This is a laboratory based study. Stool samples from animals will only be needed.

Attachment 1.

Date 16.02.88

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Dr. S.Q. Akhtar

Trainee Investigator (if any) Mr. Sirous Shabani

Application No. 88-005(P)

Supporting Agency (if Non-ICDDR,B)

Title of Study Isolation of Campylobacters from domestic animals.

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: 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, (hospitnl, 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 Yes No NA
7. Check documents being submitted herewith to Committee:
   ___ Umbrella proposal - Initially submit a overview (all other requirements will be submitted with individual studies).
   ✓ Protocol (Required)
   ✓ Abstract Summary (Required)
   NA 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)
   NA 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)

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

S. Q. Akhtar
Principal Investigator

Sirous Shabani
Trainee

SECTION I : RESEARCH PROTOCOL

1. Title : Isolation of Campylobacters from domestic animals.

2. Principal Investigator : Dr. S.Q. Akhtar

   Co-Investigator : Mr. Sirous Shabani

3. Starting Date : February, 1988

4. Completion Date : July, 1988

5. Total Direct Cost : US$1,100.00

6. Scientific Programme Head: _____

This protocol has been approved by the Laboratory Sciences Division (LSD).

Signature of the Scientific Program Head

Date: Febr. 8, 1988

7. Abstract Summary :

Campylobacters are documented as human and animal pathogen throughout the world. This study plans to investigate the presence of Campylobacter species in healthy and diarrhoeic animals in Bangladesh. Attempt will be made to isolate Campylobacters from 400 domestic animals including cattle, goat, dog, pig, chicken and duck. Stools samples or rectal swabs from these animals will be cultured in selective medium and then characterised following standard procedure. This research is expected to contribute new information regarding the occurrence of Campylobacter species in different domestic and farm animals.

This proposal is for a foundational descriptive study of potential role of Campylobacter species as an etiologic agent of diarrhoea in different animal species in Bangladesh.

8. Reviews :

(i) Ethical Review Committee : _____

(ii) Research Review Committee : _____

(iii) Director : _____

SECTION-II : RESEARCH PLAN

A. INTRODUCTION

1. Objective:

The objective of this research is to isolate and identify different species of Campylobacters from different domestic animals. This would specify which species of the genus Campylobacter are more prevalent in domestic animals of Bangladesh and cause diarrhoeal illness.

2. Background:

Campylobacters are frequently isolated from different animals in different parts of the world.(4,6,7,8,9). Campylobacter has been known as veterinary pathogen since 1909 (1,3) but only from 1947 it has been known to cause human disease (2,4).

There are different species and subspecies within the genus Campylobacter, but most frequent pathogenic subspecies is Campylobacter jejuni. However, the development of appropriate methods for isolating Campylobacters from stools has demonstrated their importance.(5).

Host range of this organism is very wide. It has been isolated from man, poultry birds, animals and environment. The gastrointestinal tracts of animals, both wild and domestic, constitute the reservior for infection (8).

Broiler chickens are the largest potential source of C. jejuni infection in many countries (Skirrow 1982). A few outbreaks and some sporadic infections have been associated with the consumption of incompletely cooked chicken (11).

C. jejuni has been incriminated as the causative agent of enteritis in humans (4,6,8,12,13). Although many sources of human infection with Campylobacters have been suggested, the real source of infection has not been clearly defined. Studies have shown that C. jejuni can be found in the gut of many lower animals, particularly avians (14,15,16). Evidence also suggests that the infection may be acquired from contaminated foods and raw milk (4,15,17,18). Vibrionic hepatitis due to C. jejuni has been found in chickens (14,19), chickens have also been incriminated as the source of human infection (15). To evaluate the role of poultry as a potential source for human infection, the frequency of isolation and antimicrobial sensitivity of C. jejuni were studied. C. jejuni was detected in 96% of classical samples from 26 live broilers, 84% of 25 processed birds ready for sale, 55% of 200 caged laying hens and 25% of 200 freshly laid eggs (20).

