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ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date Jan 12, 1984
2/2/84

Principal Investigator Dr M.I. Huq

Trainee Investigator (if any)

Application No. 84-007

Supporting Agency (if Non-ICDDR,B) 98

Title of Study: Characterisation of the parahaemolytrions isolated from clinical cases and environment in respect of production of toxin/toxic factors, haemolysin and enzymes produced by them.

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 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)
(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 Ctte. for review.

The study involves use of Microbial stock cultures and no human subject is involved.

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

12 FEB 1984

Principal Investigator

Trainee

SECTION 1 - RESEARCH PROTOCOL

1. Title : Characterisation of the V. parahaemolyticus isolated from clinical cases and environment in respect of production of Toxin/ Toxic factors, haemolysin and enzymes produced by them.

2. Principal Investigator : Dr M.I. Huq

   Co-investigators : Dr K.M.S. Aziz
   Mrs. Khaleda Haider
   Mr. Q.S. Ahmed
   Dr K.A. Al-Mahmud

   Consultant : Dr Ivan Ciznar

3. Starting Date : May 1, 1984

4. Completion Date : April 30, 1986

5. Total Direct Cost : US$ 45,011.00 1st year
   US$ 30,800.00 2nd year

6. Scientific Program Head

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

Signature of the Scientific Program Head K.M.S. Aziz

Date 29/4/84

7. Abstract Summary:

Vibrio parahaemolyticus is an enteric pathogen and a marine organism reported now from several countries. Though many studies have been done during the last decades to understand the pathogenic mechanisms of V. parahaemolyticus, its enteropathogenicity is yet be clearly understood. A thermostable direct hemolysin was first sought to be responsible for human pathogenicity. A lethal toxin has also been described by Honda et al which was found to be identical to direct haemolysin. The author also recently described a cholera like enterotoxin which caused CHO cell elongation. The V. parahaemolyticus also produces protease like activity. Till to-day the roles of these haemolysins, toxins and related enzymes

in the pathogenicity of V. parahaemolyticus are not quite understood.

Very little works have been done on the Vibrio parahaemolyticus isolated in Bangladesh from clinical cases and environment and the works done so far were related to isolation and identification of the strains and its ecological and epidemiological patterns. Inspite of ecological differences in various parts of Bangladesh V. parahaemolyticus has been isolated from almost all parts in varying number in different times of the year.

On the basis of the data available from our previous study done in different parts of Bangladesh we would like to look into the relationship of the different isolates from variable ecological niche to the pathogenic mechanism produced by them. We plan to study the production of toxin/toxic factors, haemolysins, cholera like enterotoxins, stable toxins and enzymatic factors by different Vibrio parahaemolyticus isolated from patients and environment especially water. Various established animal models including the newly developed SAM model along with the different cell lines, CHO cells Y1 adrenal cells and Hela cells will be used. As Vibrio parahaemolyticus has been found to cause invasion of the gut minicing Shigella, invasive properties will also be looked at using Standard Sereny test as well as Hela cell monolayer.

8. Reviews:

a. Research Involving Human Subjects: _____

b. Research Review Committee: _____

c. Director _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objectives:

The overall objective of this proposal is to investigate the pathogenic mechanism of the V. parahaemolyticus in causing disease in human. Studies done in different countries has inplicated different types of toxins/toxic factors, hemolysin etc to be responsible for causing disease in human; but the exact nature of their mode of action and the exact factor responsible is yet debated. We would plan to isolate the toxin/toxic factors hemolysin and enzyme factors from isolates of different environmental and ecological parameter as well as from patients and look at the pathogenic mechanisms of these different factors in causing diarrhoea in human being. We would also plan to look at the invasive characters if any produced by these organisms which may be responsible for causing invasive diarrhoea.

2. Background:

In recent years Vibrio cholerae serotypes other than 01 have been isolated from a variety of sources in nature as well as from human beings as has been recognised as an important aetiologic agents of choleraic diarrhoea [1,2]. These vibrios have biochemical reactions identical to V. cholerae but do not agglutinate with O Group I antisera. On the basis of these findings they have been variously named as non-agglutinable varios, non cholera vibrios and choleriform vibrios[3,4].

