Attachment 1.

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ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date JULY 2, 1986

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Principal Investigator DR. ANWARUL HUQ Trainee Investigator (if any) MS. ANWARI AKHTAR

Application No. 86-026P Supporting Agency (if Non-ICDDR,B) _____

Title of Study ISOLATION AND CHARACTERIZATION OF PLESIOMONAS SHIGELLOIDES 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).

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
   (b) Use of organs or body fluids Yes No
4. Are subjects clearly informed about (NA)
   (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: (NA)
   (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)

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

Anwarul Huq
Principal Investigator

Anwari Akhtar
Trainee

86-0269
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SECTION I - RESEARCH PROTOCOL (Pilot)

1. Title : Isolation and characterization of Plesiomonas shigelloides from aquatic environments of Bangladesh.

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

2. Principal Investigator : Dr. Anwarul Huq

   Co-Investigator : Mr. Q. Shafi Ahmad

   Trainee-investigator : Ms. Anwari Akhtar
   M.Sc. student from Microbiology
   Department of Dhaka University

   Consultant and : Dr. K.M.S. Aziz

3. Starting : August 15, 1986

4. Completion Date : Feb 14, 1987

5. Total Direct Cost : US $ 4975.00

6. Scientific Program Head : Dr. David A. Sack

This protocol has been approved by the Disease Transmission Working Group.

Signature of Scientific Program Head : David A. Sack

Date : 30 Jun 1986

7. Abstract Summary

This study will be carried out at the selected study sites to assess the prevalence of Plesiomonas sp. in aquatic environments of Bangladesh. Selected physiochemical parameters of the water bodies will also be determined. Analysis on the collected data will be done to see the seasonality of these organisms in the environment.

8. Reviews

a. Ethical Review Committee : _____

b. Research Review Committee : _____

c. Director : _____

## ECTION II - RESEARCH PLAN

### A. INTRODUCTION:

#### 1. Objective:

The main objectives of this protocol is to:

a. findout the prevalence of *Plesiomonas* sp. in aquatic environments of Bangladesh.

b. correlate the counts of *Plesiomonas* sp. with some physicochemical parameters.

c. findout whether environmental strains are enterotoxigenic.

d. correlate the production of hemolysin to enterotoxicity.

e. antibiotic sensitivity pattern on the isolated *Plesiomonas* sp. will be studied.

#### 2. Background

*Plesiomonas* sp. is motile, facultatively anaerobic, gram-negative bacilli, belongs to the family Vibrionaceae. They are found primarily in surface waters, soil and occasionally in human faces (1).

*Plesiomonas shigelloides* is the lone species so far been recognised under the genus *Plesiomonas*. *P. shigelloides* was previously classified under the genus *Aeromonas*. But it is now known to be a separate genus because of having distinguishable characterstics from the genus *Aeromonas*, which includes the production of ornithine decarboxylase, the lack of production of deoxyribonuclease, extracellular proteases and the ability to ferment inositol but not mannitol (1).

It has been investigated by several investigator that P. shigelloides is the caausative agent of diarrhoea in human (1,2,3,4,5 and 6). It is rarely found as normal flora in the human intestine (1). It is also responsible for sporadic diarrhoea of fresh water fish in the tropics (7).

P. shigelloides are occasionally found to cause systemic infection in both immunocompromised and non-compromised patients. Such infections include septicemia, meningitis in neonates, cellulitis, septic arthritis, endophthalmitis, and acute choleocystitis (1).

A few reports, of Plesiomonas infection, however have been documented as severe forms of diarrhoea with acute colitis or choleralike illness (2,13). Rolston et al in 1984 have noted that patients with gastrointestinal malignances may be more susceptible to gastroenteritis caused by P. shigelloides than normal individuals and these infections may be more severe than those seen in normal individuals (5).

Plesiomonas shigelloides was isolated as the sole pathogen from gall bladder bile and wall in a 58-years old woman with acute cholecystis (8). The patient developed an unusual post-operative complication characterized by culture-negative discharge from the wound in combination with extensive abdominal cellulitis and febrility (8). An 83-year-old male presented with chronic diarrhoea and malnutrition associated with P. shigelloides overgrowth in the small intestine (1).

