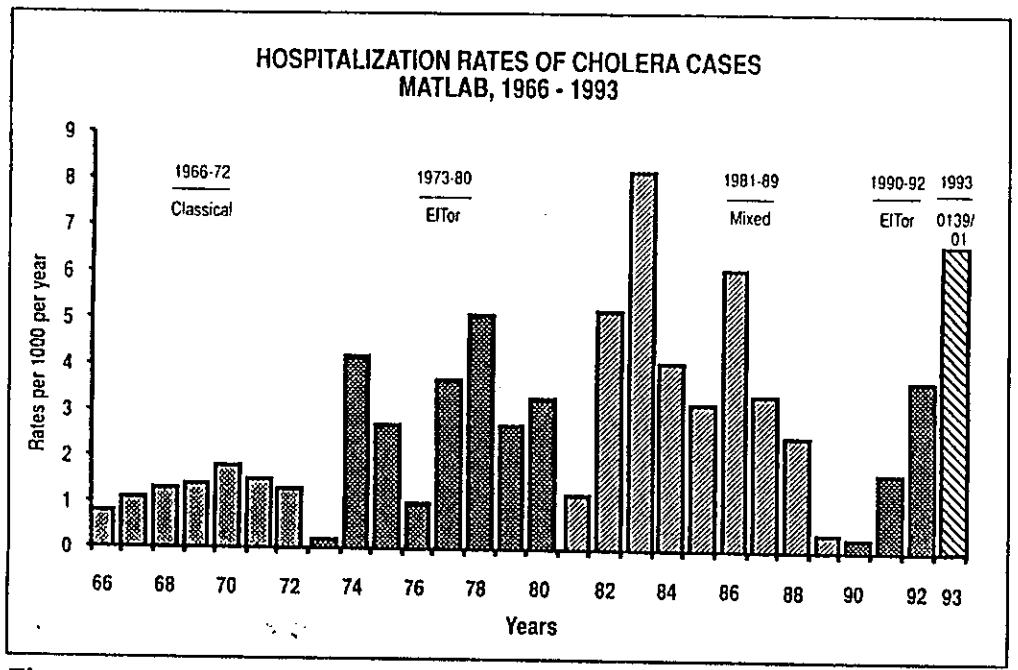

Figure 2

With the introduction of V. cholerae O139 in late 1992, it has been possible to follow some of the epidemiologic features of the two virulent vibrios, both in the two hospitals and

the population they serve. Figure 4 indicates the isolation of O1 and O139 vibrios, beginning in January I993. It can be seen that O139 predominated in the spring outbreak, whereas O1 returned in greater numbers in the fall. In 1994, there was only a small Spring outbreak, most of which was due to O1 vibrios.

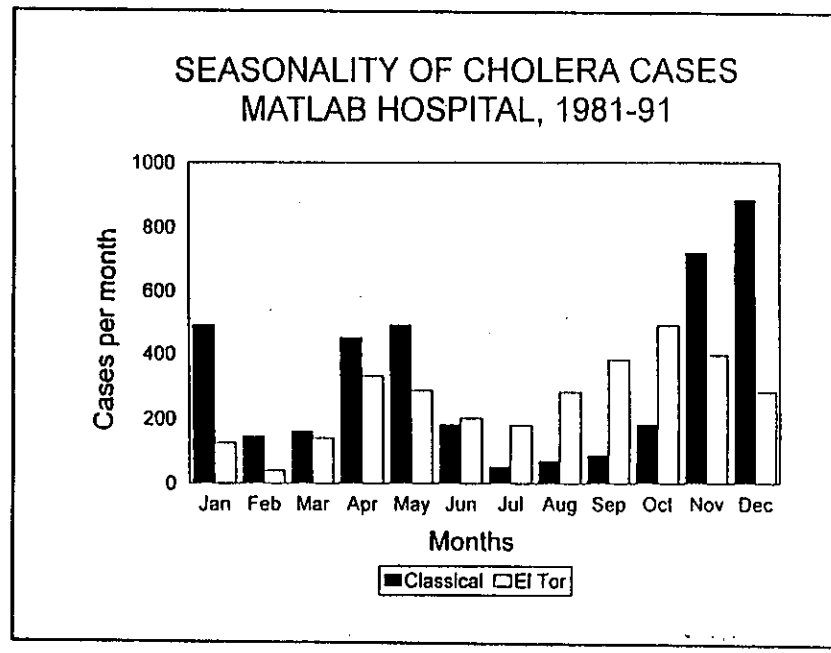

Figure 3

Transmission studies in families in Matlab were done by identifying index cases at the hospital, and then visiting the homes of the index cases for ten consecutive days to obtain rectal swabs and water samples for culture. These studies showed that O139 vibrios could be cultured from 8% of surface water samples used by the families (5/62 samples positive) and from 5% of tubewell water samples stored in the home (9/180 samples positive). None of 57 samples of tube well water were positive at the water source, indicating that contamination was occurring during storage in the home. Transmission of infection among family members was high; among 45 index case families, 60 of 236 family members became infected (25%), and 16 (27%) of those infected developed diarrheal disease. Children under the age of 4 years had the highest secondary infection rate, 37% (14/38 children) (21).

In the southern part of the country, in which classical O1 vibrios were predominant (62), the new O139 strain has completely replaced the O1 vibrios, whereas in the northern part of the country, O1 vibrios continue to be the predominant strain. (See Figure 1, Appendix 1).

In all areas of the country, most of the cases and deaths were in adults over the age of 15 years, which is distinctly different from epidemics caused by V. cholerae O1 in this endemic area. Age-specific attack rates from Matlab confirmed the high rates in adults, and suggest that prior exposure to V. cholerae O1 was not protective against the new O139 vibrio (10).

The Geographic nformation System (GIS), mentioned previously is in the area where the 200,000 people who are under surveillance live; it has been mapped in detail, with the help of satellite imaging. Cases of cholera (or other defined illnesses) can be identified both in relation to time and place. This in turn can be related to distances from surface waters (rivers, ponds, canals), latrines, markets, etc.

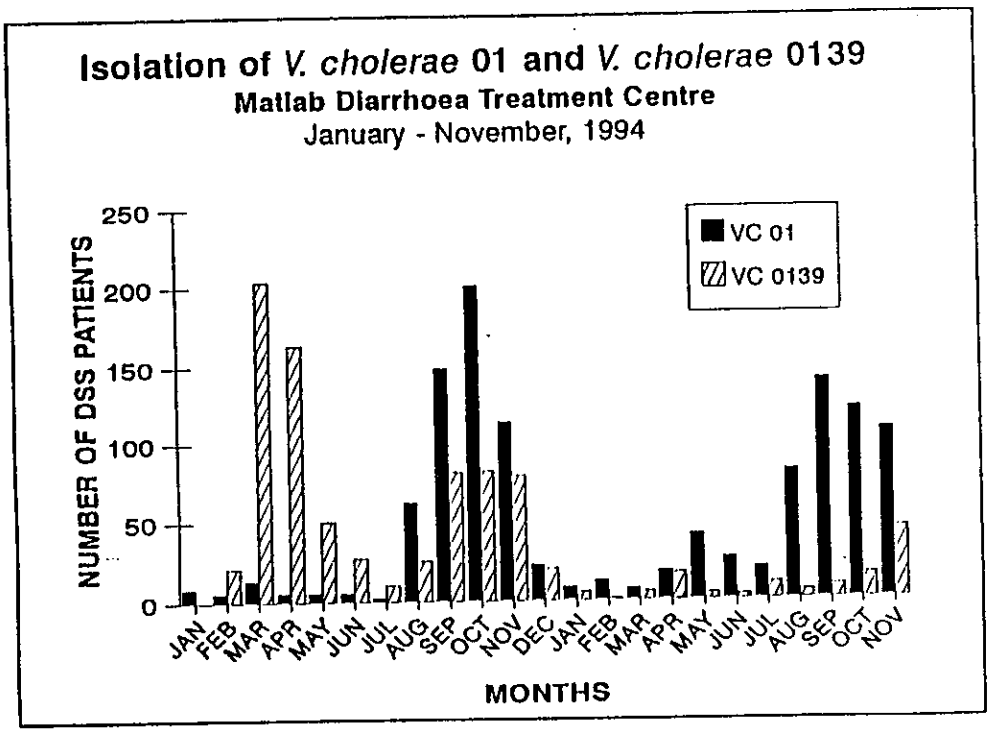

Figure 4

Clinical Studies:
(10, Appendix 2,3) Detailed clinical studies were done on patients admitted to both the Dhaka and Matlab hospitals, as well as on patients seen by the Epidemic Control Preparedness Program. All of these studies confirmed that the clinical illness is in every way identical to cholera caused by O1 strains. Colonization of the small bowel occurs, large amounts of cholera toxin and vibrios are excreted in the stool, and severe dehydration results from massive losses of electrolyte-rich diarrheal stool.

