Attachment 1. 5

Date _____

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr. M.I. Huq

Application No. 82-047

Title of Study Ecology and survival of Vibrio cholerae and related pathogenic Vibrios in the aquatic environment of Bangladesh during cholera epidemic and inter-epidemic periods.

Trainee Investigator (if any) _____

Supporting Agency (if Non-ICDDR,B) _____

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).

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.

257 264
278 298 299 386
304 305, 348,

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

Principal Investigator

Trainee

82-017

21/9/82

SECTION I - RESEARCH PROPOSAL

1. Title : Ecology and survival of Vibrio cholerae and related pathogenic vibrios in the aquatic environment of Bangladesh during cholera epidemic and inter-epidemic periods.

2. Principal Investigator : Dr. M.I. Huq

   Co-Investigators : Drs. R.R. Colwell, K.M.S. Aziz, A. Huq, P.A. West

3. Starting Date : October 1, 1982

4. Completion Date : September 30, 1983

5. Total Direct Cost : U.S.$ 24,489.00

6. Availability of Funds :

7. Scientific Program Head :

This protocol has been approved by the DTWG Working Group.

Signature of the Scientific Program Head : [signature]

Date : 17.9.82

8. Abstract Summary :

Newly developed methods for the detection of V. cholerae O1 in the aquatic environment of Bangladesh during inter-epidemic periods will be evaluated. These methods involve the use of a fluorescent-labeled anti-serum specific for V. cholerae O1, and examination for stained cells using epifluorescent microscopy, in conjunction with recovery media for resucitating stressed cells in the water.

The survival of V. cholerae O1 in the aquatic environment will be studied under conditions which more closely approach field conditions, by use of submersible diffusion chambers. Environmental parameters effecting the survival of V. cholerae O1 in the aquatic environment

will be examined. The virulence and biological activity of stressed cells of V. cholerae O1 recovered from the aquatic environment during inter-epidemic periods will be evaluated.

Ecological studies currently in progress concerning the distribution of V. parahaemolyticus in the aquatic environment will be completed.

Molecular genetic techniques will be employed to compare characteristics of strains of V. cholerae O1 isolated from the aquatic environment during inter-epidemic and epidemic periods of cholera.

Amalgamation of the results from these studies will yield significant insight into the role of the aquatic environment as an extra-intestinal reservoir of V. cholerae O1 during inter-epidemic periods.

9. Reviews :

a. Ethical Review Committee : _____

b. Research Review Committee : _____

c. Director : _____

d. BMRC : _____

e. Controller/Administrator : _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objectives: The major objectives of this research project are to determine: 1) morphological, cultural and survival properties of Vibrio cholerae serovar O1 in the aquatic environment during inter-epidemic and epidemic periods of cholera; 2) physiochemical and biotic parameters influencing the distribution of V. cholerae and related pathogenic vibrios in differents aquatic environment (sweet, brackish & sea water); 3) the efficacy of recovery media and a direct microscopic technique for the detection of "stressed" cells of V. cholerae O1 in the aquatic environment; 4) specific niches in the aquatic environment which harbour V. cholerae O1 between cholera epidemics; 5) toxigenic potential of "stressed" cells grown under very low nutrient conditions; and 6) molecular genetic characterization of V. cholerae O1 strains isolated during the inter-epidemic and epidemic periods of cholera.

The project described herein has been designed to evaluate the potential of toxigenic and so-called non-toxigenic strains of V. cholerae O1 to maintain viability and capability to produce toxin(s) in the aquatic environment, particularly between cholera epidemics. Evidence that the aquatic environment is the source of strains associated with cases of cholera each season, will be sought using ecological and molecular genetic investigation procedures. Commensurate with these objectives will be the evaluation of newly-developed methods for detection of V. cholerae O1 in environmental samples. A two-year study period will, accordingly, permit analysis of the properties of isolates collected during at least three inter-epidemic periods.

Evaluation of the properties of V. cholerae O1 in the natural aquatic environment at times of high, as well as low, incidence of cholera in the local community will provide an important contribution to the overall epidemiological understanding of the disease and its control. The study will, therefore, be of considerable relevance to the diarrhoeal disease research directives at the centre.

2. Background : Considerable advances have been made in the understanding, at the molecular level, of the pathogenesis of cholera. Comparatively little progress, however, has been made in elucidating the factors involved in the inevitable development of cholera epidemics in Bangladesh each year.

The seasonality of cholera in Bangladesh can be documented from epidemiological data. Cholera due to the eltor biovar of V. cholerae O1 occurs in the post monsoon period and coincides with the fall in water level, and changing air temperature. The incidence of cholera in communities then falls with the start of the cooler season. Nevertheless, epidemiological data has failed to indicate the principal reservoir of V. cholerae O1 during the inter-epidemic period. Alternatives reservoirs suggested to date are man, animals, the local aquatic environment, and a combination of all of these.

