Attachment 1.

Date May, 16, 1988

ETHICAL REVIEW COMMITTEE, ICDDR,B.

90

Principal Investigator Md. Zeaur Rahim Trainee Investigator (if any) May, 16, 1988

Application No. 88-14(P) Supporting Agency (if Non-ICDDR,B) X

Title of Study Formulation and evaluation of improved isolation medium for Aeromonas.

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 NA
   (b) From parent or guardian (if subjects are minors) — Yes No NA
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 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)

We 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

X
Trainee

88-014(P)
16/5/88

SECTION - I : RESEARCH PROTOCOL

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

2. Principal Investigator : Md. Zeaur Rahim

   Co-Investigator : Dr. M.S. Islam

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

3. Starting date : June 01, 1988.

4. Completion date : December 31, 1988

5. Total direct cost : US$ 4,738.00

6. Scientific Program Head :

   This protocol has been approved by the Laboratory Sciences Division.

   Signature of Scientific Program Head : _____

   Date : May 15, 1988

7. Abstract Summary :

Various approaches will be made to formulate a selective and differential medium for qualitative and quantitative estimation of Aeromonas spp. through processing several clinical as well as environmental samples. Performance of the new medium will be compared with blood agar ampicillin, TTGA, MacConkeys agar and SS agar. Various enrichment technics for isolation of Aeromonas from clinical specimens 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 in a medium suitable for selective growth of Aeromonas spp.

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

2. Background :

Aeromonas spp. have gained considerable importance after it has been 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 immunocompetent patients, specially from children with diarrhoea. A. hydrophila was isolated from specimens of diarrhoeal stools as a suspected pathogen in 1961 (1) and in 1964 (2).

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 and thereafter in 1960 (4,5). These media utilised the haemolytic property but non-haemolytic Aeromonas would not be isolated with these media. Another selective medium called RS was formulated

by Shootts and Rimler (6). Using RS medium, several researches have been conducted in the past (7,8,9,10). Similarly, MacCoy and Pilcher formulated medium was not found suitable in routine work (11). Several other media were developed using ampicillin (12,13). Since ampicillin sensitive strains are coming up now a days (14), ampicillin containing medium would adversely affect their isolation.

For better isolation of Aeromonas from stool, attempts were made using blood agar plate containing 10 mcg/ml ampicillin (15,16). Resistance to ampicillin and haemolytic property of the organism were the main selectivity of these medi . However, all Aeromonad colonies are not haemolytic, and 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 Aeromonad colonies. Besides, 10 mcg/ml ampicillin is not sufficient to inhibit Enterobacteriaceae. Hence, stool inoculated plates after overnight incubation shows profuse growth of enteric bacteria which could easily overgrow Aeromonas. Moreover, Proteus is highly motile on blood agar ampicillin medium and covers the entire plate within 24h of inoculation by the swarming motility. These problems make reading of blood agar ampicillin medium time consuming and not good for use in routine work.

Palumbo et al. 1985 published information on selective medium for quantitative detection of Aeromonas (17). 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.

In the Clinical Microbiology Section of ICDDR,B. blood agar ampicillin is being used for isolating Aeromonas from diarrhoeal stools. Problems of using blood agar ampicillin has been discussed above. TTGA (Monsur's medium) was initially developed for isolating Vibrio cholerae from the stool. In ICDDRB this medium is extensively being used for isolating Vibrios from clinical as well as from the environmental specimens. But there is no sharp definition to differentiate Aeromonas from non-01 Vibrios on TTGA. In practice, oxidase positive colonies are being picked up as non-01 Vibrios and subsequently identified either as Vibrio spp or Aeromonas spp. using a set of biochemical reactions. If the reaction suggests that the test strain is Aeromonas spp (resistant to Vibriostatic compound and only lysine, or both lysine and arginine positive), then further biochemical reaction is tested to differentiate Aeromonas spp A. hydrophila, A. sobria, and A. caviae. Thus it takes a longer to identify Aeromonas from TTGA and Aeromonas colonies cannot be identified on TTGA agar medium alone. Hence, TTGA cannot be used for quantitative estimation of Aeromonas from environmental and clinical specimens.

In addition to TTGA, Aeromonas is also being picked up from other enteric media like MacConkey's and Salmonella-Shigella agar. Since Aeromonas includes lactose postive (lactose utilizer) strains, lactose positive Enterobacteriaceae would be confused

with lactose positive Aeromonas on MacConkey's and SS agar media. Moreover, Gracey et al. (1982) reported 2% inhibition of Aeromonas strains on MacConkey's agar (3).

