Attachment 1.

ETHICAL REVIEW COMMITTEE, ICDDR,B. DHAKA-12

Date JULY 28, 1986

Principal Investigator DR. ANWARUL HUQ Trainee Investigator (if any)

Application No. 86-027 Supporting Agency (if Non-ICDDR,B) WHO

Title of Study: STUDY OF THE SEASONAL DISTRIBUTION OF VIBRIO CHOLERAE, PLANKTON AND WATER CHEMISTRY IN NATURAL WATERS IN MATLAB, 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:
   (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:
   (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)
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 (NA) — Yes No
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   X Protocol (Required)
   X 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.

ANWAR

Principal Investigator

Trainee

SECTION I - RESEARCH PROTOCOL

1. Title : Study on the seasonal distribution of *V. cholerae*, plankton and water chemistry in natural waters in Matlab, Bangladesh.

2. Principal Investigator: Dr. Anwarul Huq

   Co-Investigator : Dr. Rita Colwell

   Consultant : Dr. David A. Sack

3. Starting Date : September, 1986

4. Completion Date : August, 1988

5. Total Direct Cost : US $ 78,780.00

6. Scientific Program Head :

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

   Signature of the Scientific Program Head : David A. Sack

   Date : 30 Jun 1986

7. Abstract Summary :

V. cholerae, Seasonal distribution of various genera of zooplankton and phytoplankton with emphasis on copepods in some ponds and river waters in Bangladesh will be studied. Quantitative analysis of various plankton in different water will be determined. Through measurements, total surface area offered by these copepods in the natural environment for bacterial attachment will be calculated. Physical and chemical

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective

a. Determine a correlation that may exist between the appearance and disappearance of *V. cholerae* and copepods or other plankton species in the natural environment.

b. To be able to forecast a 'bloom' of *V. cholerae* in the environment and the likelihood of an ensuing epidemic.

c. To generate data on the ecology of plankton or other organisms in water and *V. cholerae* with the chemical and physical parameters effecting their multiplication in the aquatic environment.

2. Background

Particularly in the last two decades many investigators have attempted to come up with firm conclusions about the mechanisms of how *V. cholerae* is transmitted and its reservoirs. The cell of *Vibrio Cholerae* 01 has beenisolated at places in the United States, England and other countries where chances of contamination of water from human sources is very unlikely (Blake *et al.*, 1980, Colwell *et al.*, 1981, Sandera *et al.*, 1983, Lee *et al.*, 1984).

The association of *V. cholerae* and planktonic crustacean copepods has been established through laboratory microcosm studies (Huq *et al.*, 1983). This unique relationship may be

influenced or controlled by important physical and chemical characterstics of the environment. Studies have shown that cells of V. cholerae multiply and proliferate in high numbers in the presence of live copepods but not in the presence of dead copepods (Huq et al., 1983). There is circumstantial evidence that cells of V. cholerae may be protected by various plants and animals in the aquatic environment (Kaper et al 1979, Nalin et al., 1979 and Spira et al., 1981). Preveious studies by the author showed that high water temperature (≥25C) and high pH (≥ 7.5) significantly enhanced the multiplication of V. cholerae (Huq et al., 1984). An attempt was made to speculate and associate the appearance of V. cholerae 01 and with that of copepods from the evidences of the two different studies (Huq et al., 1983, Huq et al., 1984b).

The seasonality of cholera in the endemic areas is facinating. In a two year survey in Dhaka, Bangladesh in 1964-66 it was shown that peaks of cholera occurred during November and December. In Matlab, which is only 25 miles away from Dhaka, the peaks were in January and February. About 120 miles away in Calcutta, cholera peaks were in May and June (Martin et al., 1969). Peak cholera time has now shifted to September - November in all three places (Feachem, 1982). The elucidation of the environmental factors affecting and/or effecting the seasonal patterns of cholera presents a great challenge. Direct evidence from a single study covering major environmental and cultural aspects of V.

cholerae would be valuable. The seasonality of cholera in Bangladesh appears to be geographically related and outbreaks are often localised (Glass et al., 1982, McCormack et al 1969). Isolates of V. cholerae with diverse seasonal distribution showed different phage types (Glass et al., 1985) indicating the lack of a common source of infection.

