ERC/RRC Meeting of April 5 25th March, '85

REVIEW BOARD ON THE USE OF HUMAN SUBJECTS, ICDDR,B.

29

Principal Investigator M.S. Islam Trainee Investigator (if any) _____

Application No. 85-010 Supporting Agency (if Non-ICDDR,B) _____

Title of Study The Role of the Aquatic Flora on on the long term survival of Vibrio cholera in the environment, a mechanism for the mainteriance of endemic cholera.

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

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

1. Source of Population:
   (a) Ill subjects Yes No NA
   (b) Non-ill subjects, Yes No NA
   (c) Minors or persons under guardianship Yes No NA
2. 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
3. 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)
4. 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 NA
   (f) Right to refuse to participate or to withdraw from study Yes No NA
   (g) Confidential handling of data Yes No NA
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes No NA
5. Will signed consent form be required:
   (a) From subjects 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 Board:
   ___ Umbrella proposal - Initially submit a 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 summar
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 sensitiv areas.
3. An indication as to when the questionnaire will be presented to the Board for review.

We agree to obtain approval of the Review Board on the Use of Human Subjects for any change involving the rights and welfare of subjects before making such change.

M. S. Islam
Principal Investigator

Trainee

SECTION 1 - RESEARCH PROTOCOL

1. Title: The role of the aquatic flora on the long term survival of Vibrio cholera in the environment, a mechanism for the maintenance of endemic cholera.

2. Principal Investigators: MD. SIRAJUL ISLAM, PROFESSOR DAVID J. BRADLEY, DR. BOHUMIL S. DRASAR, DR. K.M.S. AZIZ AND MD. IMDADUL HUQ.

3. Starting Date: 1st January, 1985 (tentative). Actual time will be started from the date of approval.

4. Completion Date: 31st December, 1986

5. Total Direct Cost: U.S. $18,697.22

6. Availability of Funds:

7. Scientific Program Head:

This protocol has been approved by the *Disease Transmission* working group.

Signature of Scientific Program Head: .....

(Dr.David A. Sack)

8. Abstract Summary:

This study will examine the hypothesis that cholera maintains its endemic grip on an area through the ability of the 01 serotype of Vibrio cholerae to survive and multiply on surfaces of aquatic flora (water plants and phytoplankton) within the environment. To achieve this objective laboratory experiments will be carried out to predict about the possible role of aquatic flora as reservoirs or sites of multiplication of the pathogenic vibrios in the environment. Then environmental samples will be collected from highly endemic cholera areas of Bangladesh for the 01 serotype of V. cholera during the non-cholera season. Selection of sampling sites will be based on the presence of optimal physico-chemical conditions for the survival of V. cholera; samples taken will reflect the diversity of the aquatic environment and will include water, plants, silt and phytoplankton.

9. Reviews:

a) Research Involving Human Subjects: .....

b) Research Review Committee: .....

c) Director: .....

d) [illegible]

e) [illegible]

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective:

The primary objective of the project is to investigate whether Vibrio cholerae 01 can survive and multiply on plant surfaces in the aquatic environment for long periods of time thereby providing a mechanism for the maintenance of endemic cholera.

2. Background:

The water borne diseases in the strict sense are those where the organism reaches the water by excretal pollution and will affect those who ingest this polluted water (Bradley, D.J., 1970). Cholera is one of the water borne diseases.

Cholera is still of public health importance in certain parts of the world today. In Bangladesh there are some endemic areas where a cholera epidemic occurs almost every year and the epidemic follows a distinct seasonal pattern. During an epidemic the causative agent Vibrio cholerae 01 is isolated from the patient as well as from the environment, but when the epidemic is over, the Vibrio cholerae 01 cannot be isolated from the environment (McCormack et al, 1969). So it needs to be determined how these organisms maintained themselves in the environment during the interepidemic period, or where the reservoirs or sites of multiplication of the pathogenic vibrios in the environment during the interepidemic period are and what are the mechanisms by which endemicity is maintained in these areas.

