Attachment 1.

Date JULY 7, 1986

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator DR. ANWARUL HUQ Trainee Investigator (if any) _____

Application No. 86-025P Supporting Agency (if Non-ICDDR,B) 29

Title of Study ISOLATION AND CHARACTERIZATION OF AEROMONAS SP. FROM AQUATIC ENVIRONMENTS OF BANGLADESH

Project status:
(X) 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). (NA)

1. **Source** of Population: (NA)
   (a) Ill subjects Yes No
   (b) Non-ill subjects Yes No
   (c) Minors or persons under guardianship Yes No
2. Does the study involve: (NA)
   (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
3. Does the study involve: (NA)
   (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
   (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 (NA)
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   ✓ Protocol (Required)
   ✓ Abstract Summary (Required)
   ___ Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   ___ Informed consent form for subjects
   ___ Informed consent form for parent or guardian
   ___ Procedure for maintaining confidentiality
   ___ Questionnaire or interview schedule *

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

(PTO)

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

Anwar
Principal Investigator

Salina Parveen
Trainee

AUG 4 - 1986

86-025P
4/8

SECTION 1 - RESEARCH PROTOCOL (Pilot)

1. Title: "Isolation and characterization of Aeromonas spp. from aquatic environments of Bangladesh."

   (The work of this protocol will be submitted by the Trainee Investigator as a dissertation for the partial fulfilment of M.Sc. (Thesis) degree of the Dept. of Microbiology, University of Dhaka).

2. Principal Investigator: Dr. Anwarul Huq

   Co-investigator: Mr. Zeaur Rahim

   Trainee Investigator: Ms. Selina Parveen
   M.Sc. Student from the Microbiology Department of the Dhaka University.

   Consultant (Thesis Advisor): Dr. K.M.S. Aziz

3. Starting date: August 1986

4. Completion date: May 1987

5. Total direct cost: US $ 4995.00

6. Scientific Programme Head:

   This portocol has been approved by Disease Transmission Working Group

   Signature of the Scientific Programme Head: Dan K Sack

   Date: 30 June 1986

7. Abstract Summary:

This study will be carried out from the selected study sites to assess the presence of Aeromonas spp. and their counts during the study period and to correlate the incidence of these organisms with respect to some physico-chemical parameters. Isolated strains will be tested for drug sensitivity pattern, enterotoxigenicity and haemolytic activity.

8. Reviews: (a) Ethical Review Committee _____

(b) Research Review Committee _____

(c) Director: _____

SECTION II - RESEARCH PLAN

A. INTRODUCTIOIN:

1. Objective:

The main objective of this protocol is to:

a. Study of the variation of *Aeromonas* spp. in different months of the study period in some aquatic environments of Bangladesh.

b. Correlate the counts of *Aeromonas* spp. with some physico-chemical parameters and

c. Test of their drug sensitivity pattern and enterotoxigenicity.

2. Background:

Cells of *Aeromonas* spp. are motile, facultatively anaerobic gram negative bacilli under the family *Vibrionaceae*. They are found primarily in surface water, and also in human faeces (1).

Aeromonas has been regarded as an autochthonous inhabitant of the aquatic environment and found in fresh water (3) as well as in brackish water (4).

Aeromonas hydrophila is a potential animal pathogen known since 1891, when Sanarelli isolated these organisms from the blood of frog(2). In some vertebrates it has been shown to cause a variety of disease, e.g. stomatitis, pneumonia, and septicemia (5). These organisms, causes ulcertative dermatitis and pneumonia in bottle-nosed doiphin(6) and skin ulcer in codfish.

Moreover, these organisms were found responsible causing death of a large number of fish and alligators in Lake Apopka, Florida(7). The Aeromonas species are also associated with bovine abortion(8).

During the past decade, these organisms isolated from clinical patients as well as environments has been confirmed as enterotoxigenic agents. Sanyal and co-workers first demonstrated the enterotoxicity of A. hydrophila strains isolated from diarrhoeal stool samples and environmental sources (9,15). There are reports published frequently in recent years on the isolation of Aeromonas species (19,21).

