STUDIES WITH MEDIA RECENTLY AVAILABLE FOR USE WITH THE MEMBRANE FILTER IN THE ENUMERATION OF COLIFORM ORGANISMS IN WATER

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by

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A study was undertaken of two media recently proposed for use, in conjunction with the membrane filter technique, for the estimation of the coliform bacteria in water supplies.

The first, developed by McCarthy, was found to be inadequate for use with natural, untreated waters. Experimental work was done in an attempt to render it more suitable, but no improvement could be made, and it was concluded that the basic formula was not suitable for further development.

The second, recently available commercially, and known as M-Endo broth M.F., gave coliform determinations at 37°C approximating very closely the estimates obtained by the conventional 5-tube decimal dilution procedure. Not all of the organisms which grow upon this medium are coliform, and it is considered that the ecology, distribution, and sanitary significance of the non-coliform flora must be established before the medium can be accepted for routine use.

A direct method, using this medium with the membrane filter technique, for the estimation of Escherichia coli in water supplies was also attempted. The results obtained were not satisfactory, in that in a number of instances, samples known to contain E.coli gave negative results, and organisms other than E.coli were included in the counts obtained.

The opportunity was taken to compare lauryl tryptose broth with MacConkey's medium in the standard 5-tube decimal dilution procedure for the estimation of the coliform density of water supplies. Both media gave essentially similar results, although the addition of a pH indicator to the lauryl tryptose broth is recommended.

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INTRODUCTION

During the past five years membrane filter techniques for the quantitative estimation of the coliform group of organisms in water have been developed. A satisfactory filtering procedure has by now been well established and suitable apparatus and filter material are commercially available.

The difficulty has been, and still is, in the formulation of a suitable medium upon which to place the inoculated membranes for the growth and enumeration of the coliform group of organisms. It is a simple matter to grow organisms of this group, but due to the limited surface area available it becomes necessary to suppress the growth of the rest of the water flora to prevent the coliform organisms, especially if present in small numbers, from being overgrown and inhibited. It is also necessary to color the colonies in some way to render them visible against the white surface of the filter. Black-colored filtering material is now available, but upon autoclaving it becomes excessively brittle and difficult to handle. Exposure to ethylene oxide vapor or to ultraviolet light may be used satisfactorily, but these operations are somewhat cumbersome and are not suitable for routine use. The dyes and stains used have been such that the colonies are colored and those of the coliform organisms differentiated on

a color basis from those of other organisms capable of growing upon the medium used.

Gas production from lactose is used in the various tube-dilution methods as one of the criteria upon which the recognition of the coliform group of organisms is based, and indeed, by definition, the ability to produce gas from lactose is one of the most important diagnostic characteristics of the group. Since there is no possibility of being able to assess gas production from coliform organisms growing upon the filter surface, other distinguishing properties must be utilized in their differentiation. The recognition of coliform organisms when grown upon solid media has been carried out for many years, generally by one of three methods:

1. The production of colonies of characteristic appearance upon a medium containing eosin and methylene blue.
2. By the use of a pH indicator in the medium which contains lactose, enabling those organisms which produce acid from this sugar to be recognized.
3. The production of colonies of characteristic appearance upon a medium containing basic fuchsin and sodium sulphite.

All three of these systems of differentiation have been utilized in the media developed for use with the membrane filter and will be discussed further.

PART I

THE USE OF EOSIN AND METHYLENE BLUE

Review of the Literature

Eosin-methylene-blue agar was developed by Levine in 1918 (19) from a formulation by Holt-Harris and Teague (12) who used it as a plating medium following enrichment for the isolation of enteric pathogens. The medium was incorporated into the 1923 edition of Standard Methods (38) and remains the solid medium most widely used for the isolation of coliform organisms. Descriptions of coliform colonies as they appear on this medium are given by Levine (20) and by Howard and Thompson (13). The composition of the medium is as follows:*

Bacto-peptone	1.0 g.
Lactose	1.0 g.
Dipotassium phosphate	0.2 g.
Agar	1.5 g.
Eosin Y	0.04 g.
Methylene blue	0.0065 g.

Adjust pH to 7.1

The mode of action of E.M.B. agar is considered by Wynne et al. (51) to depend upon two factors: the formation by eosin and methylene blue of a methylene blue eosinate,

*Ingredients per 100 ml.

made up of one molecule of each of the dyes, which acts as an acidic dye, and the production by the bacteria of a low pH which enables the cells of the colony to take up the acidic dye. Colonies of bacteria which do not ferment lactose are colorless because their cells, in the alkaline environment maintained by them, do not take up the stain. Levine (21) has shown that a sugar-free base is necessary in the preparation of the medium if differentiation of coliform types is required because the addition of fermentable carbohydrate causes all coliform colonies to take on a metallic sheen.

Prescott et al. (32) have reviewed the literature concerning the specificity of this medium. Identification of colonies is subject to an error, usually less than 10%, and which is on the safe side, false positive reactions being encountered with a much greater frequency than false negatives. Standard Methods (39) in accepting confirmatory identification purely by the appearance of colonies typical of Escherichia coli recognizes the accuracy of the medium in this respect. On the other hand, in requiring the complete identification of two or more colonies most likely to be coliform if typical E.coli are not present, the inability of the medium to indicate with satisfactory accuracy the presence of coliform organisms other than E.coli is recognized.

It should be noted that apart from the dyes used no other inhibitory agents are present, but even so, Mallmann

and Darby (23) have suggested that the medium may not support the growth of all coliform strains.

Attempts have been made to utilize this type of medium in conjunction with the membrane filter procedure for the enumeration of coliform organisms in water. In 1952, the U.S.P.H.S. Environmental Health Center, (E.H.C.) (47) published details of the preparation of a medium having the following composition:*

Peptone (Albimi M)	4.0 g.
Yeast autolysate	0.6 g.
Dipotassium hydrogen phosphate	0.7 g.
Sodium chloride	0.5 g.
Bacto-bile salts no. 3	0.1 g.
Lactose	2.0 g.
Methylene blue	0.1 g.
Eosin Y	0.5 g.

Bile Salts are included in order to inhibit non-coliform organisms, and a 2-hour preliminary enrichment of the cells obtained by pre-incubating the membranes upon pads soaked in lactose broth is recommended before transfer to the differential medium. In addition, the filter material used must be dyed with methylene blue before filtration of the sample takes place. Even so, coliform colonies do not show their characteristic appearance, apparently due to the fact that the methylene-blue-eosinate complex cannot diffuse through the pores in the filter material (17). A similar formulation, with the exception that the concentration of bile salts is doubled, is given in the Technical Bulletin (41).

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*Ingredients per 100 ml.

Neither has received much attention due, no doubt, to the atypical appearance of the colonies and the extra manipulation involved in the dying of the filter material.

PART II

THE USE OF pH INDICATORS

Review of the Literature

These have been incorporated into liquid broths and solid plating media used for the recognition of coliform organisms ever since the beginnings of water bacteriology. The broth developed by MacConkey (22) is still used in Britain for the enumeration of coliform organisms in water, and the same medium solidified with agar has been of value in the isolation and differentiation of coliforms and enteric pathogens. Lactose is provided as a source of fermentable carbohydrate and the presence of organisms fermenting this sugar is recognized by the change of color in the medium due to the production of acid. In the case of MacConkey broth, inverted Durham fermentation vials are used to detect gas production. Until fairly recently, the indicator employed was neutral red, but this has now been largely replaced by brom-cresol-purple which gives a sharper color change and has been shown to be less toxic, especially to the streptococci (1).

