tachment 1. Date _____

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

Principal Investigator M.I. Huq. Trainee Investigator (if any) _____

Co-investigator : Anwarul Huq, K.M.S.Aziz.

Application No. ~~79-012~~ Supporting Agency (if Non-ICDDR,B) _____

Title of Study ~~Comparative Study of Vibrio parahaemolyticus and related organisms in Teknaf and Matlab Waters.~~

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 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 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.

Principal Investigator

Trainee

79-012
Rec'd 15/10/79

SECTION I - RESEARCH PROTOCOL

(1) Title : Comparative Study of Vibrio parahaemolyticus and Related Organisms in Teknaf Estuary and Matlab Waters.

(2) Principal Investigator : M.I. Huq

(3) Co Investigators : Anwarul Huq, K.M.S. Aziz

(4) Starting Date : October 1, 1979

(5) Completion Date : September 30, 1980

(6) Total Direct Cost : U.S. $ 10,638.00

(7) Availability of Funds :

(a) Scientific Director's Remarks :-

(b) Controller's Remarks :-

(8) Abstract Summary :

Isolates of NAG V. parahaemolyticus and other related organisms will be obtained from water, sediment, fishes and other filter feeders from Teknaf estuary and coastal areas. Their virulence factors, biological activity and phenetic character will be gathered. Attempts will be made to find out the cultural character of V. parahaemolyticus collected from marine environment and comparison will be made with the those collected from fresh water environment.

Beside all those informations, a report will be made of V. parahaemolyticus of this region on which very little work has been done, and whatever done, not focused properly.

This research may establish some interesting information particularly the virulence factors of the organisms.

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective : Ultimate goal of this research work is to focus some light as an addition to the world of Vibrio parahaemolyticus information. Characterisation of Vibrio parahaemolyticus from this region has not yet been done, and if done, not focused properly. It is hoped that the findings of this work will help to define the disease process if there is any difference, caused by the strains from marine environment of Teknaf and the fresh water environment of Matlab.

2. Background : In different earlier studies the ecology of Vibrio parahaemolyticus and other related organisms in different marine environment has been shown by different workers. Distribution of these organisms in the Atlantic Ocean of South Carolina and Georgia by Kaneko and Colwell(1974), annual cycle in Chesapeak Bay by Kaneko and Colwell(1978), in the middle Pacific Ocean by Aoki(1967), absorption of these organisms onto Chitin and Copepods by Kaneko and Colwell(1975), isolation of V. parahaemolyticus from the North-west Pacific by Baross(1968), from the marine environment in Washington by Baross(1970). Occurrence, of these organisms on mussles, and Oysters and in estuarine waters in the Netherlands by Kampelmacher(1972), from the blue crab in Chesapeak Bay by Krants et al., (1969) and reported from many other parts of

the marine environments of the world. At Cholera Research Laboratory, Dacca, admission cases with diarrhoea caused by vibrios, other than V. cholerae O group I belonged to the Heiberg group I, II, V, VII. Almost 80-90% of the strains belong to Heiberg group V and VII were found to be V. parahaemolyticus (Huq I, 1979 in preparation). Since this organism has been confirmed facultatively halophilic by Sakazaki et al.,(1963), this occurrence may show some higher rate than already found in Dacca and Matlab hospitals of Cholera Research Laboratory where patients poured in from 100% non-marine environment. Inactivation of Vibrio parahaemolyticus in distilled water found by Lee(1972). This may lead to a question that V. parahaemolyticus strains found in (Dacca/Matlab) i.e. in fresh water may be assumed as inactivated to some extent, still causing cholera like diarrhoea. Keeping this in mind, one can easily assume that the V. parahaemolyticus found in marine environment may have some virulence character different from that of fresh water ones and on the activity of some interesting phenomenon like non-adherence on the brush border membrane of rabbit small i intestine (Huq et al., 1979), Spira et al.,(1978). It is generally accepted that V. parahaemolyticus inhabits coastal waters, especially at the mouth of the rivers. Kaneko et al.,(1974), studies of the distribution of V. parahaemolyticus in the open sea are few and contradictory Kaneko(1974). Attempts will be

