Attachment 1.

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Date 1-3-87

Principal Investigator Dr.Khurshed A. Chowdhury

Application No. 87-006(P)

Title of Study A study to elucidate the enteropathogenic effects of oral Plesiomonas shigelloides infection in a Rabbit Model. (A PILOT PROTOCOL)

Trainee Investigator (if any) NIL

Supporting Agency (if Non-ICDDR,B)

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 NA Yes No
   (b) Non-ill subjects NA Yes No
   (c) Minors or persons under guardianship Yes (No)
2. Does the study involve:
   (a) Physical risks to the subjects NA Yes No
   (b) Social Risks NA Yes No
   (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) NA Yes No NA
   (b) Use of fetal tissue or abortus Yes No NA
   (c) Use of organs or body fluids Yes No NA
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 and 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 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.

Khurshed A. Chowdhury
Principal Investigator

MAR 3 1987

Trainee

87-006(P)
1.3.87

SECTION I - RESEARCH PROTOCOL (Pilot)

1. Title : A study to elucidate the entero-pathogenic effects of oral Plesiomonas shigelloides infection in a rabbit model.

2. Principal Investigator : Dr. Khurshed A. Chowdhury

   Co-Investigators : Dr. A.S.M.H. Rahman
   Mr. Zeaur Rahim

3. Starting Date : As soon as funding is available

4. Completion Date : Seven months after the starting date

5. Total Direct Cost : US $ 4,597.00

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

This protocol has been approved by the Epidemiology & Laboratory Sciences Division

Signature of Scientific Program Head : David A Sack

Date : 26.2.87

7. Abstract Summary:

Inspite of the fact that a considerable number of isolates from diarrhoeal patients have been identified as Plesiomonas shigelloides - the exact role of this bacterium as a causative agent of human diarrhoea is still a controversy. We propose to study the enteropathogenicity of P. shigelloides in an animal model. Identified strains of P. shigelloides will be fed to different groups of rabbits and evaluation will be made on clinical, immunologic, cytotoxic as well as histopathological basis, to determine the extent and nature of the intestinal lesions which may correlate with known mechanism of diarrhoea.

8. Reviews:

a. Ethical Review Committee : _____

b. Research Review Comittee : _____

c. Director : _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective:

   a. To elucidate the role of P. shigelloides as the pathogen for diarrhoeal disease.

   b. To evaluate the histopathologic effect of P. shigelloides infection in the rabbit intestine.

2. Background:

Plesiomonas shigelloides is the only one species of the genous Plesiomonas under the family Vibrionaceae. Plesiomonas is facultatively anaerobic, asporogenous, gram negative rod with polar lophotrichus flagella.

P. shigelloides may be important as a diarrhoeal pathogen in humans being. Although epidemiological information of plesiomonas diarrhoea is very minimal, in Japan two water borne epidemics have been associated with the isolation of P. shigelloides from the stools of diarrhoeal patients. P. Shigelloides was isolated from the stools of two patients with gastrointestinal malignancies (1). An oyster-associated outbreak of diarrhoeal disease possibly caused by P. shigelloides was also reported (4). Holmberg, et al. reported that, P. shigelloides may be a cause of enteric disease in normal host and the disease may be acquired from eating uncooked shellfish and also may be the cause of traveler's diarrhoea (3). P. shigelloides has been isolated from a number of animal species, humans, as well as water and sludge (5).

Ljungh A. and Wadstrom T. stated that - strains of P. shigelloides may be able to cause diarrhoea in some cases and they suggested possible role of a heat-stable enterotoxin in the mechanism of causing diarrhoea (2). A case of human diarrhoea has been reported in which fecal microscopy revealed numerous white cells suggesting mucosal invasion by the bacteria (6). P. shigelloides was isolated from about 6% of human stools specimen submitted for culture at Dhaka hospital of ICDDR,B and only 4% stools had Plesiomonas as the sole pathogen (Mr. Z. Rahim's unpublished data). Yet its role as a human pathogen is not clear since isolates have not yeilded positive result in the rabbit ileal loop test and do not produce enterotoxin (7,8). However, from the available information — it can be assumed that P. shigelloides may be associated with diarrhoeal disease in some cases. The purpose of this study is to determine if — P. shigelloides is able to produce illness in a rabbit model and to determine if oral feeding of the bacteria leads to pathologic changes in experimental animals.