Vibrio parahaemolyticus is widely distributed thoroughout the world especially in warm coastal water and differs mainly with the classical non 01 V. cholerae on being halophilic (does not grow without the

presence of salt) (5). Their main habital is in estuarine waters, sea fish and shell fish in which they propagate. In Bangladesh the major outbreak due to V. parahaemolyticus occurred in 1975 and epidemiologic studies implicated the epidemic with ingestion of contaminated water and fish(6,7). Though V.parahaemolyticus did not cause any big epidemic since 1975, it is isolated frequently from clinical cases at hospitals in Dhaka & Matlab. Microbiological studies have characterised the clinical & environmental isolates and its occurrence in relation to environmental and biochemical parameter has been established (8,9).

Many studies has been done during the last 15 years to understand the pathogenic mechanisms of V.parahaemolyticus. Its enteropathogenicity has first been demonstrated by feeding broth cultures of a clinical isolates to human volunteer and later a close relationship between Kanagawa phenomenon positive strains and human pathogenicity has also been established (10). During the same time it was also established that almost all the strains isolated from human patients were Kanagawa phenomenon positive while the environmental isolates were Kanagawa negative(11). Obara isolated a thermostable hemolysin from culture filtrates of Kanagawa phenomenon positive strains & suggested that this hemolysin might be responsible for human pathogenicity (12). Zen Yoji purified this thermostable hemolysin and proposed the name enteropathogenic toxin which was later designated as thermostable direct hemolysin by Muwatani (13). A lethal toxin has also been described by Honda et al which was found to be identical to the thermostable direct hemolysin produced by this organism(14). The lethal toxin showed cardiotoxicity in various experimental animals such as mice, rats and guineapig.

Sakazaki et al studied reactivity of ligated ileal loops of rabbits and showed that culture filtrates of both Kanagawa positive and negative strains cause fluid accumulation (15). It is well established that only Kanagawa phenomenon positive strains produce the thermostable direct hemolysin, so these results suggests that the thermostable direct hemolysin is not the only factor with enteropathogenic activity. Recently another factor has been described in culture filtrates by Honda et al (16) which

causes changes in CHO cells similar to V. cholerae and E. coli LT. So this findings suggests that the culture filtrates of V. parahaemolyticus contains an enterotoxin other than the thermostable direct hemolysin. Yamagase (17) reported that V. parahaemolyticus produces phospholipase A, lisophospholipase, glycerophosphryl choline diesterase and heat labile direct hemolysin. The heat labile hemolysin has been isolated from both Kanagawa positive and negative strains. Till to-day the roles of these hemolysins and related enzymes in the pathogenicity of V. parahaemolyticus are not quite understood.

The enterotoxigenic properties of V. parahaemolyticus & NAG vibrios have been studied in more detail during the last decade by using various animal models as well as the cell line assay (18). However the variations of experimental models in different laboratories and lack of internationally accepted -and standardised method for measuring enterotoxicity has been the only drawback in the understanding of the pathogenicity of these virbios. Factors like inoculum size, time of incubation, source and species of laboratory animal, choice of model and defination of positive response, which can profoundly affect the observed results may vary from laboratory to laboratory. So we are to be very particular on reporting the enterotoxigenicity according to the responses obtained in various models.

Very little work has been done on the extracellular and intracellular products of the halophilic Vibrios. Except for a few we do not know about the various enzymes elaborated by them and their roles in the toxic properties of the organisms. The cytotoxins need to be characterised. As some of these organisms causes extra intestinal infections and septicaemic conditions, the properties responsible for invasiveness need to be identified.

B. SPECIFIC AIMS:

1. To isolate and characterise the V.parahaemolyticus isolated from patients with watery and/or bloody diarrhoea as well as from different environmental sources.