Demographic and epidemiological studies are providing a greater insight into the spread of the disease. For example, the incidence of cholera is apparently higher in a fishing community than an agricultural community. Thus, increased contact with the aquatic environment, containing V. cholerae O1, may result in increased incidence of disease in this section of the community (data summarized from unpublished original data compiled by Dr. R.I. Glass and co-workers at ICDDR,B Dacca).

For these, and other reasons outlined below, the present study has been designed to elucidate the potential and significance of V. cholerae 01 to persist in selected aquatic environment between cholera seasons.

Evidence is now rapidly accumulating which strongly suggests that V. cholerae 01 strains can be maintained for prolonged periods of time in aquatic environments. Traditionally, epidemiologists have considered that the human intestine was the sole reservoir of V. cholerae 01 and that extra-intestinal survival was limited (Pollitzer, 1959; Felsenfeld, 1974). More recent reports of ecological studies in England (Bashford et al., 1979; Lee et al., 1982), America (Colwell et al., 1977; Blake et al., 1980; Colwell et al., 1981) Australia, (Rogers et al., 1980) and Japan (Fukimi, 1979) now strongly suggest that V. cholerae exists in the aquatic environment and its presence is not necessarily dependent on the input of faecal material (Feacham, 1981). Indeed, the hypothesis has been made that the aquatic environment is the natural reservoir of V. cholerae (Colwell et al., 1977).

Conclusions from ecological studies reported to date on V. cholerae (01 and non-01 serovars) indicate: survival and growth of non-toxigenic serovars is strongly influenced by water temperature (West, 1980; Lee et al., 1982; West and Lee, 1982; unpublished data of The Sea Grant Program working group on Vibrio cholerae); association of V. cholerae with planktonic members of the aquatic environment results in prolonged survival of the pathogen outside the human intestine (Huq, et al., 1982); strains can remain viable and substrate-responsive under conditions of low temperature and low nutrient but fail to grown on nutrient-rich media, suggesting a non-lethal stressing process (Singleton et al., 1982a, 1982b). The latter observation may indicate that traditional isolation methods, using alkaline peptone water and TCBS agar, may not recover V. cholerae 01 during the inter-epidemic period if the organisms are subjected to stress conditions and/or non-optimal growth conditions of salinity, temperature, etc. This has been

reinforced by the observations at the University of Maryland and a collaborating clinical laboratory in Louisiana where a fluorescent-labeled antibody/epifluorescent microscopy method has shown that cells in alkaline peptone water fail to grow on nutrient-rich plating media (H.-S. Xu, N.C. Roberts and R.R. Colwell, unpublished results).

The nutrient and temperature conditions reported by Singleton et al. (1982a, 1982b) which cause cells of V. cholerae 01 to become "stressed" resemble features occurring in the climate and environment during the monsoon period, especially in estuaries, suggesting that cells of V. cholerae in the aquatic environment of Bangladesh may fail to be recovered by the use of inhibitory media and high incubation temperatures. A previous study under the auspices of ICDDR,B was initiated by W.M. Spira (Principal Investigator) and concluded that V. cholerae 01 was rarely isolated from over 400 environmental samples cultured on starch agar after enrichment in alkaline peptone water. From recent data gathered at the University of Maryland, it appears stressed cells would fail to be cultured by this method. A pilot study has been started to detect V. cholerae 01 in the aquatic environment of Bangladesh during the inter-epidemic periods (ICDDR,B-University of Maryland collaborative research protocol). The fluorescent-labeled antibody/epifluorescent microscopy method will be used, in conjunction with recovery using non-inhibitory resucsitation media, for detection of the organism.

The toxigenicity of stressed V. cholerae 01 cells has not been fully evaluated. In vitro culture of V. cholerae 01 under low nutrient conditions has been performed at ICDDR,B (Dacca). These cells remain toxigenic when determined by the rabbit ileal loop technique (Table 1). However, the toxigenicity of stressed cells recovered from the aquatic environment during inter-epidemic periods is not known and is of great significance to the overall understanding of the epidemiology of the disease.

Much of the data on the ecology of V. cholerae 01 have been drawn from studies in cholera non-endemic areas. The application of these conclusions and techniques to the study of the ecology of V. cholerae 01 in Bangladesh environs is, therefore, now required and is expected to yield significant insights into the epidemiology of the disease in endemic and non-endemic global areas.

