Attachment 1.

Date Jan. 25, 1988. 28/1/88

ETHICAL REVIEW COMMITTEE, ICDDR,B.

Principal Investigator Md. Zeaur Rahim Trainee Investigator (if any) X

Application No. 88-003 Supporting Agency (if Non-ICDDR,B) _____

Title of Study Formulation and evaluation of improved isolation media for Aeromonas and Plesiomonas.

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

Circle the appropriate answer to each of the following (If Not Applicable write NA).

1. Source of Population:
   (a) Ill subjects 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:
   (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 N.A
   (b) From parent or guardian (if subjects are minors) Yes No N.A
6. Will precautions be taken to protect anonymity of subjects Yes No N.A
7. Check documents being submitted herewith to Committee: N.A.
   ___ 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. N.A
2. Examples of the type of specific questions to be asked in the sensitive areas. N.A
3. An indication as to when the questionnaire will be presented to the Cttee. for review. N.A

(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.

Zeaur Rahim
Principal Investigator

Trainee

88-503
28/11/88

SECTION - I : RESEARCH PROTOCOL

1. Title : Formulation and evaluation of improved isolation media for Aeromonas and Plesiomonas.

2. Principal Investigator : Md. Zeaur Rahim

   Consultants : Dr. Bradford A. Kay
   Dr. Anwarul Huq

3. Starting date : March 01, 1988

4. Completion date : February 28, 1989

5. Total direct cost : US$ 18,070.00

6. Scientific Program Head :

This protocol has been approved by the Laboratory Sciences Division

Signature of Scientific Program Head : [signature]

Date : Jan 25 1988

7. Abstract Summary :

Various approaches will be made to formulate selective media for isolation of Aeromonas and Plesiomonas from clinical and environmental sources. Several clinical as well as environmental samples will be processed for testing suitability of these media. Performance of the new media will be compared with those used now-a-days. If the test results are statistically significant, the new media will be judged promising. Various enrichment technical for isolation of Aeromonas and Plesiomonas from clinical specimen will also be tried.

8. Reviews :

a. Ethical Review Committee : _____

b. Research Review Committee : _____

c. Director's signature and remark, if any : _____

SECTION - II : RESEARCH PLAN

A. INTRODUCTION

1. Objective:

a. to find out an ideal concentration of bacto-peptone, sodium chloride, sodium taurocholate, glycogen, DL-ornithine, pH indicator suitable for selective growth of Aeromonas.

b. to test the suitability of the new medium for isolation of Aeromonas from clinical and environmental sources.

c. to evaluate the activity of the new medium relative to existing ones.

d. to find out an ideal concentration of bacto-peptone, sodium chloride, sodium taurocholate, specific pH indicator, and fermentable carbohydrate suitable for selective growth of Plesiomonas.

e. to test suitability of this medium for isolation of Plesiomonas from environmental and clinical sources.

f. to evaluate the activity of new Plesiomonas medium relative to exhisting ones.

2. Background :

Aeromonas spp. have gained considerable importance after it was known to cause life-threatening diseases in humans; like septicemia, meningitis, endocarditis, peritonitis etc. Earlier, this bacterium was considered as the opportunistic pathogen in the immuno-compromised host, but currently it is

being often isolated from immunologically normal patients, specially from children with diarrhoea. A. hydrophila was first isolated from specimens of diarrhoeal stools as a suspected pathogen in 1961 (1). In 1964, Rosner isolated this bacterium from stool of a diarrhoeal patient and proved its enteropathogenicity from immunological point of view (2). The isolation rate of Aeromonas from diarrhoeal stools has been found to differ in different geographical settings. Among different age groups, incidence was notably higher in children (3). Aeromonas induced diarrhoeal incidence shows distinct seasonality with highest incidence in summer months and lowest in the winter months (3).

For isolation of Aeromonas from clinical and environmental sources, several media have been formulated time to time. The first medium for Aeromonas was developed in 1946 by Gutsell and Snieszko for isolation of A. salmonicida (4). This was a very simple medium containing tryptic digest of caesin, yeast extract and sodium chloride. This medium was devoid of inhibitory agent and its selectivity was not so prominent. Eddy in the year 1960 formulated another selective medium containing peptone, 5% oxalated blood and 30% dialized skim milk (5). After inoculation, this medium was usually incubated at 30 °C for 48h. Aeromonad colonies appear caesinolytic and haemolytic on this medium (5). All aeromonad colonies are not haemolytic, therefore isolation of non-haemolytic aeromonad colonies may have some problems if used this medium for isolation.

Shotts and Rimler formulated another selective medium called RS (6). This medium contained sodium deoxycholate and novobiocine as inhibitory agent and maltose as ferentable carbohydrate. Aeromonas produced yellow colony on this green colored medium. 94% presumptive identification of Aeromonas was possible if this medium was used. Using RS medium, several researches have been conducted in the past by Chen et al. 1975 (7); Kaper et al. 1979 (8); and Fliermans et al. 1977 in USA (9). However, non-selectivity of RS medium for isolation of Aeromonas from estuarine environments was reported by Davis and Sizemore in 1981 (10). After this report, use of RS medium became very limited.

McCoy and Pilcher formulated another selective and differential agar medium for isolation of Aeromonas (11). This medium contained sodium lauryl salfate as inhibitory agent, glycogen as fermentable sugar and bromothymol blue as pH indicator. Other than Aeromonas, some member of Vibrionaceae and Enterobacteriaceae produced yellow colonies on this medium. As a result, practical use of this medium was not so satisfactory. Moreover, this medium was used as pour plate having 2% agar layed over the inoculated plate. Thus it lost popularity to work in the field as well as in routine work.

In the year 1979, Rippey and Cabelli formulated another new medium for enumeration of Aeromonas from natural water samples (12). This medium contained trehalose as fermentable carbohydrate and ethanol and ampicillin as selective inhibitor. In the same year, Rogol et al. formulated another medium

containing xylose as fermentable carbohydrate and ampicillin as selective inhibitor (13). These two medium were developed with an idea that all Aeromonas strains are resistant to ampicillin. Since several ampicillin sensitive strains are coming up now a days (14). These medium would adversely affect their isolation.

