Attachment 1.

Date _____

REVIEW BOARD ON THE USE OF HUMAN SUBJECTS, ICDDR,B. 99

Principal Investigator Shahjahan Kabir

Trainee Investigator (if any) _____

Application No. 80-013

Supporting Agency (if Non-ICDDR,B) _____

Title of Study Composition and immunological properties of the outer membrane proteins of Vibrio cholerae.

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: NA

(a) Ill subjects Yes No
(b) Non-ill subjects Yes No
(c) Minors or persons under guardianship Yes No

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

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

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 Board:
   ___ Umbrella proposal - Initially submit overview (all other requirements will be submitted with individual studies)
   ___ Protocol (Required)
   ___ Abstract Summary (Required)
   ___ Statement given or read to subject ... nature of study, risks, types of q... ions to be asked, and right to refu... to participate or withdraw (Requ...
   ___ 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 compl... prior to review, the following inform... should be included in the abstract s...
   1. A description of the areas to be covered in the questionnaire or interview which could be consid... either sensitive or which would constitute an invasion of privac...
   2. Examples of the type of specific questions to be asked in the se... areas.
   3. An indication as to when the ques... naire will be presented to the Re... for review.

I agree to obtain approval of the Review Board on the Use of Human Subjects for any ch... involving the rights and welfare of subjects before making such change.

Shahjahan Kabir

Principal Investigator

Trainee

80-013
Rec'd 3/3/80

SECTION I - RESEARCH PROTOCOL

1. Title : Composition and Immunological properties of the outer membrane proteins of Vibrio cholerae.

2. Principal Investigator : Shahjahan Kabir

3. Starting Date : March, 1980

4. Completion Date : February, 1981.

5. Total Direct Cost : $ 18,600.00

6. Availability of funds :

a) Scientific Director's Remarks :

b) Controller's Remarks :

7. Abstract Summary :

The study plans to analyse the outer membrane proteins of Vibrio cholerae from several strains belonging to both the biotypes (classical and El Tor) and the serotypes (Ogawa and Inaba). Vibrio cholerae will be grown under various cultural conditions. Membrane proteins will be isolated from the bacteria by sequential treatment with lysozyme and Triton X-100. The composition of the membrane proteins will be analysed by polyacryiamide gel electrophoresis in the presence of sodium dodecyl sulphate. Antisera to the outer membrane proteins will be raised in rabbits and the immunochemical composition of the outer membrane proteins will be analysed by immunodiffusion and immunoelectrophoresis. The prophylactic potential of the outer membrane proteins would be studied in rabbits against experimental cholera. Rabbits immunised with the outer membrane proteins will be challenged with live vibrios of both biotypes and serotypes. The degree of protection will be measured by the intestinal fluid

response in the ligated ileal loops. The prophylactic potential of the outer membrane proteins would be compared with those of the whole cell vaccine and the cholera toxoid.

8. Reviews :

a) Research involving human subjects : _____

b) Research Committee : _____

c) Director : _____

d) BMRC : _____

e) Controller/Administrator : _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective : The long term objective of these studies is to control diarrhoeal disease caused by the toxin-secreting, non-invasive bacteria such as Vibrio cholerae through immunological research and vaccine development. Hence, research will be carried out to develop new and improved immunogens with primary emphasis on those obtained from bacterial somatic structures. Experiments will be performed on small laboratory animals to determine the protective capacity of the somatic antigens against cholera. It is anticipated that information obtained from such studies will make significant contribution to the design of studies to immunise human beings against enteric bacterial infections.

Background : Vibrio cholerae, the causative agent of cholera, is a Gram-negative bacterium which colonises the small intestine. It secretes an exotoxin which binds to the ganglioside receptor and causes an outpouring of the fluid into the gut lumen. The currently available vaccine against cholera is partly effective for a short duration.

In cholera endemic areas the population acquires a significant degree of specific immune protection due to exposure to Vibrio cholerae antigens(1). Thus it has been observed that with increasing age the rate of reinfection is lowered as well as the incidence of the disease. Also, from volunteer studies it has been observed that convalescents from induced cholera were resistant to homologous challenge for upto 12 months(2). These findings suggest that it should be possible to induce effective immunity against cholera.