Although there is limited information on the distribution of the Plesiomonas sp. it appear to be ubiquitious. Report from Japan has documented Plesiomonas associated with diarrhoea and isolation of these organisms from dogs, cats, fresh-water fish, river water and sludge from

wet river beds (6,10). Most reports suggest that the source of infection is contaminated surface water. This include to epidemics of diarrhoea in Japan that were attributed to water contaminated by P. shigelloides (6). Another report of diarrhoea possibly related to consumption of contamninated fresh water fish in Zaire (7). Rutala et al reported cases of diarrhoea related to consumption of potentially contaminated oysters (11).

The association of these organisms with water may also explain a seasonal incidence of infection due to P. shigelloides. Seasonal variations in the numbers of organisms detectable in surface water samples have been found in Japan and Europe (10,12). The organisms could be detected only in the warm season, presumably because it does not grow in temperature below 8 °C (12). Cases of P. shigelloides associated with diarrhoea in Bangladesh seem to be clustered in the warm season (2).

There are many reports on drug sensitivity pattern of Plesiomonas shigelloides isolated for clinical sources (14). Reinhandt in 1985 reported that isolates Plesiomonas sp. were found to be sensitive to many antimicrobial agents e.g. ciprofloxacin, enoxacin, norfloxacin, tetracycline, trimethoprim-sulfa-methoxozole and chloramphenical. All isolates produced $\beta$- lactamase and were uniformly resistant to ampicillin (14).

3. Rationale:

Reports on the isolation of environmental Plesiomonas have been made from other countries and especially during warmer months these organisms seems to be more common. In Bangladesh, we have several warm months and clinically these organisms are not uncommon, its logical at these stage to know more about these organisms especially from the aquatic environment which we know very little.

B. SPECIFIC AIM:

The specific aim of this protocol is to:

i. Estimate the incidences of Plesiomonas sp. in the aquatic environments of Bangladesh.

ii. Identify the isolated strains of Plesiomonas sp. to species level.

iii. Enterotoxigenicity and homolysin production of the environmental isolates will be tested.

iv. Attempt will be made to correlate the incidences of Plesiomonas sp. with the selected physico-chemical data accumulated during the study.

v. Antibiotic sensitivity pattern will be examined.

C. MATERIALS AND METHODS:

1. Sampling sites: Two sites, one each at Buriganga river at Shadharghat area and Dhanmondi Lake near Kalabagan Lake Circus and one site at Matlab will be included in the study.

2. Samples: Four kinds of sample such as water, water plants, plankton and soil sediment will be collected from each sampling sites. One sample of each kind from each site except plant will be collected from 3 different sites each month. In case of plants, 2-3 different types of plants will be collected depending on the availability at the time of collection.

3. Sampling procedure: Surface water will be collected monthly from the sampling sites in presterilized 4 oz. glass bottles for bacteriological analysis. Soil sediments will be collected with the help of ICDDR,B constructed core sampler and will be transported to the laboratory in presterilized 4 oz. glass bottles. Water plants such as water hyaceinth and any other available plants will be collected in plastic bags. Caution will be taken to avoid any external contamination. Plankton samples will be collected by towing .77 u net for 1/2 hour at each site. Samples will be transported to the laboratory in insulated foam box at approximately 22 - 25 C for processing within 4 hours of collection. Matlab samples will be primarily processed at Matlab and the plates will be sent to Dhaka for further processing.

4. Analysis of the samples:

i. Measurement of Physicochemical parameters.

Turbidity will be measured by sacchidisc, dissolved oxygen tension and conductivity by portable oxygen meter (YSI model 57), temperature by mercury thermometer and pH by corning pH meter (Model - 7).

ii. Bacteriologycal analysis of the samples:

Bacteriologyical analysis of water, plankton, plants and soil sediments will be carried out for the isolation and identification of Plesiomonas sp. All the collected samples will be processed for the qualitative analysis of the Plesiomonas sp. within four of collection. Attempt will also be made to quantitate the Plesiomonas sp.

Qualitative bacteriological analysis for Plesiomonas spp.:

For qualitative estimation, water will be filtered using membrane and the membrane will be transferred in alkaline peptone water for enrichment. Soil samples and homosinized plant and plankton samples will also be enriched in alkaline peptone water for six hours and then streaked on to Inocitol Brilliant Green Bile Salt agar (IBB) plesiomonas media (Merck). Whitish to pinkish is suspected colonies will be picked for further biochemical tests for final identification. Isolates will be stocked for subsequent tests. Approximately 100 representative isolates will be tested for various biological tests.