It is clear from these data that cholera is now a disease of two serogroups, and all future studies (environmental, clinical, and immunological) will need to consider both organisms.

Laboratory Studies: (Appendix 2,3) Two colony variants of V. cholerae O139 have been detected, when grown on Luria agar, gelatin agar, or taurocholate-tellurite-gelatin agar. These are opaque and translucent colonies, the former having colonies possessing a capsule, and the latter with little or no capsule. There is difficulty in developing a vibriocidal assay for V. cholerae O139 because the organism cannot be easily killed in serum, which is attributable to the presence of the capsule. However, a vibriocidal assay has now been developed by increasing the amount of complement added, and decreasing the bacterial inoculum (unpublished data).

Diagnostic Assays: Both polyclonal rabbit antisera and monoclonal antibodies (MAbs) (10,50) have been made to the O139 vibrio. The polyclonal sera has been used for diagnostic purposes in Dhaka and sent to many of the neighboring countries in an attempt to assist their recognition of the new vibrio.

For the production of MAbs (50), female BALB/c mice were immunized with an acetone extract of V. cholerae O139 strain 1838 four times at weekly intervals. Four days after the last dose, spleen cells from two immunized mice were fused with SP2/O myeloma cells using standard procedures, optimized in the Dhaka laboratory. Six hybridomas secreting antibody specific to the LPS of V. cholerae O139 were cloned twice by limiting dilution. Tissue culture supernatants and ascites fluid produced in pristane primed mice were screened for reactivity in an ELISA to LPS of both V. cholerae O139 and O1, Inaba and Ogawa serotypes. Five Mabs, ICL9, ICL11, ICL12, ICL13, and ICL14, were specific for V. cholerae O139. All could be used in direct slide agglutination for detection of V. cholerae O139. No cross-reactions were observed with other V. cholerae serogroups (O2-O138) or other enteropathogens tested. The MAbs can also be used in indirect immunofluorescence tests, and can be used to inhibit the motility of V. cholerae O139, using dark-field microscopy. During a one-year evaluation of these MAbs in the clinical laboratory, all cases diagnosed as V. cholerae O139 by a rabbit polyclonal antiserum were also detected by these MAbs, establishing their utility as highly sensitive and specific diagnostic reagents. With these MAbs, it should now be possible to screen for the V. cholerae O139 serogroup in epidemic and endemic diarrhea cases and in environmental and food samples.

Coagglutination Assay: Two of the MAbs, ICL11, and ICL12, of IgG3 and IgG2b isotypes respectively, have been adsorbed to Staphylococcus aureus Cowan I and tested for the detection of V. cholerae O139 directly from fecal samples (51). For this purpose, fecal samples were filtered (O.65 um pore membrane filter) heated for 2 minutes, and then tested by slide agglutination. The coagglutination test was positive in 109 of 293 watery diarrheal stool samples. Compared to culture as the standard (which was positive in 118 of the samples) the test showed a 92% sensitivity, 100% specificity, and 100% positive and 96% negative predictive values. No cross-reactions were detected with V. cholerae O1 or with the 137 other non-O1 V. cholerae serogroups tested. The test, however, failed to detect less than 10(7) CFU of bacteria in samples; stool samples having few vibrios by culture were negative by this method.

Bengal SMART (sensitive membrane antigen rapid test) test: Using MAb ICL12 as the detecting reagent, this colorimetric test has been developed by New Horizon Diagnostics (Columbia, Maryland, USA) (29). The test is similar to the previously developed cholera SMART assay for detection of V. cholerae O1 (51A). It is a colloidal gold-based MAb ICL12 and rabbit anti-O139 polyclonal antibody sandwich assay which can detect bacteria in faecal samples within 10 minutes. Of 189 watery diarrheal stool samples tested by Bengal SMART, 87 were positive. Of these, 3 were culture negative, but all tested positive for cholera toxin in the PCR assay using primers specific for ctxA of the CT operon. The result indicates that these three SMART positive/culture negative samples possibly contained low numbers of bacteria or dead bacteria which could not be detected by culture. The Bengal SMART test showed 100% sensitivity and 99% specificity as compared to culture for detection of the organism from faecal samples. These results show that Bengal SMART can detect very low levels of bacteria in fecal samples, and appears to be more sensitive than the ICL11 and ICL12 based coagglutination test.

Molecular Studies of V. cholerae: The restriction fragment length polymorphisms of ctxA and rRNA genes (ribotypes) were compared in 27 isolates of V. cholerae O139 from patients in Bangladesh and India, 48 isolates of V. cholerae O1 from patients and 21 V. cholerae isolates from surface waters in Bangladesh, which included two O139 and 19 other non-O1 isolates. Ribotyping of the isolates after digestion of their chromosomal DNAs with BglI revealed that all 29 isolates of O139 vibrios belonged to a single ribotype, suggesting a clonal nature of the infection. However, the O139 vibrios comprised two ctxA genotypes, and carried three or more copies of the ctx gene, and the chromosomal locations of these copies were unlike those of the El Tor or classical vibrios. Analysis of the restriction fragment length polymorphisms of the rRNA genes suggested that V. cholerae O139 isolates are more closely related to El Tor strains of V. cholerae O1 than were 19 other non-O1 vibrios and 33 classical V. cholerae O1 isolates that were studied (22, Appendix 4).

Development of DNA probe systems for rapid detection of culturable Vibrio cholerae in environmental samples.

Dr. Morris and colleagues at the University of Maryland School of Medicine/Baltimore Veterans Affairs Medical Center have been responsible for development of a family of non-radioactive DNA probes which permit rapid identification and quantitation of V. cholerae in environmental samples. Briefly, samples are plated onto non-selective media, incubated for 8 hours, and colonies transferred to Whatman 541 filters by colony lift. As previously reported (73, Appendix 5) filters are microwaved, treated with proteinase-K, and then hybridized with alkaline phosphatase-labeled oligonucleotide probes. Colonies of interest are readily identifiable on the filter, based on the presence of a blue color; by counting the number of blue dots on the filter, the number of probe-positive organisms in the original samples can be directly calculated. Total time from sample collection until results are obtained is less than 12 hours, including an 8-hour incubation period. If desired, probe-positive colonies can be identified and picked for further study by matching up blue dots on the filters with colonies on the original plates.

Probes which are currently available are as follows:

1) CTAP: CTAP is a 23 bp cholera toxin-specific alkaline phosphatase-labeled probe derived from the region of the ctsA gene which is specific for V. cholerae. Unlike previous probes, CTAP does not hybridize with E. coli heat labile enterotoxin (LT) or with V. mimicus CT-like toxin. In one series of studies (46), CTAP correctly identified 100% of toxigenic V. cholerae O1 (n=64) and non-O1 (n=11), but was negative for nontoxigenic V. cholerae (n=205), LT-positive E. coli (n=22) and other Vibrio species.

2) O1SAP: O1SAP is a 21 bp alkaline phosphatase-labeled probe derived from the rfbS gene sequence from V. cholerae O1. In preliminary screening, this probe hybridized with 40 strains of V. cholerae O1, including El Tor and classical biotypes of both Inaba and Ogawa serotypes. In contrast, the probe failed to hybridize with over 20 V. cholerae O139 Bengal isolates or 40 other non-V. cholerae (39).

3) O139AP: The group at the University of Maryland has identified a 35kb insertion which appears to be responsible for the shift from the O1 to the O139 Bengal serogroup, and the biosynthesis of the O139 Bengal capsular polysaccharide (17, 17A). This region has been cloned, and approximately 20kb sequenced. Several candidate probes have been derived from this region. All provide excellent discrimination and differentiation between O1 and O139 strains; however, there is some cross-reactivity with other non-O1 V. cholerae strains. Studies to identify probes with optimal sensitivity and specificity are in progress, and should be complete by the time work on this project is underway.

DNA fragments from the above 11 kb region do not hybridize with O1 strains (over 50 strains tested); however, fragments do hybridize with some other non-O1 V. cholerae strains, and with V. damsela. Candidate oligonucleotide probes from the 11 kb fragment, including the junctional region, are now being synthesized, and their sensitivity and specificity as probes for V. cholerae O139 evaluated. Based on these results with the fragment probes, it should be possible to identify probes sequences which will, at a minimum, permit differentiation of O1 and O139 strains.