Another study on chicken campylobacteriosis in Calcutta (INDIA) has determined that the caecal contents of 50 (62.5%) country chickens harboured C. jejuni indicating a high carriage rate of this enteric pathogen. Along with C. jejuni one sample also yielded C. coli in contrast. C. intestinalis, a closely related species, could not be recovered from any of the caecal samples

examined. Exclusion of this nonthermophilic Campylobacter in chicken caeca appears to be related to the higher body temperature of poultry fowls (28).

Varying isolation rates of C. jejuni from chicken has been reported from different countries. In some instances (3) the incidence of C. jejuni in broiler chicken was as low as 1-8 percent, while in other cases, above 83 percent isolation rate have been reported (22,23).

C. jejuni is very common in the sheep population; C. fetus is less commonly recovered. In cattle, thermophilic Campylobacters, C. fetus and C. hyointestinalis are commonly isolated (24).

Van Damme and Laumers isolated C. jejuni in 29% of animals in Central Africa; highest isolation rates were found in pigs (44%); in chicken , rate was 38%. Campylobacters were isolated from sheep, duck and other birds from zoological gardens (25). C. fetus is well known as a cause of abortion and infertility in cattle and sheep. Another species of Campylobacter, closely related to C. fetus, is now suspected of causing similar disease. Fetuses from 100 cases of abortion and from 39 pregnant slaughtered sows were examined by bacteriological methods, Campylobacters were isolated from 47% of the aborted litters, but from only 6% of the litters of pregnant slaughtered sows (26). C. jejuni has been isolated from the intestine of several dogs at Glasgow. Study showed that experimental infection with C. jejuni in dogs give rise to only mild disease (27).

3. Rationale :

The reasons for the lack of identification of etiology of diarrheal specimens are undoubtedly numerous and multifactorial. However, a potential major contributer to this group is undoubtedly the existence of unrecognized pathogens. And during last few years many newly described etiologic agents of diarrheal diseases have been identified. But regarding campylobacter infection, one of the major etiologic agents of diarrhea in different animals; we do not have that much study in Bangladesh.

Since this research will be conducted for a Masters thesis in veterinary science, the study would concentrate on the isolation and identification of these micro-organisms from different animals in Bangladesh. It is expected that the results of this protocol would significantly add to the knowledge of previously unrecognised etiologic agents of diarrhoeal diseases in animals in Bangladesh.

B. SPECIFIC AIMS

1. To isolate and identify Campylobacter species from healthy and diarrhoeic animals.

2. To investigate the association of Campylobacters with diarrhoeal illness in animals.

C. METHODS OF PROCEDURE

For this investigation four hundred stool samples will be collected from different animals:

Cattle= 100; Goat= 100; Dog= 20; Pig= 30; Chicken= 100; Duck= 50.

1. Collection of samples:

Transport medium (modified Cary-Blair) will be used for transporting the collected samples. Stools or rectal swabs will be collected in transport medium with sterile cotton swabs. Another swab will be kept in sterile test tubes. Collected samples will be transported to the laboratory as soon as possible.

2. Inoculation and incubation of the sample:

Campy-BAP (Chart: 1-A), a highly selective and widely used medium for the isolation of Campylobacters will be used for this study (4). As soon as the samples are received in the laboratory, those will be inoculated on the selective agar plates, then incubated microaerobically in a candle jar (10) for 48 hours at 42-43 °C. Plates will be initially read at 48 hrs (chart: 1-B). Plates not yielding Campylobacters will be incubated till 72 hrs. An extra reading at 72 hrs may yield a few more positives.

Suspicious colonies should be checked for oxidase and catalase production and smeared if positive. The smear would be Gram stained with carbol fuchsin (1:5 dilution) as the counterstain. Campylobacter should be suspected if vibrio like bacteria are seen at the early stationery phase of growth. Organisms may be S-shaped or spindle-shaped. With cultures age, or if grown under unfavourable conditions, coccoid forms are frequently present. Motility would be observed with a darkfield or phase-contrast microscope.