For isolation of Aeromonas from stool, papers were separately published by Kay et al. (15) and Robinson et al. (16) in the year 1985. Basically these two media were simple blood agar plate containing 10 mcg/ml ampicillin. Resistance to ampicillin and production of prominent haemolytic zone around the colony on blood agar plate, were the two important characters exploited while developing these media. All Aeromonad colonies are not haemolytic, as a result several non-haemolytic colonies would be undetected unless cytochrome oxidase test is done. Moreover, some haemolytic E. coli colonies are also confused with haemolytic Aeromonas colonies. Besides, ampicillin 10 mcg/ml of medium is not sufficient to inhibit other Enterobacteriaceae. Hence the stool inoculated plates after incubation shows profuse enteric bacteria. As a result, each and every colony grown in the medium needs to be screened for cytochrome oxidase irrespective of haemolytic activity. These problems make reading of blood agar ampicillin medium time consuming and not good for use in routine work.

Palumbo et al. 1985 published another selective medium for quantitative detection of Aeromonas (17). Ampicillin resistance and starch hydrolysing ability of Aeromonas were utilised to develop this medium. Practical use of such medium is not convenient because after overnight incubation, inoculated plates are flooded with Lugol's iodine solution to detect starch hydrolysing colonies which were provisionally taken as Aeromonas. Subsequently such colonies are confirmed as Aeromonas following test of cytochrome oxidase and conventional biochemical reactions. Addition of iodine on the plate for in situ test of starch hydrolysis may contaminate the plates. Thus, steps followed for enumeration of Aeromonas from such plates were not convenient for practical use in routine work.

Considering all these problems discussed above, and following basic principles of fermentation of carbohydrate, decarboxylation of amino acids and inhibition of Enterobacteriaceae by chemical agent, formulation of a nutrient mixture will be tried having bacto-peptone, sodium taurocholate, sodium chloride, DL-ornithine, glycogen, bromothymol blue and bacto-agar.

Enrichment for Aeromonas :

Most of the previous workers followed direct plating for isolation of Aeromonas (18). In the recent years, von Graevenitz and Bucher (1983) used alkaline peptone water (APW) and trypticase soy broth with ampicillin (TSB, 30 mg/l) (19). They found better results in APW than TSB. Millership and Chattopadhaya (1984) attempted to isolate Aeromonas and Plesiomonas from human faeces through direct plating as well as through enrichmept in APW (20). They found improved isolation

rate enriching stool specimens in APW. Further attempts will be made to find out better enrichment media.

Plesiomonas shigelloides has been isolated from stools of patients with diarrhoea (18) and assumed its association with diarrhoea, but there is no direct evidence of causing diarrhoea by this organism. Plesiomonas can grow well on all the enteric media like; Salmonella-Shigella agar (SS), modified SS agar (21), xylose lysine deoxycholate agar. It's recovery is poor on these media when in mixed culture. Thus attempts were made to develop selective medium for Plesiomonas. Inositol brilliantgreen bilesalt (IBB,22) and Plesiomonas differential agar (23) are the two such media. These two media (PDA, IBB) contains bilesalt as inhibitory agent and inositol as the only differential fermentable carbohydrate. Of these two media, performance of IBB has been proved better than PDA in a recent publication (24). On IBB, test for cytochrome oxidase is delayed, and hence false negative results appear. Besides, both PDA and IBB are not autoclaveable and cannot be stored beyond 96h. For avoiding all these problems, formulation of a new selective medium for Plesiomonas will be tried in the protocol. The new medium will contain bacto-peptone, Sodium chloride, sodium taurocholate and pH indicator (neutral red and brilliant green). Fermentable carbohydrate will be either lactose, inositol, or xylose etc. and will be tried to select one that gives better result. In the medium, the quantities of each of the ingredients will be same as other enteric media. The new medium should be autoclaveable and could be stored at 4 ^{o}C at least for two weeks.

Enrichment for Plesiomonas :

In addition to direct plating, several enrichment media for Plesiomonas isolation were formulated. Cooper and Brown examined the utility of gram negative (GN) broth and Rappaport broth as enrichment media for Plesiomonas (25). They reported that GN broth was a better enrichment medium for this organism than Rappaport broth (25). Nair and Millership (26) formulated a Plesiomonas enrichment medium called bile salt brilliant green (BSBG) broth. They compared this enrichment medium with alkaline peptone water (APW) while working with two hundred nineteen faecal specimens examined parallel. They recovered P. shigelloides from only one BSBG enriched specimen. APW failed to allow recovery of Plesiomonas from the same specimen. Nair and Millership then artificially seeded normal stools with Plesiomonas and enriched in BSBG broth containing various concentrations of brilliant green. They reported that BSBG containing 0.001g/l brilliant green was a superior enrichment broth for the isolaion of Plesiomonas than was APW.

In a recent study, we evaluated the use of a single enrichment broth effective for the isolation of both Plesiomonas and other Vibrionaceae. We selected bile peptone broth (BPB) and alkaline peptone water (APW) for comparison. In this experiment we found that BPB as enrichment is superior than APW (unpublished Data).

3. Rationale :

There are few media for isolation of Aeromonas and Plesiomonas from clinical and environmental sources. Results of preliminary work (unpublished data) suggests that these media needs further

improvement. Thus this protocol has been designed for formulation of new selective medium for Aeromonas and Plesiomonas.