This methodology is simple, reproducible, and relatively inexpensive, particularly when compared with conventional microbiologic techniques. These probes are currently being employed in ongoing environmental studies in Peru. During the 6 months of sampling for which complete data are available (November, 1993 - April, 1994), a total of 82 environmental samples were evaluated by probe and conventional microbiologic methods. In 26 of the samples (32%) V. cholerae was detected by probe. Non-quantitative conventional selection of sucrose positive colonies from TCBS yielded only 12 samples (15%) that were positive for this organism. These results are not surprising, as the probe data indicated that only 0.5%-10% of sucrose-positive colonies grown on TCBS were V. cholerae; as it is not possible to run biochemical assays on every sucrose colony on a TCBS plate, there is a reasonable possibility that V. cholerae present in the sample will be missed if nothing other than conventional microbiologic techniques are used.

Animal Protection Studies: Studies in animals have been done to confirm the epidemiological observations that prior exposure to V. cholerae O1 seemed not to be protective against infection with V. cholerae O139 (10). Oral immunization of adult rabbits with either V. cholerae O1 or O139 resulted in strong protection to homologous challenge, while no heterologous protection could be detected. (2,3), thus corroborating the epidemiologic data.

Environmental Studies: Ten Site Study of Ponds and Rivers: A three-year study of ten sites was carried out in Matlab, Bangladesh by Dr. Anwar Huq and colleagues from the University of Maryland from February 1987 to January 1990. Eight ponds and two river sites were selected on the basis of various levels of human use; water and plankton samples were collected every two weeks. Studies done include 1) bacteriological analysis for Vibrio, Aeromonas and Plesiomonas, 2) identification of zooplankton and phytoplankton, and 3) measurement of approximately 30 chemical parameters of the water. Hours of sunshine and

rainfall data were recorded. In addition to the regular sampling plan, water and plankton samples were also collected from pond or river site when a cholera case was reported. Rectal swabs were also obtained from all family members of the patient to detect any non-symptomatic carriers.

The data obtained from this 10 fixed site sampling indicate that the highest average water temperature, 32°C, was recorded in June and water turbidity was lowest during September through November. The most fascinating part of the study was the demonstration, for the first time, of the presence of V. cholerae O1 in the aquatic environment year round, as detected by a fluorescent antibody technique in plankton. In addition, among all the genera of zooplankton and phytoplankton examined, copepod nauplii were found to have the closest association with V. cholerae. Over 63% of the total plankton samples were positive for V. cholerae by FA. However, a greater number of water samples were culture positive compared to plankton samples. The viability of V. cholerae O1 in the culture-negative plankton samples, unfortunately, was not further pursued.

A 22-kDA protein band was observed in all the environmental isolates but not in the clinical isolates (32) from this study. When these environmental strains were passaged through rabbit-ileal loops, a significant decrease in protein bands including the 22-kDA band, was observed, suggesting an environmentally induced change (13).

Studies of algae: It had been previously demonstrated that toxigenic V. cholerae O1 can enter the mucilaginous sheath of a blue-green algae Anabaena variabilis, persist there for more than 15 months (34), and also maintain their progeny but become nonculturable. More recently, a field study was carried out to determine what role, if any, blue-green algae play as a reservoir of V. cholerae O1 in the aquatic environment of Bangladesh (36, Appendix 6). Blue green alga, Anabaena species, were collected from a pond in Dhaka city every 15 days for one year, and cultured for vibrios with enrichment techniques; subsamples were preserved in 4% formalin for detection of non-culturable forms by a fluorescent antibody technique. V. cholerae O1 was not recovered by culture; however it was detected by immunofluorescence in the mucilaginous sheath of Anabaena species in 16 of the 24 algal samples examined. V. cholerae could not be identified during the months of January, February, June, or July, when very few Anabaena were present in the plankton samples. This study demonstrates an association of V. cholerae O1 and the blue-green algae Anabaena in the natural aquatic environment in Bangladesh, which could be important in the survival of O1 vibrios in the environment.

Studies of V. cholerae O139: Additional surface water sampling in Matlab and Dhaka during the O139 epidemic revealed that 5/54 water samples were positive for toxigenic O139 vibrios. Non-culturable forms of V. cholerae O139 were also detected in association with copepods (33A, Appendix 5).

4. RESEARCH DESIGN AND METHODS

Sentinel Surveillance System

Geographic areas of study: Four sentinel surveillance areas, each with a population of about 200,000, have been selected on the basis of a) geographic distribution, b) risk of being affected by epidemic outbreaks as determined by historical information, c) access both during the monsoon and dry months, and d) availability of appropriate medical facilities. (See Figure 5, Appendix 7). **Water and sanitation facilities in all these areas are not known in detail; available information is provided below. For the country as a whole, 85% of the population has access to tubewell water, meaning that they are within 100 meters of a tube well. On the other hand, the percentage of the population that have sanitary latrines is estimated at about 15%.**

1. The northern-most site is in Gangachara, in the District of Rangpur. This part of the country is relatively dry, and has a relatively cold winter season. **The population is estimated to be about 175,000; there are approximately 1,400 tubewells (1 per 125 persons). No specific data are available for the number of sanitary latrines.** Cholera outbreaks continue to be due to V. cholerae O1; **during 1989, the attack rate during the epidemic season was estimated at about 9.6 per 1,000.** The ECPP team of ICDDR,B has visited the area several times, and has established a diarrhea treatment center there.

2. The central part of the country will be represented by Matlab, where cholera surveillance has been going on for approximately 30 years. A diarrhea hospital and laboratory are in place. (See description of facilities). **The population is known to be 200,000; there are approximately 1,700 tubewells (1 per 117 persons). Only 6% have sanitary latrines.** Both O1 and O139 vibrios are presently causing cholera; **twice-yearly cholera epidemics occur on a regular basis.**

3. The southern-most site is in Bakerganj, in the District of Barisal. **The population is estimated to be 340,000; there are approximately 3,000 tubewells (1 per 113 persons), and approximately 11,500 sanitary latrines (approximately 3% of the population).** This area is traditionally known for its frequent cholera outbreaks, and the ECPP team of ICDDR,B has visited it many times. In this area, V. cholerae O139 has essentially replaced O1, which was predominantly of the classical biotype. **During the 1993 epidemic, the attack rate was estimated to be 10 per 1,000.**

4. The western-most site is in Jessore; this area rarely has cholera outbreaks and is chosen because of that feature. **The population is estimated to be about 200,000; no specific information on numbers of tubewells or sanitary latrines was available at this time. It is assumed that these parameters will be similar to the country as a whole.**

From sites number 1 and 3, it is expected that about 200 cases per year would receive treatment (incidence rates conservatively estimated at 1:1000 per year) and about 45 cases would be seen on the days of surveillance. In all, about 140 cases would be expected to

have been detected during each year. For site number 2 (Matlab) an incidence rate of 3-4: 1000 is expected, based on data obtained over the last 27 years (See figure 2). An incidence rate of <1:1000 is expected for site number 4, with the likelihood that there will be no cholera cases.

Most cases would be expected to occur during cholera seasons which generally last for 2 to 3 months, and during these times about 20 to 30 cases should be seen at each site per cholera season. Figure 3 depicts the seasonality of cholera in site number 2 (Matlab).

Clinical Surveillance activities:

The proposed surveillance will be facility-based and conducted in the government-owned thana (sub-district) health centers which offer outpatient services for primary health care and family planning and have 31 inpatient beds. (Matlab is the exception as this is an ICDDR,B diarrhea treatment facility) Though these centers serve a larger catchment population, defined unions (administrative subdivisions of thanas) with a combined population of approximately 200,000 (based on the Government of Bangladesh census and the government's annual Geographic Observation and Rectification report) will be identified as the "sentinel surveillance unions." **Persons residing in these unions who come for care at the thana complex will be included in the surveillance.**

