ttachment 1.

Date 2.4.82

ETHICAL REVIEW COMMITTEE, ICDDR,B.

rincipal Investigator Md. Zeaur Rahim

pplication No. 82-017

itle of Study Investigation of the extent of fecal pollution by enumeration of fecal coliforms & fecal streptococci.

Trainee Investigator (if any) X

Supporting Agency (if Non-ICDDR,B) X

Project status:
( ✓ ) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

rcle 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)
(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:
   (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)
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.

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

Zeaur Rahim
Principal Investigator

[redacted] 15 JUN 1998
Trainee

SECTION I - RESEARCH PROTOCOL

1. Title : Investigation of the extent of fecal pollution by enumeration of fecal coliforms and fecal streptococci.

(The work of this protocol will be submitted by the Principal Investigator as a dissertation for the partial fulfilment of M. Phil degree of the University of Dacca.)

2. a) Principal Investigator: Md. Zeaur Rahim, Research Fellow of ICDDR,B

b) Co-Investigators: K.M.S. Aziz, Md. Imdadul Huq, Md. Sirajul Islam

3. Starting date: 3rd May, 1982

4. Completion date: 30th April, 1983

5. Total Direct Cost: Tk. 85480.00

US $ 4274.00

6. Scientific Program Head:

This protocol has been approved by the D.T.W.G Working Group.

Signature of Scientific Program Head: Hamad

Date: 2/4/82

7. Abstract Summary:

Monthwise enumeration of fecal coliforms and fecal streptococci will be carried out from the selected study point to assess the seasonal quantitative variation of the indicator bacteria. Source of pollution will be determined by the ratio of fecal coliforms to fecal streptococci. Physicochemical parameters of the water bodies will be determined along with the enumeration of the indicator bacteria to determine whether the former possess any correlation wtih the latter.

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 main objective of this protocol is to:

   a) Isolate, enumerate and identify fecal coliforms (FC) and fecal streptococci (FS) from water body (two ponds and Buriganga river) to detect fecal pollution and assess the extent thereof.

   b) Ascertain the time of the year when the counts of fecal coliforms and fecal streptococci are high in the water and the relationship, if there is any, with seasonality of diarrhoeal diseases.

   c) Correlate the counts of coliforms and fecal streptococci with physicochemical factors.

### B. BACKGROUND:

A large number of enteropathogenic organisms can be found in polluted water (1). Usually it is not possible to examine a water sample for the presence or absence of all these organisms. Certain species of bacteria survive for a considerable period in the water and others die-out early and the water may be polluted continuously. At this situation scientists look for the presence or absence of special bacteria which are always present in the feces and whose normal habitat is the intestine of man and higher animals. Bacteria of this category is known as fecal indicator. One such bacterial group is fecal coliforms.

Four genera namely Escherichia, Klebsiella, Enterobacter and Citrobacter belonging to the family Enterobacteriaceae together constitute coliforms (2), which may be defined as aerobic, facultative anaerobic,

gram negative, nonspore-forming rods that can ferment lactose with formation of gas when incubated at 35±0.5°C or 44±0.5°C for 24 - 48 hours (3).

Water serves as the vehicle for the transmission of almost all the enteric pathogens. But the enteric pathogens can survive less in water than other fecal flora. So, the sanitary standard of a water body is judged by the absence or presence of fecal flora - mainly the coliforms. The coliforms are the normal inhabitants of intestine of man and other animals (1). Thus isolation of coliforms from a water source serves as the potential indicator of fecal pollution. Fecal pollution of water is dangerous, because that may act as vehicle of pathogens causing enteric diseases. Since large quantities of water in being used for drinking and other essential purposes in our daily life, we should be cautious about the quality of such essential material.

Feces get exposed to the nature after defecation. When sanitary latrine is the place for defecation, the excreta go to the safety tank. Then, due to leakage and/or over flow surface water get contaminated (4.5). Fecal contamination in these cases have been documented by the high count of fecal coliforms. There are a number of reports of epidemics (4) caused by the supply of contaminated ground water. In Comerio Puerto Rico ground water have been incriminated as the cause of Shigella epidemics (6). During the epidemic no Shigella was isolated from the water sample but the coliform count was very high. The sutdy revealed total and fecal coliforms were 4900/100 ml

and 23/100 ml of water respectively.