Several investigators have isolated the A. hydrophila from the natural water samples(20). Membrane filter method was used by Rippey et al. for the isolation of A. hydrophila from natural water samples (3). The densities of the organism correlated significantly with total phosphorous, chlorophyll a and turbidity. Moderate correlations were found with dissolved phosphorous, kjeldahl nitrogen, and dissolved organic carbon. Little or no correlation was observed with ammonia, orthophosphate, pH, alkalinity, or dissolved oxygen (16).

Reports of isolation of Aeromonas spp. from surface water and other environmental sources in Bangladesh are available (17), but we do not know about the seasonal variation of these organisms in aquatic environments of Bangladesh.

3. Rationale:

Seasonality of any organisms is very imoportant. Species of Aeromonas have been isolated from the environments of Bangladesh but seasonality is not known very well. This study will attempt to isolate the various species of Aeromonas and correlate its seasonality of appearance with water quality in the environment.

B. SPECIFIC AIM:

The specific aim of this protocol is to: -

1. Isolate the Aeromonas spp. from the aquatic environment of Bangladesh.

2. Test the enterotoxicity of isolates.

3. Correlate the counts of Aeromonas spp. with pH temp, turbidity and dissolved oxygen.

4. Test of drug sensitivity pattern of characterized strains.

C. MATERIALS AND METHODS:

1. Definition of sampling sites:

Samples will be collected from Dhanmondi Lake near Kalabagan Lake Circus and Sadarghat pier of the river Buriganga from Dhaka and one sites will be included from Matlab near Matlab station.

Water of Dhanmondi Lake is used by many people for bathing, cooking and washing. Portion of the lake attached to the sampling point, is being used for rearing fish by the Fisheries Department of the Government of Bangladesh.

Water in this area is frequently contaminated with human faces from the water transports. Passengers also use this water for bathing washing and even drinking. People of the nearby localities, use water for bathing, drinking and washing of utencils and soiled clothes of patients as well. A big drain carying city domestic waste water falls into the river near Sadarghat pier. So, this point is highly polluted from different sources.

2. Samples:

Four kind of samples namely, water, selected water plants (Telenthera philoxeroides, Eichhornia crassipes and Pistia stratiotes) plankton and soil sediment will be collected from the sampling sites.

3. Sampling procedure:

Surface water and soil sediment will be collected monthly from the sampling sites in presterilized 4 oz glass bottles for bacteriological analysis. Soil sediment will be collected with the help of ICDDR,B constructed core sampler. The core sampler consists of an aluminium pipe of one inch diameter with segments fo 3-feet each. Water plants will be collected in polythene bag. Plankton samples will be collected by towing plankton net for half an hour at each site. Samples will be stored in wide neck test tube. All samples will be transported to the laboratory in an insulating foam box provided with some cool packs to maintain a similar temperature as in the water environment.

4. Analysis of the samples:

a. Measurement of physico-chemical parameters:

Turbidity will be measured by sacchidisc, temperature by mercury thermometer, dissolved oxygen by portable oxygen meter and pH by corning pH heter (model-7).

b. Bacteriological analysis of the samlples:

Bacteriological analysis will be carried out for the isolation and identification of Aeromonas spp. All the collected samples will be processed for the quantitative analysis of Aeromonas within four to six hours of collection (11). MacConkey plates will be used for the spread plate technique.

c. Quantitative analysis of Aeromonas spp. from water, soil sediment, plankton and water plants:

Water samples will be diluted from 10^{-1} to 10^{-3}. 0.1 ml of neat water sample will also be inoculated in addition to dilution (10^{-1} to 10^{-3}). Soil sediment will be diluted from 10^{-1} to 10^{-3}. From each dilution 0.1 ml of samples will be spread plated on MacConkey's agar, following spread plate method of inoculation (17).

Plankton samples will be homogenized by using taffton tipped tissue grinder and these will be processed in the same way as plant.