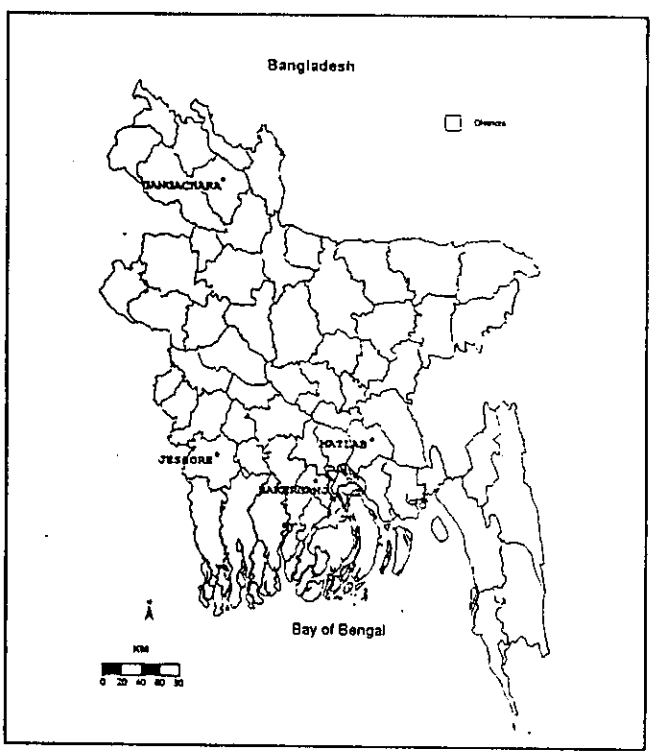

Figure 5 Surveillance Sites

Survey strategy: **Clinical surveillance will be carried out every two weeks for three successive days during each survey period with staff (physician epidemiologists) rotating among the sites to avoid staff worker bias, and with a systematic rotation to avoid "day bias" (e.g. first survey starting Sunday, second survey starting Monday, etc.) (In addition to their duties as epidemiologists, they will teach the government staff about proper case management of diarrhea.)**

Information from patients residing in the identified unions (including temporary guests) attending the facility for diarrhea during the three survey days will be recorded on precoded data forms. All such patients (or parents) who give their informed consent will be included in the survey. (With this type of survey in which there is only benefit and no risk, we expect nearly universal cooperation.) The study surveillance staff will administer a detailed questionnaire (Appendix 6) to collect personal data and information about signs and symptoms of illness, prior medical history, breast feeding status in children under 5 years,

and previous therapy. A detailed physical examination including vital signs and the state of hydration will be performed.

Definitions: Diarrhea will be defined as at least three watery or loose motions, or loose motions with blood in a 24-hour period. Assessment of state of hydration will use WHO criteria and patients will be treated according to standard case management strategies as defined by WHO and the ICDDR,B. A case of cholera will be defined as a patient who comes to the treatment facility because of acute diarrhea (without blood) of less than 7 days duration and has a positive fecal **identification** of V. cholerae O1 or O139.

Although not a primary objective of this study, other diarrheal illnesses will be recorded, though not studied microbiologically. Persistent diarrhoea will be defined as diarrhea with or without blood, with symptoms lasting 14 days or more. Dysentery will be defined as diarrhea with a history of blood during the episode or observable blood in the stool at the time of clinic visit.

Laboratory support of clinical surveillance: Fecal specimens will be obtained from survey patients with acute watery diarrhea for isolation of V. cholerae using Cary-Blair transport media and will be returned to the enteric microbiology laboratory at the ICDDR,B in Dhaka (or at Matlab for this surveillance site). V. cholerae will be isolated following alkaline peptone water enrichment, using TTGA agar and will be identified using standard techniques for identifying biotype and serotype (including agglutination with antisera to O139) (72). All isolates will be saved for further molecular studies in comparing clinical isolates with environmental isolates. Isolation for other pathogens will not be carried out (except in the case of Matlab where a limited number of other pathogens (Salmonella and Shigella) are routinely sought.

Rapid diagnostic tests will also be carried out in the field sites on selected patients, particularly early in a suspected epidemic period. The SMART test for detection of O1 and O139 vibrios (made by New Horizons Diagnostics)(29) and a coagglutination test (51) will be used for these purposes. Data from these rapid tests (which have already been validated at the Dhaka hospital [50,51, 51A) will be included in the data base together with the culture results.

A computerized data base system for managing microbiology (and other clinical laboratory) records has been in place since 1986 and more than 9000 specimens are added to the system each month. This system will be used to manage the laboratory results. Patient identifiers included with each sample will allow linkage of the laboratory results with the clinical information.

Environmental Studies

The search for V. cholerae will primarily involve surface water sampling, but will particularly focus on examination of plankton. Samples of water, sediment, plankton (zooplankton and phytoplankton), and algae will be collected every two weeks year- round from two selected ponds and two river sites from each of the surveillance areas. The sites

selected will represent typical village water bodies, (meaning that people use them for bathing, washing clothes, taking water for cooking and occasionally for drinking) but they will be separated from market areas or the hospital, to avoid unusual effects of excessive contamination from these sources.

Sample collection and processing: Surface water will be collected (5 cm below the surface) using pre-sterilized 1 liter bottles. Sediment samples will be collected by means of a core sampler and stored in 4-oz glass bottles. Samples for DNA probe analysis will be directly plated on the non-selective media on site. In addition all collected samples will be transported to the laboratory in an insulated foam box with temperature maintained (if necessary with ice packs) in situ. The team will take transport and plating media and supplies to the field so that samples will be processed at the field site, generally within 4 hours of collection. (All procedures are now routinely performed by the environmental microbiology staff at ICDDR,B, based on methods introduced by Colwell and the University of Maryland laboratory during the past 20 years.)

Using a 20 µm mesh size net, plankton samples (both phytoplankton and zooplankton) will be collected from water samples. Using a larger mesh plankton net, zooplankton and phytoplankton will be separated in the laboratory to be used for direct detection of *V. cholerae* O1 and O139 and for speciation of various members of the plankton population. Among the aquatic flora, species of blue green algae (Anabaena variabilis, Microcystis aeruginose, Microcystis flos-aquae, Lygbia species, Nostoc species, Merismoopedia species, Ocillatoria species, Nodularia species) will be identified. In addition green algae and higher aquatic plants (Eichornia crassipes [water hyacinth], Luodwigia repens, Monochoria hastata, Marsilea quadrifolia, Ipomoea aquatica, Lemna minor (duckweed) will also be collected and identified. Among the aquatic fauna, copepods, (Acartia tonsa, Pontellopsis regalis, Pleuromamma species, Labidocera aestiva, Centropages furcatus, various crustacean larvae, and other young adults in plankton form, will be enumerated for quantitative analyses to determine the association of *Vibrio cholerae* with these organisms.

In addition to plankton and hydrophites, fish, oyster, crabs and snails, etc. will be collected whenever possible and these specimens will also be tested to directly detect V. cholerae on surface structures. The identification of fauna and flora will be carried out in collaboration with the University of Dhaka, Dr. K. Muniruzzaman of the Botany Department, and Dr. S. Ali of the Zoology Department, respectively.

Physicochemical measurements will be made at each sampling site at the time of sample collection; this will include temperature, dissolved oxygen, pH, salinity, Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD), and dissolved oxygen content. The number of hours of sunlight, the rainfall and the ambient temperature will be obtained from the weather bureau.

Sediment samples will be shaken vigorously for three minutes before aliquots are taken for microbiology examination. Plankton samples will be homogenized in a teflon tipped

tissue grinder (Wheaton Scientific, Millville, NJ) using a steadfast stirrer (Model 300 Fisher Scientific).

The samples collected will be tested directly, but an aliquot will also be stored frozen in a specimen bank. The specimen bank will allow PCR studies to be done at a later date; this will be particularly important during time periods just prior to epidemics, which at present are unpredictable. Most of the banked specimens will not be tested, but the availability of the set of specimens should prove useful for more detailed studies in the future.

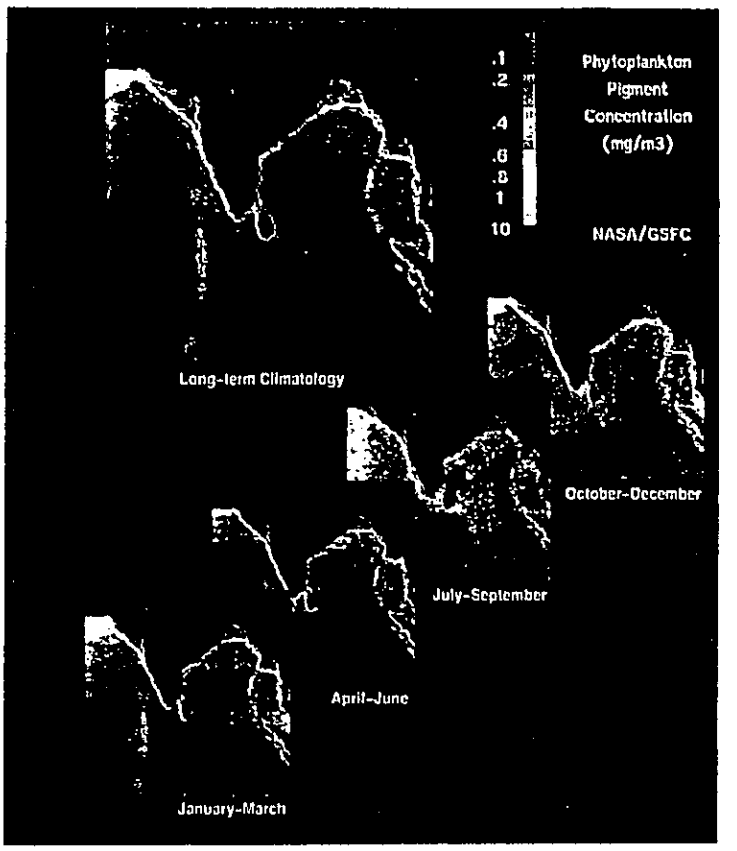

Figure 6

Geographic Evaluation of the Sites: **The Matlab area has been completely mapped using Geographic Information Systems techniques, so that all locations of rivers, ponds, canals, schools, mosques and houses are known. The other three sites will have locations of rivers, ponds, canals determined, although complete mapping, as has been done in Matlab, will not be possible.**

Remote Sensing of Phytoplankton Blooms With the assistance of collaborators, satellite data of Bangladesh, and particularly the areas of our surveillance will be available. In this study, chlorophyll measurements by remote sensing will be related to environmental water sampling within periods of epidemic cholera at the surveillance sites. (18,19, Figure 6, Appendix 8).