2. Using well defined newly developed method we would aim at isolating different toxin/toxic factors such as cholera like enterotoxins, direct hemolysin, heat labile haemolysin cytotoxin, cytolysin and enzyme factors such as proteases.

3. To find out the degree of production of these factors by these different isolates basing on their properties to be liberated at different times under different experimental/cultural conditions.

C. MATERIALS & METHODS

Isolation & Characterisation:

V. parahaemolyticus will be isolated from clinical cases attending the ICDDR,B hospital by using already established standard method. Both TTGA and TCBS will be used as isolation medium and BP(T) containing 2% NaCl will be used as enrichment. All the water samples will be concentrated by millipore filter techniques and enriched in BP(T) for 8 hours before plating onto TTGA & TCBS medium. Culturally suspected colonies will be confirmed by biochemical test using different carbohydrates containing media supplemented with 2% sodium chloride. Biochemically confirmed strains will be characterised serologically using available typing sera. During the process of characterisation of the haemolysin produced by different strains we will be needing antihaemolysin which will either be produced here at ICDDR,B using techniques set up in Dr Takeda's Laboratory or be brought from there to work on the isolates here in ICDDR,B.

As we intend to look into the production of toxin/toxic factors produced by different isolates of V. parahaemolyticus, the cultures will be grown in Heart Infusion broth pH 7.4 with added NaCl 2-4% to have maximum toxin production. The cultures will be grown in shaker bath as well as in still culture for 18-24 hours at 30°C. The resulting growth will be centrifuged and supernate tested. As different toxic factors are produced at different time of incubation samples will be taken out at 10 hours to look at cytolysin, 14-16 hours to look for preteases and 18 hours to look for cytotoxin by using techniques already established and used by different workers.

a) Test for cytotoxic and cytotonic responses will be performed on Y_1 cell response was read as cytotonic like, after exposure to the supernatant heated at 56°C for 10 mins; cells had a refractile rounded morphology. Cytotoxic activity is indicated by rounding shrinking, granule formation and vaculation of the Y_1 adrenal cells (19).

b) Hemolytic activity - Cytolytic activity against rabbit crythrocytes was determined by using the method Bernheimer & Schwartz (20). One hemolytic unit was defined as the reciprocal of the dilution that caused the release of 50% of the hemoglobin in the standardised (0.7% vol/vol). Crythrocyte suspensions

c) Protease activity - Protease activity was assayed by the uzocasein substrate method described by Krager and gray (21). One unit of activity was defined as the reciprocal of the dilution that yielded, under standard assay conditions, a reaction mixture containing enough dictotised peptide to give an absorbence of one at 440 nm.

Both the supernate and the cell deposits will be looked for toxins. To look at the cell bound toxins the deposits will be washed with saline

twice and the cells broken either by sonication or treating with polymixin B. The endotoxin thus liberated from the lysing cells will be tested by using LAL (lymulus amebocyte lysate) test, modification of Difco pyrotest(22). Alternatively the Ouchterlony's immuno diffusion will be performed using 1.2% agarose with modification using LPS extracted by phenol-water as a control (23). Animal models to be used for testing the culture supernates and sonicates are a) Adult rabbit loop; b) Infant rabbit, c) suckling mice and d) sealed adult mice (SAM) model (24). Among the cell lines CHO cells, Y_1 adrenal cell will be used. Hela cell lines will also be used for testing the cytotoxic activity (similar to cytotoxic activity (similar to cytotoxic activity shown by Shiga bacillus (25). As V. parahaemolyticus has been found to cause dysentery like syndrome in clinical patients, the isolates will be tested for invasive character by using standard Sereny test (26) as well as Hela cell monolyer. All the assays involving animal models and cell line are well established in ICDDR,B and is used somewhat routinely by different workers.