Study of coliforms can be implicated as the epidemic signal related to enteric diseases. In Rome, New York, in the years 1974 and 1975, there was an outbreak of giardiasis affecting 4800 - 5300 individuals (7). Rome's raw water showed higher coliforms count prior to the outbreak. Between January 17 and March 15, 1974 there was an approximately 1200 cases of acute gastrointestinal illnessess in Richmond Hieghts, Fla (8). In this case, epidemiologic investigation revealed that tap water consumption was associated with illness. Further it was found that one of the two wells providing water to the community had been continuously contaminated with excessive level of coliforms. The source of contamination was traced by dye studies to the septic tank of a church and a day care centre located approximately 46 m (150 ft.) away from the well. Thus the study of fecal coliforms can be utilised to trace the probable source of fecal contamination.

Enteric pathogens and indicators of fecal water pollution are subject to sedimentation after entering into a waterbody (9). These bacteria have been shown to persist in higher number in bottom sediments than the water column (10) and can persist for a considerable period of time (10, 11, 12). Since sands adsorb bacteria very loosely (13), most of the bacteria including fecal coliforms would probably be released into the water column during initial sediment disturbance caused

by the dredge cutter blade. After dredging sediment bound fecal coliforms are released into the water column due to which count of fecal coliforms is reduced in the soil sediment after dredging than that of the count before dredging.

There is a marked seasonality of diarrhoeal disease. The disease is usually common in summer months in Calcutta (India) and the early winter in Bangladesh (14). In the United States, the diarrhoeal epidemics are usually in the peak during summer months (4). In every geographical area of the world, the waterbody receive significant amount of fecal contamination at a particular season of the year, which is the main reason of the seasonality of diarrhoeal disease. During this period high fecal contamination can be perceived with higher count of fecal coliforms.

Coliforms survive for a lesser period than FS (15,16). Therefore only fecal coliforms are not sufficient to assess the hygienic status related to fecal pollution of any water body. For this reason along with fecal coliforms, study of fecal streptococci should be done. In addition to fecal pollution, the latter gives clue regarding recency (15,17) as well as remote fecal pollution (15,16).

Fecal streptococci are present in the feces of human and wormblooded animals (18), therefore their presence in water indicate fecal pollution with the feces of wormblooded animals. They can survive longer than coliforms and other bacterial pathogens (15,16,18), although some of the species

of fecal streptococci die off rapidly (15). FS cannot multiply in the nature or in the fecally polluted water. Therefore either they will die off or their number remian unchanged for a considerable period of time in the waterbody receiving fecal pollution (18).

Feces of livestock are distinguished by significant proportions of S. bovis and S. quinus (18). Higher numbers of these two organisms are associated with pollution from meat processing plants, during wastes and run-off from feedlost and farmlands (17). On the other hand, feces of human beings characterized by the presence of enterococci, but the exact speciation of enterococci from the source has not yet been ascertained accurately. S. faecalis subsp. liquefaciens constitute about 25 percent, S. faecalis of about 40 percent and S. duran constitute about 4 percent of the total streptococcal population (18). Little is known about the importance of the remaining 31 percent of the Streptococci in relation to fecal pollution indicator. Quantities of S. bovis and S. equinus in the feces of livestock are usually high (19). Percentage of these species together ranges from 18.9 percent for pigs to 66.2 percent for cattle (19). The remaining population is primary enterococci, of which 18 percent have been identified as S. faecalis subsp. liquefaciens.

Feces of human origin may be specified not only by the speciation but more simply by the determination of the ratio of the number of FC to FS (18). Human feces are characterized by higher number of coliforms than streptococci but animal feces contain higher number of streptococci than

the former. Therefore, the source (human or animal) of fecal contamination of a waterbody can be determined by the FC to FS ratio. When the ratio exceeds 4.0, it indicates contamination with human feces and pollution with animal feces are indicated when the ratio is less than 0.7 (17). Besides, speciation of streptococci provide more specific source.