Among other environmental factors, salinity has long been considered important for the long term survival of V. cholerae in water (Singleton et al., 1982, Miller et al., 1982).

The isolation of V. cholerae in the Chesapeake Bay in the U.S.A. was dependent on salinity and temperature (Colwell et al., 1981). Other studies done in the United States and Australia (Hood et al., 1982, Kaper et al., 1978, Salmaso et al., 1980 and Rogars et al., 1980) caused Miller and his collegues, (1985) to hypothesise that aquatic reservoirs play an important part in all cholera epidemics.

The disappearence of V. choleare 01 from water at particular times of the year still remains unanswered. Various hypotheses have been put forward. It has been hypothesised that V. cholerae is an autoohthonous member of the bacterial community of brackish water (Colwell et al., 1977). As such, it may well interact with other organisms which affect its seasonal appearance (Colwell et al., 1984), in the aquatic environment.

Faechem et al. (1981) concluded that the implications of various discoveries for the epidemiology and control of cholera remains to be elucidated and more comprehensive research should be accomplished from the molecular level to community level. The isolation of different phage types of V. cholerae from a single outbreak calls for further investigation to examine the homology of the strains at the molecular level.

3. Rationale

The overall model that we would like to test is shown in Figure 1. Implications of the model are that copepods play a key role in the cholera transmission since the bacteria shed into the water are maintained and perhaps amplified by high concentrations of copepods. Past studies have suggested that only a few copepods with V. cholerae cells attached to them may carry the requisite infectious dose for clinical cholera. A colonized copepod may contain up to 10^4 cells of V. cholerae, and being nearly invisible, may be swallowed by an individual when drinking water (Huq et al., 1984a). The chance consuming this "Vibrio capsule" increases during the periods of high concentration of copepods in water.

However important copepods may be, it is likely that they are not the only factor important in Vibrio maintenance and transmission in the environment. Copepods do, however, have seasonal changes (Svedrup et al., 1947) and may correlate with cholera seasonality. Others organisms such as aquatic plants and animals certainly should not be ignored and hence

some of these will be included for examination in this protocol. Nevertheless, our concentrated effort will be given to plankton and the physical and chemical properties of water.

By the end of the study we expect to have accumulated a detailed data bank on the chemistry of water, prevalence of environmental *V. cholerae* and plankton populations. In addition, data on sunshine and rainfall will be collected from government sources and will be included in the analysis. A careful analysis of all these parameters should allow for a more complete understanding of the ecology of *V. cholerae* in the environment. These results may suggest reasons for the marked seasonality of infections by this organism. Finally, these results may also suggest future areas of research which will contribute to a more complete knowledge of the human and environmental interactions of this elusive microorganism.

B. SPECIFIC AIMS

Hypotheses to be tested:

a. That zooplankton and specifically copepods are an important link in the maintenance and transmission of *V. cholerae* and perhaps its multiplication in the environment.

b. That copepod blooms correlate with and preceed cholera epidemics in Matlab.

c. That certain physical and biological parameters will predict copepod bloom.

C. METHODS OF PROCEDURE

Sampling site: The sampling sites for this study are crucial to its potential success. *V. cholerae* is a member of the brackish or marine environment. Laboratory experiments have shown that at least 0.01% salinity is required for *V. cholerae* to survive beyond 24 h (Miller *et al.*, 1982). In an environment which has salinity below the minimum required level but in which the incidence of *V. cholerae* is high must have some protective mechanism to help in the maintenance of these organisms.

Matlab is 45 km southeast of Dhaka and lies in the delta formed by the Rivers Meghna and Ganges. It is intersected by numerous tidal rivers and canals. Most of the year river salinity levels remain below 0.01% (Huq, unpublished data). Seasonal water levels vary more than 14 feet from the high to low mark. There will be 10 sampling sites chosen in Matlab within a five mile radius around the Matlab Field Station of ICDDR,B. Sites will be selected at rivers and ponds which are heavily used for domestic purposes.