B. SPECIFIC AIMS

1. To develop a new selective medium for isolation of Aeromonas from clinical and environmental sources.

2. To evaluate the performance of the new Aeromonas medium relative to exhisting ones.

3. To develop a new selective medium for isolation of Plesiomonas shigelloides from clinical and environmental sources.

4. To evaluate the performance of new Plesiomonas medium relative to exhisting ones.

C. Methods of Procedure

a. Aeromonas Medium

Quantity of ingredients in the new Aeromonas medium:

At present blood agar ampicillin medium (15,16) is used in our laboratory for isolating Aeromonas from diarrhoeal stool. Resistance to ampicillin and production of prominent haemolytic zone around the colony on the blood agar ampicillin plate were the two most important characteristics exploited for developing these medium. All Aeromonad colonies were not haemolytic, as a result, several non-haemolytic colonies would be undetected cytochrome oxidase test is done. More over some haemolytic E. coli colonies are confused with haemolytic Aeromonas. Besides, ampicillin 10 mcg/ml of medium is not sufficient to inhibit other

Enterobacteriaceae. Hence stool inoculated plates after incubation shows profuse enteric bacteria. As a result, each and every colony grown on the medium needs to be tested for cytochrome oxidase irrespective of their haemolytic activity. Proteus on this medium is highly motile and covers the whole plate after overnight incubation. These makes reading of blood agar ampicillin medium time consuming and not suitable for routine work. Using this medium isolation rate of Aeromonas is 33% from diarrhoeal. The new medium has been designed with an idea of having better performance relative to Blood agar ampicillin. The new medium should have the following ingredients :

The new Aeromonas medium should have the following ingredients:

Bacto - peptone

Sodium chloride

Sodium taurocholate

Glycogen

DL-Ornithine

Bromothymol blue

Bacto-agar

The quantity of bacto-peptone, sodium chloride, sodium taurocholate and bromothymol blue will be determined accordingly these are used in other enteric media like; MacConkey's agar, Salmonella-Shigella (SS) agar, taurocholate tellurite gelatin agar (Monsur's medium, 27) and TCBS agar. Quantity of DL-ornithine and glycogen will vary from 0.5 to 2%. The exact quantity will be determined by trial and error

method. In this medium Aeromonas would produce yellow colony. Most of the Enterobacteriaceae do not grow on it. Those grow on it would produce tiny either green or blue colony. This medium would be a solid medium which could be sterilized by normal autoclaving (at 121 °c for 15 min at 15 lbs pressure/sq. inch). The detailed procedure has been summerised in the attached flow chart No. 1 (Development of a new medium for Aeromonas).

Results of preliminary work indicate that this medium is promising. The detailed protocol has been written to do a systematic study for judging suitability of this medium for processing clinical and environmental samples. The superiority of this medium will be tested through following procedures:

1. Growth inhibition of pure culture of coliforms: Ten pure culture each of E. coli, Klebsiella spp., and Citrobacter spp. will be grown overnight into BHI broth and serially diluted into normal saline. 0.05ml culture from appropriate dilution will be plated on BHI (brain heart infusion) agar and new Aeromonas medium following spread plate method of inoculation. After overnight incubation of each plate at 37 °c, difference of counts on BHI and new medium will indicate the extent of inhibition of coliforms on the new medium. On this medium, three fold inhibitioin of coliforms is desired. Each experiment will be repeated thrice to reduce the technical error. Procedure as described by Rippey and Cabelli will be followed in this experiment.

2. Comparative growth inhibition of Aeromonas on the new medium:

Comparative counts of 25 Aeromonas spp. (each of A. hydrophila, A. sobria and A. caviae) isolated from clinical and environmental sources will be studied on BHI agar and new Aeromonas medium. Plating method will be same as above experiment. Counts higher in BHI and lower in new medium will indicate that the latter medium is inhibitory for Aeromonas. Any difference of counts in these two media (BHI and new Aeromonas medium) will be tested statistically (Chi squire test). Each experiment will be repeated thrice to reduce any technical error.

3: Description of colonial morphology of other enteric bacteria on the new Aeromonas medium:

Ten strains each of A. hydrophila, A. sobria, A. caviae, V. cholerae 01, V. cholerae non 01, V. mimicus, V. fluvialis, V. furnisii, V. plagicus, V. parahaemolyticus, E. coli, Klebsiella, Enterobacter, Citrobacter, Proteus, Pseudomonas and Acinetobacter isolated from clinical and environmental sources will be streaked on the new medium. After overnight incubation at 37 °c, colonial morphology (shape, size, color, surface of the colony) of each species will be tebulated.

4. Comparative detection ability of Aeromonas by the new Aeromonas medium relative to other enteric Media:

Stool from normal individual with known coliform counts will be diluted 1:10 and divide into several portions. One portion will be used as control and add test strains (one strain each of A. hydrophila, A. sobria and A. caviae isolated from clinical and environmental sources) to others in various ratios to coliform counts. From each dilution, 0.05ml specimen will be plated on to

MacConkey's agar, SS agar, blood agar ampicillin and new Aeromonas medium. After overnight incubation of each plate at 37°c, rate of recovery of Aeromonas in each plating medium at the highest coliform counts will be noted. Medium that helps Aeromonas recovery at the highest coliform counts will be considered as the best. Each experiment will be repeated thrice to reduce the technical error.

5. Growth inhibition of Vibrionaceae:

Five strains each of V. cholerae 01 (available serotypes and biotypes), V. cholerae non 01, V. mimicus, V. fluvialis, V. furnisii, V. plagious and V. parahaemolyticus isolated from clinical and environmental sources will be used in this experiment. Overnight culture of each strain in BHI broth will be serially diluted in sterile normal saline and 0.05ml culture from appropriate dilution will be plated simultaneously on BHI agar and new Aeromonas medium. After overnight incubation, difference of counts of each species on these two media (BHI agar and new Aeromonas medium) will give an idea of extent of inhibition by the new medium relative to non-inhibitory BHI agar. Each experiment will be repeated thrice to reduce the technical error.

6. Comparative isolation of Aeromonas from diarrhoeal stool:

250 stool or rectal swab specimens collected from diarrhoeal patients and submitted in the Diagnostic Microbiology for culture will be used in this experiment. Each of the specimens will be plated on to new Aeromonas medium, blood agar ampicillin, MacConkey's agar and SS agar plate and incubated at 37°c for 24h.

After overnight incubation, typical Aeromonad colonies will be picked up and identified by conventional biochemical reaction (Popoff 1984). Isolation rate on the new medium will be compared with other media to be used in this experiment.

The above steps has been summerised in the attached chart No. II.