Satellite remote sensing from the coastal zone color scanner (CZCS) (chlorophyll measurements, in operation from 1979-1986) and Advanced Very High Resolution Radiometer (AVHRR) (measuring sea surface temperatures [SSTs] have been used to detect phytoplankton blooms (19,50)and to calculate primary productivity (20). Real-time AVHRR images are currently used to follow potentially toxic phytoplankton blooms (confirmed with targeted sampling) associated with fish and shellfish poisonings (59).

In the future, images from SeaWiFS (the Sea Viewing Wide Field Sensor) scheduled for launch fall, 1994, and containing two additional blue and one additional green spectral bands for improved estimates of chlorophyll and chlorophyllous organisms will be available

for following phytoplankton blooms, potentially improving prediction and early warning of cholera outbreaks.

Methods for detection of V. cholerae

Environmental samples will be processed to detect both culturable and non-culturable V. cholerae by the following methods: a) DNA probes, b) standard bacteriologic techniques, including total viable counts c) immunomagnetic techniques, d) immunofluorescent (FA) methods, and e) PCR.

All samples will be screened by DNA probes, cultured bacteriologically, and assayed by FA. PCR will be done later on saved specimens that are negative by the other techniques. The sensitivity and specificity of the immunomagnetic bead technique will be determined early in the studies in order to decide whether it should be used routinely to concentrate vibrios prior to their further identification by the other methods. With these techniques we will be able to determine the presence of both culturable and non-culturable V. cholerae O1 and O139, and to correlate their presence with the clinical pattern of cholera geographically.

DNA probes:

Screening of environmental samples: Quantitative data on viable, culturable bacteria will be obtained by DNA probe techniques, with standard microbiologic techniques used as a back-up. Each sample will be serially diluted (or concentrated where appropriate), and each sample plated on selective and non-selective media (see section below). Non-selective media will be used to determine total viable counts and to screen for the presence of rugose colonies; filters prepared by colony lifts from these plates will be probed with DNA probes for cholera toxin, and the O1 and O139 antigen. Use of probes has several advantages:

a) As colonies of interest are directly counted, it is possible to obtain direct quantitative data for the organisms of interest. Standard microbiologic methods generally involve use of MPN methods, or direct counting from selective media. Our data suggest that actual counts can exceed those obtained with selective media such as TCBS by two orders of magnitude; i.e. while selective media reduce overgrowth of other organisms, they also reduce counts of the organism of interest. Use of probes also obviates the need for further testing of colonies seen on selective media: as noted in preliminary data, studies from Peru suggest that in screening environmental samples, only a minority of sucrose-positive colonies on TCBSA media are actually toxigenic V. cholerae.

b) It is possible to immediately, and quantitatively, identify toxigenic V. cholerae O1 and O139 present in samples. Outside endemic areas/epidemic settings, over 50% of V. cholerae O1 may not be toxigenic. By screening filters with CTAP, O1SAP, and O139AP probes, we can directly quantitate the number of toxigenic O1 strains, toxigenic O139 strains and toxigenic non-O1 strains in each environmental sample.

c) The methodology is simple and rapid, and permits screening of a much larger number of samples than would be possible by using standard microbiologic techniques. As there is no necessity for picking colonies or other biochemical identification, a much larger number of samples can be processed. It has been demonstrated that the techniques are easily applicable to field conditions, and they should be able to be used without difficulty in Bangladesh.

Bacteriologic Culture Techniques: The standard bacteriology plates will include GTTA, TCBS, and L agar (noninhibitory agar when used with colony blots was found to give a higher yield even though colonies could not be selected).

Homogenized plankton samples will be cultured onto the standard agar both directly and after enrichment in bile peptone water (BPW). Soil will be cultured after enrichment in BPW. These samples will also be tested using direct nucleic acid extraction and PCR (65).

To determine bacterial counts of vibrios, 100 to 1000 ml of water (volume depends of turbidity) will be filtered through a 0.22 um membrane filter (with prefilters if needed for turbid water). The filters will be placed onto the various media and incubated for 18-24 hours at 37C. Vibrio-like organisms will be picked and confirmed using standard biochemicals as V. cholerae and will be tested using antisera for O1 and O139.

To search for rugose colonies on a sample of specimens, one filter from the water sample will be subjected to chlorine 0.5 mg/liter for 2 minutes and then enriched with BPW for 6 hours prior to plating on TTGA. Strains recovered will be examined further on blood agar or L agar to determine if characteristic morphology is seen. A diagnostic antiserum specific for rugose colonies (from Dr. Glenn Morris) will be used to confirm the rugose phenotype.

Immunomagnetic Capture: Another rapid and sensitive method for the detection of V. cholerae O1 and O139 from the environmental samples will be used which is based on capture of the bacteria with immunomagnetic particles (37,49). The particles will be coated with either of two different monoclonal antibodies specific for the O-antigenic polysaccharide of the LPS of V. cholerae O1 (69) and O139 (50). Captured bacteria will be detected by an enzyme immunoassay with LPS-specific rabbit antisera (37). An additional method which may increase sensitivity is to use a membrane bag filled with immunobeads; this is then used much like a Moore sway, and placed in a body of water for varying periods. In this system, bacteria can enter the bag and become attached to the immunobeads, but the immunobeads do not escape. Vibrios are then separated and assayed by the usual methods.

Fluorescent Antibody Techniques: Water and plankton samples will be tested by fluorescent microscopy following procedures described previously (8,30). To detect viable but non-culturable V. cholerae O1 and O139 cells, the following method, combining DFA (direct fluorescent antibody (27) and DVC (direct viable count) will be employed (12, Appendix 9). To a one ml sample of concentrated water or homogenized plankton, 50 µl of 2% filter-sterilized yeast extract and 10 µl nalidixic acid (1 mg/ml stock solution) will be added. The mixture will be agitated gently in a microfuge tube and incubated for 6-18 hours at 25°C in the

dark. After incubation, each sample will be fixed by adding 2% formaldehyde. Formalin-fixed samples will be transported to the University of Maryland for analysis, where direct fluorescent antibody assays will be done using specific, monoclonal antibody prepared against V. cholerae O1 and O139 serogroups. (51A) V. cholerae O1 and O139, in both the culturable and non-culturable state, will be used as controls.

PCR Assays:

Extraction of DNA: Isolation of nucleic acids from aquatic environments will be done following the methods of Sommerville et al. (65). Concentration by filtration: After collection, water samples will be transferred to sterile 2-liter Nalgene flasks (Nalgene Labware DN., Nalge/Sybron Corp., Rochester, N.Y.) and aseptically pumped through Sterivex-GS filters (Millipore Corp., Bedford, Mass.) via a peristaltic pump. Filters will be washed with 10 ml of sterile SET buffer (20% sucrose, 50 mM EDTA, 50 mM Tris hydrochloride [pH 7.6]). Excess buffer is forced off the filter by using an air-filled 50-ml syringe, the inlet and outlet capped, and the filters stored at -20°C until processed.

Cell Lysis and Nucleic Acid Extraction: Nucleic acids will be extracted essentially by the method as described elsewhere (57) with modifications for extractions to be accomplished within the Sterivex-GS filter housing.

Purification and concentration: Nucleic acids will be purified and concentrated from the crude lysates by ethanol precipitation, as described in Sambrook, et al.(57).

Amplification of DNA by PCR: PCR will be carried out as described by Shirai et al. (60) with modification as follows. Three sets of oligonucleotide primers, flanking 302-, 564- and 777-bp sequences within the *ctx* gene, will be synthesized. DNA samples, extracted either from the water or plankton, will be used for amplification of the *ctx* gene.