The suspected colonies will be identified following standard procedure (please see chart 2).

Identification criteria

1. To assess growth at 42°C and 25°C:

Make a light suspension (Mac Farland) of Campylobacter in sterile saline. Inoculate 0.5 ml of this suspension into each of two Brucella broths. Incubate one at 42°C and the other at 25°C for 48 hours.

Growth at 42°C, but not at 25°C, indicates the presence of C. jejuni. C. intestinalis will grow better at 25°C than at 42°C.

2. To assess tolerance to glycine:

Stab-inoculate the glycine medium. Growth occurs mostly near the surface of the medium to give a cloudy appearance when the test is positive (at 48 hours). C. jejuni will give a positive result.

3. To assess tolerance to 3.5% NaCl:

Inoculate the salt medium and incubate for 48 hours. Record growth or no growth. C. jejuni should not grow.

4. To assess H_2S production:

Heavily inoculate a Kligler's Iron agar slant, attach a strip of lead-acetate paper to the tip of the tube and incubate for 48-72 hours. C. jejuni will usually produce slight growth on the slant but an alkaline/alkaline (K/K) reaction. The butt of the medium should not be blackened by H_2S; although, the lead acetate

paper should. If results are negative (No blackening of the leadacetate paper), repeat with another heavy inoculation from a fresh culture.

5. Hippurate hydrolysis:

Hippurate hydrolysis test will be performed to confirm C. jejunni and to differentiate between C. jejuni and C. coli.

C. jejuni is able to hydrolyse hippurate and gives a dark violet color by the production of glycin, which C. coli does not.

6. Nalidixic Acid:

Inoculate a blood agar plate for confluent growth with a swab from a suspension of Campylobacter. Then, place a 30 ug disc of nalidixic acid on the plate, and incubate in the candle jar. C. jejuni is usually sensitive, and ssp. intestinalis is usually resistant. (Please see chart 3 for differential features of Campylobacter species.)

D. SIGNIFICANCE

The results of this investigation may identify previously unrecognized agent of diarrhoeal diseases in Bangladesh. Such findings should help identification, treatment, prevention and control of Campylobacter induced diarrhea.

Significance of different subspecies of Campylobacter.

C. fetus:

First isolated in 1909; an important cause for abortion in cattle. Never isolated from humans.

C. intestinalis :

Cause abortion in cattle and sheep, but may also be a commensal.

Rare cause of human disease.

An opportunist that causes systemic illness (bacteremia, meningitis, endocarditis, phlebitis, etc.) in debilitated or immuno-suppressed hosts.

C. jejuni :

Cause abortion in sheep. Pathogen and Commensal of fowl.

Presumed to be pathogen in swine, monkeys, dogs, and cats.

Causes diarrheal disease in humans. In developed countries, isolated from 3% - 10% of patients with diarrhea only rarely from healthy indivduals. In developing countries, isolated more frequently from both ill and healthy individuals, especially children. Clinical illness is frequently severe with dysenteric stools, fever, and abdominal pain. Infection may also mimic acute appendicitis or acute colitis. Stools from acutely infected individuals frequently show blood and polymorphonuclear leucocytes (pus cells) on microscopic examination.

(For characteristics of the biotypes of C. jejuni, please see chart 4).

C. mucosalis:

Considered to cause ileitis in Swine; has not been isolated from other species, pathogenicity is not confirmed.

E. FACILITIES REQUIRED:

No additional facilities will be required other than those which now exist in the Department of Laboratory Services of ICDDR,B.

F. COLLABORATIVE ARRANGEMENTS:

This protocol is a collaborative one between the Department of Microbiology and Hygiene of Bangladesh Agricultural University and ICDDR,B. Mr. Sirous Shabani will carryout the study in the facilities available at ICDDR,B Microbiology Branch under Dr. S.Q. Akhtar's supervision for the partial fulfilment of his Master's degree. It is expected that such collaboration would continue as a base for program leading to higher degrees for students from Bangladesh Agricultural University, Mymensingh.