Both antibacterial as well as antitoxic immunity are developed in the host after an infection of cholera(3, 4). Although there are controversies as to the relative protection provided by antibacterial versus antitoxic immunity, recent studies on the immunity against cholera in man indicated that the predominant immune mechanism was antibacterial rather than antitoxic in nature(5). This was based on the findings that although purified cholera toxoid was antigenic when administered parenterally and orally, it failed to provide protection against challenge. However, clinical cholera conferred formidable protection against both homologous and heterologous challenge.

Cholera vaccines, composed of killed vibrios and administered parenterally, do induce immunity. However, the protective capacity is of limited duration, being 40-60% during the first 2 months and 10-20% after 6 months(1, 6). Besides, the whole cell vaccines are known to be reactigenic i.e., induce pain and fever.

Cell wall antigens consisting mainly of lipopolysaccharides(LPS) of both Ogawa and Inaba serotypes have been isolated and tried in the field(7). The Inaba antigen and the whole cell monospecific Inaba vaccines gave protection against infection by Vibrio cholerae Inaba El Tor biotype. The monovalent Ogawa vaccine was ineffective although it induced vibriocidal antibody titer rises against both Ogawa and Inaba serotypes. In a field trial conducted in the Philippines, significant protection was provided by a monovalent Inaba whole cell vaccine against Ogawa infections(8). These results indicate that Ogawa vaccines do not confer protection against infection by Inaba. However, Inaba vaccines cross protect against Ogawa infections. Both the whole cell vaccine as well as the LPS enriched antigen can induce fever and pain in the host. Considering all these problems associated with the currently available cholera vaccines it would be desirable to have

a new and improved somatic antigen which would(a) be nontoxic (b) cross protect against all biotypes and serotypes and (c) confer longer immunity.

The Protein antigens of Vibrio cholerae :

Vibrio cholerae, like other Gram-negative bacteria such as Escherichia coli, possesses three distinct layers: the outer membrane, the inner or cytoplasmic membrane and the peptidoglycan(9). The outer membrane contains surface components such as proteins and lipopolysaccharides and it is most likely that these components would interact with the host in order to elicit antibacterial immune response.

Proteins comprise about half the weight of 'the outer membrane of Gram-negative bacteria. There is evidence in the literature that protein antigens shared by both Inaba and Ogawa serotypes might contribute to the vibriocidal activity in antisera raised against live vibrios(10). But, these authors did not characterise the protein antigens. Besides, very little is known about the composition and immunological properties of the outer membrane of Vibrio cholerae.

Structure of the outer membrane of Gram-negative bacteria :

The outer membrane of Gram-negative bacteria is composed of proteins, phospholipids and LPS, the relative amounts of which depend on growth conditions. The protein composition of the outer membrane is relatively simple and depending on the strain and growth conditions, 16 to 25 protein bands are usually resolved by SDS-polyacrylamide gel electrophoresis. However, two or five polypeptides are present in greatest amount. These include three or four proteins with molecular weights in the range of 40,000. These proteins are resistant to solubilisation in SDS and depending on the temperature of solubilisation exhibit different mobilities during electrophoresis. In addition to these major outer membrane proteins of higher molecular weights another major protein called lipoprotein is present several Gram-negative bacteria(11). This protein exists in both bound and free-form and has been detected in Escherichia coli Salmonella and Shigella.

Outer membrane proteins as immunogens :

There are several studies related to the composition of the outer membrane proteins of Gram-negative bacteria such as Escherichia coli and Salmonella(12). But, comparatively little is known about

their immunological properties. Recent studies indicate that the outer membrane proteins of Escherichia coli and Salmonella can act as surface antigens(13, 14, 15). Thus antisera raised against the outer membrane proteins of Salmonella have been found to render protection against experimental Salmonellosis(16). Also, antigenic cross reactivity among several serotypes of E. coli, Salmonella typhimurium and Shigella have been reported (14). Recently, Geyer et al(17) have isolated a LPS-binding cell surface protein from Salmonella minnesota which cross reacted with several enteric strains such as Salmonella typhimurium, E. coli and Shigella. These findings suggest that some of the outer membrane proteins of Gram-negative bacteria because of their presence on the cell surface possess antigenic properties.