Samples: Three types of samples will be obtained for study such as water, plankton and plants/animal. Emphasis in this protoool will be given to water and plankton. Plants, such as water hyacinth and duck weed, and animals, such as shrimp, crabs or fish, if available at the site, will be sampled for study.

One half-hour plankton tow will be made at each sampling site using a 0.77 um mesh plankton net at the daily time when the

highest numbers of zooplankton are near the surface of the water. Plankton will be collected in 4 oz. wide-neck bottles for processing. Aliquotes of plankton samples will be immediately fixed in 3% formaldehyde for later indentification and electron microscopy. Water will be collected from 5 cm below the surface in 250 ml steriled nalgene bottles.

One of the bottles will be chilled immediately for chemical analysis in the laboratory and other will be used for bacteriological analysis. These samples for microbiological analysis will be processed within four hours of collection. They will be transported at a temperature similar to that of the environment of collection.

Bacteriological methods: One gram (wet weight) of plankton will be homogenized in 9 ml of PBS after first washing twice with sterile PBS. Plankton homogenates will be put in 10 ml of 2x alkaline peptone water (APW) for the enrichment of V. cholerae. Before enrichment, 0.1 ml of homogenized plankton will be directly plated in duplicate on TCBS agar, and incubated for 18 h at 37C. After 6 hours of enrichment in APW at 37C, homogenates will be streaked on to TCBS and also incubated at 37C for 18-24 h. The following day, suspected colonies of V. cholerae from the pre-enrichment plankton samples will be counted and will be picked for subculturing onto galatin agar (GA) plates with discs containing 10 ug of vibriostatic compound (2,4-diamino-6,7-diisopropyl-pteridine).

From the plates inoculated from the APW enrichment broth, two suspected V. cholerae colonies will be picked and streaked on to separate GA plates with 10 ug discs of vibriostatic compound and incubated at 37C for 18-24 hours. All suspected colonies will be serologically tested by slide agglutination with V. cholerae 01 antisera. After serological confirmation, colonies will be stocked in blood agar base slants for subsequent testing. Suspected non 01 V. cholerae will likewise be stocked. Plant and animal samples will be homogenized and processed in the same way as the plankton samples.

Water samples for culture for V. cholerae will be obtained in 40 ml amounts and enriched in 20 ml of triple strength APW. After enrichment they will be plated on TCBS and incubated at 37C for 18-25 h. Non 01 and 01 V. cholerae isolates will be stocked.

Chemical analysis of water sample: Temperature, dissolved oxygen (DO) tension and pH will be measured on site at the time of collection by using a portable DO and pH meter Collected water will be chilled and taken to the laboratory for chemical analysis for acidity, alkalinity, bromine, chloride, chlorine, sodium chromate, hexavallent chromium, copper, fluoride, hardness (calcium and magnesium), iron, manganese, nitrogen (nitrate and nitrite) phosphate, silica, sulphate, hydrogen sulphide, suspended solids and turbidity. These tests will be done by using a portable HACH machine (Hach Chemical Company, P.O. Box 907, Ames, IOWA 50010,

U.S.A). which uses spectrophotometric and titration methods for analysis. Beside the chemical information, we will procure data on the total rainfall, and sunshine for the area from the meteriological department of the Government of Bangladesh.

Case-control study: A case-control study has been planned during the 2 year study period. In addition to the 10 fixed samplings sites, additional samplings will be required throughout the study period. When a case of cholera is reported at the Matlab Treatment Centre, Tow field assistants will go to the village of the patient and collect water, plankton, and other samples from the pond(s) or water sources used by that patient. These specimens will be processed for V. cholerae isolation in the same way as mentioned elsewhere in this protocol. Water plankton and other samples will be collected and tested for their physical and chemical parameters as described. When a positive V. cholerae isolation is made, sampling for all specimens will be done every week until the specimens becomes negative for V. cholerae serovar 01. A maximum of 2 cases can be done in a week, with the aim of doing 200 case-control studies in two years. A control water source used by asymptomatic inhabitants of a non-cholera village will be identified and will be selected simultaneously with each case included in the study. Controls will be selected a minimum of 3 to " villages away from the case village. Samples from control sites will be processed in the same way as the specimens from

the case villages. Strains of V. cholerae isolated from the patients will be later tested in homology studies.