Chart 1-A

Composition of Campy - BAP (4):

Brucella agar Base	1 liter
Sheep Blood	100 ml
Vancomycin	10 mg
Trimethoprim	5 mg
Polymyxin B	2500 IU
Amphotericin B	2 mg
Cephalothin	15 mg

Chart 1-B

The colonial morphology takes two forms:

i. Colonies are nonhemolytic, grayish, flat, wet, glossy, and spreading.

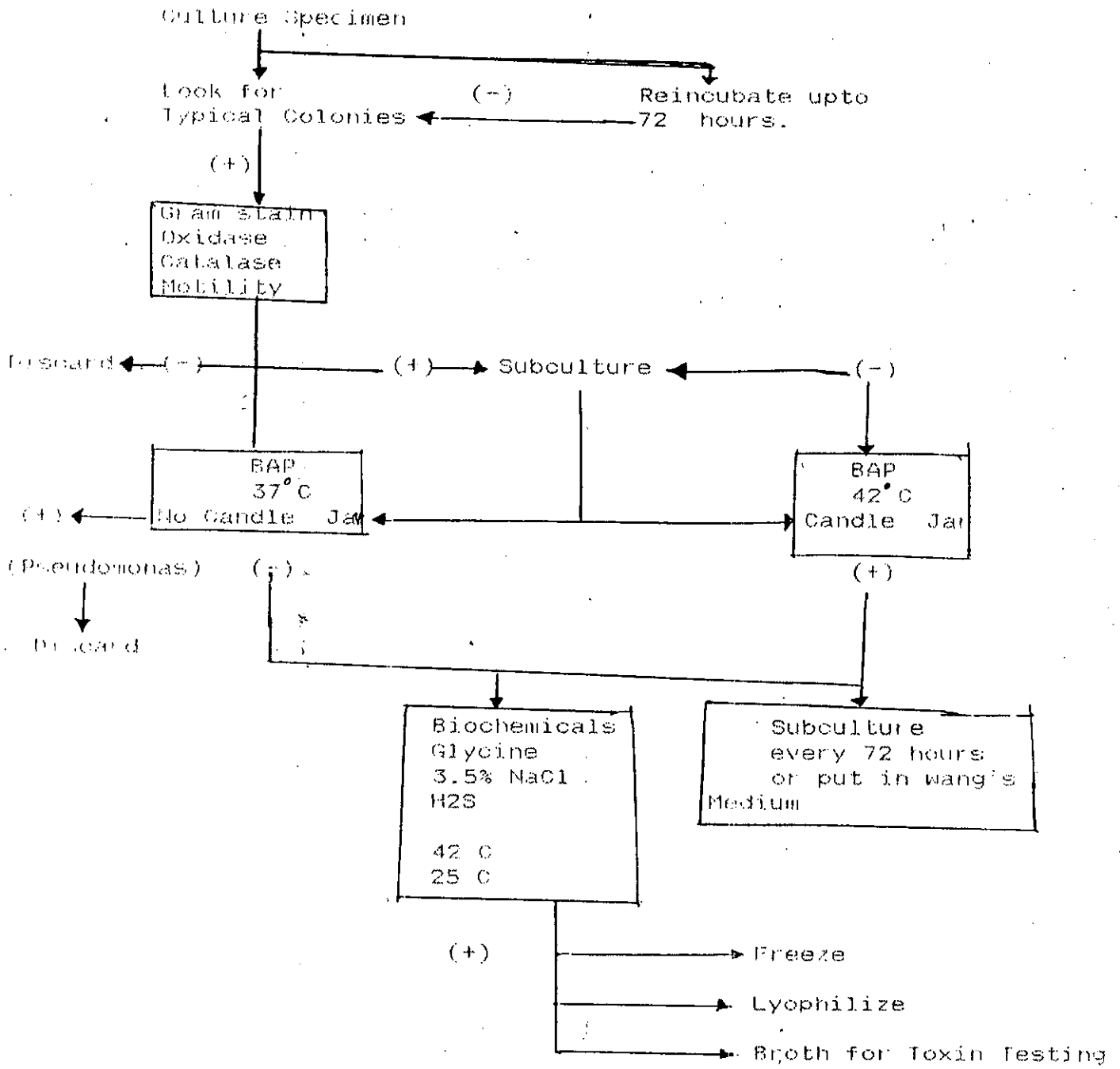
They resemble drops of water and spread along the direction of the inoculating loop. These colonies are easy to recognize and with most clinical specimens growth extends into the second and third quadrants (indicating and high bacterial concentration in the stool).