Taylor (42), and Taylor et al. (43) have utilized a triple-strength MacConkey broth containing twelve times the

normal amount of brom-cresol-purple in conjunction with the membrane filter technique for the enumeration of coliform organisms in water. Later these authors (44) reported further progress in the study. Using the membrane filter procedure they compared this medium, which requires no enrichment, and the EHC modified Endo medium described later with a 5-tube dilution technique in which primary incubation was at 42°C. In both cases the two membrane filter media gave higher counts but, as the authors point out, the high incubation temperature of the dilution procedure used may well have prevented the growth of some coliform organisms.

Goetz and Tsuneishi (9) have described the use of a modified Gassner (8) medium for the same purpose. The formula contains lactose as a source of fermentable carbohydrate and, with the exception of the peptone present, contains no buffer. Two dyes are used: a pH indicator, ink blue, which is blue in acid; and a yellow dye, fast mordant yellow, which partially inhibits cocci and spore-formers, but does not affect the development of salmonellae and coliform organisms. Pre-treatment of the filter material with the yellow dye is necessary. The acidity produced by the fermentation of the lactose stains the colonies and their immediate environment blue, while other colonies appear greenish-yellow in color. The addition of an oxidation-reduction indicator is recommended to distinguish the slow from the rapid lactose fermenters, since the latter will turn blue before the redox

potential has dropped low enough to give the colonies a red color. On the other hand, the slow lactose fermenters will turn red and will not develop the blue color appreciably. No results obtained with this medium are given, and it does not appear to have been used subsequently by other workers.

McCarthy (26), realizing the toxicity of the modified type of Endo medium described later, formulated a MacConkey type of medium for use with the membrane filter procedure in the enumeration of coliform organisms in water. The selective action depends upon the use of bile salts and a low concentration of ethyl violet, and the recognition of lactose fermenters is facilitated by the inclusion of a pH indicator brom-cresol-purple. Methylene blue is included to color the filter material in order to provide a background against which colonies can be seen more easily. After 14 hours incubation at 35°C in an atmosphere saturated with water vapor, coliform colonies are colored yellow or pinkish-yellow and those of non-coliform organisms blue. The formula is given below:*

Bacto-tryptose	1.5 g.
Sodium chloride	0.5 g.
Lactose	1.0 g.
Bacto-bile salts	0.5 g.
Brom-cresol-purple	0.15 g.
Methylene blue	0.001 g.
Ethyl violet	0.00175 g.

With both samples of well and river water counts were obtained which were higher than those on the EHC modified

*Ingredients per 100 ml. medium

Endo medium and which approximated those calculated from the standard 5-tube dilution procedure with subsequent confirmation of positive tubes in 2% brilliant green bile broth. In the case of the river and highly polluted well waters, however, there was often a spreading overgrowth of the filter surface due to the presence of non-coliform organisms, and the medium could not be regarded as satisfactory for the examination of natural raw water supplies.

Experimental and Discussion

It seemed probable that some improvement could be made in the medium, and a good deal of time was spent in an attempt to render this medium more satisfactory. Bacto-bile salts no. 3 were employed in lieu of the ordinary preparation and a range of concentrations tested to determine the optimal amount required. In addition, the effect of brilliant green and sodium lauryl sulphate was determined over a wide range of concentration for each material both singly and in combination with each other and the bile salts. This was accomplished by inoculating a number of membranes with a polluted water known to contain both E.coli and Aerobacter aerogenes according to the filtering procedure presented in Standard Methods (39). The equipment and filter material were obtained from the Millipore Filter Corporation.* A

*Watertown, Mass.

basal medium containing the tryptose, sodium chloride, brom-cresol-purple, and methylene blue was prepared in concentrated form. Solutions of suitable strength of lactose, ethyl violet, brilliant green, and the bile salts no. 3 were prepared and all were sterilized separately in the autoclave at 121°C for 15 minutes.

The complete medium was prepared from the components in the concentrations under test and the volume adjusted with sterile distilled water to a constant level, the concentration of base remaining constant. Sterile absorbent pads in sterile 50 mm. Pyrex petri dishes were wetted with a fixed amount (1.7 ml.) of the medium, the inoculated membranes rolled upon their surfaces and incubated in the inverted position in an atmosphere saturated with water vapor at 35°C for 14 - 18 hours. It was found that a series of membranes could be inoculated successively from one water sample without the need for resterilization of the apparatus each time, provided that the entire inner surface of the glass container in contact with the sample was rinsed with 30 ml. of sterile water between each inoculation. On thirty occasions a second sterile membrane was inserted and the container again rinsed thoroughly with sterile water. When incubated upon the coliform medium no colonies developed, showing that there is no danger of carrying over coliform organisms from one filtration to the next.

No improvement over McCarthy's original formula could be made -- the brilliant green, ethyl violet, and sodium lauryl sulphate bringing about little improvement in the restriction of colony size and inhibition of non-coliform organisms, except in concentration sufficient to reduce the viable coliform population considerably.

It appears that the failure of this medium lies in its inability to inhibit the growth of non-coliform organisms and to restrict the growth of the coliforms to the extent that their colonies remain discrete, while at the same time allowing all coliform cells deposited upon the filter surface to develop into colonies. Ethyl violet, brilliant green, and sodium lauryl sulphate are known to be most active against gram-positive organisms, and their ineffectiveness indicates that the interfering organisms are gram-negative. A large number of non-coliform colonies were picked from the filter surfaces and were shown to be decolorized in the gram staining procedure. It also became evident that a concentration of bile salts which would inhibit these organisms would also prevent many coliform cells from growing upon the filter surface; and in all probability this effect would be enhanced by their suspension, and consequent attenuation, in water for long periods.

Such inhibitory effects have been overcome to some extent by the preliminary incubation of inoculated filters for a period of 2 hours upon a medium such as lactose broth

containing no inhibitory agents. A modification of this method which is described later involves the use of two absorbent pads, superimposed upon each other, the upper being impregnated with a plain lactose broth and the lower with the selective medium. During the time taken for the selective medium to reach the cells they have been resuscitated by exposure to the lactose broth and are better able to withstand the toxic effect of the inhibitors used in the selective medium. A modification of this method was tested in which the medium described was prepared in the form of a 1.5% agar gel, cooled to 45°C, and a layer superimposed upon another solidified agar medium containing the nutrient base and larger amounts of brilliant green, sodium lauryl sulphate, and bile salts. It was considered that the diffusion of the inhibitors in the lower medium through the upper layer to the filter surface would take place slowly enough to permit initial colony development but would later restrict the growth of the coli-aerogenes bacteria and inhibit the growth of other organisms present. Each inhibitor in the lower medium was tested over a wide range of concentration. Different thicknesses of surface medium, restricted only by the physical limitations of the 50 mm. petri dish used, were also employed. It appears that the inhibitors used, if present in sufficient amounts, are able to diffuse through the agar rapidly enough to completely inhibit colony development, the effect being identical to that of an homogenous medium

containing larger amounts of inhibitors. It has been observed that, especially in the case of the Endo type media referred to later, colony development upon the filter surface is not a gradual process but rather that there is a long lag period extending for about 10 hours during which no growth is visible. Subsequently, growth is extremely vigorous, and during the next few hours colonies of 1 - 2 mm. in diameter develop. Presumably the 10-hour period is sufficient for the inhibitors in the lower medium to diffuse to the filter surface and to prevent subsequent colony development.

It appeared that further study of this type of medium would not be profitable, and attention was directed to the Endo type of medium described in Part III.

PART III

THE USE OF BASIC FUCHSIN AND SODIUM SULPHITE

Review of the Literature

Endo, in 1904, (5) proposed a medium for the isolation of typhoid organisms consisting of nutrient agar containing lactose and an indicator system composed of basic fuchsin and sodium sulphite. It is still widely used as a plating medium for coliform organisms and is recommended for this purpose in Standard Methods (39) which gives details of its composition and preparation. The formula is reproduced below:*

Dipotassium phosphate	0.35 g.
Bacto-peptone	1.0 g.
Agar	2.0 g.
Lactose	1.0 g.
Sodium sulphite	0.25 g.
Basic fuchsin	0.05 g.