There are four suggested mechanisms:-

a) carrier status in animals,
b) carrier status in man,
c) continuous transmission in man, and
d) an environmental reservoir.

a) Carrier status in animals:

Vibrio cholerae 01 have been isolated from domestic animals in a non-endemic area (Sanyal et al, 1974) but only when cholera is in the human population. They have not been isolated in the non-cholera season. These animals are therefore picking up the vibrios passively and cannot act as a reservoir for the organisms.

b) Carrier status in man:

Only one chronic carrier has been demonstrated to date (Azurin, et al, 1967) and this is in spite of some very extensive studies on populations exposed to cholera (Yen, 1964). Since relatively large numbers of carriers would be required to give the observed epidemiological pattern of the diseases, this theory has very little credibility as a mechanism for the maintenance of endemic cholera.

c) 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), adaptation of the organism to high salinity waters and its limited survival in potable water (Miller, Drasar & Feacham, 1982), and the sensitivity of the organism to acid conditions (Pollitzer, 1959). Epidemiological studies of the transmission of cholera in Bangladesh, the main primary endemic focus of cholera, show that even in the cholera season and under extremely poor sanitary conditions, the transmission of cholera from person to person is limited (Mosley & Khan, 1979).

d) Environmental reservoirs:

Since this is the alternative to be examined, it would be useful to briefly list the principal reasons for thinking this alternative to be the correct one, and why criticism against this mechanism is considered inadequate.

i) Some survival studies have shown that V. cholerae can survive for extended periods of time in the right environment (Altukhov et al, 1975; Sayamov, 1978).

ii) DNA homology studies (Citarella and Colwell, 1970) and numerical taxonomy (Colwell 1970; Sakazaki et al, 1967) have shown V. cholerae 01 to be very closely related to non 01 Vibrio cholerae. Vibrio cholerae non 01 strains are found throughout the year, in the environment, in cholera endemic areas.

iii) Support for this mechanism is provided by the observed epidemiology of the disease; the epidemic season in Bangladesh being initiated with "multiple simultaneous outbreaks of the disease with no apparent correlation" (Martin et al, 1969).

iv) Studies in Chesapeake Bay U.S.A. (Kaper et al, 1979) and Kent U.K. (Bashford et al, 1979) have revealed the 01 serotype of Vibrio cholerae alongside non-01 V. cholerae in the environment without any apparent association with clinical disease. In addition to this, there have been many environmental isolations of V. cholerae made in areas that whilst being old cholera areas, have been free of the disease for a number of years (Read & Pandit, 1941; Venkatraman, 1941).

v) The environment can be considered as a large number of dynamic micro-environments. It would be impossible to examine all of these for V. cholerae and therefore we can never say that V. cholerae does not reside in the environment. This constraint does not apply to a human population which yields a finite number of clinical specimens to be examined. The environmental hypothesis needs to be critically examined therefore, in order to develop a directed programme of environmental sampling.

The main source of evidence used to refute this hypothesis comprises laboratory studies in which the survival time of V. cholerae in river or tank water is measured. Survival times in these studies are usually short (Cheng, 1963; Lahiri et al, 1939; Konchady et al, 1969) and this is taken as an indication that survival in river systems is short (Mukherjee et al, 1961). This interpretation of the survival studies is considered inadequate primarily because a sample of river water represents only a tiny fraction of the total number of habitats in the aquatic environment. Further studies have concentrated on the examination of potable water which may not be relevant in terms of survival.

Adhesion to surfaces: In aquatic environments, surfaces will absorb nutrients, and this will have the effect of promoting bacterial growth at those surfaces (Heukelekiam and Heller, 1940). Many bacteria have evolved to take advantage of this phenomenon by developing specialized adhesion mechanisms that enable these organisms to adhere, sometimes very tenaciously, to such surfaces.

An example of this was observed by Zobell and Rittenberg (1938) whilst working with chitinoclastic bacteria. They found that "one culture covered the chitin strip with a heavy orange growth while the surrounding menstrum remained quite colourless. When a loopful of the medium was transferred to another tube of chitin media no growth developed."