B. SPECIFIC AIMS:

1. To determine if P. shigelloides cause pathological changes in the intestine of rabbits given oral inoculation of this bacteria.

2. To determine if any pathological changes seen are plausibly related to diarrhoeal symptoms.

3. To compare the pathological changes induced by different strains of P. shigelloides.

C. MATERIALS & METHODS:

Preparation of bacterial inoculum:

We will identify the bacterial strains as follows: P. shigelloides strains NCTC-10363 obtained from Dr. A. Lindberg will be called as strain # 1; clinical isolate of P. shigelloides from the treatment center, International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) will be called as strain # 2 and P. shigelloides isolated from the water of environmental microbiology study at ICDDR,B will be called as strain # 3, and freshly clinical isolates of Shigella Sonnei called as strain # 4. Selected strains of bacteria will be preserved at -20°C in glycerol broth. When required for animal challenge studies BHI broth will be inoculated from the frozen stock and incubated for 4 hours at 37°C. A lawn will than be inoculated onto BHI agar and incubated for 18 hours. Simultaneously, the BHI broth will be tested for purity on MacCankey agar. The BHI agar lawn will be harvested with 1 ml sterile PBS and transferred to a test tube. The optimal density of the bacterial suspension will be determined and the numbers of bacteria will be estimated from a previously determined growth curve. An appropriate dilution will be made in BHI broth and this will be used to inoculate the rabbits. Generally 10^{10} bacteria will be used. The concentration of the bacteria will be confirmed with stndard colony counts using serial dilutions on BHI agar.

Conditioning of rabbits:

Young New Zealand White rabbits (2-2.5 kg) of either sex will be obtained from the Animal Resources Branch at the ICDDR,B. All the

rabbits that will be used in the experiment will be starved for 36 hours. During the period of starvation the animals will drink plain water except the group receiving shigella sonnei will be given tetracycline with drinking water. In subsequent experiments if no pathology is seen with this conditioning, ampicillin in the drinking water may be included in the conditioning. The test bacteria are resistsant to ampicillin.

Administration of inoculum:

At time 0, cimetidine (50 mg/kg of body weight) will be administered intravenously. At 15 and 30 minutes, 15 ml of a solution of 5% $NaHCO_3$ will be administered by gastric tube. Immediately after the second $NaHCO_3$ dose the bacterial inoculum will be given. The bacterial inoculum will be followed 30 minutes later by I.P. administration of 2 ml of tincture of opium.

Group of Rabbits

Rabbits will be divided into groups (n-5) according to the bacterial stran used, and according to the type of assessment. Group A, receiving bacteria 1,2,3,4 or receiving sterile broth will be sacrificed 18 hours after inoculum. These rabbits will therefore be in groups A1, A2, A3, A4 and A5. Group B will be sacrificed on day 3 with subgroups corresponding to the strains. Group C will be sacrificed on day 7. We will therefore need 75 rabbits for the entire study.

Assessment of the Challenge:

Clinical: All inoculated rabbits will be observed (until sacrifice) for sickness/diarrhoea detected by fecal staining of the perineum. Body temperature will be recorded once a day at 9 A.M. and sickness/diarrhoea, drowsiness or time of death, if any, will be recorded.

Bacteriology: To detergmine the duration of excretion, rectal swab specimens will be obtained daily for seven days of surveillance. The swabs will be plated onto salmonella shigella agar containing ampicillin (10 ug/ml). Typical colonies will be confirmed as P. shigelloides.