The virtue of this medium lies in its indicator system. E.coli colonies developing on it produce dark red, flat, button-like colonies with a distinct metallic sheen, while other coliform types are nucleated, pink or red in color, and possess no such luster. A great deal of attention has been paid to the amounts of indicator and sulphite

*Ingredients per 100 ml. medium

required, and they have been found to be critical. Care is also needed in selecting the source of the fuchsin as not all lots are suitable.

Endo attributed the appearance of the colonies on this medium to acid formation, but this alone does not produce the deep red color and sheen typical of E.coli colonies. Other workers believed the reaction to be similar to that of Schiff's reagent with aldehyde, but the two are not apparently identical. DeBord (4) considered the effect to be caused by the combined effect of acid and aldehyde, since in his experiments neither alone could produce the characteristic color effect. Margolena and Hansen (25) concluded from their study that the effect was best explained by the theory of Neuberg and Nord (30) who postulated that the sulphite traps small amounts of acetaldehyde, an intermediate product of fermentation, as it is produced by coliform organisms; and the medium then acts as a reagent to indicate the presence of accumulated products. Thus it would appear that the diagnostic characteristic of gas production from lactose is replaced by the production of acetaldehyde.

In the search for a suitable medium for the enumeration of coliform organisms in conjunction with the membrane filter technique, it was natural that attention should be given to this medium of Endo which has proved to be so useful as a differential medium in the past.

Schutz and Kruse (36) credit the Russians with using it in this connection in 1934, when they incubated inoculated membranes upon an Endo type of agar.

The application of this type of medium to the membrane filter technique was initiated in America by the U.S.P.H.S. Environmental Health Center (E.H.C.) (2, 15) which in 1951 published details of the preparation and use of EHC modified Endo medium, the composition of which is as follows:*

Lactose	2.0 g.
Dipotassium hydrogen phosphate	0.7 g.
Neopeptone	2.0 g.
Sodium sulphite (anhydrous)	0.27 g.
Basic fuchsin	0.09 g.
Ethyl alcohol	1.5 ml.

Basic fuchsin itself is inhibitory and plays a part in regulating the size of colonies developing on the filters. The only other material present likely to inhibit growth is the ethyl alcohol used as a solvent for the fuchsin. It is probable that the concentration used is sufficient to bring about some reduction in spreading and to decrease colony size since Floyd and Dack (7) have shown that double this amount is effective in inhibiting the spreading of Proteus.

Nevertheless the authors found that this medium was inhibitory to coliform organisms, a high proportion of those present in fresh sewage failing to grow. In order to overcome this difficulty, a preliminary 2-hour enrichment,

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*Ingredients per 100 ml.

obtained by placing the inoculated membranes upon pads soaked in lactose broth prior to incubation on the differential medium, was necessary and resulted in a doubling of the sheen-producing colonies obtained. With this procedure a good agreement was found between coliform counts so obtained and those resulting from the use of 5-tube M.P.N. determinations with subsequent confirmation according to Standard Methods (39).

It has been pointed out (15) that the classical description of coliform colonies on Endo agar does not apply to those developing on the membrane filters using the modified EHC procedure, since on the latter all of the E.coli and most of the A.aerogenes and intermediates develop the metallic sheen. Clark and Kabler (3) further state that a few of the A.aerogenes and intermediates may be missed by this procedure, but consider that the increased accuracy obtained compensates for this small loss.

Neumann (31) examined several different types of media for the same purpose and found this EHC procedure the most satisfactory but not entirely suitable for all of the water sources tested, since other organisms of no known sanitary significance were not inhibited.

Kabler and Clark in 1952 (17) also considered this medium to be the best available at that time.

Streicher (40) in 1951 suggested that as had been found with the original Endo medium the fuchsin : sulphite

ratio was of great importance. Later Clark and Kabler (3) and the workers at the Environmental Health Center (48) confirmed this view and published methods for titrating the indicator system to give more satisfactory and consistent results.

Goetz et al. (10) have also attempted to correlate this membrane filter procedure using EHC Endo media with M.P.N. determinations using the 5-tube dilution technique described in Standard Methods (39). Only a very few sets of results are presented but fairly good agreement was obtained. A modification of the EHC technique is presented employing what the authors describe as a nutrient schedule procedure which is less time-consuming and gives rather better results. In the method described the filter is incubated upon a pad made up of a thin leaf of porous paper containing a lactose broth above a heavy disc containing the differential Endo medium. The two pads, after being impregnated with their respective media, are dried, placed together and sterilized, and require only moistening before use. The principle is that the inhibitory materials in the lower pad will not reach the attenuated coliform cells until they have been resuscitated and are more resistant to their effects. The method, despite the advantages claimed by its originators, does not appear to have been much used by later workers.

Presnell et al. (33) using sea water also compared the two methods of enumeration using the 95% confidence

limits of the M.P.N. determinations developed by Velz (49). Briefly this takes into account the bias of 5-tube M.P.N. determinations. By multiplying the M.P.N. obtained by factors of 0.3 and 2.9, a range is obtained. It is considered that any coliform count obtained with the membrane filter which falls within this range is not significantly different. By this manipulation 87% of the samples examined were in agreement.

Thomas and Woodward (45) have analyzed statistically the results obtained by three groups of investigators (33, 52, 50) who have compared the same two methods of enumeration, the M.P.N. determinations again being assessed on the basis of the 95% confidence limits. They concluded that the 5-tube dilution technique as presented in Standard Methods gives results higher than those obtained with the EHC medium in conjunction with the membrane filter by a factor of 1.0 - 1.9 with an average of 1.3. In addition, their analysis indicated that the two methods of enumeration do not measure the same groups of organisms. The differences were considered to be minor and in the opinion of the authors there was no reason why the 5-tube dilution method should be favored more than the membrane filter technique described.

In 1955, Thomas et al. (46), using finished waters, carried out further experiments and statistical analyses comparing the degree of control which could be exercised upon such waters using the two methods of enumerating

coliform organisms, and concluded that the membrane filter method could give equally good, if not better, control of treatment processes.

The first qualitative report on the types of colonies found upon filter surfaces incubated on EHC modified medium was published by Jeter et al. (14) who in addition typed, using the IMViC tests, a large number of the organisms concerned. They found that of 370 sheen-producing organisms isolated, only 92% produced gas from lactose broth; but, even so, coliform counts obtained were lower than those obtained by the conventional 5-tube dilution methods with subsequent confirmation. This discrepancy, however, was not due to the exclusion from the Endo medium of one particular IMViC type as all were represented. In addition, of 361 colonies not producing sheen, 4% produced gas in lactose. Again all IMViC types with only one exception (+ - + +) were represented. Mention is also made of sheen-producing colonies which upon subculture would produce no gas from lactose broth in 48 hours at 35°C and which were, of course, counted as coliform. Thus it appears that the medium is not absolutely satisfactory in differentiating coliform organisms, and it seems that the ability to produce gas from lactose is not correlated perfectly with the property of forming acetaldehyde. Nothing is known about the distribution and sanitary significance of these non-coliform, sheen-producing organisms; but, obviously, if present in large numbers in a water supply, a false

assessment would be arrived at using this membrane filter method. This work tends to support that of Thomas and Woodward (45) which has already been reviewed and who concluded that the two methods of enumeration are not measuring the incidence of the same groups of organisms.

In order to overcome some of the difficulties and discrepancies discussed, various modifications of the technique and media used have been suggested from time to time.