If V. cholerae does strongly absorb to mucin or chitin or any other surface in such a way, then clearly sampling of water alone will have little chance of picking up the organism.

Robert Koch in 1884 suggested that V. cholerae may have an environmental reservoir but it was not accepted because most of the survival studies have shown that the organisms have only limited potential for environmental survival (Felsenfeld, 1974; Pollitzer, 1959). Recently isolation of V. cholerae 01 from a number of places suggest that the organisms may be long term inhabitants of some aquatic environments (Blake et al, 1980; Rogers et al, 1980). These findings are also leading the microbiologists to think about the possible ecological niche of V. cholerae in the aquatic environment. The survival of V. cholerae during the inter-epidemic period can be explained by such an ecological niche.

Moreover the organisms have been isolated from estuarine birds (Lee et al, 1982), and ducks (Bisgaard and Christensen, 1975; Bisgaard, Sakazaki and Shimada, 1978). These might reflect its presence in other estuarine plants and animals that are being eaten by birds and ducks, such as duck-weeds, crustaceans etc. The means by which Vibrio cholerae survive during the inter-epidemic period remains to be clarified, but at present plants or animal reservoirs seem quite likely.

Aquatic fauna as reservoirs: Hood et al, (1981) isolated a typical V. cholerae 01 from oysters collected from unpolluted estuarine waters in Florida (U.S.A.). Some studies have shown that V. cholerae 01 and non-01 can produce chitinase and they can adsorb to and multiply on chitinous fauna such as crab, shrimp

and zooplankton (Huq et al, 1983; Colwell et al, 1977; Kaneko, T. and Colwell, R.R., 1975, 1978; Kaper et al, 1979; Nalin, D.R., 1976).

Aquatic flora as reservoirs: Most of the work regarding the association of vibrios in the environment was with different kinds of aquatic fauna. Islam, M.S. & Aziz, K.M.S. (1978, 1981) for the first time reported the association of vibrios with some aquatic flora, mainly macrophytes from Bangladesh. They found the association of vibrios with some water plants namely Eichhornia crassipes (water hyacinth), Monochoria hastata, Ludwigia repens and Marsilea quadrifolia. They suggested that the enzyme mucinase which is secreted by vibrios (Schneider, D.R. & Parker, C.D., 1982) may be responsible for the association with water plants because the role of mucinase in the environment is to degrade naturally present mucin and mucin is present in the plant cell, so this may be one of the factors which helps to make the association with water plants. Recent survival studies of V. cholerae 01 with mucin in the London School of Hygiene and Tropical Medicine, support the above hypothesis (unpublished data). It is also found that V. cholerae 01 can compete better with other resident bacteria and survive longer on plant surfaces than in water and on sediments (Islam, Drasar & Bradley, 1984). Spira et al, (1981) found that virulent V. cholerae biotype El Tor can associate with water hyacinth.

Islam et al, (1983) found that the percentage of isolation of vibrios is always higher from plant surfaces than water and sediments, in a survey of three ponds in Dhaka, Bangladesh.

All these previous studies have left open the possibility that the maintenance of cholera infection during the inter-epidemic period in Bangladesh and elsewhere could be due to persisting aquatic reservoirs of V. cholerae which had gone undetected in the non-cholera season, and at times when cholera infections are not occurring locally, because the vibrios are associated with selected micro-habitats that have been inadequately sampled, such as plant surfaces, sediments, zooplankton etc. (Feachem et al, 1981).

So the vibrio-plant and vibrio-plankton is a comparatively new approach, and it is necessary to look for vibrios in hitherto unexplored areas of the environment.

3. Rationale:

Whereas many of the key aspects of the epidemiology of cholera were worked out well over a hundred years ago by John Snow (1965), long before Koch first demonstrated the causative organism of the disease (Koch, R., 1884), other aspects of the epidemiology still remain a mystery. One of the more puzzling of these is the habitat of the vibrio in the non-cholera season when it seems to disappear from both the human population and the environment. It now appears however, that an answer to this problem may be within our reach as recent studies have suggested that V. cholerae may be able to survive and multiply within the environment as part of the normal flora of specific micro-habitats. (Colwell et al, 1979; Islam et al, 1983; Spira et al, 1981; Lee et al, 1982; Huq et al, 1983). This study will attempt

to demonstrate the nature of such an ecological niche.