Frequency of Sampling: Routine sampling will be done every 15 days. This frequency of sampling is considered important because in Bangladesh sudden climatic changes occure with subsequent changes in the aquatic environment are noticed throughout the year. As previously mentioned, sampling for the case-control studies will be more frequent.

Bacterial homology test: The estimated one hundred strains of V. cholerae 01 isolated from the case control studies will be tested to see the homology by cell membrane protein analysis in SDS polyacrlyamide gels. The centre is fully equipped and capable of doing this at present, so no extraordinary facility will be required for this test.

Duration of study: Since this is an ecological study involving seasonal distribution, initially we propose it to continue this study for at least two years.

E. FACILITIES REQUIRED

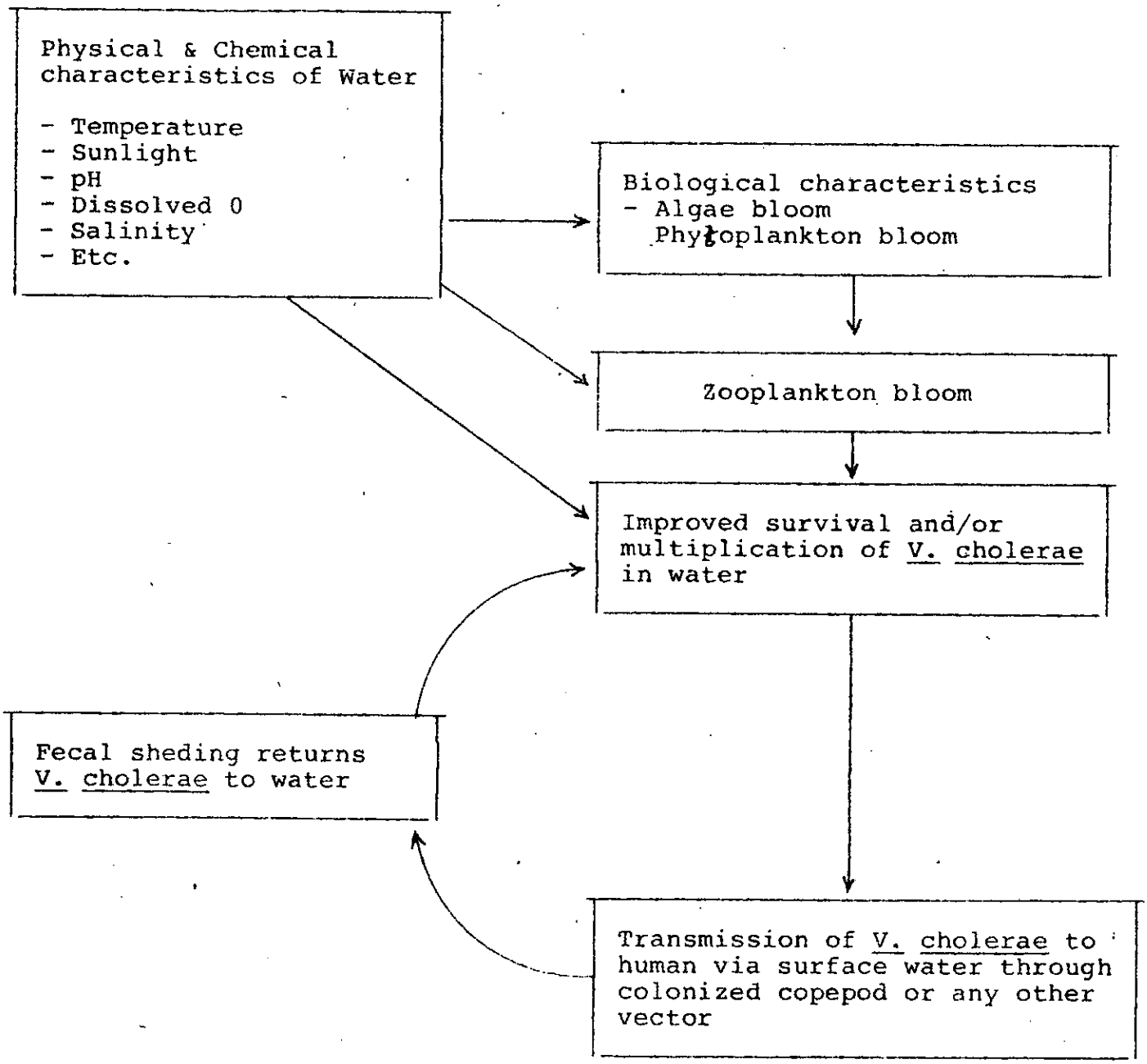
None

F. COLLABORATIVE ARRANGEMENT

Two graduate students as research trainees from the Microbiology department of the University of Dhaka will be identified to work on this project. Part of the data collected from this project will be used by those students for their dissertation as partial fulfillment for the Master degree in Microbiology. These two students will come with strong background in Botany and Zoology and will be able to handle the identificatioin of both phyto and zooplankton.

Also PI intends to go to the United States with the plankton samples to be saved for the scanning electron microscopy. Modern technology developed for specific organisms to be identified by scanning will be applied to these plankton. This will be done at the collaborating laboratory in the United States.