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. The exact quantity of the above mentioned ingredients will be determined by trial and error method which has been summerised in attached flow chart 1, (Development of a new medium for Aeromonas). The new medium would be a solid one which could be sterilized by autoclaving.

Like other selective and differential media, the new Aeromonas medium should have the basic growth promotic substances like; bacto-peptone and sodium chloride and sodium taurocholate as inhibitor of enteric bacteria. In TTGA medium, sodium taurocholate has been successfully used to inhibit enteric bacteria. Glycogen will be used as fermentable polysaccharide and bromothymol blue as pH indicator. Aeromonas can utilize glycogen to produce yellow colored colony. V. parahaemolyticus can partially utilize glycogen and produced yellow colored colony on DL-ornithin defficient medium and hence confused with Aeromonad colonies. DL-ornithine will be used in the medium to mask the production of yellow colored colony formation. Inhibition of yellow colored colony formation by V. parahaemolyticus due to addition of DL-ornithine can be explained as follows: Having decarboxylase enzyme, V. parahaemolyticus can produce ammonia due

to decarboxylation of ornithine which turns yellow colonies of *V. parahaemolyticus* into bluish-green one. DL-ornithine containing medium inhibits growth of *Vibrio cholerae* which has been observed during the preliminary work.

In the preliminary work, the above mentioned ingredients were used having following concentration:

	gm/l
Bacto-peptone	10.00
Sodium chloride	10.00
Sodium taurocholoate	05.00
Glycogen	10.00
DL-ornithine	20.00
Bromothymol Blue	8 ml from 0.5% stock soln.
Agar	15.00
pH	7.7

394 stool specimens were simultaneously inoculated onto new *Aeromonas* medium (having above mentioned composition of different ingredients) alongwith blood agar ampicillin, MacConkey's agar, Salmonella-Shigella agar. The following isolation rates were obtained.

Name of the Media	Samples yield positive	% (n = 394)
New *Aeromonas* medium	125	32
Blood agar ampicillin	93	24
MacConkey agar	53	1
SS agar	36	9

Results of preliminary work indicate that this medium is promising. Further work is needed to determine the exact concentration of each of the above ingredients.

Rationale:

There are few media for isolating Aeromonas from clinical and environmental sources, but each of those had practical demerits. This protocol has been designed to formulate a selective and differential medium for quantitative as well as for qualitative estimation of Aeromonas from clinical as well as from the environmental specimens.

B. SPECIFIC AIMS

1. To develop a new selective and differential medium for qualitative estimation of Aeromonas from clinical specimens and quantitative estimation of Aeromonas from environmental specimens.

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

C. Methods of Procedure

a. Aeromonas Medium

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 will be streaked on to the new Aeromonas medium and non-inhibitory brain heart infusion (BHI) agar. Inhibition on the new medium relative to BHI will be studied observing the growth condition (good growth, poor growth, and no growth) in these two plating media.

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. plagious, 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 tabulated.

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

Stool suspensions inoculated with various dilutions of the culture of test strains (one strain each of A. hydrophila, A. sobria and A. caviae isolated from clinical and environmental sources will be used separately) will be streaked on to the new Aeromonas medium, Blood agar ampicillin, TTGA, MacConkeys and SS agar. The medium, which can detect Aeromonas from the artificially inoculated stool having highest dilution (lowest count) of test strain will be judged superior.

5. Growth inhibition of Vibrionaceae:

Five strains each of V. cholerae O1 (available serotypes and biotypes), V. cholerae non O1, 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, TTGA 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.

After overnight incubation, following criteria should be noted in case of each plating medium used and score will be tabulated:

a. number of isolated colonies of Aeromonas spp. appearing on each medium.

b. appearence of a particular selective marker on the colony.

c. growth of Proteus.

d. swiftness of oxidase test done by taking Aeromonad colonies directly from the plate.

Particular plating medium having highest score interms of above heading will be judged superior.

Enrichment technic for isolation of Aeromonas from the stool:

Stool suspensions inoculated with different dilutions of test strains (one strain each of A. hydrophila, A. sobria and A. caviae isolated from clinical and environmental sources will be use separately) will be inoculated into five enrichment broths [bile peptone (pH 8.8), bile peptone tellurite (pH 8.8), bile peptone with ampicillin (10 mcg/ml, pH 8.8), Alkaline peptone (pH 8.8), and Alkaline peptone with ampicilline (10 mcg/ml, pH 8.8)] and incubated at 37 °C. Cultures from each enrichment broth will be streaked on to new Aeromonas medium after 4 h and 24 h. of incubation. Particular enrichment medium that helps to recover Aeromonas from stool having highest dilution (lowest counts) of test strain will be regarded as the best.