B. SPECIFIC AIMS

1. To reveal whether the organism can selectively attach to and grow on plant surfaces in both the cholera and the non-cholera season, thereby providing a mechanism for the maintenance of endemic cholera.

2. To identify candidate environmental micro-habitats that might support growth of the organism.

3. To establish the relationship of the survival of Vibrio cholerae with some physico-chemical conditions.

METHODS OF PROCEDURE:

Overall strategy

The study will be divided into three phases; a laboratory phase, a field work phase and a subsequent laboratory phase.

LABORATORY STUDIES

a) Survival Studies

This will involve studies directed at measuring the effect of PH, salinity, temperature and dissolved oxygen on the shape of the survival curve of V. cholerae in the presence of plants and sediments with different substrates, for example mucin, chitin etc. Ten strains will be tested against these variables. This study will be done by simple experiments involving the addition of a number of organisms to a measured volume of suspending fluid, and the subsequent monitoring of viable numbers over a defined period of time. A standardized technique will be developed for these experiments.

The standardized experimental technique

Standard methods will be used for the following procedures:

Organisms will be used, processing of plants and sediments, preparation of innoculum, preparation of suspending fluid, innoculation of suspending fluid, storage of bottles, counting procedure and sampling time-table. These procedures are described below.

Organisms to be used in the study: Six Bangladeshi (3 environmental and 3 clinical) isolates, two Australian (environmental isolates), and two Tanzanian (clinical) isolates of 01 serogroup of V. cholerae will be used for the experiments. These isolates were lyophilized after primary isolation. They will be reconstituted and grown overnight in alkaline peptone water at 37 ^{o}C. Aliquots of 1.0 ml will be mixed with 0.1 ml glycerol and will be stored at -70 ^{o}C. Multiple aliquots of each isolate will be prepared so that isolates that will be used for the later studies will not be subjected to repeated thawing and re-freezing.

Processing of plants and sediments: A portion of plant materials will be taken in a glass made homogenizer and a measured amount of PBS will be added and then homogenized manually until all plant parts are completely and uniformly disintegrated. Then appropriate decimal dilutions in PBS will be prepared. 0.1 ml portion of each dilution will be spread onto duplicate plates of nutrient agar and thiosulphate citrate bilesalt sucrose agar (TCBS). A definite amount of sediments will be collected with the help of a core sampler and then decimal dilutions will be prepared in PBS.

Preparation of inoculum: An aliquot of each bacterial suspension will be removed from the -70 ^{o}C freezer, and will be allowed to come to room temperature. The suspension will be innoculated directly onto a nutrient agar plate and incubated overnight at 37 ^{o}C. After incubation a loopful of the resulting growth will be removed from the plate and suspended in 100 ml M9 medium and incubated in a shaker incubator at 37 ^{o}C at 200 rpm. After a definite period of time, optical density of bacterial growth in M9 medium will be taken until it reaches up to the desired level.

Preparation of suspending fluid: Preparation of suspending fluid will vary though some aspects of its preparation will be standardized. Glass distilled water will be used for all experiments. The acidity of this distilled water which is about 5.0 will be removed by first autoclaving the water, thus removing much of the disolved CO_2 , a major contributing factor to the acidity, then bringing the water up to pH 8.5 with the use of 0.05 M NaOH.

Innoculation of suspending fluid and storage of bottles: All survival studies will be initiated by adding 5.0 ml of innoculating suspension to 500 ml suspending fluid. The bottles to be used will be made of borosilicate glass and have a total volume of 640 ml. These bottles will be kept at room temperature (about 25 ^{o}C) and placed near the window so that they get sunlight.

Counting procedure: A spread plate technique will be used for all bacterial counts. This will involve innoculating 0.1 ml of the bacterial suspension, or a log dilution of this suspension to 9 cm TCBS and nutrient agar plates. The diluent to be used is phosphate buffer saline of pH 7.3.