Roots of some water plants will be homogenized in electrical blender and 0.1 ml of sample will be spread plated on MacConkey's agar.

d. Speciation of Aeromonas:

Inoculated plates will be incubated at 37C for 18-24 hours. After incubation, morphologically typical colonies of Aeromonas spp. (10) will be picked up and streaked on MacConkey's agar plate for pure culture. Pure Aeromonas strains will be identified following biochemical reactions described in Bergey's Manual (18), sensitivity to 0/129, dehydrogenation of arginine, fermentation of glucose (with gas), mannitol, salicine, utilization of arabinose, hydro lysis of esculin, VP reaction and growth in peptone water containing 0%, 3%, 6% of NaCl.

e. Test of Haemolytic activity:

To test the haemolytic activity a small portion of a bacterial colony will be spotted on blood agar plate and incubated at 37 °C for 18-24 hrs. After overnight incubation, typical haemolytic zone will appear around the spotted colonial growth of the haemolytic strains.

f. Test of enterotoxicity:

Strains of Aeromonas will be tested for enterotoxicity:

i. Adult rabbit ileal loop will be used for such test (12). In short, this will be performed in rabbit ileal loops by the method of De and Chatarjee (1953). Rabbit's weighing 1.5 - 2.0 kg will be starved for 24 hours with only water to drink. The intestine will be exposed by laparatomy; beginning near the ileocaecal junction. 10 cm loops will be tied with an interval of 5 cm between

each. Usually six loops will be made in one rabbit. The test organism will be inoculated in the loop as 1 ml of a 10^{3} saline dilutioin for 4 h nutrient broth culture, containing about 10^{5} bacterial cells. The first loop will always be used as a positive control with cell free culture filtrate of a V. cholerae strain (569B) and the last as a negative control with broth medium to be used for culturing Aeromonas spp. for toxin production. Each strain will be tested in 2-3 rabbits. The animals will be sacrified after 16-18 hrs. and the reaction in the loops will be noted. The length of each loop and the volume of fluid in it will be measured to determine the fluid secretion per unit length. If there will be anuy fluid in any of the gut between loops or any negative reaction in the positive control loops, the results will be discarded.

ii. Test of enterotoxicity by using suckling mice:

Preparation of culture fluid: For enterotoxin assay 5ml of Trypticase soy broth with 0.6% yeast extract in 25 ml Erlenmeyer flasks will be inoculated with the Aeromonas strains to be tested and incubated at 37 °C and 300 rpm in roller drum. Cellfree preparations will be made by centrifuging the cultures at 10,000 x g for 30 min at 4 °C followed by filtration throught a membrane filter (0.22 ug diameter). Supernatant fluids will be stored at 4 C and tested within 1 day after preparation.

Suckling mouse test:

Test solution (100 ul) containing 2 drops of 2.5% pontamine skyblue dye per ml will be administred intergastrically into the milky white stomach of 2 to 4 days old suckling mice with 1 ml syringe. At least three mice will be used in each test. After incubation for 3 h at 28 °C, the animals will be killed by cervical dislocation and small and large intestines will be removed. A ratio of intestinal weight to remaining boidy weight will be determined. A ratio >0.080 will be considered positive and <0.080 will be considered negative.

g. Antibiotic susceptibility testing:

All strains isolated from the environments will be tested for sensitivity against common antibiotics following single antibiotic disc diffusion method (14). The antibiotics to be tested are ampicillin, chloramphenicol, tetracycline, streptomycin, gentamicin, trimethoprim-sulphamethoxazole and kanamycin.

D. SIGNIFICANCE

The prevalence of Aeromonas spp. in aquatic environment of Bangladesh will be studied throughout the study period which will help us to know the fluctuation of Aeromoinas counts in the aquatic environment during the study period and also help us to correlate the counts with physichochemical parameters. The .pa

antibiotic sensitivity pattern will be provide additional information as possible threat of environmental isolates for human.

E. FACILITIES REQUIRED

No extra facilities required other than normal and presently available.