In contrast, V. parahaemolyticus is usually associated with enteritis in the population of the coastal areas and the epidemiology of the disease directly reflects the marine origin of the causative agent. V. parahaemolyticus has an obligate requirement for the sodium ion (Lee, 1972; Reichelt and Baumann, 1974. Nevertheless, strains phenotypically indistinct from V. parahaemolyticus have been isolated from the freshwater areas of Dacca and Matlab. Ecological studies are in progress to investigate the apparent survival of V. parahaemolyticus in these regions and require completion in order for statistical analysis of data to be performed.

Plasmids are a relatively recent discovery in Vibrio species and the role of many of these extrachromosomal elements remains to be defined. Only a few plasmids fround in V. cholerae have been studied extensively. Two sex plasmids have been described (Bhaskaran and Sinha, 1971; Bhaskaran et al, 1973) which appear to cause a decrease in toxin production (Sinha and Srivastava 1978). Until recently, antibiotic resistance (R) plasmids were rarely found in V. cholerae 01 (O'Grady et al., 1976). However, strains carrying R plasmids have begun to feature in cholera outbreaks, notably in Tanzania (Mhaulu et al., 1979) and in Bangladesh (Glass et al., 198). The plasmids belong to incompatibility group C (Threlfall et al., 1981). Interestingly, these plasmid-carrying strains fail to be isolated after the outbreaks. The reason for the rapid emergence and subsequent disappearance of these R plasmid-containing strains of V. cholerae 01 is not known. The possibility that the strains acquire, and lose, plasmids in the aquatic environment requires

investigation. It is possible that antibiotic-resistant V. cholerae 01 strains appear in the aquatic environment before emerging in the human population, thus providing an early indication of disease which would not respond to a regime or tetracycline treatment.

The rapid emergence of molecular genetic characterization techniqes as routine laboratory methodologies is now allowing their application to other fields of research. The present study will amalgamate the disciplines of microbial ecology and molecular genetics to provide a greater understanding of one aspect of the epidemiology of cholera, i.e., the role of the aquatic environment in the spread and maintenance of disease in the local human population.

3. Rationale: Climatic and aquatic environmental conditions appear to strongly influence the epidemiology of cholera and therefore, imply that these parameters must also effect the properties of the causative agent, V. cholerae serovar 01. Recent studies have demonstrated that V. cholerae 01 can become stressed, and subsequently non-culturable, under conditions which resemble those of the aquatic environment in inter-epidemic periods. Accordingly, the conclusions from previous ecological studies and survival experiments may now need to be revised. Failure to isolate the organism may, in fact, be attributable to the use of lethal recovery techniques rather than an absence of organisms in the water.

The present study will incorporate recovery procedures to overcome the possibility of sub-lethal stressing of cells in the aquatic environment. Studies are also designed to assess the significance of V. cholerae 01 in the aquatic environment during inter-epidemic as well as epidemic periods of the disease.

Studies on V. parahaemolyticus, currently in progress, will be completed. This study will be the most detailed examination to date of the distribution

of this pathogen in the aquatic environments of Bangladesh.

B. SPECIFIC AIMS

1. Evaluation of non-selective recovery procedures for the isolation of V. cholerae O1 from waters and other aquatic environmental samples during the inter-epidemic period.

2. Detection of stressed cells of V. cholerae O1 in the aquatic environment using a newly-described fluorescent-labeled antibody technique and determination of morphological properties of these cells.

3. Comparison of in situ survival patterns of V. cholerae O1 in waters of Dacca & Matlab during the inter-epidemic and epidemic periods.

4. Identification of specific forms of plankton associated with survival of V. cholerae O1 in the aquatic environment.

5. Determination of the toxigenicity of V. cholerae O1 strains isolated during the inter-epidemic periods by the recovery techniques.

6. Confirmation that toxigenic strains of V. cholerae O1 remain viable and capable of producing significant amounts of cholera toxin when cultured under conditions of low nutrient.

7. Plasmid analysis of V. cholerae O1 strains isolated from the aquatic environment throughout the study period in order to detect the emergence, or the reservoir, of multiple-antibiotic resistant(MARV) strains of V. cholerae O1.