FIGURE 1


Physical & Chemical characteristics of Water
- Temperature
- Sunlight
- pH
- Dissolved 0
- Salinity
- Etc.
Biological characteristics
- Algae bloom
Phytoplankton bloom
Zooplankton bloom
Improved survival and/or multiplication of V. cholerae in water
Fecal sheding returns V. cholerae to water
Transmission of V. cholerae to human via surface water through colonized copepod or any other vector

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ICDDR,B
BUDGET PROPOSAL
PROGRAM NAME : DTWG
PROGRAM HEAD : Dr. D. A. Sack
PROTOCOL:
PRINCIPAL INVESTIGATOR: Dr. Anwarul Huq
CO-INVESTIGATOR: Dr. Rita R. Colwell
PROTOCOL NO.: STARTING : September, 1986
BUDGET CODE: COMPLETION : August 1988

BUDGET SUMMARY

A/c	CATEGORY	EXPENSE 1986 .00	EXPENSE 1987 .00	EXPENSE 3rd year .00	TOTAL PROJECT COST
3100	Local Salaries	0	0	0	25824
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	5000
3700	Supplies & Materials	0	0	0	12272
3800	Other Costs	0	0	0	700
4800	Inter-departmental	0	0	0	34984
	TOTAL DIRECT COST	0	0	0	78780
0000	Indirect Cost, 31%	0	0	0	24421.8
	TOTAL OPERATING COST	0	0	0	103201.8
0300	Capital Expenditure	0	0	0	0
	TOTAL PROJECT COST	0	0	0	103201.8

PERSONNEL REQUIREMENT - LOCAL (A/c 3100)

		No. of positions	Man Months	$ Amount
(A)	Existing	0	0	0
(B)	New Recruitments	4	96	9024
(C)	Allocated from other area	4	72	16800
	SUBTOTAL	8	168	25824
(D)	Separations	0	0	0
(E)	Allocated to other area	0	0	0
	SUBTOTAL	0	0	0
	TOTAL	8	168	25824

LOCAL STAFF: (A) EXISTING

Job designation	No. of position	Man month	Rate per month	$ Amount
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
	0	0	0	0
TOTAL	0	0		0