If rabbits die, cultures will be obtained of intestinal contents to determine if P. shigelloides is present in the gut using the same methods as for rectal swabs.

Pathology: Rabbits that die following inoculation as well as sacrificed rabbits at the interval of 18 hours, 3 days and 7 days after inoculation will be routinely autopsied and gross lesion will be recorded if any. Segments from each level of intestine will be ligated and the lumens will be infused with 10% buffered neutral formalin. The ligated sections will then be placed in individual vials of 10% buffered neutral formalin for fixation. Portions of liver, spleen and one mesenteric lymph node will also be included for fixation. Well fixed tissues will be processed for routine histopathological examination. While doing

histopathological evaluation, inflammatory changes e.g. congestion, edema, necrosis, ulcers, exudates etc. in the different level of intestine will be recorded and graded according to their severity. A comparison of the extent of the lesions among the groups will be performed. Where needed - Photomicroscopic pictures of the identified lesion will be taken for documentation. To insure an objective evaluations, the sections will be read ina randomized and blinded manner.

Antibody detection:

Three rabbits will be selected from the group "C" for antibody titre assessment. Preimmunized sera will be collected from these three rabbits before the oral feeding of bacterial strains and the sera will be kept at - 20 ^{o}C to be used as control at the time of assessment. These three rabbits will be killed after 28 days and sera will be collected by conventional method. Antibody detection will be measured by tube agglutination against homologus bacterial strain.

Cytoxin production assay:

Culture filtrate will be prepared from all three strains of *P. shigelloides* for examination of cytoxic effect by using: (a) Chinese hamster ovary (CHO) cell assay (9) and (b) Adrenal cell assay (10).

Data Analysis

From the symptomatology and pathological lesions observed, a conclusion should be possible as to the pathogenic potential of

these bacteria for rabbits. If a disease process is detected, a description of the type of pathology should help characterize the type of disease e.g. invasive, secretory, etc. If lesions do occur, examination of the other organs will determine if the infection is limited to the intestine. Because this is a descriptive study statistical analysis will not be used in the assessment of clinical symptoms or bacteriology. For evaluation of pathological changes, a grading system of 0-3 will be used to express severity of the changes observed. Groups of rabbits challenged with the three strains can then be compared to the control group using chi-square analysis.

SECTION III DETAILED BUDGET

1. PERSONNEL SERVICES

Name	Designation	Effort%	Taka	US $
1. Dr.Khurshed A. Chowdhury	Princ. Investigaor	50	93,150	
2. Dr. ASM Hamidur Rahman	Co-Investigator	20	23,000	
3. Mr. Zeaur Rahim	Co-Investigator	20	20,100	
4. Mr. KM Shafiullah	Tech. Officer	10	8,000	
5. Mr. Arjun C. Das	Sr. Animal Tech.	10	5,000	
6. Mrs. S. Pashi	Hist. Technician	30	29,500	
7. Mr. A. Rob	Hist. Technician	30	12,432	
8.	Lab Attendant	50	13,500	

2. SUPPLIES & MATERIALS

Item	Amount required	Unit Cost in Tk.	Project requirement Taka	US $
Rabbit, young	100	450	45,000	1,500
Maint. & Post-experimental care	100	40 Rabbit/day	24,160	805
Anaesthetics, chemicals (cimetidin)				50
Gloves, syringe, needle and plastic animal feeding tube (stomach tube).				100
Surgical instruments, Blades etc.				50
Histological specimens	400	100	45,000	1,333
Stains (H&E, PAS)				150
Paraffin				100
Reagents & chemicals				100
Imersion Oil, Lens Paper etc.				50

Bacteriological Media	300	15	4,500	150
Microbiology Lab support			4,500	150
Toxin assay	48	21	1,008	34
Antibody detection	3	250	750	25
Equipmentcal specimens	None			
Patients	None			
Out patient	None			
ICDDR,B Transport	None			
Travel	None			
Transportation of things	None			
Rent, Communication & Utilities	None			

Total =Tk. 1,46,418 $ 4597

Project requirement: US $ 4,597.00

(1 US $ = 30 Taka)

REFERENCES

1. Ralston K.V.I. and Hopfer R.L.: Diarrhea due to Plesiomonas shigelloides in Cancer Patients. J. Clin. Microbiol. Sept. 1984, p-597-598, Vol. 20(3).