C. MATERIALS AND METHODS

a. Environmental sampling. The following sites will be sampled: Dacca, Buriganga River between Swarighat and the water purification plant; Matlab, Dhonogoda River within two miles east and west of the ICDDR, B Canal mouth, as well as the canal itself; Teknaf, the Naf River and its estuary, and the sites where previous sampling have been performed.

b. Sampling procedures. Top and middle level water samples will be collected in presterilized 500 ml plastic bottles and transported to the laboratory for processing. Representative samples will be retained and frozen for chemical analysis (alkalinity, nitrate, nitrate, chloride, copper, calcium, magnesium and sulphate) at a later date. Samples of plankton will be collected using a No. 20 (77 μ mesh) net after towing for sufficient time to obtain 0.5 to 1 g wet weight material. A portion of each sample will be fixed in formaldehyde for examination by microscopy, as well as identification of plankton to the genus level. Fish will be obtained as fresh as possible from commercial markets and the intestinal contents, and gills, sampled for V. cholerae 01 and related pathogenic vibrios.

c. Determinaton of aquatic environmental parameters. Turbidity will be determined by secchi disk. Water temperature, salinity, pH and dissolved oxygen content are determined polarographically using portable electronic meters.

d. Bacteriological methods. The methods for isolation of V. parahaemolyticus from aquatic environmental samples have been described in ICDDR, B protocol "Comparative study of Vibrio parahaemolyticus and related organisms in Teknaf and Matlab waters", and will be used without modification in the present study.

The methods for recovery of V. cholerae 01 from the aquatic environment have been summarized in Figure 1. These methods differ from more traditional schemes which do not include resucsitation steps. Two recovery broths, salt tryptone broth (STB) and brain heart infusion (BHI) broth will be compared with alkaline

peptone water (APW) enrichment and TTP enrichment (Monsur). The composition of each recovery broth is given below:

i) STB

Solution A		Solution B	
NH_4Cl	- 1.0	$MgSO_4.7H_2O$	- 0.02
NH_4NO_3	- 0.2	NaCl	- 10.0
Na_2SO_4	- 0.4	Yeast extract	- 0.2
K_2HPO_4	- 0.6	Tryptone	- 4.0
KH_2PO_4	- 0.2	Water	- 100 ml
Water	- 100 ml		

Adjust pH of both solutions to 7.6, autoclave separately and mix aseptically when cool. Aseptically dispense the mixed solution in 15 ml volumes in sterile universal bottles.

ii) BHI

Use Difco (or other brand) brain heart infusion broth made to full strength according to the manufacturers' instructions. Adjust pH to 7.2 before autoclaving. Dispense in 15 ml volumes in universal bottles then autoclave.

iii) APW

Dissolve Bacto-peptone (10 g/l) and NaCl (10 g/l) in water and adjust pH to 8.6. Dispense in 15 ml volumes in universal bottles and autoclave.

iv) TTP

Dissolve Bacto peptone 10 g/l, Nacl (10 g/l) and Na-Taurocholate 5 g/l in water and adjust pH to 9.0. Dispense in 15 ml volumes in universal container and autoclave. Add K-Tellurile solution to a final concentration of 1: 100000 while using.

The selective plating medium for isolation of V. cholerae 01 is TTGA agar (Monsur). Colonies typical of V. cholerae 01 will be picked and streaked for purity on gelatin agar. Sensitivity to the vibriostatic agent (0/129) will be tested on the gelatin agar plate using discs saturated with 10 µg and 150 µg 0/129. Strains sensitive to 0/129, oxidase-positive and producing gelatinase will be screened

for possession of the 01 antigen with 01 polyvalent antiserum. Strains giving a positive slide agglutination will be identified biochemically as V. cholerae using the protocols currently in use at ICDDR, B centre. Resistance to antibiotic compounds will be determined by disc diffusion, as well as minimum inhibitory concentration, methods described by Collins and Lyne (1976).

Other environmental samples (sediment, plankton and plant) will be enriched in the recovery media detailed in Figure 1 following homogenization in a solution of each enrichment broth.

e. Epifluorescent microscopy. A fluorescent-labeled antiserum specific for V. cholerae 01 has been prepared at the University of Maryland and used to stain heat-fixed slides of subcultures collected at various stages of enrichment outlined in Figure 1. These slides can be kept frozen until examination. Stained slides will be examined using a standard brightfield microscope equipped with an epifluorescence condensor, quartz halogen lamp, band filter, beam splitter and barrier filter to produce coherent light around 560 nm wavelength to detect fluorescent isothiocyanate-labeled antiserum bound to cells of V. cholerae 01.

f. In situ survival studies. Polycarbonate membrane-plexiglass survival chambers, based on the original design of McFeters and Stuart (1972), will be used to determine the behavior of V. cholerae 01 placed in the aquatic environment during the inter-epidemic and epidemic cholera periods. An exploded diagram of a chamber is presented in Fig. 2. A chamber consisting of three sections of machined plexiglass (A,B,C) which are held together by six stainless steel nuts and bolts running through the surrounding body of the central lumen. A polycarbonate membrane filter (Nucleopore Co.) of 0.4 µm pore size and 75 mm diameter is positioned either side of the central spacer (C) and held in place by the endplates (A and B) when the chamber is bolted together. Thus a central enclosed area is formed with a capacity of 20 ml and closed at both ends by membrane. Polycarbonate membranes have the advantage of being less biodegradable

than cellulose membranes. Two 18-gauge stainless steel hypodermic needles (D and E) are fitted into the top of the central spacer to allow filling and withdrawal of samples. Caps for the needles are made by filling cut-down ends of plastic syringes with epoxy resin. These chambers are available at the University of Maryland and have been used by one of the investigators to illustrate the survival characteristics of non-01 V. cholerae in waters of England (West, 1980; West and Lee, 1982). Chambers will be suspended in waters of the Matlab and Dacca areas.