There are several precautions to be adopted when ratios are considered (17), because the ratios may be altered due to several reasons as follows:

a) bacterial densities can be altered drastically when the pH of the sample is below 4 and above 9.

b) due to limited survival capability of some of the fecal streptococci it is essential to sample close to the pollution source to obtain reliable ratios. It is essentially true for the highly sensitive S. bovis and S. equinus.

c) it is difficult to use ratios effectively when mixed pollution sources are present.

The physicochemical characters of a water body may have direct bearing on the fecal bacterial flora in the water. In addition to pH, various other chemical constituents of the water may also affect their servival and multiplication.

3. Rationale: The rationale underlying this research is to ascertain the time of the year exhibting high fecal contamination of the water of Buriganga river and two other ponds under investigation. The

study will also elucidate if there exists any correlation between the counts of fecal coliforms and streptococci and the physicochemical characters of the water bodies.

B. SPECIFIC AIM:

The specific aim of this research is:

1. To count fecal coliforms and fecal streptococci from the soil sediments and water columns collected from three points of the Buriganga river and two other ponds under investigation.

2. To correlate the counts of the above bacteria with the seasonality of diarrhoeal diseases.

3. Correlate the physicochemical factors with the counts of the above bacteria.

4. To identify the isolated strains of fecal coliforms and fecal streptococci upto the species level.

C. METHODS OF PROCEDURE:

1. Sampling points: Samples for bacteriological analysis will be collected from the Buriganga river and two ponds of village Chunkutipara, which is situated on the bank of the river Buriganga. From the Buriganga river samples will be collected from three points viz; Sadarghat steamer station, Babubazar ghat and Farashganj ghat.

2. Kinds of samples: Two kinds of samples namely, water and soil sediments will be collected from the sampling sites.

3. Sampling procedure: Water samples will be collected from the depth of 1 meter by Hach dissolved oxygen sampler and taken into a sterile

4 oz glass bottle for bacteriological analysis and those for chemical analysis will be collected following the sample procedure into NELGENE plastic bottles. Soil sediments will be collected with the help of ICDDR,B constructed core sampler and taken into a sterile 4 oz glass bottle. From each sampling point water and soil sediments will be collected 5-6 yards away from the point of the bank of the river touching water. Then the samples will be carried from the field to the laboratory in an insulating foam box provided with ice bags.

ANALYSIS OF THE SAMPLE:

Chemical analysis: Using HACH Machine Model DR-E1/2 only the water samples will be analysed for the detection of acidity, alkalinity, carbondioxide, chlorine, chloride, copper, hardness (calcium, magnesium), ammonia nitrogen, nitrate nitrogen, nitrite nitrogen, inorganic phosphate, organic phosphate, sulfate and pH. Turbidity will be detected by sacchi disc, dissolved oxygen by portable oxygen meter and pH by corning pH meter (model 7).

Bacteriological analysis of the sample: Bacteriological analysis will be carried out for the isolation and identification of total coliforms, fecal coliforms and fecal streptococci. All the collected samples will be processed for the quantitative as well as qualitative analysis of the above mentioned bacteria within four to six hours of collection.

Quantitative bacteriological analysis for total coliformsm fecal coliforms and fecal streptococci: Quantitative bacteriological analysis will be carried out by Standard Most Probable Number (S-MPN), Millipore Filtration technique (MFT) and spread plate method, following standard methods (3).

Qualitative isolation and identification:

a) Fecal coliform: After incubation , colonies of fecal coliforms will be blue to black with metellic sheen on mFC agar plate as well as on the membrane filters, placed on sterile pads soaked with mFC broth. Morphologically different typical fecal coliform colonies will be picked and then for pure culture they will be streaked on Eosin Methylene Blue agar plate before studying further biochemical properties for identification. Fecal coliforms will be identified by using Kligler Iron Agar (KIA) slant, the IMViC (indole, methyl red, voges proskaur, citrate) test, lysine and ornithine decarboxylase broth, arginine dihydrogenase broth, malonate, rhamnose and sorbitol fermentation.

b) Total coliform: After incubation colonies of total coliform will be red to pink with metallic sheen on mFC agar plate as well as on membrane filters, placed on sterile pad soaked with mFC broth. After incubation morphologically dissimilar typical total coliform colonies will be picked up from each plate and then they will be purified and identified.

c) Fecal streptococci: After incubation colonies of fecal streptococci will be dark red to pink on KF streptococcus agar plate depending on species. Typical dissimilar colonies will be picked up from each plate

and the following procedure will be carried out for identifying different species of fecal streptococci.