Detection of Amplified DNA. Following amplification, 10µl of the PCR products will be analyzed by electrophoresis on a 1.0% agarose gel in TAE(1x) buffer for 1 hour.

Detection of PCR-amplified products with the alkaline phosphatase labeled oligonucleotide probe: The PCR-amplified products (as described above) will be analyzed by hybridization with the V. cholerae CT DNA as a probe. The sequence of the CT probe is 5'-ACTATATTGTCTGGTCATTCTACT-3' (60). Before hybridization, the DNA will be incubated in 0.3 M NaOH for 5 min at 70°C and 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°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, 0.5% 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.

Additional molecular studies linking strains: V. cholerae isolates from the patients with cholera and from the environment will be characterized genetically and phenotypically to determine whether they are related, thereby suggesting a causal relationship.

The strains will also be compared with lyophilized strains in the ICDDR,B collection (23). These strains are from endemic and epidemic cases of cholera collected since 1965 from the Dhaka and Matlab hospitals, as well as strains from epidemics in various rural districts of Bangladesh; they include both classical and El Tor isolates from 1965 to the present, and several hundred isolates of O139 vibrios since 1993. In addition, the collection includes approximately 200 strains from various geographic locations in Asia, Africa and the Americas.

Analysis of restriction site polymorphisms.

High molecular weight chromosomal DNA will be isolated from strains grown overnight in T1N1 broth as described by Stull et al (68). Approximately 5ug 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 ug of DNA. The digested DNAs will be electrophoresed in 0.8% agarose gels in Tris-borate-EDTA buffer by a standard method, and transferred onto nylon membranes (Hybond-N, Amersham) by Southern blotting (64).

The rRNA gene probe is a 7.5 kb BamHI fragment of pKK3535 which is a pBR322 derived plasmid containing an E. coli ribosomal RNA operon consisting of one copy each of the genes coding for 5S RNA, 16S RNA, 23S RNA, and tRNAGlu. The DNA probe for cholera toxin genes consists of a 554 bp XbaI-ClaI fragment encoding 94% of the A-subunit of cholera toxin cloned into pBR325 through EcoRI linkers. The recombinant plasmid pCVD27 has been kindly provided to us as transformed culture by Dr. James B Kaper, Centre for Vaccine Development, Baltimore, MD. The recombinant plasmids will be prepared, digested with appropriate restriction enzymes, and the inserts will be separated by electroelution from agarose gel as described by Maniatis et al (47).

The probe DNAs will be labeled by random priming (24) using [a-32P]-deoxycytidine triphosphate (3000 Ci/mmol, Amersham International plc., U.K.) and a random primers DNA labeling system (Bethesda Research Laboratory). Southern blots will be hybridized and washed under stringent conditions. Autoradiographs will be developed from the hybridized filters using Kodak X-Omat AR film (Kodak Rochester, NY), with intensifying screens at -70°C for 24 hr.

Plasmid analysis

Plasmids will be prepared by the alkaline lysis method of Birnboim and Doly (7) from a 1.5 ml overnight culture of the bacteria in Luria-Bertini (LB) broth. The extracted plasmid DNA will be electrophoresed in a 0.7% agarose gel by standard methods (47). The gels will be stained in 0.5% ug/ml aqueous ethidium bromide for 20-30 min and photographed on type 57 Polaroid film using short wave ultraviolet light. Plasmid size

030800

will be extrapolated from a plot of band migration distance versus the log of molecular size (base pairs) using supercoiled DNA standards of known size.

Multilocus Enzyme Electrophoresis (MEE)

Isolates will be assayed for allelic variation in 12 enzyme loci as described by Selander et al (58).

Antimicrobial susceptibility tests

The antimicrobial susceptibility tests will be done by the method of Bauer et al. (6) using commercially available disks (BBL Microbiology Systems).

Data analysis of molecular studies:

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. Vibrio strains isolated throughout the study will be compared to see whether there are shifts in clonality. The vibrio strains isolated during this study will be compared to isolates in the ICDDR,B culture collection to determine how they have changed over the past 30 years.

Genetic and phenotypic data obtained from the study will be analyzed using computer programs as we have described previously. (45,58.23A). Genetic distances between pairs of isolates will be calculated as the proportion of loci at which dissimilar alleles occur, i.e. the proportion of mismatches and this will be expressed by a coefficient as described by Nei (48). Dendrograms will be generated using the data to determine divergence and evolutionary relatedness among different clones of V. cholerae.

Overall Data Analysis

Summary analysis of the clinical surveillance. Based on the survey samples, data will be extrapolated to estimate rates of cholera in the survey unions monthly, quarterly and annually. For these rates the estimated numbers of cholera cases will be based on the number of cases from the defined unions detected during the surveillance days divided by 0.214 to correct for sampling 6 out of 28 days. The population of the defined unions will be the denominator. Age and sex specific rates will also be calculated based on numbers of cases in the appropriate subgroups but because of low numbers in subgroups, these rates will be calculated annually. Age groups will include <5 years, 5 - 14, 15 - 45, and > 45.

Data from each sentinel site will be plotted temporally to determine seasonality of cholera, overall diarrhea cases, dysentery and persistent diarrhea, and in-hospital cholera and diarrhea case fatality rates in the various surveillance sites. These data will be reported

to the Government of Bangladesh CDD program manager (Diarrheal Disease Program), as well as to the managers of the thana medical centers in the study.

Predicting cholera illness incidence: Since the goal of this study is to understand the patterns of cholera's seasonality in relation to environmental factors and the detection of V. cholerae in culturable or nonculturable form, the data will be computerized to develop a model suitable for predicting cholera illness incidence. We plan to regress cholera illness incidence against the risk factors in order to identify which risk factors prove to be associated with increased cholera incidence, i.e., outbreaks. Obviously, events preceding outbreaks will be more important than those which occur subsequently, since only these will have predictive power.

We will use Poisson regression to carry out the analysis. Since cholera illness incidence follows seasonal patterns (see Figure 3) there is correlation structure in the dependent variable. We will accommodate this correlation structure into the Poisson regression by using the general estimating equation (GEE) approach (75). Since both the dependent and independent variables are time dependent, the regression will be done in the "event-history" framework. For example, let xj(t) be the jth risk factor at time t, and let y(t) be the cholera incidence, within a sentinel surveillance area, at time t, where time is measured in months. In the regression, we will look for correlation between y(t) and x_j (t-**tau**) for various time lags **tau** > 0. This will be carried out in a multivariable fashion for all the important risk factors. For example, if PCR results from river water always show a rise in the number of positive samples during the month prior, **tau** = 1 month, to an outbreak, then we will want to quantify the strength of association between this risk factor and cholera incidence in the regression model.

We will use regression diagnostics for multicollinearity (41) to eliminate highly correlated risk factors from the model. For example, culture of water and culture of water plants may not reveal any additional information, therefore, the sample which can be obtained more simply and consistently will be used.

Intermediate events will also be sought to determine if a chain of events leads to a higher probability of a cholera outbreak. For example, high temperature and sunlight may lead to an algal bloom which might sometimes (but not always) be followed by a cholera outbreak. In this case the algal bloom would be enhancing, but by itself not be sufficient to lead to an outbreak, and might require other factors as well. The Poisson regression employs a multiplicative model, and thus, is well-suited for quantifying the effects of risk factors that operate along a single causal pathway (43). If necessary, we will also use additive (linear) event-history models in order to explore whether selected risk factors are redundant in causing outbreaks (43). Thus, the search for a predictive model will attempt to understand mechanisms as well as look for statistical associations.

In our analysis, we will exploit the contrasts in risk factors across the four sentinel surveillance areas. Koopman and Longini (44, Appendix 10) show that inter-area variation of risk factors can be much more important than intra-area variation for identifying important risk factors for infectious disease transmission. The fourth sentinel surveillance area that

experiences very low cholera incidence will provide key information for the such contrasts. For example, two risk factors that differ between sites 1-3,(that experience high seasonal cholera incidence), and site 4, (that experiences very low cholera incidence), are average number of tubewells per square km, and average water level during the monsoons. Site 4 has a higher density of tubewells and lower water levels during the monsoons. These risk factors will be examined in the Poisson regressions to see if they are predictive of cholera illness incidence.