g. Toxin production by environmentally-stressed strains of V. cholerae 01. Isolates recovered from the aquatic environment during the inter-epidemic period will be screened for cholera toxin production and diarrhoeogenic potential in the rabbit Ileal loop test, chinese hamster ovary assay and enzyme-linked immunosorbent assay using the schemes currently in use at the ICDDR, B Centre. In addition, these strains will be cultured in vitro under conditions of high and low nutrient levels in a defined salts medium (Singleton et al., 1982a) to determine the minimum level of nutrient required to maintain viability and biological activity in cells of V. cholerae 01. An initial study at the ICDDR, B Centre has demonstrated that strains of V. cholerae 01 remain viable, and capable of producing fluid accumulations in rabbit ileal loops, when maintained in nutrient levels as low as 100 µg/L tryptone (West, Huq and Colwell, unpublished data, Table 1).

h. Plasmid characterizations. DNA restriction fragment analysis can be used to investigate the molecular identity of plasmids. Restriction enzymes endonucleases which recognize a specific base sequence in DNA and effect double stranded scissions within or near the recognition sequence, will be used to produce DNA fragments that can be separated on the basis of size by agarose gel electrophoresis. A considerable number of different restriction endonucleases with different base sequence specificities have been characterized and some are

available commercially. When treated with a restriction endonuclease, plasmids with identical base sequences yield an identical set of DNA restriction fragments while plasmids with unrelated base sequences yield different sets of restriction fragments. Non-identical plasmids which have a large region of base sequence homology in common yield identical fragments plus fragments unique to each plasmid. Several instructional texts are now available for these molecular genetic studies. The present study will employ methods, or modification of methods, described in the manuals compiled by Davis et al. (1980) as well as those techniques reviewed by Old and Primrose (1981).

Strains of V. cholerae 01 will be screened for plasmids using the rapid "in the well lysis" technique and horizontal agarose gel electrophoresis method recently developed by Newland (1981) at the University of Maryland. This procedure is currently in use at ICDDR, B Dacca. Plasmids from strains will be characterized by molecular weight, as well as restriction enzyme digest analysis. Large scale purification of plasmid DNA from V. cholerae strains will be prepared from approximately 500 ml of cultures prepared following the method of Guerry et al. (1973). DNA in the plasmid enriched supernatant fluid of the lysates will be purified by cesium chloride-ethidium bromide dye bouyant centrifugation. Following centrifugation, DNA bands will be visualized by viewing the tubes under UV illumination, and the covalently closed circular (CCC) DNA plasmid band will be collected by fractionation (Crosa et al., 1975; Falkow et al., 1975). The CCC DNA will be dialysed to remove CsCl and ethidium bromide. Purity of the CCC DNA preparations will be tested by gel electrophoresis of the DNA. If necessary, the DNA will be further purified by recentrifugation. Duggleby et al. (1977) report that, in general, only a single dye-bouyant density centrifugation step is necessary to obtain CCC DNA sufficiently pure for restriction enzyme analysis. CCC DNA solutions will be concentrated by dialysis against Ficoll and the concentrated DNA solutions will be redialysed against buffer. Approximately

1 µg quantities of DNA will be digested with restriction endonucleases as described by Causey and Brown (1978). A control reaction mixture minus enzyme will be run to give a preparation of undigested DNA.

Lambda DNA, whose restriction fragments profile is well characterized (Gottesman and Adhya, 1977), will be used as a control DNA for the digestion procedure and to provide restriction fragment molecular weight standards. The CCC DNA digests and undigested CCC DNA will be electrophoresed on horizontal agarose (0.5-1.0%) gel slabs approximately 30 cm in length. Following electrophoresis, gels will be stained with ethidium bromide solution and photographed under UV light. Plasmid DNA will be initially digested with EcoRl and Hind III endonuclease. Small molecular weight plasmids which have few restriction sites will be digested with two or more restriction enzymes. Molecular similarity between plasmids will be examined by comparison of these restriction enzyme fragment patterns of mobility (Old and Primrose, 1981). Further studies requiringthe use of radioactive probes, such as the probe for cholera toxin genes described by Kaper et al. (1981) will be performed at the University of Maryland. Standard methods described by Davis et al. (1980) and Old and Primrose (1981) will be employed for radioactive studies.