Identification of streptococci:

a) After incubation, from the membrane filters and spread plates typical fecal streptococcal colonies will be picked up and then inoculated into brain heart infusion (BHI) agar slant and BHI broth which would be incubated at 35°C for 24 hours.

b) After 24-48 hours of incubation at 35±0.5°C in BHI agar slant, catalase test will be done.

Catalase test:

A loopful of bacteria from the BHI agar slant,will be transferred to a clean glass slide and a few drops of freshly prepared 3% hydrogen peroxide will be added to the smear. Visible oxygen as buble will indicate the presence of catalase enzyme. Positive catalase test will indicate non streptococcal species.

Identification of Fecal Streptococci to species:

This identification to species is performed using the additional biochemical tests described to differentiate and confirm the group Q streptococci, the bovis - equinus group and the enterococci. The entero-

cocci can be separated as S. faecium and S. faecalis varieties or into groups according to original source.

Speciation in brief:

Group Q streptococci and enterococci are separated from the bovis equinus groups by positive growth at 10 and 45°C and are then varified by growth in 6.5% Nacl and at pH 9'6. The enterococci are separated from the group Q streptococci by reduction of methylene blue in milk. Subsequently the enterococci are either speciated or separated by origin using additional biochemical. The Streptococcus bovis and S. equinus are verified by hydrolysis of strach and separated by lactose fermentation. The detail procedures are described as follows:

Separation of Enterococci and group Q Streptococci:

From the BHI broth culture 1 drop of growth will be transferred to each of the two BHI broth tubes.

One tube will be placed in a 45±0.5°C water bath and the growth will be observed (as evidenced by turbidity) within two days. The other tube will be placed in a 10±0.5°C water bath and check for growth within 5 days. Growth at 10 and 45°C indicates that the culture is a potential member of the enterococcus or Q groups. On the other hand, S. equinus and S. bovis exhibit growth at 45°C but not at 10°C.

Confirmation of Enterococcus group:

Confirmation of enterococci will be achieved by testing their growth in 6·5% Nacl and at BHI broth having pH 9·6 and observing for reduction of 0·1% methylene blue in milk. Positive reaction in all cases confirms the presence of the enterococcus group. Positive reaction in 6·5% Nacl and pH 9·6 BHI broth and no reaction in 0.1% methylene blue indicate the tentative identification of group Q.

Separation of Enterococcus group by species:

The enterococcus group can be separated into species by observing the reduction of potassium tellurite and 2,3,5 triphenyl tetrazolium chloride and the fermentation of D - sorbitol and glycerol (Fig. 3).

Identification of group Q Streptococci:

The group Q streptococci are initially separated from the Enterococcus group and tentatively identified by growth at 6.5% Nacl in BHI broth and BHI broth at pH 9.6 but no growth in 0.1% methylene blue in milk ( as shown in Fig. 2).

The detail method of speciation of fecal streptococci will be shown in Fig. 1,2,3.

Correlation of fecal coliform count with seasonality of diarrhoea:

The monthwise faecal coliform counts may be correlated with the monthly incidence of diarrhoea cases in Dacca, data of which are available at ICDDR,B.