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

Comperative Performance of Enrichment Media:

100 rectal swab specimens collected from the diarrhoeal patients will be inoculated on to the new medium before and after enrichment into five enrichment broths mentioned above and incubated overnight a 37 C. The enrichment broth which gives higher number of isolation will be considered as the best.

Elevated temperature enrichment for Aeromonas :

Another set of enrichment broths (APW, APW + 10 mcg/ml ampicillin, BPB, bile peptone tellurite 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.

Processing of Environmental Samples Using New Medium:

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

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 (25, 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 (1). Recovery percentage of each medium will be compared.

$$\text{Recovery \%} = \frac{\text{Presumptive counts on the test medium}}{\text{Confirm counts using kaper's medium}} \times 100$$

D. SIGNIFICANCE

If the new medium is established, this will help to know the actual rate of isolation of Aeromonas from the clinical specimens. This media will also help in qualitative and quantitative aestimation of Aeromonas from the environmental samples.

E. FACILITIES REQUIRED :

No additional facilities is required.

F. COLLABORATIVE ARRANGEMENT :

Not at the moment.

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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%	1/2 yr	-	-
Dr. M.S. Islam	NOB	Co-investigator	10%	1/2 yr	-	-
Dr. Bradford A. Kay		Consultant	-	-	-	-
Dr. Anwarul Huq		Consultant	-	-	-	-
To be named	GS3	Lab Tech	100%	1/2 yr	-	-

2. Supplies and Materials:

Culture media	3,350	-
Chemicals	600	-
Membrane filter	100	4,050

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:

Local (Dhaka-Teknaf-Dhaka) — 88

8. Transportation of things — N.A.

9. Rent, Communication and Utilities — Not applicable

10. Information Services (Library and Publications) — 500

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	-
2. Supplies and materials	4,050
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	88
8. Transportation of things N.A.	
9. Rent, Communication and Utilities N.A.	
10. Information Services (Library & Publication)	500
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%).

V

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

V

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

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).

V

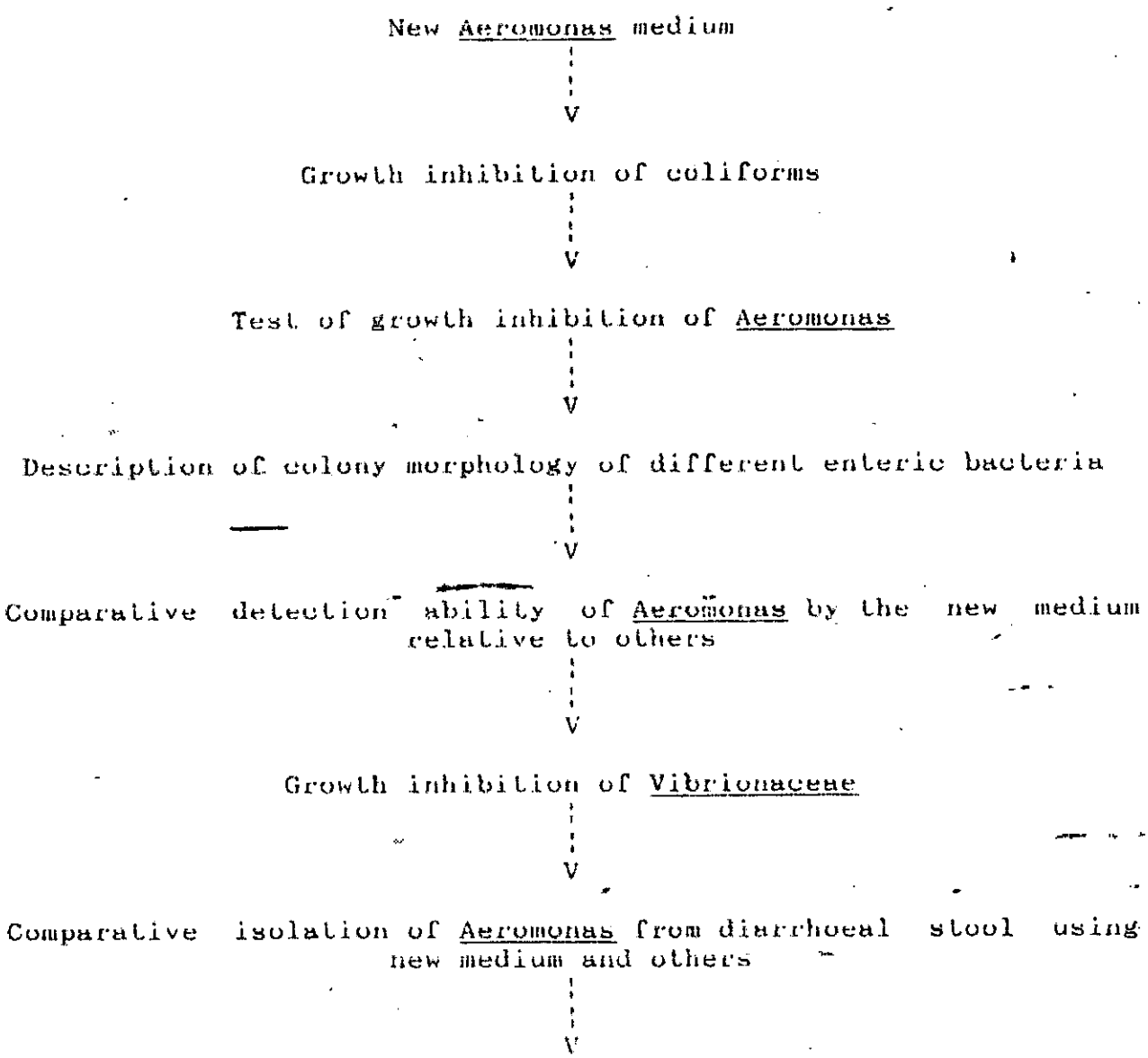
Streak culture of A. hydrophila, A. sobria and A. cavine on the medium.