Automatic micro pipettes (Oxford) will be used for the measurement of volume.

Bacterial counts will be derived from macroscopic counts of individual colonies. The dilution will be chosen for the count that will give between 30 and 300 colonies per plate. Each count will be carried out in duplicate.

Sampling time table: A sampling time table will be used with 3 days interval after the first count at the time of innoculation. The counting of any one sample will be discontinued after failure to recover V. cholerae on two consecutive samplings.

b) Study of adsorption and multiplication of V. Cholerae with water plants by isotope labelling

The use of labelled isotope in microbiology is a well established technique. When bacteria can not be isolated by a technique which is available for the detection of the bacteria, the use of radio labelled markers which can be incorporated into the organism presents another way of studying the organism even when it is available in a small number. The high sensitivity of the technique enables the obtaining of positive results with as few as a million organisms (Antia, N.H. 1978).

The adsorption, and multiplication of bacteria to surfaces in aquatic environment has been investigated by microbial ecologists for several decades. The multiplication of bacteria in a particular environment at a given time can be studied by radio isotope labelling. It is well known that cells undergoing division duplicate their DNA, whereas non-growing cells do not. Therefore, cells which are multiplying will incorporate the radioactive material with their DNA and become radioactive. So investigations will be carried out to see the adsorption and multiplication of V. cholerae on plant surfaces by labelling the organisms with [3H] thymidine.

This study will help to provide clues as to the location of the organisms in artificial aquatic ecosystems and the degree of selective partitioning of bacteria between water and plant surfaces. This study will demonstrate whether they remain in the water as floating organisms or they stick to the plant surfaces or settled to the bottom and stick to the sediments, after inoculation into the aquatic environment. Some experiments have been carried out in the Department of Medical Microbiology and Department of Tropical Hygiene, London School of Hygiene and Tropical Medicine. The results demonstrate that after inoculation in the water of artificial aquatic ecosystems, they migrate from water column and stick to the plant surfaces where they get some survival advantages and prolonged their survival time. Whether V. cholerae can multiply on plant surfaces need to be determined. Study is in progress to examine the multiplication of the organisms on the plant sufaces in the artificial aquatic ecosystems.

Procedures: Bacteria will be prepared for isotope labelling by growing the cells overnight at 37 ^{o}C in alkaline peptone water of PH 8.5. The bacteria will then be washed three times and then innoculated into 10 ml M9 glucose minimal salt medium, containing 20% Difco casamino acid. M9 medium will be supplemented with 0.1 ml tritiated thymidine [3H] (1.0 mci/ml) and incubated at 37 ^{o}C in a shaker incubator at 200 rpm. The optical density (OD) of M9 medium with bacteria will be checked by spectrophotometer at 550 nanometers (nm).

When the OD will indicate 10^{9}/ml organisms then 5.0 ml will be taken and washed thrice with PBS by centrifuging at 10,000 rpm for 30 minutes.

The labelled cells will be innoculated into 500 ml 0.1% sea-salt

solution with sediments. Plants will be added in sea-salt solution and subsequently plants, sea-salt solution and sediments will be sampled every one hour interval.

Plants sample: 0.1 gram of plants materials will be taken in a homogenizer and 0.9 ml PBS will be added and homogenized properly. 0.1 ml of the homogenized suspension will be soaked in duplicate Whatman filter papers (2 x 2 cm).

Sea-salt solution: 0.1 ml of sea-salt solution will be taken and soaked in duplicate filter papers.

Sediments sample: 1.0 gram of sediment will be taken with the help of a laboratory made core sampler and mixed with 9.0 ml PBS in a whirl-mixer for 5 minutes. 0.1 ml of the aliquot will be soaked in duplicate filter papers.

All the filter papers will be air dried and then washed thrice in 5% trichloroacetic acid (TCA) and again dried in 37 ^{o}C for one hour. After drying, the filter papers will be placed in scintillation vials and 10 ml of toluene based scintillation fluid will be added. Finally the counts of tritiated thymidine will be taken with the help of a computerized scintillation counter.

