

Principal Investigator: _____ Trainee Investigator (if any): _____
 Application No. 24-029 Supporting Agency (if Non-ICDDR,B): _____
 Title of Study: Incidence, enterotoxigenicity
antibiogram and biochemical properties Project status:
of Aeromonas hydrophila isolated from
diarrhoeal patients
☐ 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:
 - Will signed consent form be required?
 - From subjects Yes ☒ No ☐
 - From parent or guardian (if subjects are minors) Yes ☒ No ☐
 - Does the study involve:
 - Physical risks to the subjects Yes ☒ No ☐
 - Social risks Yes ☒ No ☐
 - Psychological risks to subjects Yes ☒ No ☐
 - Discomfort to subjects Yes ☒ No ☐
 - Invasion of privacy Yes ☒ No ☐
 - Disclosure of information damaging to subject or others Yes ☒ No ☐
 - Does the study involve:
 - Use of records, (hospital, medical, death, birth or other) Yes ☒ No ☐
 - Use of fetal tissue or abortus Yes ☒ No ☐
 - Use of organs or body fluids Yes ☒ No ☐
 - Are subjects clearly informed about:
 - Nature and purposes of study Yes ☒ No ☐
 - Procedures to be followed including alternatives used Yes ☒ No ☐
 - Physical risks Yes ☒ No ☐
 - Sensitive questions Yes ☒ No ☐
 - Benefits to be derived Yes ☒ No ☐
 - Right to refuse to participate or to withdraw from study Yes ☒ No ☐
 - Confidential handling of data Yes ☒ No ☐
 - Compensation &/or treatment where there are risks or privacy is involved in any particular procedure Yes ☒ No ☐
- 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 signature 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:
- 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.
 - Examples of the type of specific questions to be asked in the sensitive areas.
 - An indication as to when the questionnaire will be presented to the Cttee. for review.

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

Zia/Rahim

Principal Investigator

13 JUL 1984

Trainee

SECTION I - RESEARCH PROTOCOL

1. TITLE : Incidence, enterotoxigenicity, antibiogram, and biochemical properties of Aeromonas hydrophila isolated from diarrhoeal patients.
2. PRINCIPAL INVESTIGATOR : Md. Zeaur Rahim
3. CO-INVESTIGATORS : Dr. Nigar S. Shahid
Dr. A. S. M. Hamidur Rahman
4. CONSULTANT : Dr. K. M. S. Aziz and
Prof. S.C. Sanyal
5. STARTING DATE : 1st of August 1984
6. COMPLETION DATE : 31st August 1985.
7. TOTAL DIRECTO COST : US\$ 5,313.00
8. SCIENTIFIC PROGRAM HEAD

This protocol has been approved by Disease Transmission Working Group.

Signature of the Scientific Programme Head: K. M. S. Aziz

Date: 26/8/84

9. ABSTRACT SUMMARY:

Incidence and seasonality of Aeromonas hydrophila associated diarrhoea will be studied in 4% surveillance patients. The isolated A. hydrophila strains will be tested for enterotoxicity, cytotoxicity, drug resistance pattern against common antibiotics and biochemical properties. Attempts will be made to explore the existence of any correlation between enterotoxicity and biochemical properties so that enterotoxigenic strains may be detected by simple biochemical tests. The antibiotic resistance pattern will be examined to delineate the feasibility of developing a selective medium for isolation of the organism inhibiting other enterobacteria.

A. INTRODUCTION

1. Objectives:

To find out the incidence and seasonality of A. hydrophila associated diarrhoea. Further, the enterotoxicity of the strains and biochemical behaviour will be studied to explore the possibility of any correlation between these characters and their antibiogram will be done to examine the feasibility of developing a selective medium for isolation of this organism inhibiting other enterobacteria.