taken to have some water collections from the high sea area only if the concentration of V. parahaemolyticus found high at the estuaris. Yasunaga and Kuroda(1964) isolated V. parahaemolyticus from fish tuna, caught in the open sea of the Indian Ocean. Aoki(1967) also reported of this organisms from sea water, plankton and fish samples collected from the open sea in South-east Asia. In sea or in the river mouth, the occurrence of V. parahaemolyticus rate is high during the warm months, Horie, S. et al.,(1967). In Bangladesh the warm months starts from April, hence the immediate start of work is needed. The association of Vibrio parahaemolyticus with zooplankton has proved fascinating. During winter, V. parahaemolyticus is not found at all is not true but the rate of occurrence was noted very low, Kaneko et al.,(1973).

3. Rationale : The rationale behind this proposed work is to give some idea of V. parahaemolyticus of this region which is very little known till now. The cultural character of Vibrio parahaemolyticus isolated from two different environment(fresh water and marine water) does behave same or have some difference in respect of their virulence and toxicity in the animal model? This pre-determination regards in respect of less infection rate of V. parahaemolyticus in Teknaf region then that of the internal part of Bangladesh, while it is established or generally accepted that V. parahaemolyticus inhabits coastal waters, Kaneko(1974). Identification

of these pathogen and an understanding of their disease process may help in better way in classification of these organisms and also in the diagnosis.

A similar type of work for the Vibrio parahaemolyticus will be carried out in Matlab, a fresh water environment. The study will start simultaneously in Teknaf and Matlab from where the data will be compared for the results of marine and fresh water environment.

B. SPECIFIC AIMS

The specific aims of this research are :

1. To find out the real characters of V. parahaemolyticus and related organisms of the Teknaf estuary and coastal areas.

2. Vibrio parahaemolyticus and related organisms will be isolated from the marine aquatic sources using procedures which minimize the likelihood of any physical or bio-chemical character loss.

3. Compare the pathogenic potential of organisms isolated from the marine environment with those isolated from non-marine environment using different laboratory model system.

4. Enough phenatic characters will be examined to give a representative sample of the properties of the organisms.

C. MATERIALS AND METHODS

1. Sampling in Teknaf : Samplings will be done at the Naf river, its estuary, off the shore Teknaf beach especially rocky and sandy shore where lot of fauna are existing. Samplings will also be done where the small fishing boats deposit their fishes at one place. Plankton, water and mud will be procured and swabs from the filter feeders will also be taken because it is generally accepted that organisms present in water gets concentrated in the gills of these animals. Samples from the landlocked areas in or around Teknaf where salinity is high and the water body present in between closely situated islands will be taken. Details of the sampling locations will be fixed after sight seeing in well advance of the starting of the work.

Sampling in Matlab : Sampling will be done in the areas where previous records of V. parahaemolyticus incidence are existing. The same plankton, water, mud and the swabs from the filter feeders will be taken as in Teknaf.

2. Sampling Procedures : Water samples will be collected by the Cholera Research Laboratory made sampler at 2-5 meter depth and from the surface where depth is low in presterilized narrow neck 500 ml. NALGENE plastic bottle. They will be kept at normal air temperature until back to the laboratory.

Plankton samples will be collected with the plankton sampling net with a no. 20(77 µm mesh) and mainly for zooplankton no. 15(94 µm mesh) will be used. After each time of use of the plankton net, will be rinsed with 70% alcohol and then with steriled distilled water for at least three times. The plankton samples will be transferred to steriled wide mouth 4 ozs. glass bottles. Plankton net will be towed for 10-15 minutes.

Fish and shrimp collected off Teknaf are caught by small country boat fishermen or by trawl, and the gills of these animals will be swabbed for isolation of bacteria with presterilized cotton swab in 5 ml. Monsur's Bile pepton broth, pH 8.4 to 8.5 for enrichment and on back to the laboratory, 6 hours incubation at 37°C.

Where possible, core samples will be taken using Cholera Research Laboratory core sampler. Core samples will be collected in presterilized universal container with 5 ml. pepton water made in sterile sea water.

Dissolved oxygen and temperature will be determined by KENT portable oxygen meter model 1520 and turbidity with Sacchi disk. Salinity will be measured by Mercuric nitrate titration method(standard method) pH value will be determined

in the laboratory using CORNING pH meter mod. 7. Sampling procedures will be same in Matlab as in Teknaf.