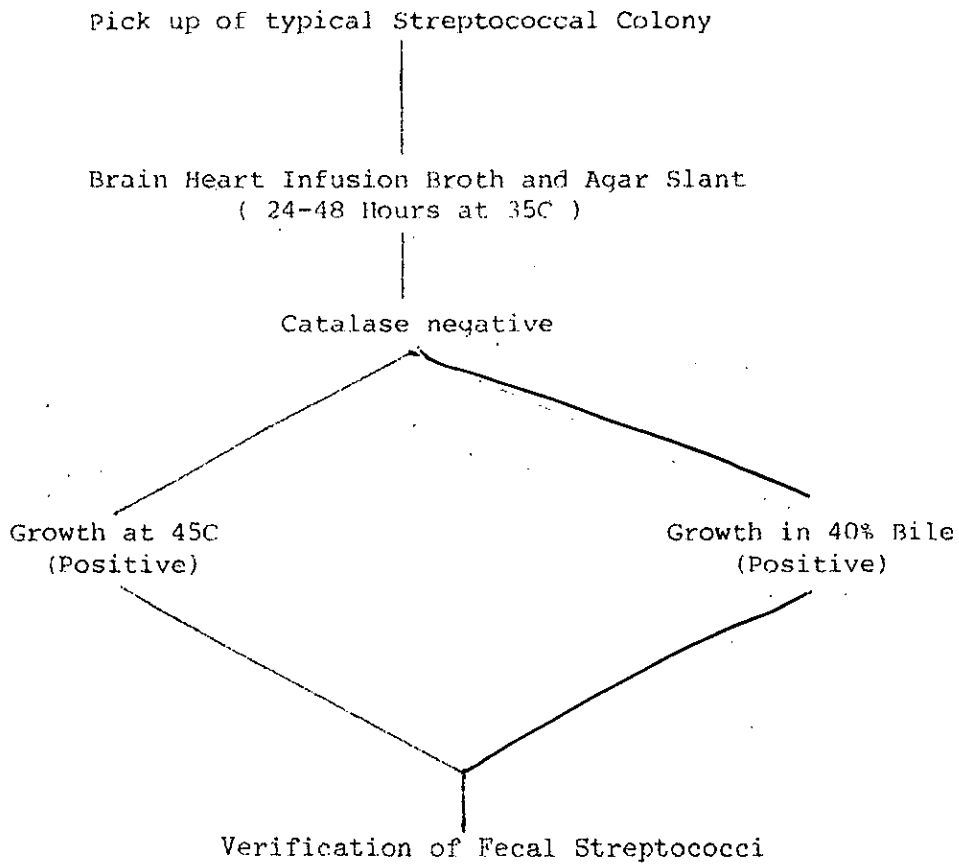


Figure 1 : Verification Procedure for Fecal Streptococci

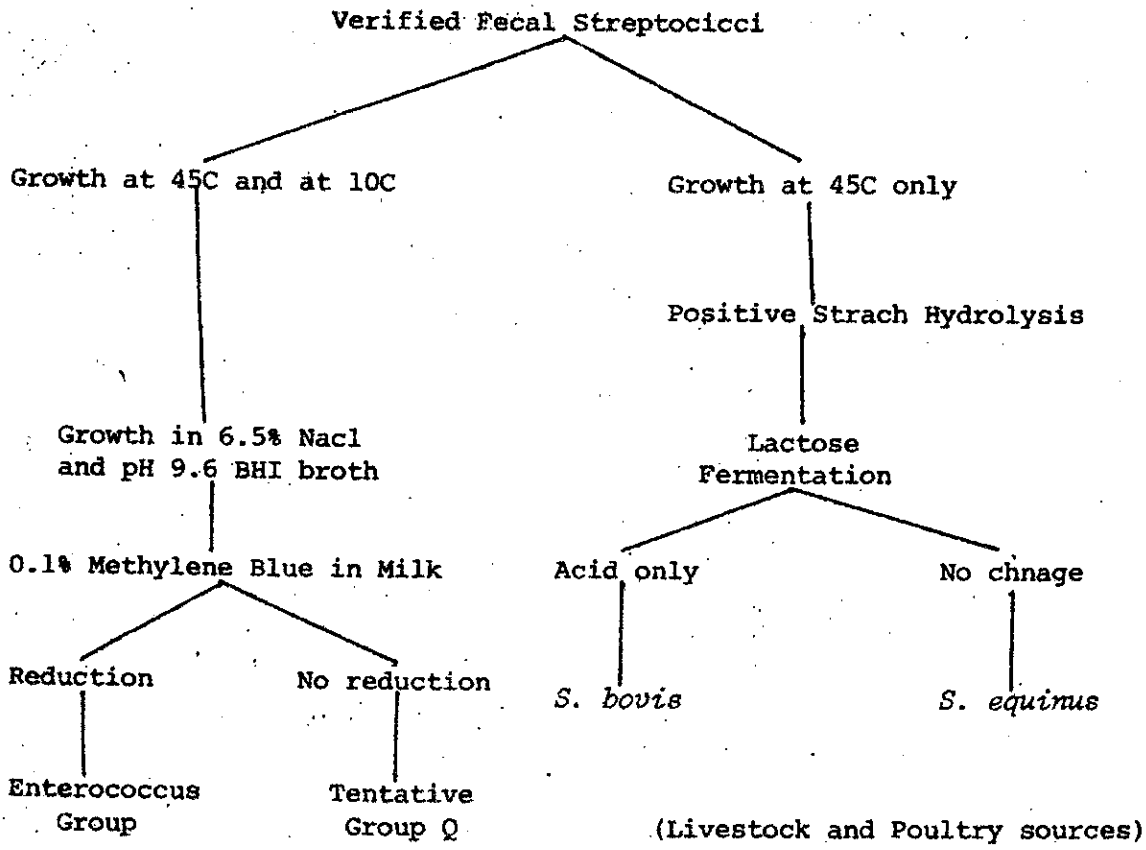


Figure 2: Identification of Fecal Streptococci, General procedure.