Kabler (16) used the same formula as that described above except that the lactose in the modified Endo medium was autoclaved separately and the dye content reduced slightly. During the period 1952 - 1953 he examined 1,706 samples of water using this medium with the membrane filter technique, comparing the results obtained with the standard 5-tube dilution test, again using the 95% confidence limits of the M.P.N.

Of his samples 74% showed agreement, although as pointed out by McCarthy (26), if completely negative samples, i.e. those showing no coliforms by either method, are omitted, only 64% are in agreement. He also found that in eighty-five samples, coliform organisms could be detected by the standard tube-dilution method, none being observed upon the membrane filters. In addition, two samples showed the presence of coliform organisms by the membrane filter technique and gave zero M.P.N. determinations. It would seem that coliform organisms are being missed by this modification of the method.

Levin and Laubausch (18) reported on the testing by both methods of 207 samples of drinking water delivered to "Hotel" trains under emergency conditions. Of these three showed the presence of coliform organisms with the confirmed tube-dilution method, two of them producing coliform-like colonies upon the membrane filter. These authors also autoclaved the lactose in the Endo medium separately, and in an attempt to render the medium less inhibitory, reduced the dye-sulphite content to 0.7 of the amount producing optimal results in a titration procedure.

One of the difficulties experienced in the counting of coliform colonies developing upon the filter surface is that many non-coliform organisms also grow and if present in large enough numbers may crowd out the coliforms present. In an attempt to reduce this "background" population, Yee et al. (52) used oxine (8-hydroxyquinoline) as an inhibitor for these organisms. Details of the composition and preparation of this medium, known as LCC Endo, may be found in the Technical Supplement (41) distributed by the Millipore Filter Corporation. The results obtained were not consistent, a reduction in non-coliform population being found only with certain waters. In addition, the authors found that only 81% of sheen-producing colonies on this medium produced gas from lactose and that 22% of non-sheen producing types did ferment the sugar with the production of gas. A statement is made that if the filter surface is allowed to

dry out after incubation, then more colonies will develop the characteristic sheen, but no evidence is presented that these are coliform organisms.

The addition of brilliant green known to be particularly effective against gram-positive organisms has also been recommended for reducing the numbers of non-coliform organisms growing upon the filter surfaces and is suggested in Standard Methods (39) for this purpose in conjunction with the EHC modified Endo medium. No published data appear to be available concerning its performance.

Hajna and Damon (11) used 0.02% sodium desoxycholate in order to bring about the same effect in a medium known as M-HD Endo broth (41). It utilizes fuchsin and sulphite as the indicator. Yeast extract, casitone, and thiopeptone are also included, presumably to overcome the toxic effect of the fuchsin and desoxycholate against attenuated coliform organisms. No enrichment is required. Ethyl alcohol in a concentration of 1.5% is also present. The authors state that false positive reactions (the presence of sheen-producing non-coliform colonies) are eliminated, but no comparison with M.P.N. determinations was made. The method was used only qualitatively to detect the presence or absence of coliform organisms. Out of 147 samples examined by this method and the standard 5-tube confirmed M.P.N. procedure, fifty were positive by both methods and seventy were negative. In eight samples the membrane filter method gave

negative results, while coliforms were found by the tube-dilution test, and the reverse was true in seventeen cases.

McCarthy (27) later carried out further studies using the same medium but on a quantitative basis. He found that it gave good results combined with the membrane filter technique in the examination of samples of diluted fresh sewage. Comparisons were made using the standard 5-tube dilution method. When water samples were used, a rather different picture was obtained, the counts with membrane filter method being considerably lower. In addition, he found that considerable numbers of organisms not producing the characteristic sheen upon this medium were, in fact, coliform; and by picking off all of the colonies on a particular filter into lactose broth, he was able, on the basis of gas production in that medium, to double his original coliform counts. If incubation was continued for a total period of 48 hours (at presumably 35.5°C) a doubling of the count also resulted, and he concluded that the medium left much to be desired.

In the same year Slanetz and Bartley (37) described the use of another modified Endo medium which does not require preliminary enrichment and which is known as M-Endo broth, the formula of which follows:*

Again yeast extract and either neopeptone or thiotone are employed, and no inhibitors apart from the fuchsin-sulphite indicator are present.

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*Ingredients per 100 ml. medium

Yeast extract	0.6 g.
Neopeptone	2.0 g.
Lactose	2.5 g.
Dipotassium hydrogen phosphate	0.7 g.
Basic fuchsin	0.1 g.
Sodium sulphite	0.25 g.

The medium was compared with others available for use with the membrane filter at that time, i.e. EHC modified Endo broth (41), the M-HD Endo broth described above, as well as the triple strength MacConkey broth suggested by Taylor et al. (43, 44), and was found to be superior. Incubation at 35 or 37°C gave similar results, and a 22 - 24 hour incubation period was required. In contrast to the experience of other workers, incubation in an atmosphere saturated with water vapor was not found to be necessary. Fifty-five samples from eight rivers, one brook, and one pond were examined, and counts of colonies showing sheen obtained on this medium in conjunction with the membrane filter technique were compared with the results of the standard 5-tube dilution procedure followed by confirmation of positive tubes in 2% brilliant green bile broth. While the membrane filter counts were generally lower than the M.P.N. determinations, good agreement was obtained if the 95% confidence limits of the latter were used in the assessment. Cultural studies of 193 sheen-producing colonies were undertaken, and 97% proved to be coliform. The authors concluded that this medium allied with the membrane filter procedure was an efficient method for the detection and enumeration of coliform bacteria in water supplies.

In addition to the media listed above, these authors, in an effort to reduce the numbers of non-coliform organisms growing upon their filters, tested the performance of a number of inhibitory agents in this respect. Penicillin, 8-hydroxyquinoline (oxine), and brilliant green were tested, but none were satisfactory. Since these materials are known to be particularly effective against gram-positive organisms, it would appear that the interfering flora growing upon these filters is predominantly gram-negative, and this is borne out by the experience of the author.

Recently a further modification of Endo's medium has been marketed by the Millipore Filter Corporation. This is known as M-Endo Broth MF^{R}, and is manufactured by Difco Laboratories. Again, no preliminary enrichment is required. The composition is as follows:*

Bacto-yeast extract	0.15 g.
Bacto-casitone	0.5 g.
Bacto-thiopeptone	0.5 g.
Bacto-tryptose	1.0 g.
Lactose	1.25 g.
Sodium desoxycholate	0.01 g.
Dipotassium phosphate	0.4375 g.
Monopotassium phosphate	0.1375 g.
Sodium chloride	0.5 g.
Sodium lauryl sulphate	0.005 g.
Sodium sulphite	0.21 g.
Bacto-basic fuchsin	0.105 g.

It will be noticed that the fuchsin - sulphite content is almost the same as in those media previously described but that two inhibitors, sodium desoxycholate and sodium lauryl

*Ingredients per 100 ml.

sulphate, have been included, presumably to inhibit the growth of non-coliform organisms. Both of these materials are known to be effective against gram-positive organisms, but it has been demonstrated that the preponderence of non-coliform organisms growing upon the filter surface are gram-negative. No less than four complex sources of nitrogen are included, presumably to supply growth factors and micronutrients. It is difficult to conceive that all of these materials are necessary and no information is available regarding their inclusion.

No results have been published concerning the use of this medium for the enumeration of coliform organisms in water supplies. Fifield et al. (6) have utilized it to count coliform organisms, using the membrane filter procedure, in pasteurized milk, comparing counts so obtained with plate counts on desoxycholate agar. Good agreement was obtained between the two methods, but it is extremely doubtful whether, with all the extra manipulation involved, the membrane filter technique is suitable for this purpose.