Preliminary studies (18) :

There are two biotypes of Vibrio cholerae : El Tor and classical. Within each biotype there are two serotypes: Ogawa and Inaba. In the preliminary study the outer membrane was prepared from all these strains according to the method described by Schnaitman(19) and the membrane proteins were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis(SDS-PAGE). All these

strains contained a major protein of molecular weight 48, 000. But they differed in composition and proportions of minor proteins. The outer membrane protein profile was influenced by the growth medium. A polypeptide of molecular weight 15, 000 was observed in the outer membrane when Vibrio cholerae ogawa 395(classical) were grown in peptone-water, whereas a protein of molecular weight 68, 000 appeared when grown in the synthetic medium. Under anaerobic growth conditions the proportions of the low molecular weight protein 13, 000 was greatly enhanced. The outer membrane contained heat modifiable proteins as the polypeptidee with molecular weights 41, 000 and 37, 000 disappeared when membrane proteins were disaggregated in sodium dodecyl sulphate at or above 60^{o}C. Immunochemical studies showed that Vibrio cholerae possessed a protein antigen in the outer membrane which was common to all biotypes and serotypes. Thus, the antiserum raised against the outer membrane of one classical strain(Ogawa 395) produced immunoprecipitation against the outer membrane preparation from different biotypes and serotypes. Toxicity of the various cell envelope components such as lipopolysaccharide, the outer membrane proteins was studied in the mouse(20). It was observed that the outer membrane did not possess any toxicity

whereas the lipopolysaccharide component of Vibrio cholerae was found to be toxic.

3. Rationale : There are several reasons which justify the present study. Firstly, the currently available whole cell vaccine against cholera has only limited effectiveness for a short duration. Hence the various cell surface components of Vibrio cholerae should be analysed in detail in order to develop new and improved immunogens. Membrane proteins comprise about 50% of the cell surface. Yet, very little is known about them. Therefore, there is good reason to analyse the outer membrane of Vibrio cholerae so as to (a) have a better understanding of the cell surface architecture and (b) explore these components as potential immunogens, first in animals and if promising, later in man.

Secondly, studies in mice indicate that membrane proteins of Vibrio cholerae are nontoxic whereas LPS,the other cell surface component,possess toxic properties. Also, the existing whole cell vaccine cause pain and fever in man. Hence it is desirable to develop an immunogen which would be nontoxic.

Thirdly, preliminary studies have demonstrated that a polypeptide of molecular weight 48,000 was present in the outer membrane of all biotypes and serotypes of Vibrio cholerae. Also, the antiserum to the outer membrane proteins of Vibrio cholerae Ogawa 395, a classical strain, agglutinated Vibrio cholerae of all biotypes and serotypes. Hence there is a good rationale to develop an immunogen which would cross react with all biotypes and serotypes.

B. SPECIFIC AIMS :

1. To study the outer membrane proteins from several strains of Vibrio cholerae belonging to both biotypes and both serotypes so as to obtain a broad spectrum of the outer membrane protein profile.

2. To study the outer membrane protein profile of other enterotoxigenic vibrios such as non-agglutinable vibrios(NAG's) and Vibrio parahaemolyticus.

3. To immunise laboratory animals such as rabbits against the outer membrane proteins of Vibrio cholerae.

4. To examine the antigenic cross reactivity of the major outer membrane protein in several strains of Enterobacteriacae by -
   a. bacterial agglutination
   b. various immunoelectrophoretic techniques.

5. To evaluate the protective capacity of the outer membrane proteins in adult rabbits against challenge with classical and El Tor biotypes of both Inaba and Ogawa serotypes.

6. To compare the efficacy of the outer membrane proteins of Vibrio cholerae as immunogens against the cholera vaccine and cholera toxoid.

7. To examine whether differences exist between the membrane protein profile of the multi-antibiotic resistant strains of Vibrio cholerae and those sensitive.

8. If the results of the above objectives are promising attempts will be made to study the immunogenecity of Shigella and enterotoxigenic E. coli outer membrane proteins.