3. Bacteriological Analysis : All the bacteriological analysis will be done as soon as possible but not more than six hours of collection. For vibrio, 100 ml. of water sample will be millipored and the filter membrane will first be placed onto sea water starch agar plate (SWSA = starch agar plate will be made of sterile sea water from Teknaf) for six hours for enrichment at 25°C. After that the membrane will be transferred onto TCBS media plate (Eiken TCBS) = YE - 5G, Peptone - 10G, Sodium citrate - 10G, Sodium thiosulfate - 7G. Dxgall Eiken-5G, Sodium cholate - 3G, Saccharose-20G, Sodium Chloride-10G. Ferric citrate-1G, Bromothymol Blue 0.04G, Thymol Blue 0.04G and Agar Eiken 15G/liter. The plates are to be incubated now at 37°C for 24 hours. Typical green colonies on TCBS as presumptive V. parahaemolyticus (P.V.P.) Colwell (1973) will be streaked onto Gelatin Agar (GA) plate for purity plate with 0/129 vibrio-static compound both 10 µg and 150 µg, incubation over night at 37°C. Gelatinase hydrolysis, oxydase positive and string, Smith, H.L. (1970) (0.5% deoxycholate) test positive and V.S.C. sensitive colonies will be picked for further biochemical test with 3% NaCl Fermentation reaction will be observed in sucrose, mannose and arabinose and manitol,

bromocresol purple broth 0.1% carbohydrate, Lysine or Ornithin decarboxylase and argenine dihydrolase reaction, growth in peptone water broth containing 0%, 3%, 8% and 10% sodium chloride, voges proskaur test and growth at 42°C. At last the Kanagawa test on Wagatsuma agar, Wagatsuma(1968). Slide agglutination with Venkat raman's 0 group I polyvalent V. cholerae antisera will also be observed.

All Vibrio parahaemolyticus and other non-agglutinin vibrio will be stocked for further required analysis on blood agar base stants in screw cap one drum culture vial.

Total viable xxx bacterial count will also be done. Pour plate-1 and plate-2 with total count agar (BBL = TSB-7.5 G, YE-25 G, Na - 0.5 gr, agar 15.0 g and 1000 ml. distilled water) will be incubated at 37°C for overnight. Collected plankton samples will be grinded up or homogenized with pistle and mortar or Tefflon tipped Elvejhen Tissue Grinder (Thomas Phi'ladilphia). Spread plates will be made from the grinded plankton onto TCBS plates and will be incubated for 24 hours at 37°C. Further characterisation for vibrio will be same as water sample. For enrichment, measured volume of grinded plankton will be added to single strength bile peptone (Monsur BP Broth - NaCl - 1%, Bactopeptone 1%, Sodium taurocholate 0.5% where 1% NaCl will not be used but the media will be prepared with sterilized

sea water). After six hours incubation at 25°C, streak plates will be made onto Eiken TCBS and will be incubated at 37°C for 24 hours where possible, sediments will be collected and will be processed in the same way as the grinded plankton will be processed. All bacteriological analysis of Matlab samples will be carried out in the same way as Teknaf but the additional NaCl will be added in culture media,

4. Screened isolates for biological activity in laboratory model system : A set of about one hundred isolates and appropriate control strains will be screened for biological activity in the following laboratory model system:

a. Rabbit Ileal loop Test: The ligated ileal loop model (De and Chatterjee, 1953) will be used for extracellular enterotoxic activity. TSBYE plus 3% salt, 18 hours shake culture supernatant will be injected into the ligated ileal loop in duplicate rabbits. Rabbits will be observed after 18 hours.

The ratio of the volume of fluid accumulation to the length of the ligated segment will be recorded.

b. The same culture filtrate will also be tested for the activity in the Chinese Hampster Ovary(CHO) cell assay, Guerrant et al.,(1974). For ST activity, the same supernatant

will be used for suckling mouse assay Dean et al., (1972) and Gianella, (1976).

D. SIGNIFICANCE

The nature of pathogenic organisms as V. parahaemolyticus of this marine environment has never been studied properly, nor the organisms been characterized. The direct significance of the proposed work is that, information of these organisms from the marine water of Bangladesh and other parts of the world will define in the ecological point of view.