Quantitative analysis for Plesiomonas spp:

10 ml of water will be passed through .22 $\mu$ membrane filter and the filter paper will be placed on the agar plate to count the colonies on the following day. Other samples will be spread plated by taking 0.1 ml homoginate.

5. Test of enterotoxigenicity:

Test of enterotoxicity of Plesiomonas will be done in various models as follows:

i. Heat-labile enterotoxin (LT): Following standard procedure, heat-Labile enterotoxin will be detected by:

(a) adult rabbit ileal loop test (16)

(b) skin permeability test (17)

ii. Heat-stable enterotoxin (ST): Will done in "suckling mice assay"(18).

6. Antibiotic susceptibility testing:

All strains isolated from environment will be tested for sensitivity against common antibiotics following standard agar disc diffusion method (19), which is a slight modificatioin of that described by Bauer et al. (20). The following antibiotics will be used: ampicillin, chloramphenicol, tetracycline, streptomycin, gentamicin, trimethoprime and sulphamethoxazole and kanamycin.

7. Test of hemolytic activity:

Hemolytic activity will be done by using blood agar media.

8. Analysis of Data: Various physico-chemical parameters and other test result will be analysed to estublish corelationship with sensitivity and also to evaluate the existance of possible pathogenic Plesiomonas sp. in the natural aquative environment.

D. SIGNIFICANCE:

The prevalence of Plesiomonas sp. in aquatic environments of Bangladesh will be studied throughout the study period which will be helpful to know about this species in the aquatic environment. The correlation between hemolysin productioin and enterotoxicity will be helpful to find out the frequency of toxin producing strains in the environment. The various biochemical tests and antibiotic sensitivity pattern will provide additional data to know in detail about this bacterium, which might be helpful for treating Plesiomonas infection and for further study.

E. FACILITIES REQUIRED:

No extra facilities required other than normal and presently available.

## REFERENCES


1. Reinhardt JH. et al 1985. Plesiomonas shigelloides associated diarrhoea. JAMA. 253(22):3294-5.

2. Huq MI. et al 1981. Microbiological and clinical studies in diarrhoea due to Plesiomonas shigelloides. Indian. J. Med. Res. 77:793-7.

3. Pastian MR. 1984. Inclusion bodies in Plesiomonas shigelloides. App. Environ. Microbiol. 47(1):216-8.

4. Pitarangisic et al, 1982. Enteropathogenicity of Aeromonas hydrophila and Plesiomonas shigelloides: Prevalence among individuals with and without diarrhoea in Thailand. Infect. Immun. 35(2):666-73.

5. Rolston KV. et al. 1984. Diarrhoea due to Plesiomonas shigelloides in cancer patients J. Clin. Microbiol. 20(1):597-8.

6. Tsukamoto T. et al. 1978. Two epidemic of diarrhoeal disease possibly caused by P. shigelloides. J. Hygllon. 80(2):275-80.

7. Van Damme LR et al. 1980. Frequent isolation of Edwardsiella tarda and Plesiomonas shigelloides from healthy zairese fresh water fish a possible source of sporadic diarrhoea in the tropics. Appl. Environ. Microbiol. 39:475-479.

8. Classon BE. et al. 1984. Plesiomonas shigelloides in acute cholecystitis a case report. J. Clin. Microbiol. 20(5):985-7.

9. Penn RG. et al. 1982. Plesiomonas shigelloides overgrowth in the small intestine. J. Clin. Microbiol. 15(5):869-72.

10. Arai T. Ikejima N, Itoh T. et al. k1980. A survey of Plesiomonas shigelloides from aquatic environments, domestic animals, pets and humans. J. Hyg. 84:203:203-211.

11. Rutala, W.A. et al. 1982. Oyster associated outbreak of diarrhoeal disease possibly caused by Plesiomonas shigelloides. Lancet. 1:739.

12. Schubert R.H.W. 1984. Genus IV: Plesiomonas in Krair N.R. (ed): Bergey's Manual of Systematic Bacteriology. Baltimore, Williams & Wilkins Co. 1:545-548.

13. Chatterjee B.D. Neogy K.N. 1972. Studies on Aeromonas and Plesiomonas species isolated from cases of choleraic diarrhoea. Indian J.Med. Res. 60:520-4.