C. METHODS OF PROCEDURE

Bacterial strains :

The study will emphasize on the strains of Vibrio cholerae belonging to both the biotypes (El Tor and classical) and serotypes (Ogawa and Inaba). Also it will include strains from non-cholera vibrios such as NAG's and Vibrio parahaemolyticus and enteropathogenic Gram-negative bacteria such as Shigella, E. coli and Salmonella.

Growth media :

The following media will be used to grow Vibrio cholerae :

1. Peptone water : A 3% solution of peptone water, pH 7.4 would be prepared as directed by Burrows et al (20).

2. Semisyuthetic or syncase : This medium will be prepared according to the procedure described by Finkelstein et al (21).

3. Synthetic : In this medium ingredients will be the same as those described in the semisynthetic medium except that casaminoacids will be replaced by 2.5 gms/liter of each of the following acids : glutamine, serine, aspartic acid and arginine.

Growth conditions :

1. Aerobic : Cultures will be incubated with or without shaking at 37°C and harvested at the stationary phase of the growth.

2. Anaerobic : Media containing bile salts will be prepared by adding 1 g(2.2 mM) sodium deoxycholate to 1 liter of syncase. The medium will be prereduced for 24h in an atmosphere of H_2 and CO_2 and the growth will have to be maintained in Gas pak jars(BBL) in a similar atmosphere at 37°C for 16h.

Preparation of the outer membrane :

Vibrio cholerae cultures(250 ml) will be grown at 37°C with shaking to a density of A_{600} 0.8 -- 1.00 and harvested at 12,000 xg for 15 min. at 0-4°C. The cell pellet will be resuspended in 25 ml of 0.1 M Tris-HCl, pH 7.8 and sheared at 4°C in a Waring blender (19,000 rev/min, 45 sec) to remove flagella as described for E. coli (22). The suspension will be diluted sixfold in the Tris-buffer and subjected to centrifugation at 12,000xg for 10 min. The cell pellet will be resuspended in cold 0.75 M sucrose - 10 mM Tris- HCl, pH 7.8 (0.7 ml sucrose solution per 10 A_{600} units of original culture). The suspension will be transferred to an Erlenmeyer flask containing lysozyme (2 mg/ml of H_2O, 0.05 ml/ml of cell suspension) and the mixture incubated in ice for 2 min. The suspension will be diluted with 2 volumes of cold EDTA (1.5 mM) and kept at 4°C for 3 h. The spheroplast will be lysed by the osmotic shock by slowly pouring the suspension into 4 volumes of cold water with magnetic stirring and will be stirred for 10 min. in the cold. The total membrane fraction will be recovered from the osmotic lysate first by centrifugation at 12,000 xg for 15 min. at 2 --4°C to remove the unlysed cells and later by centrifuging the supernatant at 48,000 xg for 30 min. The pellet will be resuspended into

0.01 M Tris-HCl (pH 7.8) containing 10 mM EDTA by a syringe and to be washed once with Tris-HCl (0.01 M), pH 7.8 containing 5 mM $MgCl_2$. The inner membrane will be dissolved from the total membrane by extraction at $0^{o}C$ for 20 min. with 2% Triton X-100 containing 5mM EDTA described for E. coli by Schnaitman(19). The outer membrane will be recovered as a pellet.

Enzyme assay : To check the purity of the outer membrane preparation, the NADH-oxidase activity will be assayed as described by Osborn et al(23). The incubation mixture for the measurement of NADH-oxidase activity will contain 50 mM Tris-HCl, pH 7.5, 0.12 mM NADH, 0.2 mM dithiothreitol and the membrane preparation (0.1-0.2 mg of protein) in a volume of 1.0 ml. The rate of decrease in absorbance will be measured at 340 nm at $25^{o}C$.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis(SDS-PAGE). SDS-PAGE will be carried out according to the method of King and Laemlli(24). The slab gels (10% acrylamide) will be case between glass plates (15 X 15 cm) to a hight of 10 cm using spacers of 1.5 mm thick. The gel solution will be overlaid with water until polymerization is completed. A stacking gel (3% acrylamide) of 1 cm height will be applied on top of the analytical gel. Electrophoresis will

be performed at 30 mA until the tracking dye would reach the end of the gel.