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ICDDR,B

BUDGET PROPOSAL

PROGRAM NAME : DTWG

PROGRAM HEAD : Dr. D. A. Sack

PROTOCOL: Isolation and characterization of Aeromonas spp. from aquatic environments of Bangladesh

PRINCIPAL INVESTIGATOR: Dr. Anwarul Huq

PROTOCOL NO.: STARTING : August, 1986

BUDGET CODE: COMPLETION : May, 1987

BUDGET SUMMARY

A/c	CATEGORY	EXPENSE 1986 .00	EXPENSE 1987 .00	EXPENSE 3rd year .00	TOTAL PROJECT COST
3100	Local Salaries	0	0	0	776
3200	International Salaries	0	0	0	0
3300	Consultants	0	0	0	0
3500	Travel: Local	0	0	0	0
3600	Travel: International	0	0	0	0
3700	Supplies & Materials	0	0	0	975
3800	Other Costs	0	0	0	100
4800	Inter-departmental	0	0	0	3042
	TOTAL DIRECT COST	0	0	0	4893
0000	Indirect Cost, 31%	0	0	0	1516.83
	TOTAL OPERATING COST	0	0	0	6409.83
0300	Capital Expenditure	0	0	0	0
	TOTAL PROJECT COST	0	0	0	6409.83

PERSONNEL REQUIREMENT - LOCAL (A/c 3100)

		No. of positions	Man Months	$ Amount
(A)	Existing	0	0	0
(B)	New Recruitments	1	12	396
(C)	Allocated from other area	0	1	380
	SUBTOTAL	1	13	776
(D)	Separations	0	0	0
(E)	Allocated to other area	0	0	0
	SUBTOTAL	0	0	0
	TOTAL	1	13	776

LOCAL STAFF: (B) NEW RECRUITMENTS

Job designation	No. of position	Man month	Rate per month	$ Amount
Trainee Investigator	1	12	33	396
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
TOTAL	1	12		396

LOCAL STAFF: (C) ALLOCATED FROM OTHER AREA

Budget	Job Desig	Level	No. of position	Man month	Rate per month	$ Amount
060401	Manager	NO-B	0	1	380	380
			0	0	0	0
			0	0	0	0
TOTAL			0	1		380

SUPPLIES AND MATERIALS		(A/c 3700)
Account	Items	$ Amount
3701	Drugs	0
3702	Glassware	200
3703	Hospital Supplies	0
3704	Stationery and Office Supplies	50
3705	Chemicals and Media	250
3706	Materials for Uniform	0
3707	Fuel, Oil and Lubricants	0
3708	Laboratory Supplies	100
3709	Housekeeping Supplies	0
3710	Janitorial Supplies	0
3711	Tools and Spares	0
3712	Non-stock Supplies	150
	SUBTOTAL	750
3713	Freight and Other Charges (30%)	225
	TOTAL	975

OTHER COSTS		(A/c 3800)
Account	Items	$ Amount
3800	Repairs and Maintenance	0
3900	Rent, Communication and Utilities	0
4100	Bank Charges	0
4200	Legal and Professional Expenses	0
4300	Printing and Publication	100
4400	Entertainment, Hospitality and Donation	0
4500	Service Charges	0
4600	Staff Development and Training	0
	TOTAL	100

INTER-DEPARTMENTAL SERVICES		(A/c 4800)
Account	Items	$ Amount
4801	Computer	100
4802	Transport - Dhaka	200
4803	Transport - Matlab	0
4804	Water Transport - Matlab	100
4805	Transport - Teknaf	0
4806	Xerox and Mimeograph	50
4807	Pathology	0
4808	Microbiology Tests	2142
4809	Biochemistry	0
4810	X-ray	0
4811	I.V. Fluid	0
4812	Media	0
4813	Patient Hospitalization - Study	0
4814	Animal - Research	400
4815	Medical Illustration	50
4817	Telex	0
4818	Outpatient Care	0
4830	Transport Subsidy	0
	TOTAL	3042