Preliminary work using pure cultures has been carried out (29). Coliform cultures of eight IMViC types were diluted and suitable portions passed through the membrane filter discs and plated on nutrient agar. After incubation at 37°C for 24 hours, colony counts were performed. Five sets of results are available for each culture. Very good agreement was obtained between these two methods, the counts obtained

with the membrane filter medium tending to be slightly lower, but no inhibition with the active cultures used was noticeable. This shows considerable improvement on the results obtained by McCarthy (26) with the EHC medium following preliminary enrichment and using similar methods. She used only one pure culture of E.coli, but found that a recovery of only 76% was obtained with this medium when compared with the colony count on brain-heart-infusion agar.

On the basis of these results it was decided to find out how this medium would perform when used to assess the coliform content of water supplies.

The advantages inherent in the membrane filter technique are considerable and have been discussed and evaluated at some length by a number of workers (10, 36, 46). The application of the procedure for the estimation of the coliform population of water supplies as indicated above, has not been successful, except perhaps in the experience of Slanetz and Bartley (37). This is reflected in the tentative approval of the technique and EHC modified media in Standard Methods (39) which approves the method only under conditions where it has been found to give results comparable to the approved tube-dilution methods.

The author is concerned with the bacteriological examination of small rural water supplies including wells, streams, springs, boreholes, and rainwater, and it is with such natural waters that present media have been found to be

inadequate. If a suitable medium were available, much time and materials could be saved by utilizing the membrane filter technique for this purpose, and it was with these facts in mind that the potentiality of the new medium was investigated.

Materials and Methods

Water samples. As many different types of natural raw water supplies were examined as possible, those known by previous testing to contain coli-aerogenes bacteria being selected for study. They comprised a number of driven and drilled wells, two rivers, and several lakes. Samples were taken in 30-ounce bottles, fitted with screw caps and plastic liners, which had been sterilized in the autoclave at 121°C for 20 minutes before use. This provided a single sample sufficient for all tests to be performed. It was well shaken before being tested -- always within 6 hours of sampling.

Membrane filter technique. The glass filtration apparatus, filter material, and absorbent pads were those supplied for the purpose by the Millipore Filter Corporation.* Filtration was carried out according to the procedure in the Tenth Edition of Standard Methods (39), the amount of sample filtered being adjusted to give less than 200 sheen-producing

*Watertown, Mass.

colonies per membrane. Duplicate inoculated membranes prepared from each sample were rolled upon the surface of absorbent pads moistened with 1.7 ml. of the medium described and contained in 50 mm. Pyrex petri dishes. The medium used was obtained in dehydrated form and was made up according to the instructions of the manufacturer.* A sufficient amount was prepared to meet the requirements for one day and was used within 6 hours. The requisite amount was weighed out on paper and transferred to a sterile 1-ounce screw-capped bottle. The necessary quantity of 1.5% ethyl alcohol in sterile distilled water was added and the powder dissolved at 45°C before being heated in the steamer for 10 minutes. Without this precaution, a black insoluble material was found to be present in the final product. Petri dishes containing the filters resting upon their absorbent pads were inverted and placed upon a perforated tray within a plastic box, the bottom of which was lined with wet cheesecloth. By this means an atmosphere saturated with water vapor was obtained. The box was placed in a "walk-in" incubator maintained at a temperature of 35°C and incubated for 18 - 20 hours.

Studies were also made of the flora developing upon filters incubated upon this medium at 44°C. For this purpose Pyrex cups were used which were 50 mm. in diameter at the base, 80 mm. in diameter at the top, and 50 mm. deep.

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*Difco Laboratories, Detroit, Mich.

Two absorbent pads were placed in the bottom of each cup and moistened with sufficient medium to just cover their surface. After the inoculated membranes had been rolled on in the usual way, the cups were covered with sterile filter paper soaked in sterile water and were sealed with a plastic cover. No sterilization of these covers was found to be necessary, there being no evidence of any contamination. The cups were incubated in a magnetically stirred water bath maintained at a temperature of 44±0.5°C. for a period of 18 - 20 hours. The weight of the cups caused the water to come within 2 cm. of their tops. There was no method whereby the temperature of the medium could be determined, but it was assumed to be that of the bath.

At the end of the incubation period colonies showing a metallic sheen were counted using a binocular dissecting microscope having a magnification of x6.6 diameters. A source of illumination as nearly vertical as possible was provided as this enabled the presence of the sheen to be recognized more easily.

M.P.N. determinations. These were used as the standard for comparison. Quintuplicate amounts of the water sample were inoculated into 10 ml. of double strength lauryl tryptose broth and quintuplicate amounts of 1.0 ml. and 0.1 ml. into 5 - 6 ml. quantities of the single strength medium. The 0.1 ml. amounts were contained in 1.0 ml. of a 1/10 dilution of the sample prepared by pipeting a 10 ml.

portion into 90 ml. sterile 0.85% saline in a dilution bottle. This was considered to be more accurate than the method whereby 0.1 ml. of sample is pipeted directly into the medium.

In addition, the same procedure was carried out using MacConkey's broth according to British practice. The medium was prepared according to the method described in the Report of the Coliform Sub-Committee (34).

In the inoculation procedure, one volume of sample was used for both sets of media, and in order to eliminate external factors as far as possible, one pipet was used for each series of portions pipeted. The same 1/10 dilution of sample was used for the inoculation of 0.1 ml. water into both media. In addition, tubes of each medium were inoculated alternately. In the case of very heavily polluted waters further 10-fold dilutions were prepared and 1.0 ml. portions again used to inoculate tubes of the single strength media. Incubation was in a "walk-in" incubator maintained at 35°C and tubes were examined after 24 and 48 hours. Lauryl tryptose broth tubes showing gas production at either of these times were recorded and subcultured, using a triple loop, into tubes of 2% brilliant green bile broth. These tubes were also examined after 24 and 48 hours' incubation at 35°C and those positive, i.e. showing gas production, recorded.

Positive tubes of MacConkey broth, recognized by the

presence of both acid and gas, were similarly treated, but a second series of subcultures in the brilliant green bile broth were prepared and incubated in a magnetically stirred water bath maintained at a temperature of 44±0.5°C. Incubation was continued for 48 hours at this temperature when positive tubes were recorded.

From the data so obtained the Most Probable Numbers (M.P.N.) of coli-aerogenes organisms and of E.coli were calculated from the tables in the Tenth Edition of Standard Methods (39).

Cultural studies. In determining the gram staining reaction and IMViC characteristics of pure cultures, the following procedures were used.

Gram's stain:The Burke and Kopeloff-Beerman modification (24) was employed.

Indole tests were carried out on 2-day old cultures in bacto-tryptone broth using Pringheim's reagent to detect the presence of indole.

For the M.R. and V.P. tests Bacto MR-VP broth was used, the tests being carried out as described in the Report (34).

In order to determine citrate utilization, Bacto Simmonds citrate agar slopes were used. Inoculation was by stab and any change of color in the indicator after 48 hours noted.

Results, Discussion, and Conclusions

A. The comparison of lauryl tryptose broth and MacConkey's broth. The results obtained using both of these media in the 5-tube decimal dilution standard procedure are given in Table I.

In all, forty-two samples of water were examined. Identical results in the confirmed test were obtained in the case of sixteen samples which contained no coli-aerogenes bacteria and, in addition, two samples containing these organisms gave the same result.

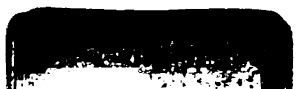
Of the remaining samples, fourteen gave higher M.P.N. determinations when the MacConkey medium was used, and in ten instances the higher count was obtained by the use of lauryl tryptose broth. The results obtained in all cases are very similar, and if the 95% confidence limits of the M.P.N. determinations are used for the comparison, there is no difference between them.