V

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

FLOW CHART II

STUDY OF CLINICAL SPECIMEN



FLOW CHART III

AEROMONAS ENRICHMENT MEDIA :

Stool artifically 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

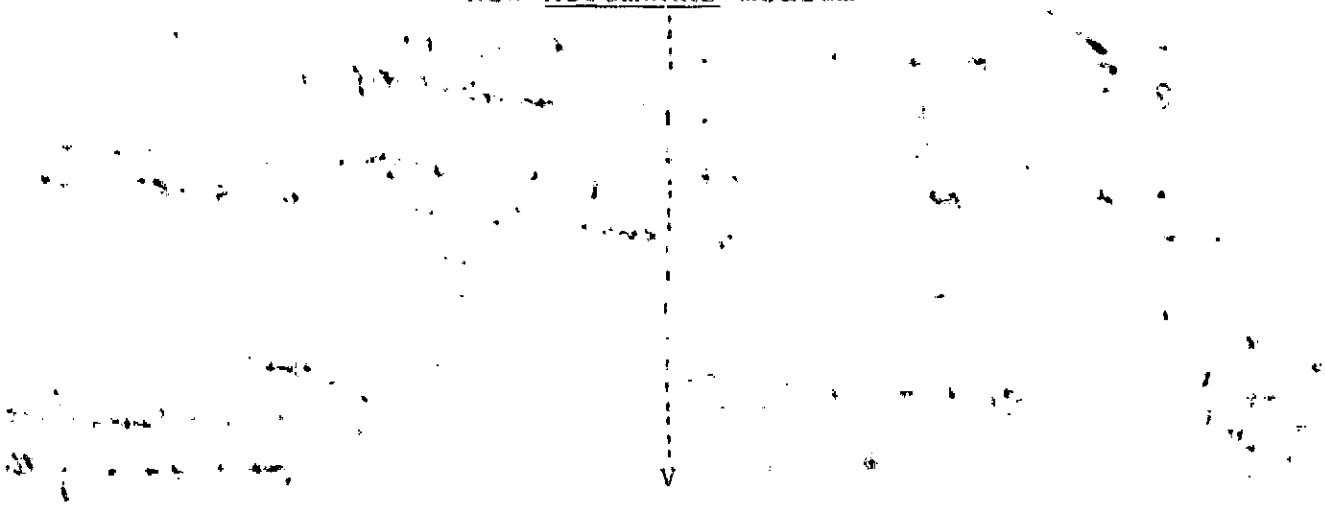
↓

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

FLOW CHART IV

STUDY OF ENVIRONMENTAL SPECIMENS :

New *Aeromonas* medium



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

which the physicians decide to admit (check total)

Cases — gr.1 + gr.2   persistent d. >14d. & during 48h of observation is found to have diarrhea (define)

Controls.   Acute d   < 4 days & on subsequent check discharge c̄ cure (define) & the total duration is not >14d

check Roy's data re: age distribution.

stratum matching.

Case 1

Next acute pt admitted ; alternate 2m – 11m ; 2m – 36m }

0-36m — check (check) surveillance age distribution of the decide how to select controls.