2. Background:

Aeromonas hydrophila is regarded as a pathogen in different vertebrates. In cold blooded animals, this bacterium causes a variety of diseases such as stomatitis, pneumonia (29) and septicemia (10) in snakes and red leg disease in frog (38). A. hydrophila has been incriminated in causing death of a large number of fish and alligators in lake appoke (54). This bacterium has also been isolated from the intestines of the American alligators (Alligator mississippiensis) which died without showing any symptom of disease (24). It has been reported to cause bovine abortion (61). In fish, it reportedly can cause furunculosis and has been isolated from kidneys and intestines of both healthy and moribund fish (35). Aeromonas induced disease in reptiles has been reviewed by Marcus (36). In human beings, this bacterium produces a variety of diseases such as septicemia (14,46), meningitis (46) endocarditis (13), corneal ulcer (23), peritonitis (50) and wound infection (28,31,54).

Rosnner (1964) isolated Aeromonas from feces of a patient with abdominal pain, fever and bloody stool (49), which gave an indication that A. hydrophila

may be an etiological agent of diarrhoea. von Graevenitz and Mensch (1968) isolated A. hydrophila from stools of patients with and without diarrhoea (59). During recent years, the role of A. hydrophila as an etiological agent of diarrhoea has been confirmed by various workers (6,8,11,25,26,43,45). The rate of isolation of A. hydrophila from diarrhoeal stool varies in different countries. In India, Chatterjee and Neogy (11), Sanyal et al. (52), and Bhat et al. (6) isolated Aeromonas and Plesiomonas in 5.0% cases of diarrhoea in Calcutta, Varanasi and Vellore respectively. Gracey et al. (1982) reported the isolation of Aeromonas spp. in 10% of 500 children attending hospital with diarrhoea in Perth, West Australia. Similar isolation rate (10%) has been found among the children with diarrhoea in Jakarta, Indonesia (25). It has also been isolated from asymptomatic controls in Thailand (43), United States (8,59), Australia and Indonesia (25).

In ICDDR,B surveillance system, all the known etiological agents of diarrhoea are being identified routinely. From the surveillance system we know the incidence and seasonality of rotavirus, Shigella, enterotoxigenic Escherichia coli and Vibrio cholerae O1 (57). Plesiomonas shigelloides has also been reported from ICDDR,B (30). According to the surveillance reports, the rate of isolation of A. hydrophila is very low (less than 1.0%). Gracey et al. (1982) reported that in Perth, West Australia, Aeromonas associated diarrhoea among the children is in the peak in the summer months (25).

It is established that, gastroenteritis caused by Aeromonas is due to elaboration of enterotoxin from this bacterium. Sanyal, Singh and Sen (1975) first demonstrated enterotoxicity of A. hydrophila strains isolated from diarrhoeal stools and environment (51). They also demonstrated that cell-free culture filtrate of the organism can cause fluid accumulation in rabbit ileal loop (1,2), increase in permeability of rabbits skin (17,19) purified the enterotoxin up to electrophoretic homogeneity (18), cloned the enterotoxin

gene(58) & also showed cyclic AMP mediated enterotoxicity (16). Most of the observations were later confirmed by different workers (7,60). Besides elaboration of additional toxic factors such as haemolysin (60) aerolysin (7), cytotoxin (15) by this organism have been reported.

Man and other animals acquire infections with drug resistant bacteria from the environment (3,4). Isolation of drug resistant A. hydrophila from human infections has been reported (21,22,40,41). Clinical isolates usually acquire resistance due to treatment of diseases with synthetic chemotherapeutic agents and antibiotics. In Bangladesh, environmental isolates of A. hydrophila showed resistance against ampicillin, septrin, chloramphenicol, neomycin, polymixin B, vancomycin and streptomycin (unpublished data). But we do not know the resistance pattern of A. hydrophila strains isolated from diarrhoeal stools in Bangladesh.