LOCAL STAFF: (B) NEW RECRUITMENTS

Job designation	No. of position	Man month	Rate per month	$ Amount
Research Officer, GS-5	1	24	190	4560
Field Assistant, GS-3	1	24	120	2880
Research Trainee	2	48	33	1584
	0	0	0	0
	0	0	0	0
TOTAL	4	96		9024

LOCAL STAFF: (C) ALLOCATED FROM OTHER AREA

Budget	Job Desig	Level	No. of position	Man month	Rate per month	$ Amount
060401	Manager	NO-B	1	12	380	4560
060401	Sr.Res.Of.	GS-6	1	24	260	6240
060401	Lab.Tech	GS-3	1	24	120	2880
010101	Secretary	GS-6	1	12	260	3120
	TOTAL		4	72		16800

PERSONNEL REQUIREMENT - INTERNATIONAL (A/c 3200)

		No. of positions	Man Months	$ Amount
(A)	Existing	0	0	0
(B)	New Recruitments	0	0	0
(C)	Allocated from other area	0	0	0
	SUBTOTAL	0	0	0
(D)	Separations	0	0	0
(E)	Allocated to other area	0	0	0
	SUBTOTAL	0	0	0
	TOTAL	0	0	0

INTERNATIONAL STAFF: (A) EXISTING

Job designation	No. of position	Man month	Rate per month	$ Amount
	0	0	0	0
	0	0	0	0
	0	0	0	0
TOTAL	0	0		0

INTERNATIONAL STAFF: (B) NEW RECRUITMENTS

Job designation	No. of position	Man month	Rate per month	$ Amount
	0	0	0	0
	0	0	0	0
TOTAL	0	0		0

INTERNATIONAL STAFF: (C) MANPOWER ALLOCATED FROM OTHER AREA

Budget	Job Desig	Level	No. of position	Man month	Rate per month	$ Amount
			0	0	0	0
			0	0	0	0
			0	0	0	0
	TOTAL		0	0		0

CONSULTANTS (A/c 3300)

Job designation	No. of days	Total Per diem	Total Honorarium	Travel cost	$ Amount
	0	0	0	0	0

TRAVEL PLAN - INTERNATIONAL (A/c 3600)

Job desig	Purpose	From/To/ From	No. of days	Transp. cost	Per diem/ others	$ Amount
P.I.	Research	Dhaka/USA/ Dhaka	28	2200	2800	5000

SUPPLIES AND MATERIALS (A/c 3700)

Account	Items	$ Amount
3701	Drugs	0
3702	Glassware	980
3703	Hospital Supplies	0
3704	Stationery and Office Supplies	420
3705	Chemicals and Media	7000
3706	Materials for Uniform	0
3707	Fuel, Oil and Lubricants	0
3708	Laboratory Supplies	470
3709	Housekeeping Supplies	0
3710	Janitorial Supplies	0
3711	Tools and Spares	0
3712	Non-stock Supplies	570
	SUBTOTAL	9440
3713	Freight and Other Charges (30%)	2832
	TOTAL	12272

OTHER COSTS (A/c 3800)

Account	Items	$ Amount

3800	Repairs and Maintenance	0
3900	Rent, Communication and Utilities	100
4100	Bank Charges	0
4200	Legal and Professional Expenses	0
4300	Printing and Publication	600
4400	Entertainment, Hospitality and Donation	0
4500	Service Charges	0
4600	Staff Development and Training	0
	TOTAL	700

INTER-DEPARTMENTAL SERVICES (A/c 4800)

Account	Items	$ Amount
4801	Computer	3000
4802	Transport - Dhaka	300
4803	Transport - Matlab	0
4804	Water Transport - Matlab	9000
4805	Transport - Teknaf	0
4806	Xerox and Mimeograph	200
4807	Pathology	0
4808	Microbiology Tests	21584
4809	Biochemistry	200
4810	X-ray	0
4811	I.V. Fluid	100
4812	Media	0
4813	Patient Hospitalization - Study	0
4814	Animal - Research	400
4815	Medical Illustration	100
4817	Telex	100
4818	Outpatient Care	0
4830	Transport Subsidy	0
	TOTAL	34984