Defination

Sweet water: Comprises river and pond water of Dacca and Matlab where salinity ranges from 0-0.1 parts per thousand during different times of the year.

Brackish water: Comprises river water connected with sea. The water is somewhat salty with salinity ranging from 4-5 parts per thousand. This type of water is available in some parts of Teknaf and also some rivers in Khulna.

Sea water: mostly water from Bay of Bengal and comprises the Teknaf estuaries. The water is highly salty with salinity ranging from 10 to 30 parts per thousand.

D. Significance

The inter-epidemic reservoir of V. cholerae 01 in Bangladesh still remains to be elucidated. Ecology studies of V. cholerae 01 in cholera non-endemic areas strongly suggest that strains can survive for long periods of time in the aquatic environment, possibly in a stressed state which may affect recovery by traditional methods. Epidemiological and demographic data on cholera in Bangladesh now indicate an intimate link between the spread of the disease and changes in the abiotic and biotic parameters of the aquatic environment. For these reasons, this study will focus on the isolation and characterization of V. cholerae 01 from the aquatic environment of Bangladesh during the inter-epidemic periods. The application of recently-developed ecological and molecular genetic techniques is expected to yield a greater understanding of the transmission of cholera. For example, strains of V. cholerae 01 isolated from the inter-epidemic periods and initial cases of cholera in the subsequent epidemic season will be compared using molecular genetic characterization procedures to determine the involvement of inter-epidemic isolates in initiation of the disease in the community. All isolates from the aquatic environment will be screened for antibiotic resistance to provide an insight into the reasons for the recent emergence of MARV strains.

E. Facilities required:

(a) Laboratory space: One working table (3 feet x 8 feet) will be required in Dacca.

(b) Animal resources: New-Zealand white rabbit - 100
Infant rabbit - 40

(c) Logistical support:

Automobile transportation in Dacca	- 800 miles
Automobile transportation in Teknaf	- 500 miles
Return trips to Teknaf (2 persons x 6)	- 12
Speedboat transport in Teknaf	- 10 Hours
Speedboat transport in Matlab	- 100 Hours

SECTION III - BUDGET

A. DETAILED BUDGET

1. PERSONNEL SERVICES

Name	Position	Percent of effort or no. of days	Annual Salary	Project Requirement Taka	Dollar
Dr. M.I. Huq	Head, Microb.	10%	$ 53,740	-	8,061
Dr. R.R. Colwell	Prof., Univ. of Maryland	5%	-	-	-
Dr. K.M.S. Aziz	Asso. Director	5%	$ 62,080	-	3,104
Dr. P.A. West	Postdoctoral Fellow,	15%	-	-	-
Mr. Anwarul Huq	Res.Officer/ Grad. Student	25%	Tk. 46,990	11,747	-
Mrs. Khaleda Haider	Sr. Res. Offic.	25%	Tk. 50,860	12,715	-
Mr. R. Rahman	Res. Tech.	100%	Tk. 45,180	45,180	-
Mr. Ansaruzaman	Res. Offic.	50%	Tk. 35,230	17,615	-
Mr. B. Hossain	Sr. Tech. (Res.)	20%	Tk. 36,640	7,328	-
				94,585	11,165

2. SUPPLIES AND MATERIALS

Media Petridishes	2,000
Laboratory supplies (Chemicals and Glasswares)	1,000
	3,000

3. EQUIPMENT

None

4. PATIENT HOSPITALIZATION - None

	Taka	Dollar
5. OUTPATIENT CARE - None		
6. ICDDR,B TRANSPORT		
1300 miles @ Tk. 4.50 per mile land transport	6,000	-
Speedboat 110 hours @ Tk. 105.00 per hour	11,550	-
	17,400	
7. TRAVEL AND TRANSPORTATION OF PERSONS		
Exchange of laboratory personnel trips between Dacca and USA for learning specialized techniques		
Dacca - College Park - Dacca		1,600
Sampligg within Bangladesh - 6 trips Dacca - Cox'x Bazar - Dacca for 2 persons	12,000	
8. TRANSPORTATION OF EQUIPMENT		
Media and Equipment (Dacca - Cox's Bazar - Dacca)	3,000	-
9. RENT, COMMUNICATION AND UTILITIES		
Guest House charges for Investigators from College Park - 60 days x US$ 30	-	1,800
10. PRINTING AND REPRODUCTION		
Xerox	5,000	-
Others	1,000	-
Publication costs	5,000	-
	9,000	
11. OTHER CONTRACTUAL SERVICES - None		
12. CONSTRUCTION, RENOVATION, ALTERATION - None		