Study of these organisms and identification may focus an understanding of their disease process which ultimately help in better way in classification of these organisms and also in the diagnosis.

Comparring the data of two different environment that is in fresh water and marine water may focus some useful information in the pipe line of V. parahaemolyticus.

E. FACILITIES REQUIRED

a. Laboratory space: One working table 3 feet X 8 feet will be required while working at Teknaf laboratory and in Dacca no additional space is required.

b. Animal Resources: Rabbit, Newzealand white 2.0 to 2,5 Kg. 100 maintained from Dec. 15 -Sep. 15.

Whito mico 2-3 days old

100 maintained from Dec. 15 - Sept. 15.

c. Logistical Support :

Automobile transportation in Teknaf	1800 miles
Return trips to Teknaf	9
Speedboat transport in Teknaf	25 hours
Speedboat transport in Matlab	48 hours

d. Specialized Requirements — None

F. COLLABORATIVE ARRANGEMENTS

This work will be carried out in collaboration with Prof. R.R. Colwell of the University of Maryland, College Park, U.S.A. who will be providing consultative services and confirm some of the representative isolats and characterise them in her laboratory. This has been arranged during the visit of one of the authors in her laboratory.

Literature Cited

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SECTION III - BUDGET

A. DETAILED BUDGET

1. PERSONNEL SERVICES

Name	Position	Percent of effort or number of days	Annual Salary	Project Taka	Requirement Dollars
M.I. Huq	Head, Microbiology Br.	20%	81,348	16,269	-
K.M.S. Aziz	Scientific Director	10%	1,26,480	12,648	-
Anwarul Huq	Research Asstt.	50%	24,384	12,192	-
Rezaur Rahman	Research Tech.	50%	27,936	13,968	-
Belayet Hussain	Research Tech.	48 days	18,432	3,402	-
Matlab		32 days	8,795	1,082	-
Teknaf	Lab. Technician	32 days	8,795	1,082	-
Teknaf	Lab. Attendant	32 days	6,144	756	-
Matlab	Lab. Attendant	48 days	6,144	1,134	-
		Taka		61,651.00	-
Per Diem				10,700.00	
		Sub- Total taka		72,351.00	-

2. SUPPLIES AND MATERIALS

Items	Unit Cost	Amount Required
Media	U.S. $	1800.00
Lab. supplies	$	1200.00
Membrane filteration materials	$	450.00
Office supplies &	$	150.00
Miscellaneous	$	150.00
	Sub-total U.S. $	3,750.00

3. EQUIPMENT

No extra equipment needed.

4. PATIENT HOSPITALIZATION

None

5. OUTPATIENT CARE

None

6. CRL TRANSPORT

1500 miles at 3.00 per mile(land transport)	Tk.	6,000.00
Speed boat 75 hours in(Matlab & Teknaf)	Tk.	7,875.00

7. TRAVEL AND TRANSPORTATION OF PERSONS

Air Travel of two persons		
Dacca - Cox's Bazar & back	Tk.	9,900.00

8. TRANSPORTATION OF THINGS

Media, plates and samples to		
and from Teknaf (Air fare)	Tk.	3,000.00

9. RENT, COMMUNICATION AND UTILITIES

None

10. PRINTING AND REPRODUCTION

Xerox	Tk.	600.00
Others	Tk.	600.00
Publication cost	Tk.	3,000.00
	Tk.	30,975.00

11. OTHER CONTRACTUAL SERVICES

None

12. CONSTRUCTION, RENOVATION, ALTERATIONS

None

B. BUDGET SUMMARY

	Category	Year 1 Taka	Year 1 Dollar
1.	Personnel	72,351.00	-
2.	Supplies	-	3,750.00
3.	Equipment	-	-
4.	Hospitalization	-	-
5.	Out patient	-	-
6.	CRL Transport	13,875.00	-
7.	Travel persons.	9,900.00	-
8.	Transportation things	3,000.00	-
9.	Rent/Communication	-	-
10.	Printing/Reproduction	4,200.00	-
	Taka	1,03,326.00	$ 3,750.00

Total $ 10,638.00

Conversion Rate $ 1.00 = Tk. 15.00