Sample size and power

Each of the four sentinel surveillance areas will have a population of about 200,000. In the Poisson regression we will be trying to detect risk factors for cholera outbreaks for areas 1-3. The baseline incidence for these areas, expected to be about 1 case of cholera per 1,000 person years of follow-up, will be used as the dependent variable. Independent variables will include, among others, total counts of a) culturable vibrios in water, b) toxigenic O1 and O139 V. cholerae, c) viable but non-culturable V. cholerae, d) vibrios associated with specific plankton, and environmental parameters such as water temperature, salinity, and pH. We expect a risk factor to be associated with a cholera outbreak if the quarterly cholera incidence has a four-fold increase about one month after the risk factor reaches a high level. We have done some preliminary sample size calculations (George, 1984) for this case, where the null hypothesis is that the risk factor in question is not associated with an outbreak, i.e., no increase in cholera incidence, and the alternative hypothesis is that there is a four-fold or greater increase in quarterly cholera incidence. We assume that number of cholera cases follows a Poisson distribution, and that the sampled baseline quarterly cholera incidence will be 0.214 x 0.025 = 0.005 cases of cholera per 1,000 person quarters of follow-up. We set the significance level at 0.05, and power at 0.90. Just considering one area, we would need about 137,000 persons under surveillance for one year follow-up using the figures given above for the power calculation. If we assume that the chance of observing the effect of the risk factor is the same for each year of the study, then the sample sizes become about 69,000, 46,000, 35,000 and 28,000 for 2,3,4 and 5 years of follow-up, respectively, in each of the three areas . For detecting two risk factors, using the Bonferonni correction (Kleinbaum, et al., 1988), the numbers are about 163,000, 82,000, 55,000, 41,000 and 33,000 for 1-5 years of follow-up, respectively, in each of the three areas. Of course, the fact that we have four populations under surveillance, increases power and decreases the required sample sizes, but precise calculations can not be made. Thus, the populations' sizes of 200,000 should be sufficient to screen for the effects of several risk factors.

Time Table for the Project

The study will take 5 years to complete. The first approximately 3 - 6 months will be used to establish the new surveillance sites, and laboratory techniques, with appropriate training of personnel. The surveillance activities will then continue for a full 4 years. The last 6 months will be used for final laboratory work and data analysis.

5. HUMAN SUBJECTS

(1) Human subjects will be included in the surveillance system, but will not be part of any experimental treatments or tests.

Gender and minority inclusion: Both sexes and all racial/religious groups are treated at the facilities and will be included in proportion to their attendance during the surveillance periods.

(2) Clinical data and stool specimens only will be obtained from patients who are hospitalized during the surveillance periods.

(3) All patients admitted during the surveillance days will be requested to be a part of the surveillance. Consent will be requested by the government physician or the ICDDR,B physician.

(4) There are no risks to participation.

(5) There are no risks.

(6) There are no risks, and no experimental devices or drugs will be used.

6. VERTEBRATE ANIMALS.

(1) Monoclonal antibodies will be produced in female BALB\c mice by standard procedures, as described in the protocol, involving immunization and collection of spleens, expansion of clones and production of antibodies from ascites. Approximately 200 mice will be used annually.

Mice will be immunized with V. cholerae 01 antigens using Freund's complete and incomplete adjuvant and will be sacrificed following the immunization. After sacrifice, the spleen cells will be used to fuse with a hybridoma cell line.

Mice will also be used to produce the monoclonal antibodies in ascites fluid. Mice are primed by intraperitoneal injection of 0.5 ml pristane, and the mice are given an intraperitoneal injection with the hybridoma cells. Prior to the development of respiratory distress, the mice are sacrificed and the ascites fluid is collected.

(2) Justification for the use of animals. Monoclonal antibodies are critical to the recognition of certain antigens on the vibrios. BALB/c mice are the standard animal in which to prepare these antibodies.

(3) The animals are maintained in a well-equipped and well-staffed animal care facility at the ICDDR,B. The facility is supervised by veterinarians well experienced in the care of laboratory animals.

(4) Anesthetics are not needed for these experiments since pain is minimal. Animals are observed daily and animals that are ill are sacrificed.

(5) Euthanasia is carried out by cervical dislocation, methods consistent with the Panel on Euthanasia of the American Veterinary Association.

INTERNATIONAL CENTRE FOR
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This represents the budget for the subcontract which the ICDDR,B is proposing in cooperation with the Johns Hopkins University application for an NIH grant (Dr. R. Bradley Sack, PI) from the United States National Institutes of Health. October 2, 1995

ICDDR,B FIVE YEAR BUDGET FOR NIH GRANT

	1st yr	2nd yr	3rd yr	4th yr	5th yr	TOTAL
PERSONNEL	242,420	258,480	275,617	293,907	313,427	1,383,852
CONSULTANT	6,000	6,300	6,615	6,946	7,293	33,154
EQUIPMENT	24,303	0	0	0	0	24,303
SUPPLIES	68,867	71,620	75,276	79,040	82,992	377,795
TRAVEL	34,316	36,032	37,833	39,725	41,711	189,618
OTHER EXP	32,825	36,186	37,945	39,786	41,734	188,476
RENT & UTILITIES	58,000	60,900	63,945	67,142	70,499	320,487
TOTAL DIRECT	466,731	469,517	497,232	526,546	557,657	2,517,683

Assumptions:
1. Salary budgeted for 7% increase annually for national staff *
2. Equipment for ICDDR,B will be purchased during first year

* 5% for international staff

K Tipping

Ken Tipping
Divisional Director, Finance
ICDDR,B

October 2 1995
Date:

J3/NIHBDGT.MJA

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74. Wright AC, Miceli GA, Landry WL, Christy JB, Watkins WD, and JG Morris. Rapid identification of Vibrio vulnificus on nonselective media with an alkaline phosphatase-labelled oligonucleotide probe. Appl Environ Microbiol, 1993; 59:541-546.

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SENTINEL SURVEILLANCE FOR CHOLERA -- NHC01

1. Date of Examination (Day/Month/Year) /__/__/__/__/__/__/

2. **Thana / Year/ Round/ Case no.** /_1_/_9_/_7_/__/__/__/__/__/
(Bakerganj=1, Chhatak=2, Jessore=3, Matlab=4)

3. Union _____ /__/__/

4. *Current Identification* ***(CID)*** *No. (For Matlab only)* /__/__/__/__/__/__/__/__/__/__/

5. *Hospital No.(For Matlab only)* /__/__/__/__/__/__/__/

Patient's name _____ (do not enter into computer)

6. Patient's age (year/month) /__/__/__/__/__/

7. Sex (male=1, female=2) /__/

8. If the interviewee is different from patient, relationship with the patient /__/
(Self=0, mother=1, father=2, brother=3, sister=4, other_____=5)

9. Religion (Muslim=1, Hindu=2, Christian=3, Buddhist=4) /__/

10. Onset of diarrhoea: Date (day/month/year) /__/__/__/__/__/__/__/__/

11. Onset time (hrs) /__/__/

12. Character of stool (watery=1, loose=2) /__/

13. Is there any blood in the stool (No=0, Yes=1) /__/

14. Frequency of stool in last 24 hrs (exact number) /__/__/

15. Vomiting (No=0, Yes=1) /__/

16. Abdominal Pain (No=0, Yes=1) /__/

17. Abdominal cramp (No=0, Yes=1) /__/

18. Feverish feeling in last 24 hrs. (No=0, Yes=1) /__/

19. General appearance (well,alert=1, restless,irritable=2, lethargic or unconscious=3) /__/

case# /__/__/__/

20. Eyes (normal=1, sunken=2, very sunken=3) /__/

21. Mouth & Tongue (Moist=1, dry=2, very dry=3) /__/

22. Skin elasticity (pinch goes back quickly=1, slowly=2, very slowly=3) /__/

23. Pulse rate (Normal=1, Rapid=2, Feeble=3, Imperceptible=4) /__/

24. Dehydration status (No=0, Some=1, Moderate/Severe=2) /__/

25. If this patient is below 3 years of age, is he/she on breast feed (No=0, Yes=1, NAP=8) /__/

26. Received any ORT (None=0, Pkt.ORS=1, LGS=2, ORS=LGS=3) /__/

27-30. Taken any medicine (No=0, Yes=1)
Capsule /__/ (27) Tablets /__/ (28) Syrup /__/ (29) IV /__/ (30)

31. Any other member of the family having diarrhoea, if yes, how many (none=0) /__/

32-36. Source of drinking water (No=0, Yes=1) Tubewell /__/ (32) Dugwell /__/ (33) Pond /__/ (34) Canal /__/ (35) River/__/ (36)

37-41. Source of water for washing utensils (No=0, Yes=1)
Tubewell /__/ (37) Dugwell /__/ (38) Pond /__/ (39) Canal /__/ (40) River /__/ (41)

42-46. Source of water for bathing (No=0, Yes=1)
Tubewell /__/ (42) Dugwell /__/ (43) Pond /__/ (44) Canal /__/ (45) River /__/ (46)

47. Rectal swab (Taken=1, Not taken because Blood=2, Refused=3) /__/

48. Hospitalized (No=0, Yes=1) /__/

49. Lab ID No /__/__/__/__/__/__/__/__/__/

50. Interviewer's signature _____ Date_____

51. Form completed /__/ Supervisor's signature _____/__/__/ Date_____

52. Data entered /__/ Data entry's signature _____/__/__/ Date_____

Consent Form

International Centre for Diarrhoeal Disease Research, Bangladesh (Cholera Hospital), Mohakhali, Dhaka is the centre for health and population research and clinical services for diarrhoea patients. We have come from the above Centre to this thana health complex for a project titled "Epidemiology and Ecology of *V cholerae* in Bangladesh". The main objective of this project is the decrease of cholera mortality and morbidity in Bangladesh. A questionnaire with clinical signs and symptoms will be administered to you or your parent/ guardian after you have been examined by a doctor in this hospital. We also would like to ask you some questions about your family and your living conditions. Rectal swabs will be taken for culture of *V cholerae* and its sensitivity.