BUDGET SUMMARY

		TAKA	DOLLAR
1.	Personnel Services	94,585.00	11,165.00
2.	Supplies and Materials	-	3,000.00
3.	Equipments	-	
4.	Patient Hospitalization	-	-
5.	Outpatient Care	-	-
6.	ICDDR,B Transport	17,400.00	-
7.	Travel and Transportation of persons	12,000.00	1.600.00
8.	Transportation of Things	3,000.00	-
9.	Rent, Communication and Utilities		1,800.00
10.	Printing and Reproduction	9,000.00	-
11.	Other Contractual Services	2,500.00	-
12.	Construction, Renovation and Alteration	-	-
	Sub-Total :	1,38,485.00	17,565.00
	Total Cost :	6,924.00	17,565.00

GRAND TOTAL: US$ 24,489.00

Conversion rate US$ 1.00 = Tk. 20.00

Personnel $ 15,894

Others $ 8,595

F. Collaborative Arrangements

This study will be carried out at ICDDR,B Dacca and at the Department of Microbiology, University of Maryland, U.S.A. in collaboration with Professor Rita R. Colwell.

Ecological sampling biochemical characterization of strains, survival studies and recovery experiments will be performed in Bangladesh. Facilities at the University of Maryland will be used for electron microscopy, computer analysis of ecological data and some aspects of the molecular genetic characterization of isolates. For covering the costs for these experiments to be done in the University of Maryland an approximate budget of US $ 7,000.00 has been proposed which will be paid by the Dept. of Microbiology, University of Maryland.

The study will introduce the latest developments in ecological and molecular genetic methodologies to the ICDDR,B Dacca. Provisions and arrangements have been requested to enable personnel to travel between College Park and Dacca for training of ICDDR,B staff in these new techniques.

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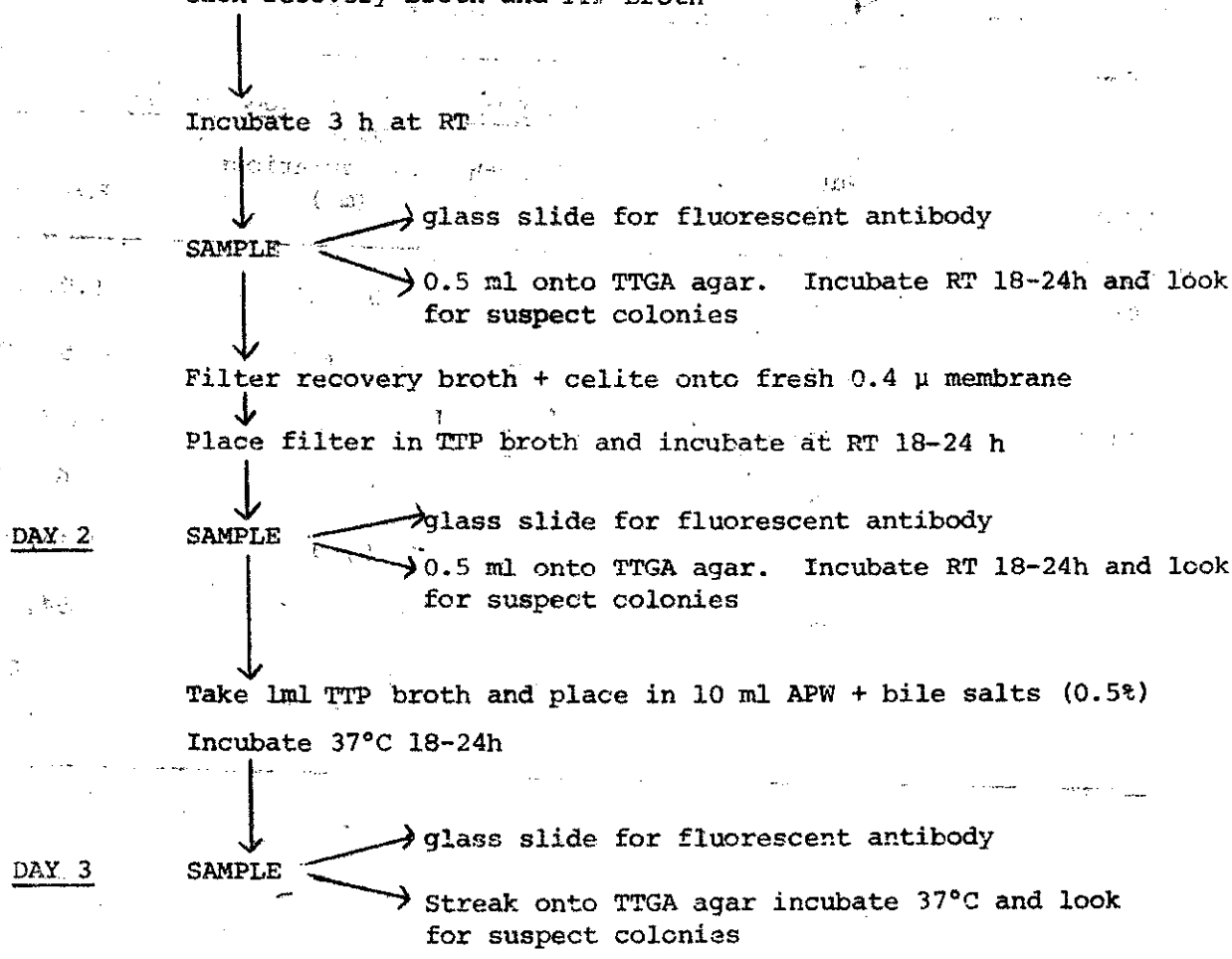
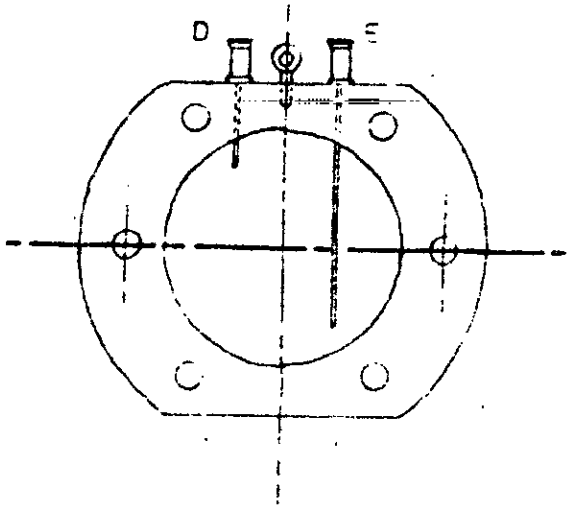
Table 1. Fluid accumulation ratios for three strains of toxigenic *V. cholerae* serovar 01 after incubation at room temperature for 21 days under high (200 µg/L tryptone) and low (100 µg/L trypton) nutrient conditions in laboratory microcosms