Enrichment technic for isolation of Aeromonas from the stool:

One strain each of A. hydrophila, A. sobria and A. caviae isolated from the diarrhoeal stools will be used in this experiment. The test strain will be grown overnight at 37°c in nutrient broth and 10-fold dilutions will be made for counts on BHI agar. Test stool specimens would be diluted 1:10 and emulsified in sterile normal saline for coliform counts on mFc agar. To evaluate the growth of small inoculum in to the test enrichment broth, 0:05ml of ten fold dilutions of test organism will be added to three 1 ml aliquots of each test enrichment broths [bile peptone (pH 8.9) and alkaline peptone (pH 8.9) water with and without ampicillin (10 mcg/ml)] and incubated at 37°c for 24h. Tubes showing growth would be subcultured to check for purity. An enrichment broth would be considered to be satisfactory if growth occurred in at least two of the three tubes to which 1-10 colony forming units/ml had been added. Each experiment will be repeated thrice to reduce technical error. Selectivity of an enrichment broth will be examined by inoculating 0.05 ml of 10 fold dilution of each test strain into 1 ml aliquot of well-mixed fecal suspension, and plated out 0.05ml on MacConkey's agar before adding test enrichment broth. This will give initial counts of fecal flora.

Afterwards all inoculated test broths containing 10 ml of enrichment broth will be incubated at 37°c for 24h. A 0.05 ml culture from the enrichment broth will be plated on the new medium following spread plate method of inoculation. From each plate both Aeromonas and fecal flora will be counted to calculate the maximum initial counts of fecal flora permiting the growth of at least one Aeromonas colony using particular enrichment medium. The detailed procedure will be same as described by Nair and Millership 1987 (24).

The enrichment steps has been summerised in chart No. III (Aeromonas enrichment media).

Elevated temperature enrichment for Aeromonas :

Another set of enrichment broths (APW, APW + 10 mcg/ml ampicillin, BPB, and BPB + 10 mcg/ml ampicillin) will be prepared as above and incubated at 44°C for 24 h. After overnight incubation, the method of inoculation will be same as above. This experiment will give an idea whether enrichment broth incubated at higher temperature helps better isolation.

Field Study:

100 rectal swab specimens collected from the diarrhoeal patients will be inoculated parallel into four enrichment broths mentioned above and incubated overnight at 37°c. After incubation, one loopful of culture will be streaked on to new Aeromonas medium for isolation and identification of Aeromonas. The enrichment broth which gives higher number of isolation will be considered as the best.

Processing of Environmental Samples Using New Medium:

The following steps have been summerised in Chart IV (Study of environmental specimens).

Stress recovery:

One strain each of A. hydrophila, A. sobria, and A. cavine isolated from clinical and environmental sources will be used in this experiment. Overnight culture of each test Aeromonas strains in BHI broth will be serially diluted in normal saline and stored overnight at -20 °c. After defrost, 0.05ml culture from each dilution will be plated simultaneously on BHI agar plate and new Aeromonas medium. Difference of counts of test strains on BHI agar and new Aeromonas medium will give an idea of extent of inhibition of cold shocked Aeromonas by the inhibitory chemicals added in the new medium. The methodology for doing this experiment will be same as described by Rippey and Cabelli (12).

Quantitative morphological confirmation of Aeromonas colonies on the new medium relative to other media:

Six environmental samples (water, soil sediment and plankton samples from the Buriganga river, water from the container used to carry fish in the local market, muscle of fresh water bivalve shell available in the Buriganga river and the root of water hyacinth) will be used in this experiment. Appropriate dilution of each environmental sample (root of water hyacinth, and the muscle of bivalve shell will be crushed in electrical blender (30) and serially diluted in normal saline in w/v) will be spreaded with the help of sterile bent glass rod on Rippey and Cabelli medium (12), peptone beef-extract glycogen

agar (11), Rimler and Shott's (R) medium (17) and incubated overnight at 37°c. After overnight incubation, 10-15 typical Aeromonad colonies from each medium will be picked up and identified as Aeromonas using Kaper's medium (28). Recovery percentage on the new medium will be compared with other media.

$$\text{Recovery \%: } \frac{\text{Presumptive counts on the basis of colony morphology}}{\text{Confirmed identification by Kaper's medium}} \times 100$$

Quantitative morphological confirmation of Aeromonas on the new Aeromonas medium by the membrane filtration technic:

Ten fresh water samples collected from different sources of Dhaka city (the Buriganga river, different ponds, Dhanmondi lake, and Crescent lake) and ten brackish water samples collected from Matlab and Teknaf will be used in this experiment. Different quantities of test water sample (to be diluted when required) from different sources will be passed through membrane filter (29, pore size, 0.45 um; diameter, 47mm) and to be placed on media like; Rippey and Cabelli (12), peptone beef-extract glycogen (11), RS(17) along with the new one. All plating media will be incubated at 37°c for 24h. After overnight incubation, 10-15 typical Aeromonad colonies (as described in case of each media) will be picked up and identified by the Kaper's medium (28). Recovery percentage of each medium will be compared.

$$\text{Recovery \% } = \frac{\text{Presumptive counts on the basis of colony morphology}}{\text{Confirmed identification by Kaper's medium}} \times 100$$

b. Plesiomonas Medium

Quantity of ingredients in the new Plesiomonas medium:

The new Plesiomonas medium should have the following ingredients like;

Bacto-peptone

Sodium chloride

Sodium taurocholate

Neutral red

Brilliant green

Lactose/Xylose/Inositol/Mannitol etc.

Bacto-agar

The quantity of bacto-peptone, sodium chloride and sodium taurocholate and pH indicator (neutral red, brilliant geen) will be determined accordingly these ingredients are added in other enteric media like; MacConkey's agar, SS agar and taurocholate tellurite gelatin agar (Monsur's medium). Fermentable carbohydrates, such as lactose, xylose, mannitol and inositol will be tried separately to select one that gives best result. The exact quantity of the fermentable carbohydrate will be determined by trial and error.

This medium can be sterilized by normal autoclaving (i.e. at 121 ^{o}C for 15 min. at 15 lbs pressure/sq. inch.) and could be stored at 4 ^{o}C at least two weeks.