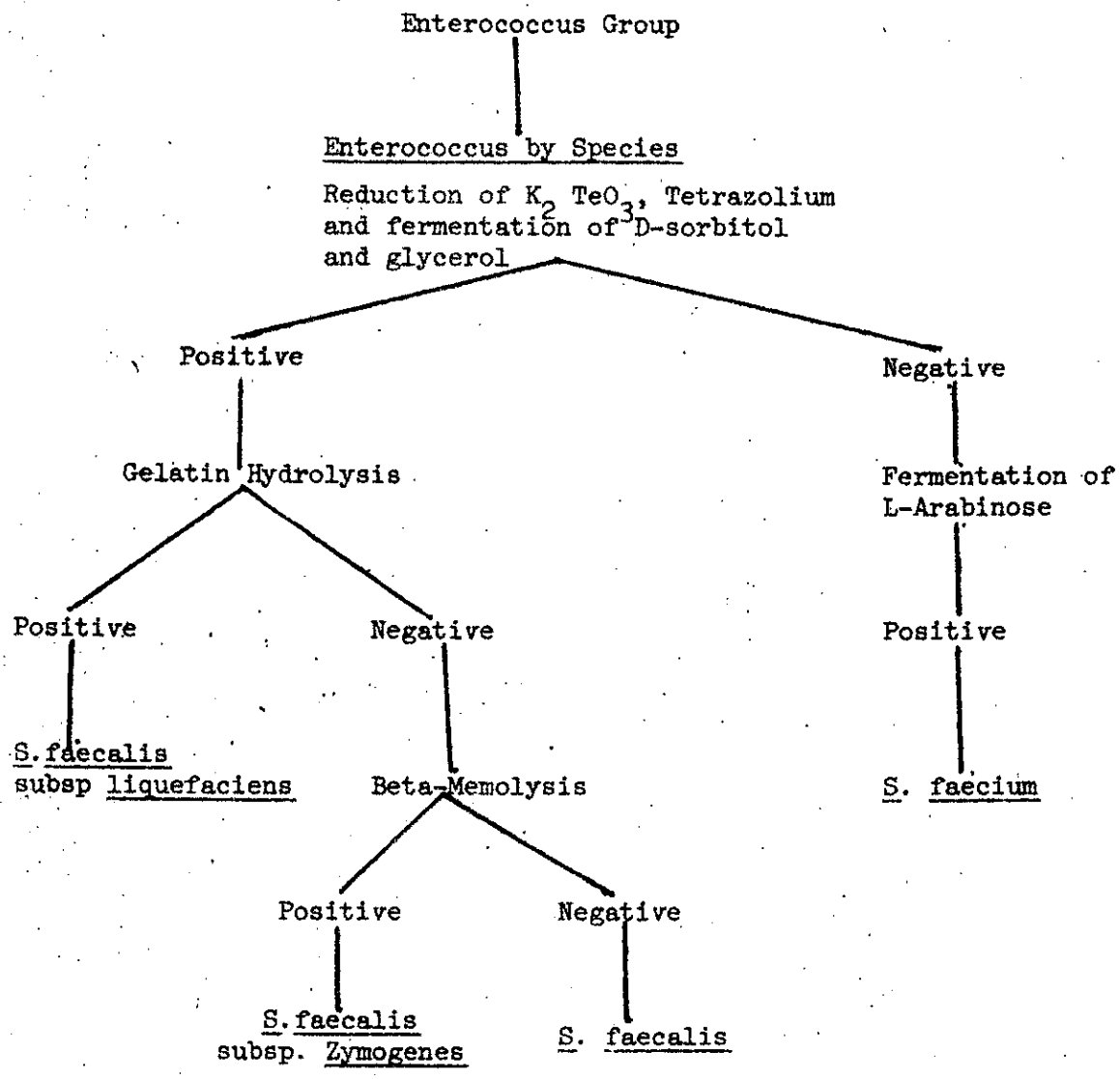


Figure 3: Identification of Fecal Streptococci: Separation of Enterococcus Group by Species and by Original source of culture.

## ABSTRACT SUMMARY

From the collected water and sediment sample of the selected study points, enumeration of fecal coliforms and fecal streptococci will be carried out monthwise throughout the year to find out the existence of relationship with seasonality of diarrhoeal disease in Dacca. Attempts will also be made to correlate the physicochemical factors of the water bodies with the counts of above bacteria.

1. Subject population : This study does not involve any population.
2. Risks : Not applicable
3. Protection against risk : Not applicable
4. Confidentiality : Not applicable
5. Privacy : Not applicable
6. Interview : Not applicable
7. Benefit : Not applicable
8. Use of hospital records : Hospital records for seasonal incidence of diarrhoea during the study period will be noted.

## SECTION III - BUDGET

### A. DETAILED BUDGET

1. PERSONALS SERVICES:

| Name | Position | % or No. of day | Annual Salary | Budget requirement Takas | Dollars |
|---|---|---|---|---|---|
