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IMPROVED MEMBRANE FILTER TECHNIQUE FOR ENUMERATION OF ESCHERICHIA COLI AND ENTEROCOCCI

MICROBIOLOGICAL CRITERIA WORKSHOP

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I. INTRODUCTION

The improved membrane filter technique described below incorporates modifications to the basic methodology already in use in many water testing laboratories. These modifications are designed to enhance recovery of the specific indicator organisms, Escherichia coli and Enterococci, and are recommendations based on extensive studies that continued over a period of ten years.

Marine waters collected from polluted and nonpolluted beaches were tested for six years. The organisms recovered were evaluated as indicators for the occurrence of swimming-associated gastroenteritis in persons bathing in those waters. Findings from the first two years showed that neither the most probable number (MPN) nor the membrane filter method (MF) for total coliforms gave results that correlated well with elevated swimming associated gastroenteritis rates (1). This led to the exclusion of these tests in the studies that followed. Fecal coliforms and their component genera, E. coli, Klebsiella sp., Enterobacter sp. and Citrobacter sp. were measured, as well as Enterococci, Clostridium perfringens, (spores), Pseudomonas aeruginosa, Aeromonas hydrophila and the pathogen Vibrio parahaemolyticus. All of these organisms recovered from the membrane filters were speciated during a period of three years, and their presence evaluated as indicators. Of the organisms examined, Enterococci densities in the water correlated best with swimming-associated gastroenteritis(1). E. coli was a weak second. Total fecal coliforms, their component flora, and other organisms tested showed no such relationship (2).

The series of studies on fresh waters carried out during 1979 through 1983 compared the indicator organisms E. coli and Enterococci to the standard fecal coliforms. They showed a correlation coefficient with swimming related gastroenteritis for E. coli to be slightly greater than Enterococci. This came as no surprise as E. coli are more numerous in feces and tolerate fresh water well, whereas Enterococci are salt tolerant and survive in marine waters better than E. coli. However, on the basis of survival quality of both

marine and fresh water, Enterococci are the most dependable as a single indicator organism.

The two best indicator organisms were the subject of laboratory studies designed to enhance their recovery rate. The improved methods presented in the procedure descriptions that follow, were developed as a result of these studies. The method for E. coli includes a resuscitation period for weakened organisms that enhances recovery rates to over 90% and quantifies them within 24 hours without requiring further subculture and identification (3). The method for Enterococci results in a 10,000 fold reduction in background organisms and appears to measure those species most closely associated with fecal wastes of humans (4). Verified recovery rates exceed those of the standard KF method by one order of magnitude.

II. METHOD FOR RECOVERY OF ESCHERICHIA COLI

A. Equipment needed

(1) Member filter holders and clamps
(2) Membrane filters - 0.45 μm pore size
(3) Plastic petri plates - 9 x 50 mm with tight lid
(4) Polyethylene film water sample bags
(5) Incubator 35°C
(6) Water bath 44.5°C
(7) Weights (to ensure immersion of petri plates in water bath)
(8) Container to hold plastic bag while immersed
(9) Forceps
(10) Burner or alcohol lamp
(11) Vacuum source
(12) Source of magnification 10 to 15 x
(13) Autoclave or ultraviolet sterilizing apparatus.
(14) Small beaker or other open mouth vessel to hold alcohol

B. Medium

mTEC Medium

Proteose peptone No. 3 (Difco)	5.0g
Yeast extract (Difco)	3.0g
Lactose (Fisher Scientific)	10.0g
NaCl	7.5g
K_2HPO_4	3.3g
KH_2PO_4	1.0g
Sodium Lauryl Sulphate (2003 Matheson, Coleman & Bell)	0.2g
Sodium Desoxycholate (Fisher Scientific)	0.1g
Brom Cresol Purple (Nutritional Biochem. Co.)	0.08g
Brom Phenol Red (Matheson, Coleman & Bell)	0.08g
Agar (Difco)	15.00g
Distilled water	1000.00 ml

Dissolve ingredients by stirring (and heating to dissolve agar if medium is to be divided into smaller aliquots prior to sterilization). Sterilize by autoclaving at 121°C for 15 minutes. Pour into 9 x 50 mm petri plates, at 4 ml.per plate. The pH of the medium should be 7.3 $\pm$ 0.1. When agar has solidified, ascertain that the lids are tightly fixed.

C. Solutions and reagents

Urease Subtrate Solution

Urea	2.0g
Phenol red	10.0mg
Distilled water	100.0ml.

Adjust pH to 5.0 ± 0.2 (straw color). Store at 6 to 8°C for a period of no more than one week.

Phosphate Buffered Saline

Can be purchased or prepared.

95% Alcohol

Can be purchased.