Suitability of this medium for processing clinical and environmental samples would be tested through following procedure.

1. Growth inhibition of pure culture of coliform :

Ten pure culture each of E. coli, Klebsiella and Citrobacter will be grawn overnight into BHI broth and serially diluted into normal saline. 0.05 ml culture from appropriate dilution will be plated on BHI agar and new Plesiomonas medium following spread plate method of inoculation (28). After overnight incubation at 37 °C for 24 h, difference of counts on BHI agar and new medium will indicate the extent of inhibition of coliforms on the new Plesiomonas medium. 2-3 fold inhibition of coliforms is desired on this new medium. Each experiment will be repeated thrice to reduce technical error.

2. Comparative growth inhibition of Plesiomonas on the new medium :

Comparative counts of 25 P. shigelloides strains isolated from both clinical and environmental sources will be studied on BHI agar plate and new Plesiomonas agar plate. Plating method will be same as above experiment. To reduce the experimental error, each experiment will be repeated thrice.

3. Comparative detection ability of Plesiomonas on the new medium relative to others:

Stool from normal individuals with known coliform counts will be diluted 1:10 and divide into several portions. One portion will be used as control and add test strains (two strains each of P. shigelloides isolated from clinical and environmental sources) to others in various ratios of coliform counts. From each dilution, 0.05 ml specimen will be plated on SS agar, Plesiomonas differential agar and inositol brilliant green bile salt agar and new medium. After overnight incubation, rate of recovery of

Plesiomonas at highest coliform counts on these media will be noted. The detailed methodology will be followed as described elsewhere (19,20). Each experiment will be repeated thrice to reduce the technical error.

4. Description of colonial morphology of different entric bacteria on the new medium :

10 strains each of P. shigelloides, A. hydrophila, A. sobria, A. caviae, Vibrio cholerae 01, V. cholerae non-01, V. mimicus, V. fluvialis, V. furnisii, V. plagious and V. parahaemolyticus, Pseudomonas spp., Proteus spp., E. coli, Klebsiella spp., Enterobacter spp.. amd Citrobacter spp. isolated from clinical and environmental sources will be streaked on new medium. After overnight incubation at 37 ^{o}C, colonial morphology (shape, size, color, surface of the colony) of each species will be described.

5. Growth inhibition of Vibrios :

Five strains each of V.cholerae 01 (available serotypes and biotypes), V. cholerae non 01, V. mimicus, V. fluvialis, V. furnisii, V. plagious and V. parahaemolyticus isolated from clinical and environmental sources will be used in this experiment. Overnight culture of each strain in BHI broth will be serially diluted in sterile normal saline and 0.05 ml culture from appropriate dilution will be plated simultaneously on BHI agar and new Plesiomonas medium. After overnight incubation, difference of counts of each species on this two media (BHI agar and new Plesiomonas medium) will give an idea of extent of

inhibition by the new medium relative to non-inhibitory BHI agar. Each experiment will be repeated thrice to reduce the technical error.

6. Comparative isolation of Plesiomonas from Stool :

250 rectal swab specimens collected from diarrhoeal patients submitted in the Diagnostic Microbiology section for culture will be used in this experiment. Each of the rectal swab specimen will be plated directly on Plesiomonas differential agar and inositol brilliant green bilesalt agar, Salmonella-Shigella agar and new Plesiomonas medium - before and after enrichment in bile peptone broth. Typical colonies from each plate will be identified by conventional biochemical test. Isolation rate of new Plesiomonas medium will be compared to judge its efficasy.

Enrichment broth for isolation of Plesiomonas shigelloides from diarrhoeal Stool :

In our previous study (paper submitted for publication in the Journal of Clinical Microbiology) with clinical specimen, we have observed that bile peptone broth is superior than alkaline peptone water. Therefore, bile peptone broth as enrichment broth for Plesiomonas will be used when necessary in this protocol.

Study of environmental samples :

To test the suitability of the new medium for isolation of Plesiomonas from the environment, ten water and plankton samples will be analysed. The same sample will be inoculated on the new medium, SS agar, IBB (Inositol brilliant gree bile salt medium) and PDA (Plesiomonas differential agar). New medium, and SS will

be incubated at 37 °C for 24 h. and PDA and IBB will be incubated at 44 °C for 24 h. After overnight incubation, typical Plesiomonas colonies from respective medium will be isolated and identified. Isolation rate in each medium will be compared. Particular plating medium that gives highest isolation will be considered as the best medium. Each experiment will be repeated thrice to reduce technical error.

D. SIGNIFICANCE

If the new media are established, this will help to know the actual rate of isolation of Aeromonas and Plesiomonas from the clinical specimens. Media for Aeromonas and Plesiomonas if judged suitable for processing environmental specimens, ecology of Aeromonas and Plesiomonas could be studied more comfortably.

E. FACILITIES REQUIRED :

No additional facilities is required.

F. COLLABORATIVE ARRANGEMENT :

Not at the moment.

REFERENCES

1. Lautrop, H. 1961. Aeromonas hydrophila isolated from human faeces and its possible pathological significance. Acta. Pathol. Microbiol. Scand. 51 (suppl.) 144: 299-301.

2. Rosner, R. 1964. Aeromonas hydrophila as the aetiologic agent in a case of severe gastroenteritis. Am. J. Clin. Pathol. 42:402-404.

3. Gracey, M., V. Burke, J. Robinson. 1982. Aeromonas-associated gastroenteritis. Lancet, ii:1304-1306.

4. Gutsell, J.S., and S.F. Snieszko. 1964. Dosage of sulfamerazine in the treatment of furunculosis in brook trout, Salvelinus fontinalis. Trans. Am. Fish. Soc. 76:82-96.

5. Eddy, B.P., 1960. Cephalotrichus, fermentative gram-negative bacteria: the genus Aeromonas. Appl. Bacteriol. 23:216-249.

6. Shotts, E.G., and R. Rimler. 1973. Medium for the isolation of Aeromonas hydrophila. Appl. Microbiology 26:550-553.

7. Chen, P.K., R.S. Ellenberger, and M. White. 1975. Selective enrichment of Aeromonas liquefaciens by using ampicillin. Am. Soc. Microbiol. Annu. Meet., Abstr. Ann. Meet., p.187.