Each interview will take about 20 minutes. All the information collected will be kept confidential. There are no risks for you in participating in this study.

If you wish to withdraw from the study at any time, your are free to do so, even then you will get the standard treatment of this disease from this hospital.

If the above conditions are acceptable to you, please sign or give thumb impression as indicated below on this form.

Signature of the investigator

Date ----------

Signature/left Thumb Impression of the patient/guardian

Date ----------

Witness----------

/30/96 93,856
AND NOTICE dated ... except that any additions or ... previously imposed remain in effect unless specifically rescinded.

201 AI39129-01A1

5 ADMINISTRATIVE CODES

Mo./Day/Yr. 68 B Mo./Day/Yr.

9/01/96 Through 08/31/01

9/01/96 Through 08/31/97

(OR PROGRAM) (Limit to 56 spaces)

EMIOLOGY & ECOLOGY OF VIBRIO CHOLERAE IN BANGLADESH EDC-2

AND ADDRESS

NS HOPKINS UNIVERSITY
N WOLFE ST
TIMORE, MD 21205

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE

NATIONAL INSTITUTES OF HEALTH
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES EXTRAMURAL

NOTICE OF GRANT AWARD

AUTHORIZATION (Legislation/Regulation)

42 USC 241 42 CFR 52
RESEARCH

10 DIRECTOR OF PROJECT (PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR) (LAST NAME FIRST AND ADDRESS)

SACK, R BRADLEY MD
JOHNS HOPKINS UNIVERSITY
615 N WOLFE STREET
SCH HYGIENE & PUBLIC HEALTH
BALTIMORE, MD 21205

(Excludes PHS Direct Assistance)
Funds Only
t costs including grant funds and all other financial participation [I]
elect one and place NUMERAL in box.)

and Wages	$ 0
enefits	$ 67,111
tal Personnel Costs	$ 67,111
t Costs	0
t	0
	2,000
	22,850
are — Inpatient	0
— Outpatient	0
s and Renovations	0
	4,000
m/Contractual Costs	716,326
lated Expenses	
ipends	0
ition and Fees	0
avel	0
L DIRECT COSTS →	$ 812,287
OSTS Rate ☆ % of S&W/TADC	$ 133,253
ROVED BUDGET	$ 945,540
	$ 0
are	$ 945,540
al Share	$

ms and Conditions Attached — ☑ Yes ☐ No

12 AWARD COMPUTATION FOR FINANCIAL ASSISTANCE

a. Amount of PHS Financial Assistance (from Item 11.v.)	$ 945,540
b. Less Unobligated Balance From Prior Budget Periods	$ 0
c. Less Cumulative Prior Award(s) This Budget Period	$ 0
d. AMOUNT OF FINANCIAL ASSISTANCE THIS ACTION	$ 945,540

13. RECOMMENDED FUTURE SUPPORT (SUBJECT TO THE AVAILABILITY OF FUNDS AND SATISFACTORY PROGRESS OF THE PROJECT)

YEAR	TOTAL DIRECT COSTS / STIPENDS	YEAR	TOTAL DIRECT COSTS / STIPENDS
a. 02	880,886	d. 05	988,499
b. 03	915,683	e.	
c. 04	951,094	f.	

14. APPROVED DIRECT ASSISTANCE BUDGET (IN LIEU OF CASH)

a. Amount of PHS Direct Assistance	$
b. Less Unobligated Balance From Prior Budget Periods	$
c. Less Cumulative Prior Award(s) This Budget Period	$
d. AMOUNT OF DIRECT ASSISTANCE THIS ACTION	$

SEP 4 1996

15. PROGRAM INCOME SUBJECT TO 45 CFR PART 74, SUBPART F, OR 45 CFR 92.25 SHALL BE USED IN ACCORD WITH ONE OF THE FOLLOWING ALTERNATIVES: (Select One and Place LETTER in box.) [B]

a. DEDUCTION
b. ADDITIONAL COSTS
c. MATCHING
d. OTHER RESEARCH (Add/Deduct Option)
e. OTHER (See REMARKS)

16 THIS AWARD IS BASED ON AN APPLICATION SUBMITTED TO, AND AS APPROVED BY, THE PHS ON THE ABOVE TITLED PROJECT AND IS SUBJECT TO THE TERMS AND CONDITIONS INCORPORATED EITHER DIRECTLY OR BY REFERENCE IN THE FOLLOWING:
a. The grant program legislation cited above. b. The grant program regulation cited above.
c. This award notice including terms and conditions, if any, noted below under REMARKS.
d. PHS Grants Policy Statement including addenda in effect as of the beginning date of the budget period.
e. 45 CFR Part 74 or 45 CFR Part 92 as applicable.
In the event there are conflicting or otherwise inconsistent policies applicable to the grant, the above order of precedence shall prevail. Acceptance of the grant terms and conditions is acknowledged by the grantee when funds are drawn or otherwise obtained from the grant payment system.

RATE ($ 195,961 X 68.00)

ENDED FUTURE SUPPORT REFLECTS TOTAL COSTS.
AWARDED UNDER TERMS & CONDITIONS OF FED. DEMO. PARTNERSHIP PHASE III.

T OFFICER: (Signature) (Name Typed/Print) (Title)
LINDA SHAH, GMO, GMB, DEA, NIAID

.1.4A
ITCAN

18. CRS - EIN 1520595110A5

19. LIST NO.:

425751

DOCUMENT NO. R1AI39129A

ADMINISTRATIVE CODE

AMT. ACTION FIN. ASST.

AMT. ACTION DIR. ASST.

v. 7/94)

y

./Proj. ECT	REC YEAR 1	FUND YEAR 1	FUND YEAR 2	FUND YEAR 3	FUND YEAR 4	FUND YEAR 5
RSONNEL	35,910	35,910	37,346	38,840	40,394	42,010
NSULTANTS	0	0	0	0	0	0
UIPMENT	4,059	4,059	0	0	0	0
PPLIES	580	580	95	99	104	108
AVEL, DOMESTI	4,740	4,740	4,930	5,127	5,332	5,545
AVEL, FOREIGN	0	0	0	0	0	0
PATIENT COST	0	0	0	0	0	0
TPATIENT COS	0	0	0	0	0	0
TER. & RENOV	0	0	0	0	0	0
IRD PARTY CO	0	0	0	0	0	0
HER EXPENSES	3,761	3,761	3,911	4,067	4,230	4,399
TAL DIRECT C	49,050	49,050	46,282	48,133	50,060	52,062
DIRECT COSTS	0	24,520	25,224	26,232	27,283	28,374
L COSTS	49,050	73,570	71,506	74,365	77,343	80,436

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./Proj. ECT	REC YEAR 1	FUND YEAR 1	FUND YEAR 2	FUND YEAR 3	FUND YEAR 4	FUND YEAR 5
RSONNEL	242,420	242,420	252,117	262,202	272,690	283,598
NSULTANTS	6,000	6,000	6,240	6,490	6,750	7,020
UIPMENT	24,303	24,303	0	0	0	0
PPLIES	68,867	68,867	71,622	74,487	77,466	80,565
AVEL, DOMESTI	34,316	34,316	35,689	37,117	38,602	40,146
AVEL, FOREIGN	0	0	0	0	0	0
PATIENT COST	0	0	0	0	0	0
TPATIENT COS	0	0	0	0	0	0
TER. & RENOV	0	0	0	0	0	0
IRD PARTY CO	0	0	0	0	0	0
HER EXPENSES	90,825	90,825	94,458	98,236	102,165	106,252
TAL DIRECT C	466,731	466,731	460,126	478,532	497,673	517,581
DIRECT COSTS	0	0	0	0	0	0
L COSTS	466,731	466,731	460,126	478,532	497,673	517,581