2. Liungh A., Wadstrom T.: Aeromonas and Plesiomonas as possible causes of Diarrhoea. Infection, Vol.13(1985)4, p-1-6.

3. Scott D. Halmberg et al.: Plesiomonas Enteric Infection in the United States. Annals Int. Med. 1986, Vol.105(5), p-690-694.

4. William A. Rutala et al. : Oyster-Associated outbreak of Diarrhoeal Disease possibly caused by Plesiomonas shigelloides. The Lancet, March 27, 1987, p-719.

5. Teruyoshi A. et al. A survey of Plesiomonas shigelloides from aquatic environments, domestic animals, pets and humans. J. Hyg. Comb. 1980, 84, p-203-211.

6. Downey D.J. and Clark J.N.T. A case of diarrhoea associated with Plesiomonas shigelloides. NZ. Med. Jour. V-97, 1984(8), p-92.

7. Ljungh A. et al. Aeromonas hydrophila in acute diarrheal disease: detection of enterotoxin and biotyping of strains. J. Clin. Microbiol. 1977,6:96-100.

8. Hostacka A. et al. Toxic factors of Aeromonas hydrophila and plesiomonas shigelloides. Zentralbl Bacteriol Microbiol Hyg. (A). 1982, 252:525-534.

9. Guerrant RL. et al: Cyclic adenosine monophosphate and alteration of chinese hamster ovary cell morphology; a rapid sensitive invitro assay for the enterotoxins of Vibrio cholerae and Escherichia Coli. Infect. Immun. 10:320-327, 1974.

10. Sack DA, Sack RB: Test for enterotoxigenic Escherichia Coli using Y_1 adrenal cells in miniculture. Infect Immun 11:334-336, 1975.

ATTACHMENT-A (OF ANNEX. I)

CURRICULA VITAE

PRINCIPAL INVESTIGATOR

1. Surname/Family Name: CHOWDHURY

   First name/other names: MHURSHED ALAM

2. Date of birth: 05-11-41

   Place of birth: Comilla, Bangladesh

   Nationality: U.S.A.

3. Degrees:

Degree	Year	Institution	Disciplines
D.V.M.	1965	B. Agricultural Univ. Bangladesh	Veterinary Medicine
Ph.D.	1969	University School of Vet. Medicine, Brno, Czechoslovakia	Veterinary Pathology

4. Academic Distinctions:

Degree	Year

5. Present post (Title, Institution, Dates):

   Title: Pathologist (Consultant)

   Institution: ICDDR,B

   Dates: 13-03-86 - present

6. Previous posts (Title, Institution, Dates):

Title: a) Professor in Veterinary Pathology

b) Staff Pathologist (Research Support)

c) Head, Veterinary Pathologist

Institution:

a) Al-Fateh University, Tripoli, Libya

b) Food and Drug Administration (FDA), U.S.A.

c) Maryland Dept. of Agriculture, MD., U.S.A.

Dates: a) Nov. 1984 - March, 1986

b) Sept. 1981 - Nov., 1984

c) Nov. 1977 - Sept., 1981

7. Academic & Research Awards, Consultant & other posts:

8. Other University & Institutional Posts:

Postdoctoral Fellow, sponsored by UNESCO.

9. Current research interests including details of projects of which Applicant is Principal Investigator:

Development of Animal Models to help understand the Pathophysiological aspect of diarrhoeal diseases.

Since I have just joined the Centre this is the first protocol in which I am P.I.

10. Publications & Communications:

Please see the attached list.