ii. A second type of colony is also nonhemolytic but is discrete (1-2 mm in diameter), convex, entire, and glistening. These colonies may be harder to see than the above type if there are many contaminating enteric organisms on the plate.

(From: Campylobacter fetus ssp. jejuni: A Laboratory Manual By: Martin J. Blaser, IEDDR,B special publication No. 7 p. 10, 11).

Chart-2

Procedure for Identifying Campylobacter



(From: Campylobacter fetus ssp jejuni : A Laboratory Manual
By: Martin J. Blaser, ICDDR,B Special Publication No.7, P.30)

Chart-4

Characteristics of the biotypes of *C. jejuni*

Tests	*C. jejuni*	*C. jejuni* biotype coli	*C. jejuni* biotype NART
Temperature (°C)			
25	–	–	–
30.5	–	+	+
37	+	+	+
43	+	+	+
45.5	d	d	+
Hippurate	+	–	–
Triphynyl terrazolium chloride (40 g/l)	S	R	S
NaCl (1.5%)	–	–	+
H_2S in iron medium	d	–	+
Nalidixic acid (30 µg/disk)	S	S	R
Metronidazole (5 µg/disk)	d	d	R

S= sensitive, R= resistant, d= variable, += growth, –= no growth
NART= nalidixic acid resistant thermophilic Campylobacter.

(From: Braude, Infectious diseases and Medical Microbiology 2nd edition, P. 312).

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7. Abstract Summary :

Campylobacters are documented as human and animal pathogen throughout the world. This study plans to investigate the presence of Campylobacter species in healthy and diarrhoeic animals in Bangladesh. Attempt will be made to isolate Campylobacters from 400 domestic animals including cattle, goat, dog, pig, chicken and duck. Stools samples or rectal swabs from these animals will be cultured in selective medium and then characterised following standard procedure. This research is expected to contribute new information regarding the occurrence of Campylobacter species in different domestic and farm animals. This proposal is for a foundational descriptive study of potential role of Campylobacter species as an etiologic agent of diarrhoea in different animal species in Bangladesh.

The study does not involve any human subject.

Item 1 through 8 are not applicable.

ICDDR,B
1988 BUDGET PROPOSAL
(In US $)

PARTICULARS

Division Name: Laboratory Sciences Division

Protocol/Branch name: Isolation of Campylobacter from Domestic Animals

Name of P.I./Branch Head/Division Head: S.Q. Akhtar

Budget Code: ..... Starting Date: February 1988

Protocol No: ..... Completion Date: July , 1988

Donor Name: ..... Grant Amount: US$1,100.00

EXPENSE CATEGORY		Column A: Actual Jan.-June 1987	Column B: Estimated Whole Yr. 1987	Column C: Proposed 1988
A/C No.	Description / Refer Page			
3100	Local Salaries 2			
3200	Intl. Salaries 8			
3300	Consultants 14			
3500	Travel Local 15			
3600	Travel Intl. 16			
3700	Supplies & Mat. 18			650
4000	Other Costs 19			50
4800	Inter Deptl. Ser. 21			400
	Total Direct Operating Cost			1,100
0300	Capital Expenditure Refer Page 22			1,100
	TOTAL DIRECT COST			