Aeromonas shares various characteristics with the members of Enterobacteriaceae and Vibrionaceae. These common characteristics and the lack of a selective isolation medium are the two main problems in isolation of A. hydrophila. Eddy (1960) used peptone water containing horse blood (5%) and dialized skimmed milk (20) for isolation of A. hydrophila. Other workers like Schubert (1967), Shotts and Rimble (1973), Rippey and Cabelli (1979) and Rogol (1979) also formulated various media (47,48,53,56). None of them is satisfactory, because the medium of Schubert cannot pick up all strains of the species, that of Shotts and Rimble can not differentiate Vibrio parahaemolyticus and Aeromonas. Rogol's medium is impregnated with ampicillin on which only ampicillin resistant A. hydrophila strains can grow. Recently we have isolated a number of A. hydrophila that are sensitive to ampicillin (unpublished data). So, the use of Rogol's medium has the possibility to miss ampicillin sensitive strains of A. hydrophila. These data indicate that there is a need to formulate a selective medium for better isolation of

A. hydrophila. In this work we will use Peptone Beef Extract Glycogen agar (38) which is without added antibiotic for the isolation of A. hydrophila.

Morphologically aeromonads are confused with the members of Enterobacteriaceae. So, the isolation rate may be low in some cases. In the environmental samples (river and pond water, and fish ulcer) the isolation rate is very high (95-100%). So, the low rate (>1%) of isolation from the diarrhoeal patient is surprising. In ICDDR,B surveillance system, for isolation of Aeromonas stool samples/rectal swabs were directly plated on MacConkey's agar. Higher rate of isolation may be possible by plating the stool samples/rectal swabs on more than one plating media before and after enrichment in bile peptone and selenite F broth (6).

Studies of different workers (7,12) as well as those conducted in our laboratory indicated that enterotoxigenicity may be related to certain biochemical characters including haemolysin production. There are also indications (12) that clinical isolates may differ in biochemical behaviour with environmental isolates. It would be interesting to study the correlation between enterotoxigenicity and biochemical behaviour of clinical and environmental isolates.

3. Rationale:

The rationale underlying this research is to find out the actual rate of A. hydrophila associated diarrhoea in Bangladesh, to test whether all of them are enterotoxigenic, to find out if there is any correlation between enterotoxin production by them and their biochemical properties, so that a simple indicator can be developed for detection of enterotoxic strains; and to see drug sensitivity pattern to have simple guidance for treatment as well as explore the feasibility of developing a selective medium incorporating an antibiotic to inhibit other enteric bacteria.

B. SPECIFIC AIM

The specific aim of this research is-

1. To find out the incidence and seasonality of Aeromonas hydrophila associated diarrhoea.
2. To see whether the rate of isolation increases using selective media following enrichment of the stool/rectal swab.
3. To study the comparative biochemical behaviour of clinical and environmental isolates and their correlation with enterotoxin.
4. To study the comparative drug sensitivity pattern of the environmental and clinical isolates and explore the feasibility of developing a better selective medium incorporating an antibiotic to inhibit other enterobacteria.

MATERIALS AND METHODS:

Isolation and identification of Aeromonas hydrophila: stool samples/rectal swabs of 4% surveillance patients will be streaked on MacConkey's agar plate and peptone beef extract glycogen agar media before and after enrichment in bile peptone and selenite both (6) and incubate the plates at 37°C. After overnight incubation characteristic aeromonad colonies will be inoculated in KIA, MIU, Citrate, Lysine, Ornithine decarboxylase and Arginine dihydrolase, sensitivity to O/129, V-P reaction, fermentation of mannitol and inositol, growth in 0% and 8% NaCl. Oxidase test will be done from growth on KIA and one colony of identified aeromonad strain will be preserved in glycerol broth at -20°C for further studies.

Drug sensitivity test:

All the strains isolated from the patient and equal number of environmental isolates will be tested for sensitivity against common antibiotics following single antibiotics disc method. Among antibiotics: ampicillin, chloramphenicol, tetracycline, streptomycin, neomycine, trimethoprim-sulfamethoxazole, gentamycin, erythromycine novobiocine and vancomycine will be use.

Test of enterotoxicity, correlation between their properties and other biochemical characters:

Enterotoxicity of A. hydrophila strains correlates with haemolysin production by them (Rahim et al. unpublished data). Further, lysine decarboxylase and VP reactions have also been correlated with enterotoxin. All the strains will be tested for the presence of haemolysin on blood agar plate containing 5.0-7.0% sheep blood, and every third haemolytic strain will be tested for enterotoxicity. Lysine decarboxylase and VP will be done routinely for identification of A. hydrophila. The environmental isolates for comparison of antibiogram, enterotoxicity and biochemical properties with those of clinical isolates will be used from our laboratory stock.