In the case of MacConkey's broth, fourteen, or 6.3% of the 226 positive tubes, did not confirm in 2% brilliant green bile broth. With lauryl tryptose, fifteen more tubes were positive initially but of the total of 241 positive tubes, thirty-two, or 13.3%, did not confirm. Thus it appears that there is very little difference between the two media but that MacConkey's broth, in this investigation, tended to give fewer false positive results, the number of tubes confirmed with both media being almost the same. It

is considered that the addition of a pH indicator, brom-cresol-purple, to the lauryl tryptose medium would be an advantage in that if tubes showing small amounts of gas with no acid production were considered negative, fewer false positive results would be obtained. In Britain, where presumptive tubes showing acid and gas production in MacConkey's broth are considered to contain coliform organisms without subsequent confirmation, this is a decided advantage, and for all practical purposes renders subculture into a confirmatory medium unnecessary.

TABLE I. Results Obtained Using the Decimal 5-tube Dilution Technique With MacConkey's Medium and Lauryl Tryptose Broth

No. of samples	MacConkey's Broth			Lauryl Tryptose Broth		
	No. of tubes positive in 48 hours at 35°C		M.P.N. Coli-aerogenes organisms per 100 ml. sample	No. of tubes positive in 48 hours at 35°C		M.P.N. Coli-aerogenes organisms per 100 ml. sample
	Presumptive test	Confirmed test		Presumptive test	Confirmed test	
1	1	1	2	4	0	0
1	3	3	7.8	2	2	4.5
1	5	5	23	6	3	6.8
1	8	6	33	5	4	13
1	6	6	33	5	5	23
1	7	7	46	13	6	33
1	8	8	64	5	5	23
1	8	8	79	8	7	49
1	8	8	79	6	6	33
1	13	11	220	12	10	140
1	12	12	490	13	11	330
1	14	14	1600	13	13	920
1	17	17	24000	16	15	12000
1	11	11	35000	9	9	13000
16	0	0	0	3	0	0
1	10	9	130	10	9	130
1	12	12	490	12	12	490
1	3	1	2	6	3	6.8
1	6	5	14	8	8	17
1	8	6	31	7	7	49
1	6	6	33	7	7	49
1	6	6	33	7	7	49
1	7	7	49	9	8	70
1	7	7	49	9	9	110
1	13	12	210	16	16	350
1	11	11	330	13	12	490
1	16	13	700	17	15	2400
42	226	212		241	209	



B. The comparison of coliform determinations by the membrane filter technique using M-Endo Broth MF and the standard 5-tube dilution procedure. The results of this investigation are given in Table II.

The examination of the membrane filter surfaces after incubation revealed the presence of a number of different colonial types, some with sheen and others without. Colonies were sharply defined, discrete, and except when very large numbers were present (in excess of 500) showed no tendency to coalesce. In the case of one sample from a lake, only a few coliform organisms were present, but the "background" flora was excessive, almost covering the filter surface; but, even so, sheen-producing colonies could be recognized. When the bacterial population was such as to cover the filter area completely, however, no sheen was recognizable in any colonies at the end of the incubation period even though M.P.N. determinations indicated that an excess of 2,400 coliform organisms were present in 100 ml. of sample.

All previous workers using the membrane filter procedure have regarded the presence of sheen as diagnostic of coliform organisms when grown upon the other modified Endo media discussed previously. Estimates of this group are made by counting the number of sheen-producing colonies present upon the filter surfaces using the standard conditions of illumination described. Standard Methods (39) also recommends this procedure by stating that "All dark colonies

having a yellowish metallic-appearing surface luster shall be counted as coliform colonies." In this investigation some difficulty was experienced in distinguishing colonies showing sheen and those which were dark red in color, especially if the incubation period was prolonged beyond 20 hours. In addition, many of the sheen-producing colonies were surrounded by a transparent periphery. In most cases colonies were 1 - 2 mm. in diameter, and this margin was very small; but in a number of samples, especially those of polluted river water, large numbers of smaller colonies (0.5 - 1.5 mm. in diameter) were present. The transparent margins of these colonies was at least one quarter of the colony diameter in width, and such colonies exhibited small, dark centers and characteristic sheen. These are included in the totals of sheen-producing colonies given in Table II. It is almost impossible to make separate counts of these two colony types as in a great many instances all gradations from one to the other can be found upon one filter surface.

Membranes were not allowed to dry out before counting although some desiccation took place during this period, since the petri dish covers were removed and a powerful ribbon-filament lamp was used as the source of illumination.

In addition to the results of the thirty samples presented, all of which were shown to contain coli-aerogenes bacteria by one or another of the methods used, a further twenty samples were examined in which no coliform bacteria could be demonstrated by either technique.

Examination of the data in Table II reveals that good agreement was obtained between duplicate membranes and that the two methods of enumeration give remarkably similar results. There does not appear to be any significant difference between them, and it is apparent that these results show much closer agreement than any previously published. With fifteen samples, the membrane filter technique gave the higher counts, and in the other fifteen trials the reverse was true. Furthermore, as previously mentioned, in the case of another twenty samples no coli-aerogenes organisms could be detected by either method. In one sample coliform bacteria could be demonstrated by the membrane filter technique while a zero M.P.N. determination was obtained by the tube dilution method. In this instance 600 ml. of sample was filtered and three sheen-producing colonies counted, and with such a small coliform population a zero M.P.N. determination is well within the limits of probability.

Thus it was demonstrated that these two methods of enumeration give very similar indices of coli-aerogenes content.

In assessing these results it has been assumed that all sheen-producing colonies are in fact coliform and that all coliform bacteria present upon the filter surface grow and produce the characteristic luster.

Previous work, using other modifications of the Endo medium has shown that this is not necessarily the case and

that only a proportion of sheen-producing colonies can produce acid and gas from lactose. Three previous investigations have estimated this figure at 92% in the case of the EHC Endo medium (14); 81% with LCC Endo medium (22); while Slanetz and Bartley (37) using M-Endo broth found that 97% of the sheen-producing organisms were coliform.

In two instances (14, 52) non-sheen-producing colonies were also studied and of these 4% and 22% were found to ferment lactose with the production of acid and gas.

This being the case, it was decided to investigate further the bacteriological flora growing upon the filter surfaces in order to determine the proportion of coliform organisms distributed among the sheen- and non-sheen-producing colonies.

Discrete, well-isolated colonies were picked from the filter surfaces into tubes of "Bacto" purple broth base to which 1% lactose had been added, inverted Durham tubes being used to detect gas production. A thin straight wire was used to make the transfers and the colonies only lightly touched. In a number of instances no growth was obtained in 48 hours at 35°C and these results have been ignored. At the beginning of the work lactose broth tubes showing growth were streaked upon Levine's E.M.B. agar, but when it became apparent that only in a very few cases were mixed cultures being obtained, confirmation in 2% brilliant green bile (B.G.B.) was used. Tubes showing gas production in this medium after 48 hours at 35°C were considered to contain coliform organisms.

A total of 514 colonies with the characteristic sheen were treated in this way. Of these, 335 or 65.2% produced acid and gas in lactose broth and gas in the B.G.B. broth. In no case were organisms found which produced acid and gas in the lactose broth and which did not subsequently confirm.

The remaining 179 cultures produced no gas in lactose broth and were not considered to be coliform. A high proportion of these (143) did produce acid in this medium, the amount being sufficient to completely change the indicator to a yellow color in 24 hours. These organisms made up 27.8% of the total sheen-producing colonies only 7% of which grew in the lactose broth without producing any visible change apart from the usual turbidity.