8. Kaper, J.H. Lockman, R.R. Colwell, and S.W. Joseph. 1979. Ecology, Serology and enterotoxin production of Vibrio cholerae in Chesapeak Bay. Appl. Environ. Microiol. 37:91-103.

9. Fliermans, C.B., R.W. Gorden, T.C. Hazen, and G.W. Esch. 1977. Aeromonas distribution and survival in a thermally altered lake. Appl. Environ. Microbiol. 33:114-122.

10. Davis, J.W., and R.K. Sizemore. 1981. Non-selectivity of Rimler-Shotts medium for Aeromonas hydrophila in estuarine environmets. Appl. Environ. Microbiology 42:544-545.

11. McCoy, R.H. and K.S. Pilcher. 1974. Peptone beef-extract glycogen agar, a selective and diferential Aeromonas medium. J. Fisheries. Res. Board. Can. 31:1553-1555.

12. Rippey, S.R., and V.J. Cabelli. 1979. Membrane filter procedure for enumeration of Aeromonas hydrophila in fresh water. App. Environ. Microbiol 38:108-113.

13. Rogol, M, I. Sechter, L. Grinberg and C.B. Gerichter. 1979. Pril-xylose-ampicillin agar, a new selective medium for the isolation of Aeromonas hydrophila. J. Med. Microbiol. 12:229-231.

14. Rahim, Z., S.C. Sanyal. K.M.S. Aziz, M.I. Huq and A.A. Chowdhury. 1985. Isolation of enterotoxigenic, hemolytic and antibiotic resistant Aeromonas hydrophila strains from infected fish in Bangladesh. Appl. Env. Microbiol. 48:865-867.

15. Kay, B.A., C.E. Guerrero and R.B. Sack. 1985. Media for the isolation of Aeromonas hydrophila. J. Clin. Microbiol. 22:888-890.

16. Robinson, J., V. Burke, P.J. Worthy, J. beaman, and L. Wagener. Media for isolation of Aeromoinas spp. from faeces. J. Med. Microbiol. 18:405-411.

17. Palumbo, S.A., F. Manino, A.C. Williams, R.L. Buchanon, and D.W. Thayer. 1985. Starch-ampicillin agar for the quantitative detection of Aeromonas hydrophila. Appl. Environ. Microbiol. 50:1027-1030.

18. Pitarangsi, C., P. Echeverria, R. Whitmire, C. Tirapat, et al. 1982. Enteropathogenicity of Aeromonas hydrophila and Plesiomonas shigelloides: prevalence among individuals with and without diarrhea in Thailand. Infect. Immun. 35:666-673.

19. Von Graevenitz and C. Bucher. 1983. Evaluation of differential and selective media for isolation of Aeromnas and Plesiomonas spp. from human feces. J. Clin. Microbiol 17:16-21.

20. Millership, S.E., and B. Chattopadhya. 1984. Methods for the isolation of Aeromonas hydrophila and Plesiomonas shigelloides from faees. J. Hyg (camb). 92:145-152.

21. Tsukamoto, T., Y. Kinosita, T. Shimada, and R. Sakazaki. 1978. Two epidemics of diarrhoeal disease possiblly caused by Plesiomonas shigelloides. J. Hyg. (London), 80:275-280.

22. Schubert, R.H.W. 1977. Ueber den Nachweis von Plesiomonas shigelloides Habs and Schubert, 1962, und ein Electivenedium, den Inositol - Brilliantgrun-Gallesalz - agar. E. Rodenwaldt Archiv 4:97-103.

23. Mannual of Clinical Microbiology, 1986. American Society for Microbiology, 4th ed., A. balows, W.J. Hauster, Jr., H.J. Shadomy (eds), P. 1080.

24. Miller, M.L. and J.A. Koburger. 1986. Evaluation of inositol brilliant green bile salts and Plesiomonas differential agar for recovery of Plesiomonas shigelloides from aquatic samples in a seasonal survey of the suwanne River estuary. J. Food. Protect. 49(4):274-277.

25. Cooper, R.G., and G.,W. Brown. 1968. Plesiomonas shigelloides in South Australia. J. Clin. Pathol. 21:715-718.

26. Nair, P., and S.E. Millership. 1987. Isolation of Plesiomonas shigelloides from nutrient broth with brilliant green: its use in screening stool samples from an African population. J. Clin. Pathol. 40:680-682.

27. Monsur, K.A. 1961. A highly selective gelatin taurocholate medium for the isolation of Vibrio cholerae. Tr. R. Soc. Trop. Med. Hyg. 55:440-442.

28. Kaper, J., R.J. Seidler, H. Lockman and R.R. Colwell. 1979. A medium for the presumptive identification of Aeromonas hydrophila and Enterobacteriaceae. Appl. Environ. Microbiol. 38:1023-026.

29. American Public Health Association. 1981. Standard method for examination of water and waste water. 14th ed., American Public Health Association, Washington, D.C.

30. Rahim, Z., KMS aziz, M.S. Islam, and M.I. Huq 1985. A preliminary survey of the relative abundance of fecal coliform bacteria in water and sediment and in the fresh water bivalve, Lamellidens marginalis of the Buriganga river, Bangladesh. Microbiol. Ecol. 11:227-280.

31. Popoff, M. 1984. Aeromonas, P. 545-548. In Krieg, N.R., J.G. Holt(eds). Bersey's Mannual of Systematic Bacteriology. Williams and Wilkins. Baltimore, London.