/Branch Head Associate Director Reviewed by Budget & Finance

Budget Code: _____

SUPPLIES AND MATERIALS-1988

A/C Code	Item Description	$ Amount
3701	Drugs (used for medication in the hospitals and field stations)	
3702	Glassware (Bottle, beaker, cylinder, petridish, aluminium seal, slides, stopper, tube etc.)	
3703	Hospital supplies (bandage, gauze, blade, bowl, catheter, cotton, needle, syringe, solution, leukoplast, towel etc.)	50
3704	Stationery and office supplies (Battery, book register, binders, files, pencil, fastener, paper, ribbon, stapler etc.)	
3705	Chemicals and media (Acid, reagent, dextrose, sodium, bactoagar etc.)	300
3706	Materials for uniform (Cloth, button etc. required for making uniforms)	
3707	Fuel, oil and lubricants (Diesel, mobil, petrol, kerosene etc.)	
3708	Laboratory supplies (Aluminium foil, bag, blade, brush, cap, container, film X-Ray etc.)	
3709	Housekeeping supplies (Aerosol, battery, wiping cloth, duster, lock and key etc.)	
3710	Janitorial supplies (Bleaching powder, brush, detol, detergent, insecticide, soap etc.)	
	Page total (balance c/f)	350

(Contd. to page No. 18)

Budget Code:_____

OTHER COST-1988

A/C Code	Accounts Description	$ Amount
3800	Repairs and maintenance (Maintenance and repairs of vehicles, equipments, furniture and building)	
3900	Rent, communication and utilities (Postage, telephone, telegram, electricity etc.)	
4100	Bank charges	
4200	Legal and professional expenses (Professional membership fee, legal fee, audit fee etc.)	
4300	Printing and publication (Printing of forms, books, journals, reprints etc.)	50
4400	Hospitality & donation (Guest house accommodation, donations, hospital food, lunch, refreshment etc.)	
4500	Service charges (Porter, labour, washing, laundry and other misc. exp.)	
4600	Staff development and training (Training course fee, training materials, stipend,scholarship, subsistence paid to the staff)	
	TOTAL	* 50

*AGREES WITH PAGE 1 A/C No.4000 COLUMN C

Budget Code: _____

SUPPLIES AND MATERIALS-1988

(Contd. from Page No. 17)

A/C Code	Item Description	$ Amount
	Page total from page No.17 (balance b/f)	350
3711	Tools and spares (Automobile spares, tyres, tubes, battery, stores required for maintenance services etc.)	
3712	Non-stock supplies (Materials not normally kept in stock and purchased only against specific requisitions)	100
	Sub-Total	450
3713	Freight and other charges Add 30% to above sub-total for imports.	200
	TOTAL	* 650

*AGREES WITH PAGE 1 A/C 3700 COLUMN C

Note: For rates please contact Supply Ext.260.
Add 10% for inflation

Budget Code: _____

**INTERDEPARTMENTAL SERVICES-1988

A/C Code	Service Area	$ Amount
4801	Computer	
4802	Transport Dhaka	50
4803	Transport Matlab	
4804	Water transport-Matlab	
4805	Transport Teknaf	
4806	Xerox and mimeograph	
4807	Pathology	
4808	Microbiology tests	
4809	Biochemistry	
4810	X-Ray	
4811	I.V. fluid	
4812	Media	200
4813	Patient hospitalisation study	
4814	Animal research	100
4815	Medical illustration	50
4817	Telex	
4818	Out patient care	
4819	Maintenance charges	
4820	Vehicle maintenance charges	
4821	Library service charges	
4822	Staff Clinic Charges - Dhaka	
	Page total (balance c/f)	400

(Contd. to page No. 21)

Budget Code: _____

**INTERDEPARTMENTAL SERVICES-1988

(Contd. from Page No. 20)

A/C Code	Service Area	$ Amount
	Page total from page # 20 (balance b/f)	400
4823	Staff Clinic Charges - Matlab	
4824	Bacteriology Test	
4830	Transport Subsidy	
	TOTAL	* 400

*AGREES WITH PAGE 1 A/C 4800 COLUMN C

** See Annexure-B for rates.