A similar study was also carried with 390 non-sheen producing colonies. Of these 54, or 14.1%, proved to be coliform in that they produced acid and gas from lactose and B.G.B. broths. A further 8.5% produced acid in the former medium and the remaining 303 cultures grew but produced no visible change.

The organisms producing acid but no gas in lactose broth were found to be short, gram-negative rods with rounded ends and a tendency to occur singly, although long rod forms in chains were occasionally isolated. In addition, a few gram-positive, paired, diplococci were isolated which produced the same reaction in lactose broth. The gram-negative organisms fermented dextrose rapidly but with no gas

production and grew in B.G.B. broth. Acid was also produced in MacConkey's medium and was demonstrated in lauryl tryptose broth to which brom-cresol-purple had been added. Growth was very sparse in all of these media, but acid production was rapid. The group is a somewhat heterogenous one as indicated by the fact that only some of these organisms could grow upon Levine's E.M.B. agar, while a few produced the characteristic sheen. Organisms tended to die out rapidly, a large proportion of cultures in sugar broths not being viable after incubation at 35°C for two days followed by a similar period at room temperature.

It would appear that although the counts obtained with the membrane filter technique and M-Endo broth MF are very similar to M.P.N. determinations obtained with the conventional tube dilution methods, the medium does not appear to be superior to any of those developed earlier insofar as the isolation of coliform organisms is concerned.

This is due to the fact that only some 65% of sheen-producing colonies growing upon the filter surfaces are in fact coliform, a high proportion of the remainder being capable of producing acid but not gas from lactose.

In view of the close agreement obtained with the two methods of enumeration, it is tempting to disregard this discrepancy, especially as the conclusions concerning the potability of the supplies examined would be the same by both methods of examination. In fact, by the examination of

volumes of water larger than 100 ml. the membrane filter technique can give added assurance that a particular supply is free from contamination.

It should be pointed out that the percentages of the various groups of organisms quoted are not indicative of their distribution in all types of supply but are averages.

In the admittedly small number of supplies examined the acid-producing organisms were associated only with serious contamination. They were found only in samples of sewage-polluted river water and in wells subject to obvious contamination from surface water and which contained large numbers of E.coli. In a number of wells containing only a very few A.aerogenes organisms they were absent, but it is obvious that if the medium is to be considered for routine use, the ecology, distribution, and sanitary significance of these organisms would need to be clearly established.

In conclusion, it is considered that when combined with the membrane filter technique for the enumeration of coli-aerogenes organisms in water, this medium leaves much to be desired. The ideal medium should permit all of the coli-aerogenes cells deposited upon the filter surface to grow out and form colonies, which may be easily distinguished by relatively inexperienced technicians from those of other organisms capable of growing upon the medium. M-Endo broth MF falls far short of this ideal although it is recognized that for certain types of investigation, as, for example,

the routine examination of finished waters, it may, after a period of trial, be found satisfactory and suitable for routine use.

The formula of this medium would indicate that the complex nitrogen sources have been included to counteract the toxic effects of the sodium sulphite-basic fuchsin indicator system to coli-aerogenes organisms. It may be that their inclusion is responsible for the possession of sheen by non-coliform organisms and that the inhibitors used are inadequate to suppress their growth. Presumably an increase in the concentration of the selective materials would lead to an undesirable increase in toxicity to coliform organisms.

It would appear that this differential indicator system relying on the use of basic fuchsin and sodium sulphite is not satisfactory and that fundamental work on the development of other systems for the regulation of colony size and for the selection and differentiation of coliform bacteria growing upon these filter surfaces must be carried out.

TABLE II. The Comparison of the Number of Sheen-Producing Colonies Developing on M-Endo Broth MF Used with the M.F. Technique and the M.P.N. of Coli-Aerogenes Organisms Calculated from the Standard 5-tube Dilution Method.*

M-Endo Broth M.F.			
No. of sheen-producing colonies counted on replicate		Calculated MPN Coli-aerogenes organisms per 100 ml. sample	MPN Coli-aerogenes organisms per 100 ml. sample calculated from the no. of tubes positive in lauryl tryptose broth which subsequently confirmed in 2% B.G.B. broth
A	B		
3	-	0.33	0
0	1	0.5	4.5
2	2	2	2
10	2	6	49
11	16	13	6.8
8	6	14	2
1	2	15	2
26	22	24	49
28	24	26	33
31	31	27	79
30	23	27	23
25	32	28	70
37	30	34	79
33	38	35	79
81	57	69	33
78	71	74	110
10	6	80	33
145	173	159	350
28	31	295	490
20	12	533	140
41	45	860	9,200
18	21	3,900	1,600
150	81	11,550	2,400
88	116	19,125	18,375
74	83	19,750	16,500
37	44	27,700	52,700
52	49	33,700	32,700
54	49	34,300	15,300
68	64	44,000	11,300
67	72	53,000	52,700

*Results are presented for those samples in which coliform organisms were detected by either method.

C. The comparison of the counts of E.coli obtained at 44°C by the membrane filter method with M-Endo broth MF and by using the 5-tube dilution procedure with confirmation of tubes positive in the presumptive test in B.G.B. broth at 44°C. In Britain, the enumeration of E.coli type I in water supplies is carried out in many laboratories as a routine procedure. This is accomplished by sub-culturing positive presumptive tubes of MacConkey's medium at 37°C into tubes of either fresh medium or 2% B.G.B. broth, and incubating these in a water bath at 44±0.5°C for 48 hours (35). This test has been shown in that country to be practically characteristic of E.coli, although infrequently some A.aerogenes strains which can produce gas from lactose at that temperature are encountered.

It is apparent that should the membrane filter technique be found adequate and replace the presumptive standard tube dilution procedure, then an alternative method would be needed to estimate the E.coli content of water supplies. This could probably be achieved by picking colonies directly from the filter surface into either MacConkey's medium or 2% B.G.B. broth, followed by incubation at 44°C. Such a procedure would, however, be extremely laborious and time-consuming, and it was decided to investigate the possibility of incubating inoculated membranes directly upon the M-Endo broth MF medium at 44°C. This was accomplished as previously described. The method used is obviously capable of considerable refinement, but it was felt that if comparative counts

could be obtained and the procedure established, then more consideration could be given to the design of suitable apparatus.

The results obtained are given in Table III. In addition to those presented, a further twenty samples were examined in which no coli-aerogenes organisms could be detected. Great difficulty was experienced in finding wells and lakes polluted with fecal material and containing E.coli in detectable numbers.

The appearance of the filters when incubated at this temperature was markedly different to those incubated at 35°C. Colonies of sheen-producing organisms were smaller, but the most striking difference was in the numbers of "background" colonies which had, except in two instances, completely disappeared. Even in these two cases, the numbers of non-sheen-producing organisms was reduced to negligible proportions, and it was possible for large volumes of sample to be filtered before confluent growth was obtained.

Nineteen samples of well water were examined which contained coli-aerogenes organisms but in which no E.coli could be detected by either method even though volumes of water considerably in excess of 100 ml. were examined by the membrane filter procedure.

In three instances small numbers of E.coli were detected by the tube dilution method, and although 200 ml. of water was examined by the membrane filter procedure, no

sheen-producing colonies were visible upon the filters after incubation.

The remaining ten samples examined gave positive results by both methods. In general the counts obtained by the membrane filter procedure were lower than those calculated from the results of the tube dilution method, but if the 95% confidence limits of the M.P.N. determinations were used in the comparison, only in one sample (*Table III) is the difference significant.

It was decided to carry out cultural studies in order to determine the specificity of both methods for E.coli type I. Colonies were picked as previously described from the filter surfaces and their gram staining and IMViC reactions determined. In the case of the tube dilution method, one tube of the highest positive dilution at 44°C was streaked on Levine's E.M.B. agar and again colonies picked and similarly identified.