0.5% formalin will be used as a control.

Field Work

The principal aim of this work will be to demonstrate *V. cholerae* in the environment in the non-cholera season. 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 (Heukekekian & 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). Thus the search for *V. cholerae* though involving some water sampling, will be based on an examination of various plant surfaces. River or marine sediments 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. The likely survival habitat will be determined in the light of laboratory results, obtained from the experiments in the London School of Hygiene and Tropical Medicine.

Environmental sampling:

Samples will be collected from Dhaka, Matlab and Teknaf and the sites will be selected on the basis of different physico chemical parameters like temperature, pH, DO_2 and salinity.

Sampling procedure:

Surface water (5cm below the surface) will be collected in pre-sterilized 500 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 core sampler and kept in 4 ounce 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 about 4 ^{o}C. Samples will be processed within 4 hours 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 to transport to London for identification.

Treatment of samples:

Plant samples will be blended for at least two minutes with an equal volume of phosphate buffer saline (PBS) to give a homogenous mixture.

Sediment samples will be shaken vigorously for 5 minutes before aliquots are taken for analysis.

Plankton samples will be processed by shaking or blending, depending on need, in the same way.

A portion of the blended plants or plankton materials will be innoculated in enrichment media (alkaline bile peptone) and incubated for 6 hours, and subsequent plating will be done onto TCBS agar. One gram of sediment will be taken and processed in the same way. 500 ml of water will be filtered through .45 µm membrane filter, and then the filter paper will be immersed in alkaline bile peptone, incubated for 6 hours and subsequent plating onto TCBS agar will be done.

Bacterial counts:

Water: 50 ml of water sample will be filtered through 0.45 µm membrane filter paper. Then the filter paper will be placed onto TCBS agar and incubated for 24 to 36 hours at 37 ^{o}C. Vibrio like organisms (yellow or green colonies) will be picked up and streaked on TTGA (Monsur, 1961) and incubated at 37 ^{o}C to see the production of gelatinase. Then the gelatinase producing organisms will be tested for sensitivity to 0/129 vibrio static compound and reactions in Kliglers Iron Agar (KIA) and Motility Indole Urea (MIU) agar. Presumptive vibrio isolates will be checked for lysin and ornithine decarboxylase, argenine dihydrolase and for fermentation of mannitol, sucrose, mannose and arabinose (Huq, M.I., 1979) and growth in 0, 3, 8 and 10% NaCl. Serology will be done if indicated. They will also be examined microscopically.

Other specimens:

Spread plating will be done taking 0.1 ml from blended or shaken samples on TCBS agar. Then incubation and procedures will be followed as above.

Determination of aquatic environmental parameters:

Water temperature, salinity, PH and disolved oxygen content of water will be determined using potable electronic meters.

Strains isolated from the environment will be characterized to species level. All isolates of Vibrio cholerae will be freeze dried/frozen in preparation of transportation to London.

Follow up laboratory studies:

The use of any remaining time after termination of field studies is largely dependant on the results of the field work plus the amount of time available for writing the thesis.

The primary outline for the proposed research was developed in 1982 during discussions between Prof. David J Bradley of the Department of Tropical Hygiene, London School of Hygiene and Tropical Medicine, and Drs. W B Greenough and K M S Aziz of ICDDR,B. This project is to serve as the basis for Mr. Islam's doctoral thesis.

D. SIGNIFICANCE:

This study will test the hypothesis that Vibrio cholerae can survive within the environment. This will not only have relevance to the maintenance of endemic cholera, but will also have significant implications for our understanding of the transmission of the disease and may influence future cholera control programs. Should V. cholerae be found to multiply within the environment then control of the disease through provision of sanitation alone will not be possible and alternative strategies will have to be sought.

The information gained about the microhabitats in the aquatic environment supporting V. cholerae 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.