D. Performance of test

(1) Filtration

(a) Sterilize filter holders

i. Autoclave,15 lbs pressure for 20 min., or

ii. Ultraviolet sterilizing apparatus for 2 min., or

iii. Place in boiling water,one to two minutes

(b) Dip forceps in alcohol and flame to sterilize

(c) Assemble filters using sterile forceps to place filter membrane in situ and adjust clamp to hold assembled filter in place

(d) Shake sample vigorously to ascertain even distribution of organisms present

(e) If less than 20 ml of sample is to be filtered, moisten filter membrane by pouring approximately 20 ml of sterile phosphate buffer into filter. If 20 or more ml of sample is to be filtered, use 5.0 ml.

(f) Using a sterile pipette transfer a measured amount of sample into the filter. Amounts tested for unknown samples should range from 0.1 ml to 50.0 ml. Recommended amounts for testing unknown sample are:

0.1 ml, 0.3 ml, 1.0 ml, 3.0 ml,

10.0 ml, 30.0 ml, 100.0 ml

Filter holders can be re-used without re-sterilization if one works from smaller to larger amounts of test samples.

(g) Rinse filter holder with no less than 20 ml of sterile phosphate buffer. (Be certain to rinse down sides of the fulter so that organisms clinging to the sides will be wash down onto the filter membrane.

(2) Culture technique

(a) Remove filter top exposing the filter pad placement area

(b) Dip forceps into alcohol and flame to sterilize

(c) Grasp filter membrane containing organisms with sterile forceps and place on surface of mTEC plate by first allowing one edge to make contact and then carefully rolling it onto the surface to avoid entrapment of air

(3) Resuscitation and incubation

(a) Carefully close plate and press edges to secure lid. Place up to 4 plates in the sterile water tight plastic bags. The open edge of the bag is then rolled tightly for several folds, and held in place by bending the flaps at either edge. Place the bag thus prepared in a 35° C incubator for the 2 hour resuscitation period

(b) Remove the bags from the incubator and immerse them in a 44.5° C water bath. Weight them to keep plates immersed. Incubate for approximately 22 hours.

(4) Biochemical tests

(a) Remove bags from water bath, dry and take plates from bags

(b) Remove top of petri dish containing the culture, and place a sterile filter membrane in it, using a forceps sterilized by method previously described. Saturate pad by pipetting approximately 1.5 ml of urease agent onto it

(c) Dip forceps into alcohol and flame to sterilize

(d) Transfer filter membrane containing colonies, and place it on top of the urease saturated filter membrane, using the same roll on technique.

(f) Allow to stand 15 to 20 minutes

(g) Using 10x magnification, count all yellow colonies and record number. This count, when adjusted on the basis of amount of water cultured, is calculated to represent the number of thermotolerant E. coli/100 ml of sample. All colonies that are dark on the mTEC plates, and are urease positive, are not E. coli.

III. METHOD FOR RECOVERY OF ENTEROCOCCI

A. Equipment needed

The same as for *E. coli*, with exception that the only incubator needed is one adjusted to 41°C.

B. Media

mE Medium

Peptone	10.0g
NaCl	15.0g
Esculin	1.0g
Agar	15.0g
Yeast extract	30.0g
Actidione	0.05g
Sodium azide	0.15g
Distilled water	1000.0ml

Autoclave at 121°C for 15 min. After autoclaving add:

Nalidixic acid	0.24g
Triphenyl tetrazolium chloride	0.15g

Adjust pH to 7.1 ± 0.1 and pour 4.0 ml amounts into sterile plastic petri dishes (9 x 50 mm) with tight lid.

EIA Medium (Esculin Iron Agar)

Esculin	1.0g
Ferric Citrate	0.5g
Agar	15.0g
Distilled water	1000.0ml

Adjust pH to 7.1 ± 0.1 before autoclaving at 121°C for 15 min. Pour 4.0 ml amounts into sterile 9 x 50 mm petri plates.

C. Solutions

Phosphate buffer - same as for *E. coli*

D. Performance of test

(1) Filtration - same as for *E. coli*

(2) Culture technique

(a) Proceed as for *E. coli*, except that filter membrane is placed on the mE plate

(b) Incubate at 41°C for 48 hours

(c) Remove plates from incubator

(d) Dip forceps in alcohol and flame to sterilize

(e) Use forceps to place membrane onto EIA plate, using the same roll on technique as previously described

(f) Incubate for 20 to 30 minutes at 41°C

(g) Count dark red to purple colonies, approximately 1-2 mm in diameter, with dark halos, reading through the bottom of the plate.

(h) Calculate the number of colonies grown from 100 ml sample as for E. coli

LITERATURE CITED

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