Due to the scarcity of supplies containing E.coli these studies were restricted to seven samples of heavily polluted river water and to one sample from a contaminated well.

One hundred and forty-six discrete colonies were picked from membrane filter surfaces to lactose broth and all produced acid and gas in that medium showing them to be coliform. Of these, 106 or 72.6% produced gas in 2% B.G.B. broth in 48 hours at 44±0.5°C, but only 54 or 50.9% of this

total gave the IMViC reactions typical of E.coli type I, the remaining 49.1% being identical to A.aerogenes except for the ability to produce gas at 44°C.

From the tubes of MacConkey's broth confirming at 44°C, only cultures of E.coli type I were obtained. From the results obtained with the membrane filter procedure it might have been expected that orgamisms of the A.aerogenes 44°C positive type would be found. Their absence may be due to the fact that only a small number of tubes were streaked, and these were of the highest positive dilution. In addition, the E.coli tended to predominate and would probably outgrow the A.aerogenes if the two were present together.

A number of isolates from the filters representing 27.4% of the total, did not produce gas when incubated in B.G.B. broth at 44°C for 48 hours and with the exception of two cultures (++--) corresponded to the IMViC classification of A.aerogenes.

Thus it appears that a high proportion of coliform organisms can grow upon this medium under the conditions of incubation described which are not true 44°C positive cultures, and this discrepancy, if found to hold good for all types of water supplies, is considered to be too large for the adoption of this procedure for routine examinations.

Again, as has already been pointed out, false negative results are obtained and in any event the performance of this medium at 37°C is such that there is considerable doubt as to its usefulness in the bacteriological examination of water.

In conclusion, it may be said that the procedure is not satisfactory, the counts obtained being much lower than those calculated from the results of the standard British procedure for the enumeration of E.coli type I in water supplies. This discrepancy is no doubt due to the combined toxic effects of the indicator system, inhibitory agents, and initial high incubation temperature used. It is greater than appears at first sight because it has been shown that of the sheen-producing colonies counted only 72% are capable of producing gas from lactose at 44°C, the remainder being coliform organisms other than E.coli type I. It is considered that these discrepancies are such as to preclude the use of this procedure for the routine enumeration of E.coli type I in water supplies.

TABLE III. The Comparison of the Number of Sheen-Producing Colonies Developing on M-Endo Broth MF Incubated at 44°C Using the M.F. Technique and the Number of E.coli Calculated from the 5-tube Dilution Method with Confirmation at 44°C

M-Endo Broth M.F.			
No. of sheen-producing colonies counted on replicate		Calculated MPN Coli-aerogenes organisms per 100 ml. sample	MPN E.coli per 100 ml. sample calculated from the number of tubes positive in MacConkey's broth at 37°C which subsequently confirmed in 2% BGB broth at 44°C
A	B		
0	0	Absent in 200 ml.	0*
0	0	Absent in 200 ml.	0*
0	0	Absent in 200 ml.	0*
0	0	Absent in 200 ml.	0*
0	0	Absent in 200 ml.	0*
0	0	Absent in 200 ml.	0*
0	0	Absent in 200 ml.	0*
0	0	Absent in 200 ml.	0*
0	0	Absent in 200 ml.	0*
0	0	Absent in 200 ml.	0*
0	0	Absent in 400 ml.	0*
0	0	Absent in 400 ml.	0*
0	0	Absent in 400 ml.	0*
0	0	Absent in 400 ml.	0*
0	0	Absent in 500 ml.	0*
0	-	Absent in 500 ml.	0*
0	-	Absent in 500 ml.	0*
0	-	Absent in 500 ml.	0*
0	-	Absent in 500 ml.	0*
0	0	Absent in 200 ml.	4.5
0	0	Absent in 200 ml.	7.8
0	0	Absent in 200 ml.	6.8
4	5	25	9.2
* 3	3	1.5	41
71	89	80	47
27	36	3,150	11,500
5	9	2,300	12,000
72	60	2,640	13,200
8	14	3,600	14,700
7	13	3,300	14,700
20	13	4,300	15,200
4	1	1,300	18,000

*Samples contained coli-aerogenes organisms as demonstrated by positive presumptive tests at 37°C.

The Present Status of the Membrane Filter Procedure in the Bacteriological Examination of Water

In Britain at the present time the membrane filter procedure has no official standing in the enumeration of coliform organisms in water and is not recommended for this purpose.

In America, where nearly all of the work done in connection with the development of this system of analysis for the bacteriological examination of water has been carried out, the mechanics of the filtration procedure have become well established and are regarded as being satisfactory. As has been indicated, a great variety of media have been developed and used for the growth, recognition, and enumeration of the coliform group of organisms. By far the most attention has been given to modifications of the Endo type of medium which utilizes basic fuchsin and sodium sulphite as an indicator system for the recognition of coliform colonies. It has become apparent that this indicator is toxic to attenuated coliform cells and is not by itself a suitable inhibitor of non-coliform organisms. Other materials such as brilliant green, bile salts, sodium desoxycholate, and sodium lauryl sulphate have been included in most formulae for this purpose; and, in an effort to overcome their toxicity to coliform organisms, complex sources of vitamins, amino-acids, and micronutrients have been supplied. This type of medium would appear to have reached the peak of its development in

the M-Endo MF broth described and which has been shown to be unsatisfactory for the examination of heavily polluted natural water supplies.

In the Tenth Edition of Standard Methods for the Examination of Water, Sewage and Industrial Wastes, a filtration procedure and a medium (EHC Endo broth) are described for use in the enumeration of coliform bacteria in water. It is unfortunate that the conditions under which it may or may not be used are not clearly defined since while it is stated that "It must be understood that this (the membrane filter procedure) is in no way a standard technique, and it cannot be considered an acceptable substitute for the tube dilution method," the next sentence reads, "When the limitations of the test are fully recognized and the difficulties of the interpretation of the results are known, the technique may be used."

In a more recent memorandum the subcommittee dealing with this section have attempted to clarify the position. The advantages of the procedure are described and the limitations discussed. The subcommittee in conclusion considers that "For the applications discussed above (the testing of water potability) and only when used with full recognition of the limitations noted, the membrane filter technique as given in the Tenth Edition of Standard Methods, or its demonstrated equivalent will yield results comparable to those of the Standard Completed Test for determining the presence of

coliform bacteria in potable waters." In addition, it is also stated that "In no instance should the membrane filter method be adopted for testing water potability until adequate parallel testing of duplicate samples by the membrane filter technique in comparison with the standard completed test has demonstrated beyond reasonable doubt the relationship of the results obtained by the two methods."

It is apparent that the official status of the procedure is by no means clearly defined. It is felt that the obvious fundamental advantages inherent in the procedure have been so great as to obscure the fact that without the development of a suitable medium for the growth, recognition, and identification of coli-aerogenes organisms, the results obtained with it are valueless.

The position is made more serious by the fact that the manufacturers of the necessary equipment and media have given the method a great deal of publicity, and media are now available and are being sold for use in this respect which are not supported by any published data. A new word has been coined for their application, the label on one bottle indicating that the contents are "for use in the detection and quantitation of coliform organisms." Presumably this is not the same thing as quantitative estimation.

It is the opinion of the author that none of the media developed so far are suitable for use with the membrane filter procedure for the enumeration of coliform organisms in

all types of water supplies. It is felt that a different approach is required to the problem and that a re-examination of the growth requirements of the coli-aerogenes group, together with information concerning the biology of the organisms associated with them in water supplies, is required, and may lead to the development of a different type of selective medium based rather upon nutritional requirements than upon the use of inhibitory materials.

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