14. Reinhart, J.F. et al. 1985. Comparative in vitro activities of selected antimicrobial agents Aeromonas species and Plesiomonas shigelloides. Ann. Inst. Pasteur. Microbiol 136 A(2):173-98.

15. American Public Health Association: 1975. Standard methods for estimation of water and waste water, 14th ed. American Public Health Association. Inc. Washington, D.C.

16. De S.N., D.N. Chatterjee, 1953. An experimental study of the mechanism of action of V. cholerae on the intestinal mucous membrane. J. Pathol. Bacteriol. 46:559-62.

17. Craig, J.P. 1965. A permeability factor found in cholera stool and culture filtrates and its neutralization by covalescent cholera sera. Nature. 207:614.

18. Gianella, R.A. 1977. Specificity of suckling mouse assay for ST and mode of action of the toxin. Gastroenterology. 72:A-39/1062.

19. Barry, A.L. and C. Thornsberry, 1985. Susceptibility tests: Diffusion test procedures, P. 978-987. In E.H. Lennette, A. Bolows, W.J. Hauster Jr., H.J. Shadomy (eds). Manual of Clinical microbiology. 4th ed. Am. Soc. Microbiol. Washington, D.C.

20. Bauer, A.W., W.M. Kirby, J.C. Sherris, M. Turk, 1966. Antibiotic susceptibility testing by a standardized single disc method. Am. J. Clin. Pathol. 45:493-496.

21. Schubart R.H.W. 1977. Ueber den Nachweis von Plesiomonas shigelloides Habs and Schuberta, 1962 and in Elecktivmedium, den Inositol Brilliantgrun-Gallesalz-Agar. E. Rodenwaldt-Arch 4:97-107.

ICDDR,B

BUDGET PROPOSAL

PROGRAM NAME : DTWG

PROGRAM HEAD : Dr. D. A. Sack

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

PRINCIPAL INVESTIGATOR: Dr. Anwarul Huq

PROTOCOL NO.: STARTING : August 15, 1986

BUDGET CODE: COMPLETION : July 31, 1987

BUDGET SUMMARY

| A/c | CATEGORY | EXPENSE 1st year .00 | EXPENSE 2nd year .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 | 3124 |
| | TOTAL DIRECT COST | 0 | 0 | 0 | 4975 |
| 0000 | Indirect Cost, 31% | 0 | 0 | 0 | 1542.25 |
| | TOTAL OPERATING COST | 0 | 0 | 0 | 6517.25 |
| 0300 | Capital Expenditure | 0 | 0 | 0 | 0 |
| | TOTAL PROJECT COST | 0 | 0 | 0 | 6517.25 |

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 |
|---|---|---|---|---|
| Research Trainee | 1 | 12 | 33 | 396 |
| | 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 | 300 |
| 3706 | Materials for Uniform | 0 |
| 3707 | Fuel, Oil and Lubricants | 0 |
| 3708 | Laboratory Supplies | 150 |
| 3709 | Housekeeping Supplies | 0 |
| 3710 | Janitorial Supplies | 0 |
| 3711 | Tools and Spares | 0 |
| 3712 | Non-stock Supplies | 50 |
| | 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 | 50 |
| 4100 | Bank Charges | 0 |
| 4200 | Legal and Professional Expenses | 0 |
| 4300 | Printing and Publication | 50 |
| 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 | 0 |
| 4802 | Transport - Dhaka | 200 |
| 4803 | Transport - Matlab | 200 |
| 4804 | Water Transport - Matlab | 0 |
| 4805 | Transport - Teknaf | 0 |
| 4806 | Xerox and Mimeograph | 0 |
| 4807 | Pathology | 0 |
| 4808 | Microbiology Tests | 2074 |
| 4809 | Biochemistry | 50 |
| 4810 | X-ray | 0 |
| 4811 | I.V. Fluid | 0 |
| 4812 | Media | 0 |
| 4813 | Patient Hospitalization - Study | 0 |
| 4814 | Animal - Research | 500 |
| 4815 | Medical Illustration | 100 |
| 4817 | Telex | 0 |
| 4818 | Outpatient Care | 0 |
| 4830 | Transport Subsidy | 0 |
| | TOTAL | 3124 |



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