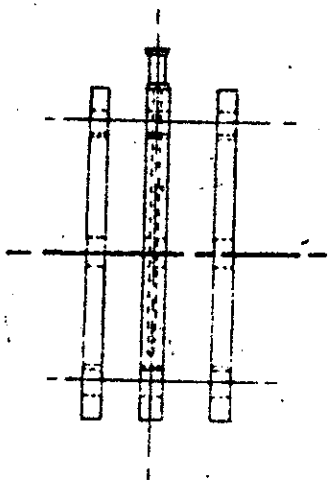
		Duplicate rabbit ileal loops		
Strain	Nutrient Level	Loop Length (cm)	Fluid Accumulation (ml)	F.A. ratio
569B	high	6.0, 6.3	6.0, 6.2	1.0, 0.98
	low	6.5, 6.6	6.2, 6.4	0.95, 0.97
LA4808	high	7.0, 6.8	7.2, 8.6	1.03, 1.26
	low	6.5,6.7	10.5, 9.2	1.61, 1.37
CA401	high	7.5, 7.1	10.5, 9.8	1.40, 1.38
	low	6.5, 6.9	10.0, 8.6	1.54, 1.25
Saline	-	6.5, 6.8	0, 0	0

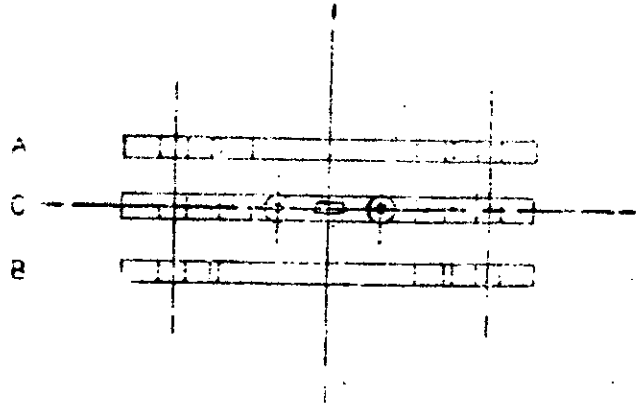
Figure 1. Recovery of Vibrio cholerae 01 from environmental samples



(RT=Room, or ambient, temperature)







MEMBRANE DIALYSIS CHAMBER	SCALE 1·2	FIGURE 2	