D. SIGNIFICANCE:

The proposed study aims at looking into the pathogenic mechanisms of V. parahaemolyticus isolated from diarrhoeal patients as well as from environment. Most of the published reports dealt mainly with the isolates from patients or environment from estuarine areas where the habitat is different from our part of the world. The clinical isolates from Dhaka will cover areas having surface water of very negligable or no salt concentration and the pathogenesis and disease pattern caused by them is expected to be different. The use of various animal models and cell line assays will allow us to detect different toxins/toxic factors such as

cholera like enterotoxins, ST like toxin, cytolysin, direct haemolysin and other haemolysin which is responsible for causing different manifestation of disease in human being. As many of these organisms has been found to cause invasive diarrhoea like Shigella sp. the properties responsible for producing these invasiveness will also be looked at under their mechanism of action in the gut. The study will show the differences between the clinical and environmental isolates in the production of toxin/toxic factors in different conditions.

E. FACILITIES REQUIRED:

a. Laboratory space - Bench space required for the working of the Principal Investigator and Co-investigators are available. Amicon filtration apparatus and chromatographic separation technique to be used are available from the past study of the Co-Investigator and to be reorganised. A 20-25 feet bench space will be needed to set up these apparatus.

b. Most of the important chemicals and chromatographic materials will be available through the courtesy of Prof. S.H. Richardson.

F. COLLABORATIVE ARRANGEMENTS:

This study will be a collaborative project between ICDDR,B Dhaka and Dr Y. Takeda of Tokyo, Japan. Technical help has also been assured by Prof. S.H. Richardson in the characterisation of different toxin/toxic factors in whose lab similar work will be initiated from January, 1984.

SECTION - III BUDGET

1. PERSONNEL SERVICES

Name	Person/ month	Project requirement Taka	US dollar
Dr M.I. Huq	25%		17,000
Dr K.M.S. Aziz	5%		4,000
Mr. Q.S. Ahmed	15%	11,280	
Mrs. Khaleda Haider	25%	14,500	
Research Officer (To be appointed)	100%	35,200	
Dr K. Al-Mahmud	15%	13,100	
Lab. Technician	50%	11,100	
		85,080	21,000

2. SUPPLIES AND MATERIALS

Media	900
Chemicals	2,200
Glass & Plastic wares	1,300
Chromatographic & filtration equipment	4,500
Rabbits, Guineapigs, mice	1,200
	10,100

3. EQUIPMENT

Millipore - Sweeney - 10 — 1,500

4. PATIENT HOSPITALIZATION - None

5. OUTPATIENT CARE - None

6. TRANSPORT (ICDDR,B)

2000 miles @ 4.50 — 9,000

7. TRAVEL & TRANSPORTATION OF PERSONS

Round trip travel North Carolina Dhaka - North Carolina	2,500
Round trip travel Tokyo - Dhaka - Tokyo.	2,200
	4,700

8. TRANSPORTATION OF THINGS:

Chemicals & other perishable items — 300

		Taka	US dollar
9.	RENT, COMMUNICATION & UTILITIES		
	Guest House charges at Dhaka, per diem & consultancy.		3,200
10.	PRINTING & REPRODUCTION	7,000	
11.	OTHER CONSTRUCTUAL SERVICES - None		
12.	CONSTRUCTION, RENOVATION, ALTERATIONS - None		

B. BUDGET SUMMARY

	1st year Taka	1st year US dollar	2nd year US dollar
1. Personnel Services	85,080	21,000	22,400
2. Supplies and Materials		10,100	6,200
3. Equipment		1,500	-
4. Patient Hospitalization -none			none
5. Transport - ICDDR,B	9,000		400
6. Outpatient Care - None			none
7. Travel & Transportation of persons		4,700	none
8. Transport of things		300	500
9. Rent, communication and utilities		3,200	none
10. Printing & Reproduction	7,000		300
11. Other contractual services - None			1,000
12. Construction, Renovation, Alterations - None			
Tk.	1,01,080	40,800	30,800
Equal to US$	4,211	=======	======

	1st year	2nd year
Grand Total	US$ 45,011	US$ 30,800
Salaries	US$ 24,545	US$ 22,400
Operational Cost	US$ 20,466	US$ 8,400

Conversion rate 1 US$ = Tk. 24.00

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