| Md. Zeaur Rahim | Principal Investigator | 100% | 24480 | | |
| Dr.K.M.S. Aziz | Associate Director | - | - | - | - |
| Dr. M. I. Huq | Head, Microbiology Br. | - | - | - | - |
| Md.Sirajul Islam | Sr. Res. Officer | - | - | - | - |
| Md. Ashraf Ali | Lab. Attendent | 15% | - | 160.00 | |

2. SUPPLIES AND MATERIALS:

| | Amount required | Cost Unit | Project requirement Takas | Dollars |
|---|---|---|---|---|
| Media and Chemicals | | | 26,000 | |
| Millipore Filters | | | | 750 |
| Hach Cehmicals | | | 14,000 | |

3. EQUIPMENT: None

4. PATIENT HOSPITALIZATION: None

5. OUT PATIENT CARE: Nil

6. ICDDR,B TRANSPORT: 4.00/mile 1000 — 4,000

7. TRAVEL AND TRANSPORTATION OF PERSONS: Nil

8. TRANSPORTATION OF THINGS: Nil

9. RENT, COMMUNICATION AND UTENCILS: Nil

10. PRINTING AND REPRODUCTION: 2,000

11. OTHER CONTRACTUAL SERVICES: Nil

12. CONSTRUCTION, RENOVATION, ALTERATION ETC: Nil

## BUDGET SUMMARY

| Category | Year - 1 | |
|---|---|---|
| | Takas | Dollars |
| 1. Personnel | 24480 | |
| 2. Supplies | 55000 | |
| 3. Equipments | - | |
| 4. Transport | 4000 | |
| 5. Printing | 2000 | |
| Total = | 85480 | |

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