SECTION-III : BUDGET

A. DETAILED BUDGET

1. Personnel Services:

Name	Level	Position	Effort	Time	Annual Salary (US $)	Total
Md. Zeaur Rahim	NOB	P.I.	50%	1 yr	2436.00	
Dr. Bradford A. Kay		Consultant	-	-	-	
Dr. Anwarul Huq		Consultant	-	-	-	
To be named	GS3	Lab Tech	100%	1 yr	1520.00	3956

2. Supplies and Materials:

Culture media	5750.00	
Chemicals	750.00	
Membrane filter	250.00	6700

3. Equipment — Not applicable (N.A.)

4. Patient Hospitalization — N.A.

5. Outpatient care — N.A.

6. ICDDR,B Transportation — 50

7. Travel and Transportation of Persons:

International (Dhaka-Washington-Dhaka) — 2800

Local (Dhaka-Teknaf-Dhaka) — 88

8. Transportation of things — N.A.

9. Rent, Communication and Utilities — Not applicable

10. Information Services (Library and Publications) — 150

11. Printing and Production — 50

12. Other Contractual Services — N.A.

13. Construction, Renovation and Alteration — N.A.

B.	BUDGET SUMMARY	US$
1.	Personnel Services	3,956
2.	Supplies and materials	6,750
3.	Equipment Not applicable (N.A.)	
4.	Patient hospitalization N.A.	
5.	Outpatient care N.A.	
6.	ICDDR,B Transportation	50
7.	Travel and Transporation of Persons	2,880
8.	Transportation of things N.A.	
9.	Rent, Communication and Utilities N.A.	
10.	Information Services (Library & Publication)	150
11.	Printing and Reproduction	50
12.	Other Contractual Services N.A.	
13.	Construction, Renovation, Alteration N.A.	

FLOW CHART I

DEVELOPMENT OF A NEW MEDIUM FOR AEROMONAS

1. Determine the conc. of sodium taurocholate (inhibitory agent)

Fixed conc. of bacto-peptone and sodium chloride and varying conc. of sodium taurocholate (0.1-0.75%).

V

Seed known counts of A. hydrophila, A. sobria and A. caviae isolated from clinical and environmental sources on this medium and non-inhibitor BH1 agar.

V

Then determine the exact conc. of sodium taurocholate which do not affect counts of Aeromonas on the new medium relative to BH1 agar.

2. Determine the conc. of glycogen (fermentable polysaccharide)

Fixed conc. of bacto-peptone, sodium chloride, and sodium taurocholate and add varying conc. of glycogen (0.1-1%).

V

Streak A. hydrophila, A. sobria and A. caviae strains isolated from clinical and environmental sources on this medium.

V

Select particular conc. that gives bright yellow colored aeromonad colony production.

3. Determine the conc. of DL-ornithine :

Fixed concentration of bacto-peptone, sodium chloride, sodium taurocholate and glycogen and varying conc. of DL-ornithine (0.1-2.0%) 2.0%).

Seed known counts of A. hydrophila, A. sobria and A. caviae from above sources on the new medium and non-inhibitory BH1 agar.

Select the particular conc. of DL-ornithine which do not affect the counts of Aeromonas strains on the new medium relative to BH1.

4. Determine the particular pH :

Fixed conc. of bacto-peptone, sodium chloride, sodium taurocholate, glycogen, bromothymol blue (10 ml per liter from 0.5% bromothymol blue stock soln.) and varying pH (7.0 to 8.0).

Streak culture of A. hydrophila, A. sobria and A. caviae on the medium.

Select particular pH that helps to produce prominent yellow colored aeromonad colony on the new medium.

FLOW CHART II

STUDY OF CLINICAL SPECIMEN

New *Aeromonas* medium

↓

Growth inhibition of coliforms

↓

Test of growth inhibition of *Aeromonas*

↓

Description of colony morphology of different enteric bacteria

↓

Comparative detection ability of *Aeromonas* by the new medium relative to others

↓

Growth inhibition of *Vibrionaceae*

↓

Comparative isolation of *Aeromonas* from diarrhoeal stool using new medium and others

↓

FLOW CHART III

AEROMONAS ENRICHMENT MEDIA :

Stool artificially contaminated with *Aeromonas* strains

↓

inoculate into
a) Alkaline peptone water (APW)
b) APW + 10 mcg/ml ampicillin
c) bile peptone broth (BPB)
d) BPB + 10 mcg/ml ampicillin

↓

o

incubate 37 C for 24 h

↓

Streak on new Aeromonas medium

↓

Reading: Particular broth medium that helps *Aeromonas* isolation at highest coliforms counts.

FLOW CHART IV

STUDY OF ENVIRONMENTAL SPECIMENS :

New *Aeromonas* medium

V

Stress recovery (-20^{o} C "Cold Shock")

V

Surface plating method for quantitative morphological confirmation of *Aeromonas* colonies on the new medium relative to other media using environmental specimen.

V

Membrane filtration method for quantitative morphological confirmation of *Aeromonas* colonies on the new medium relative to other media using environment specimen.

V

FLOW CHART V

STUDY OF SPECIMEN :

New *Plesiomonas* medium

↓

Growth inhibition of coliforms

↓

Growth inhibition of *P. shigelloides*

↓

Comparative detection ability of *Plesiomonas* from artificially contaminated stool specimens

↓

Description of colonial morphology of different enteric bacteria on the new medium

↓

Growth inhibition of *Vibrios* on the new medium

↓

Comparative isolation of *Plesiomonas* from diarrhoeal stool using new medium and others.

ICDDR,B
BUDGET PROPOSAL
(In US $)

PARTICULARS

Program name: Lab. Sciencena Division Protocol title: Formulation and evaluation of improved isolation media for Aeromonas and Plesiomonas;

P. I's name: Md. ZEAUR RAHIM

Protocol no: .....

Starting date: March 01, 988

Budget code: .....