Aeromonas enterotoxin has been detected in various models and the present study will be conducted with Bangladeshi strains using following models.

Heat labile enterotoxin

- a. Adult rabbit ileal loop test (63)
- b. Skin permeability test and (62)
- c. Chinese hamster ovarian cell line (CHO) (65)

Heat stable enterotoxin:

Test for heat stable enterotoxin will be done in "suckling mice assay". (64)

ABSTRACT SUMMARY

Incidence and seasonality of Aeromonas hydrophila associated diarrhoea will be studied in 4% surveillance patients. The isolated A. hydrophila strains will be tested for enterotoxigenicity, cytotoxicity, drug resistance pattern against common antibiotics and biochemical properties. Attempts will be made to explore the existence of any correlation between enterotoxigenicity and biochemical properties so that enterotoxigenic strains may be detected by simple biochemical tests. The antibiotic resistance pattern will be examined to delineate the feasibility of developing a selective medium for isolation of the organism inhibiting other enterobacteria.

- | | | |
|----|-------------------------|---|
| 1. | Subject population | The study will involve 4% surveillance patient for stool/rectal swab. |
| 2. | Risks | Not applicable |
| 3. | Protection against risk | Not applicable |
| 4. | Confidentiality | Not applicable |
| 5. | Privacy | Not applicable |
| 6. | Interview | Not applicable |
| 7. | Benefit | This study will help better management of patients. |
| 8. | Use of hospital records | Hospital records will be used. |

SECTION III - BUDGET

A. DETAILED BUDGET:

1. Personnel Services:

<u>Name</u>	<u>Position</u>	<u>% or no. day</u>	<u>Annual Salary</u>	<u>Project Requirement Taka Dollars</u>
Md. Zeaur Rahim	Principal Investi- gator	50%		18,000
Dr. Nigar S. Shahid	Co-investigator	5%		7,000
Dr. A.S.M. Hamidur Rahman	Co-investigator	10%		8,000
Md. Ashraf Ali	Lab. Attendant	20%		5,000
Dr. K.M.S. Aziz	Consultant			
Prof. S.C. Sanyal	Consultant			
Sub Total :				<u>38,000.</u>

2. SUPPLIES AND MATERIALS:

	<u>Amount Required</u>	<u>Unit Cost</u>	<u>Project Requirement Taka Dollar</u>
Culture media			12,000
Millipore sets			500
Chemicals and reagents			<u>800</u>

<u>Animals</u>	<u>Amount Required</u>	<u>Unit Cost</u>	<u>Project Requirement</u>	
			<u>Taka</u>	<u>Dollars</u>
Adult albino Rabbit	100	10 (US\$)		1000
Suckling mice	150		2,000	
<u>Tissue Culture</u>				
CHO	200 tests each		3,000	
3. <u>EQUIPMENT:</u>		; Nil		
4. PATIENT HOSPITALIZATION		Nil		
5. OUT PATIENT CARE		Nil		
6. ICDDR,B TRANSPORT		Nil		
7. TRAVEL AND TRANSPORTATION		Nil		
8. TRANSPORTATION OF THINGS		Nil		
9. RENT, COMMUNICATION		Nil		
10. PRINTING & REPRODUCTION			3,000	
11. OTHER CONTRACTUAL SERVICES		Nil		
12. CONSTRUCTION, RENOVATION ALTERATION		Nil		

B. BUDGET SUMMARY

Category:

	<u>Taka</u>	<u>Dollars</u>
1. Personal	38,000	
2. Supplies	17,000	2,300
3. Equipment	Nil	
4. Hospitalization	Nil	
5. Outpatient	Nil	
6. ICDDR,B Transport	Nil	
7. Travel persons	Nil	
8. Transportation of things	Nil	
9. Rent and Communication	None	
10. Printing & Reproduction	3,000	
11. Contractual services	None	
12. Construction	None	
	58,000	2,300

US\$ 2,320 + 2300.00 = 4,620

Overhead charge 693.00

Grand Total : US\$ 5,313.00

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