The slab gels will be fixed by treating them with a solution of 45.4% methanol and 9.2% acetic acid in water. The gels will be stained with gentle shaking for 2 h in 0.025% Coomassie brilliant Blue R-250 dissolved in isopropanol (25%) and acetic acid (10%) and would be destained by shaking overnight in 10% acetic acid.

Unless otherwise stated, membrane proteins will be disaggregated by mixing an equal volume of sample buffer (0.125 M Tris-hydrochloride, pH 6.9, 4% SDS, 10% 2-mercaptomethanol, 0.002% bromophenol blue) with a sample containing 10 to 100 mg of protein. The mixture will be heated at 100°C for 3 min.

Isolation of the outer membrane proteins :

i. Gel filtration in the presence of sodium dodecyl sulphate :

Membrane proteins (ca. 800 mg) will be treated with 0.05 M Tris/HCl (80 ml, pH 7.00) containing 1% sodium dodecyl sulphate (SDS) and 1 mM EDTA at 25°C for a period of 1 h. The dissolved material will be applied on to a column of Sephadex G-200 and eluted at 25°C with 0.05 M Tris/HCl,

pH 7.00 containing 1% SDS, 1 mM EDTA and 0.02% sodium azide. The flow rate will be 25 ml/h and 7.5 ml fractions are to be collected. Fractions under each peak are to be pooled, dialysed against several changes of sterile water and lyophilized.

ii. Isolation of the polypeptides from the polyacrylamide gels :

The polyacrylamide gel section containing separated protein bands will be cut into pieces and macerated by passing through a syringe. Proteins will be eluted by shaking at 37°C into 10 volumes of 0.05 M $NH_4 HCO_3$ containing 1% SDS. After 6 h, the polyacrylamide fragments will be removed by centrifugation and then washed once with a small volume of elution buffer. The combined supernatants will be dialysed against SDS in order to remove the buffer originally present in the gel. Upon lyophillisation $NH_4 HCO_3$ will be removed leaving a protein-SDS mixture which will be utilised for further studies.

Antisera :

Antisera against Vibrio cholerae membrane proteins will be prepared in rabbits by immunising each rabbit with 1 mg of Vibrio cholerae Ogawa 395 total membrane, suspended in 0.5 ml of saline and an equal volume of Freund's incomplete adjuvant. Antigens will be

administered on days 0, 14, 28 and 42. Blood will be collected one week after the last injection. The antisera will be stored at-70^{0}C until use.

Immunodiffusion :

The outer membrane proteins will be solubilised in the non-ionic detergent Tween 20. Briefly, one volume of a 0.10 M Tris-HCl (pH 8.0) containing 5% Tween 20 will be added to 1 volume of the membrane suspension. The mixture will be shaken for a period of 2 hours at room temperature and will be centrifuged at 10,000 xg. The supernatant will be subjected to immunodiffusion in 2% agarose.

Immunoelectrophoresis :

The outer membrane proteins, solibilised in Tween 20 as described above, will be subjected to immunoelectrophoresis in a barbital buffer, pH 8.2 in 2% agarose for 1 h and allowed to diffuse against antiserum to Vibrio cholerae total membrane.

Challenge studies :

Immunization : Rabbits are to be immunized subcutaneously in both flanks on days 0 and 7. They are to be challenged via ligated ileal loops on days 14 and 18.

Preparation of ligated ileal loops :

Ileal loops are to be prepared according to the method of De and Chatterje(25). Rabbits are to be fasted for 48 h to allow elimination of fecal pellet. Each segment of the ligated ileum will be challenged one half milliliter of a vibrio suspension, appropriately diluted in PBSG(phosphate buffered saline, pH 7.2 containing 0.1% gelatin). Each segment will be approximately 6 to 9 cm in length and each separated by an uninoculated segment of about 2 cm. Control loops will receive 0.5 ml of PBSG or 0.5 µg of cholera toxin in 0.5 ml of diluent. After 18 h the rabbits are to be sacrificed and the ileum excised . Loops are to be carefully measured and the ratio of the volume of fluid accumulated (in milliliters) to the length of the segment (in centimeters) will be calculated and expressed as the fluid accumulation(FA) ratio.