Completion date: ~~2811~~ Feb. 28, 989

EXPENSE CATEGORY	A/C No.	Refer Page	Column A 1st year Jan.-Dec.	Column B 2nd year Jan.-Dec.	Column C 3rd year Jan.-Dec.	Column Total Project Cost
Description			3,956			3,956
Local Salaries	3100	2	0			0
Intl. Salaries	3200	8	0			0
Consultants	3300	14	88			88
Travel Local	3500	15	2,800			2,800
Travel Intl.	3600	16	6,700			6,700
Supplies & Mat.	3700	18	0			0
Other Costs	3800	19	250			250
Inter Deptl. Ser.	4800	20	13,794			13,794
Total Direct cost			4,276			4,276
Indirect cost = 31% of total direct cost	0000		18,070			18,070
TOTAL OPERATING COST						
Capital expenditure Refer page no. 21	0300					
TOTAL PROJECT COST						

Zeaur Rahim

P.I.'s signature

Reviewed by Budget & Finance

PERSONNEL REQUIREMENT-(LOCAL STAFF) 1st/2nd/3rd year

	No. of positions	No. of man months	$ Amount
A. Direct Project/Protocol/Branch Staff at starting date Sourced from Page 3			
Add:			
B. New recruitments Sourced from Page 4			
C. Staff allocated from other area Sourced from Page 5	2	18	3,956
(i) Sub-Total	2	18	3,956
Less:			
D. Seperations Sourced from Page 6	0	0	0
E. Staff allocated to other area Sourced from Page 7	0	0	0
(ii) Sub-Total	0	0	0
(i)-(ii) TOTAL			* 3,956

*Agrees with Page 1 A/C No.3100

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ziz-13.

MANPOWER-ALLOCATED_FROM_OTHER_AREA-(LOCAL_STAFF)-1ST/2ND/3RD_YEAR

A	B	C	D	E=(CxD)	F	G=(ExF)	Endorsement
Job designation	Level	Budget code of other area	No. of posit.	No. of months	No. of man months	**Rate per month	$ Amount	of other area's P.I/ Branch Head
Assistant Scientist	NOB	200411	1	6	6	406	2436	
Lab Tech	GS III	210210	1	12	12	126	1520	
TOTAL		* 2	XXXXXXX	* 18	XXXXXXXX	* 3,956	XXXXXXXXXXX	

** Please contact Budget Office (Ext.280) for rates.

* AGREES WITH PAGE 2 ROW C

TRAVEL PLAN (LOCAL)-1ST/2ND/3RD YEAR

Job designation	Travel fr-to-fr	No. of persons	Estim. days of travel per prn	No. of person days (A)	** Perdiem Rate (B)	** Perdiem Amount (C=AXB)	*** Transport Air (D)	*** Transport Ground (E)	**** Other cost (F)	Total $ amount (C+D+E+F)
Assistant Scientist	Dhaka-Teknaf-Dhaka	ONE	3	3	8·6	26·0	5000	12·00	—	88
								TOTAL		*

** Check with Finance Ext. 214 for perdiem rates.
*** Check with Travel Ext.234 for transport cost.
**** Includes excess baggage, embarkation fee, travel tax etc.

*AGREES WITH PAGE 1 A/C 3500

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Aziz-13.

SUPPLIES AND MATERIALS-1ST/2ND/3RD YEAR

A/C Code	Item Description	$ Amount
3701	Drugs (used for medication in the hospitals and field stations)	0
3702	Glassware (bottle, beaker, cylinder, petridish, aluminium seal, slides, stopper, tube etc.)	0
3703	Hospital supplies (bandage, gauze, blade, bowl, catheter, cotton, needle, syringe, soluvion, leukoplast, towel etc.)	0
3704	Stationery and office supplies (battery, book register, binders, files, pencil, fastener, paper, ribbon, stapler etc.)	0
3705	Chemicals and media (acid, reagent, dextrose, sodium, bactoagar etc.)	5,750
3706	Materials for uniform (cloth, button etc. required for making uniforms)	0
3707	Fuel, oil and lubricants (diesel, mobil, petrol, kerosene etc.)	0
3708	Laboratory supplies (aluminium foil, bag, blade, brush, cap, container, film X-Ray etc.)	0
3709	Housekeeping supplies (aerosol, battery, wiping cloth, duster, lock and key etc.)	0
3710	Janitorial supplies (bleaching powder, brush, detol, detergent, insecticide, soap etc.)	0
	Page total (balance b/d)	5,750

Contd. to page 18

TRAVEL PLAN (INTERNATIONAL)-1ST/2ND/3RD YEAR

Job designation	Travel from-to-from	Estim. days of travel (A)	** Perdiem Rate (B)	** Perdiem Amount (C=AxB)	*** Transportation Air (D)	*** Transportation Ground (E)	**** Other cost. (F)	Total $ amount (C+D+E+F)
Assistant Scientist	Dhaka-Washington Dhaka	10	100	1,000	1,800	0	0	2,800
						TOTAL		$

** Check with Finance Ext. 214 for perdiem rates.
*** Check with Travel Ext.238 for transport cost.
**** Includes excess baggage, embarkation fee, travel tax etc.

*AGREES WITH PAGE 1 A/C 3606

Budget86.16
Aziz-13.

SUPPLIES AND MATERIALS-1ST/2ND/3RD YEAR

(Contd. from Page No. 17)

A/C code	Item description	$ Amount
	Page total from page 17 (balance c/d)	5,750
3711	Tools and spares (automobile spares, tyres, tubes, battery, stores required for maintenance services etc.)	0
3712	Non-stock supplies (materials not normally kept in stock and purchased only against specific requisitions)	950
	Sub-Total	0
3713	Freight and other charges add 30% for import	0
	TOTAL	6,700

AGREES WITH
PAGE 1
A/C 3700
COLUMN D

Note For rates please contact Supply Ext.260 (add 10% to rates for inflation)

Budg:t86.18
Aziz 13.

**INTERDEPARTMENTAL SERVICES-1ST/2ND/3RD YEAR

A/C code	Service Area	$ Amount
4801	Computer	
4802	Transport Dhaka	50
4803	Transport Matlab	
4804	Water transport-Matlab	
4805	Transport Teknaf	
4806	Xerox	50
4807	Pathology	
4808	Microbiology tests	
4809	Biochemistry	
4810	X-Ray	
4811	I.V. Fluid	
4812	Media	
4813	Patient hospitalisation study	
4814	Animal research	
4815	Medical illustration	
4817	Telex	
4818	Out patient care	
4819	Maintenance charges	
4820	Vehicle maintenance charges	
4821	Library service charges	150
4830	Transport subsidy	
	TOTAL	* 250

** Please contact Cost Office on Ext. 281. for rates.

*AGREES WITH PAGE 1 A/C 4800

Budget86.20
Aziz-13.