E. FACILITIES REQUIRED

1. Office space: No additional space required.

2. Laboratory space: No additional space required at ICDDR,B.

3. Hospital resources: Not required.

4. Animal resources: None.

5. Logistic support:

Automobile transportation in Dhaka	-	250 miles
Automobile transportation in Teknaf	-	25 miles
Return trip to Teknaf (2 persons x 1)	-	2
Speed boat transport in Teknaf	-	8 hr.
Automobile transport in Matlab	-	25 miles
Speed boat transport in Matlab	-	12 hr.

6. Major items of equipment: None.

7. Other specialized requirements: None.

F. COLLABORATIVE ARRANGMENTS

This work will be carried out in collaboration with the Departments of Tropical Hygiene and Medical Microbiology, London School of Hygiene and Tropical Medicine, U.K, Dr. Drasar and Professor Bradley specifically.

SECTION III - BUDGET

A. DETAILED BUDGET

PERSONNEL SERVICES

AME	POSITION	PERCENT OF EFFORT OR NO. OF DAYS	ANNUAL SALARY	PROJECT TAKA	DOLLAR
D. SIRAJUL SLAM	Sr.Res.Off.	100%	TK.76008.00	152016.00 (for 2 years)	---
ROF. DAVID BRADLEY	Prof, Dept. of Tropical Hygiene	5%	---	---	---
R. BOHUMIL DRASAR	Reader, Depts of Medical Microbiology and Tropical Hygiene	5%	---	---	---
DR. K.M.S. ZIZ	Associate Director, ICDDRB	5% (for 2 months)	$62,080		$517.50
R. MD. IDADUL HUQ	Scientist & Head Microbiology Branch, ICDDRB	5% (for 2 months)	$53,740		$447.83
IR.MD.ASHRAF	Lab.Attend	10% (for 2 months)	TK2400.00	TK400.00	---
				TK152416.00	$965.33

Salary will be paid from Training Branch budget.

2. SUPPLIES AND MATERIALS (For two years)

Petridishes	$ 3,000.00
Media	$ 2,000.00
Laboratory supplies (chemicals and glassware, membrane filtration materials, biological materials, antiserum, chicken cells etc.)	$ 5,000.00
Total	$10,000.00

3. EQUIPMENT: No extra equipment needed.

4. PATIENT HOSPITALIZATION: None.

5. OUT PATIENT CARE: None.

6. ICDDR,B TRANSPORT:

300 miles @ TK. 5.00 per mile land transport	= TK.1500.00
Speed boat 20 hrs @ TK.105.00 per hour	= TK.2100.00
	TK.3600.00

7. TRAVEL & TRANSPORTATION OF PERSONS:

Dhaka - Cox's Bazar - Dhaka for two persons one trip	TK.2000.00
One return trip for one of the investigators between London and Dhaka during field work. London - Dhaka - London	$ 631.25

8. TRANSPORTATION OF EQUIPMENT

Media and equipment (Dhaka - Cox's Bazar - Dhaka)	TK. 500.00

9. RENT, COMMUNICATION & UTILITIES: None.

Guest House charges for investigator from London - 14 days x US $ 30	$ 420

10. PRINTING & REPRODUCTION:

Xerox	TK.5000.00
Publication cost	TK.2500.00
Others	TK.1000.00
	TK.8500.00

11. OTHER CONTRACTUAL SERVICES: None.

12. CONSTRUCTION, RENOVATION, ALTERATION: None.

B. BUDGET SUMMARY

	CATEGORY	COST TAKA	COST DOLLAR
1.	Personnel	152416.00	965.33
2.	Supplies	---	10,000.00
3.	Equipment	---	---
4.	Hospitalization	---	---
5.	Out Patient	---	---
6.	ICDDR,B Transport	3600.00	---
7.	Travel Persons	2000.00	631.25
8.	Transportation of Equipment	500.00	---
9.	Rent, Communication & Utilities	---	420.00
10.	Printing & Reproduction	8500.00	---
11.	Other Contractual Services	---	---
12.	Construction, Renovation & Alteration	---	---
	Sub-Total =	167016.00	12016.58
	Total Cost =	6680.64 +	12016.58
	GRAND TOTAL:	US $ 18697.22	

Conversion rate US $ 1.00 = TK.25.00

Personnel $ 7061.97
Others $ 11635.25

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