D. SIGNIFICANCE :

Successful completion of the proposed studies should considerably increase our knowledge about organisation of the cell wall of Vibrio cholerae. The studies will provide specific information on the presence of common protein antigen among various biotypes and serotypes of Vibrio cholerae as well as other enteric bacteria. The study might lead to the possibility of

developing a non-toxic immunogen which would provide protection against challenges with all biotypes and serotypes of Vibrio cholerae.

E. FACILITIES REQUIRED :

1. Office space : Office space required for the Principal Investigator.

2. Laboratory space : Laboratory space required for experimental work.

3. Hospital resources : None

4. Animal resources : 100 rabbits.

5. Logistical support : none

6. Major items of equipments :

a. Ultracentrifuge

b. Electrophoretic apparatus for

i. i. Polyacrylamide gel electrophoresis

ii. Immunoelectrophoresis

iii. Crossed immunoelectrophoresis

7. Other specialized requirements :

F. COLLABORATIVE ARRANGEMENTS :

The principal investigator would maintain a collaborative research programme with the Max-Planck Institute für Immunbiologie, Freiburg, Germany which is very well equipped to perform analytical works on bacterial cell surface antigens. The principal investigator has discussed this possibility with the Director of the Max-Planck-Institute in person and would like to spend a few weeks there during the course of this study.

SECTION III - BUDGET

A. DETAILED BUDGET

1. PERSONNEL SERVICES

NAME	POSITION	% TIME USED	ANNUAL SALARY	TAKA	DOLLAR
Dr. S. Kabir	Scientist	25%	105, 000	26, 250	-
Dr. A. Mahmood	Assc. Scientist	10%	71, 000	7, 100	-
Mr. S. Ali	Technician	100%	26, 568	26, 568	-
Mr. Shafiullah	"	5%		1, 000	-
		Sub total -		60, 918	-

2. SUPPLIES AND MATERIALS

Reagents, chemicals	Tk.	Tk.	15, 000	-
	Sub total	Tk.	15, 000	-

3. EQUIPMENT

		-	$ 6, 000
Sub total	Tk.	-	$ 6, 000

4. PATIENT HOSPITALISATION

None

5. OUTPATIENT CARE

None

6. ICDDR,B TRANSPORT

None

7. TRAVEL AND TRANSPORTATION OF PERSONS

International travel		TAKA	DOLLAR
Transport	1 meeting		2,000
Per diem	10 days		500
Collaborative work	50 days		2,500
	Sub total	Tk. -	$ 5,000

8. TRANSPORTATION OF THINGS

Import of Equipments :		$ 1,000
Local shipment - none		
	Sub total -	$ 1,000

9. RENT, COMMUNICATIONS AND UTILITIES

Postage		Tk. 500.00
Telephone	- none	
Cable	- none	
Rent	- none	
	Sub total	Tk. 500.00

10. PRINTING AND REPRODUCTION

Printing forms	500	
Special reproduction	2,000	
Publication costs		500
Sub total	2,500	$ 500

11. OTHER CONTRACTUAL SERVICES

None

12. CONSTRUCTION, RENOVATION AND ALTERATIONS

None

13. ANIMAL REQUIREMENT

100 rabbits	Tk.	12,500
Sub total	Tk.	12,500

B. BUDGET SUMMARY

	CATEGORY	Year - 1 TAKA	DOLLAR
1.	Personnel	60, 918	-
2.	Supplies	15, 000	-
3.	Equipment	-	6, 000
4.	Patient hospitalisation	-	-
5.	Outpatients care	-	-
6.	ICDDR, B transport	-	-
7.	Travel Persons	-	5, 000
8.	Transportation things	-	1, 000
9.	Rent/Communication	500	-
10.	Printing & reproduction	2, 500	500
11.	Contractual service	-	-
12.	Construction	-	-
13.	Animal requirement	12, 500	-
	Grand total	91, 418	12, 500
			6, 100
	Total $		18, 600

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