A FIELD PROCEDURE FOR DETECTING
POLLUTION OF WATER USING GRAM-NEGATIVE
LACTOSE FERMENTING BACTERIA

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
Herbert S. Wright
1962

ABSTRACT

A FIELD PROCEDURE FOR THE DETECTION OF FECAL POLLUTION OF WATER USING GRAM-NEGATIVE LACTOSE FERMENTING BACTERIA

By Herbert S. Wright

A new field method for the detection of gram-negative lactose fermenting bacteria indicative of sewage pollution has been proposed. The formula for the medium follows:

| | | |
|---|---|---|
| Lactose | 5.000 | g/l |
| Tryptose | 20.000 | g/l |
| NaCl | 5.000 | g/l |
| $K_2HPO_4$ | 2.750 | g/l |
| $KH_2PO_4$ | 2.750 | g/l |
| Sodium lauryl sulfate | 0.100 | g/l |
| Brom cresol purple | 0.015 | g/l |

Coliform and pseudomonas-like bacteria of fecal origin are detected by acid production from lactose at 35 C. Quantitatively more coliform bacteria are detected than by standard procedures. The method is a one-step procedure and can be completed without the use of a laboratory. A 35 ± 0.5 C incubator is the only laboratory equipment needed. A positive test is indicated by a change in color of the medium at the end of 48 hr incubation.

A sterile dehydrated medium is placed in sterile calibrated screw-cap test tubes. For convenience the medium can be prepared in sterile tablets. The water to be tested is collected in the tubes at the site.

# A FIELD PROCEDURE FOR THE DETECTION OF FECAL POLLUTION OF WATER USING GRAM-NEGATIVE LACTOSE FERMENTING BACTERIA

by

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1962

ACKNOWLEDGEMENT

The author wishes to express his appreciation to Dr. W.L. Mallmann for his interest, guidance and counsel throughout the investigation and preparation of this manuscript.

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# INTRODUCTION

There is a need for a simple field test for the examination of water to determine its sanitary quality. It has been reported by many authors that the number of culturable coliform organisms decreased with aging of a water sample, therefore a test at the site would be desirable. A test of this kind should be used without the benefit of a laboratory, other than a 35 ± 0.5 C incubator and the services of a trained bacteriologist.

The present standard methods of testing water using the multiple tube dilution method are wholly unadaptable for use in the field. The tube containing liquid medium would be difficult to transport and the use of pipettes for inoculating the tubes with measured quantities of water would be impractical. After incubation any attempt at confirmation of the positive cultures would be impossible without a fully equipped laboratory. If a one-step presumptive medium (no confirmation required) could be found in which a positive test is based on gas production, the inclusion of fermentation vials would not be practical for use as a field test.

Another limitation which has been given increasing attention in recent years is the failure of standard methods to detect many of the atypical coliform organisms, which includes many slow lactose fermenting bacteria.

The purpose of this investigation was to develop a method whereby a dehydrated medium would be hydrated with the water of the sample at the site. It was desired that the method be a one-step procedure which would not require the use of a confirmation medium, as is now required by Standard Methods for Examination of Water and Wastewater (APHA 1960). This could be accomplished by the use of a medium containing a carbohydrate in which acid production signifies the presence of coliform organisms.

This new procedure would necessitate a change in the definition of coliform bacteria from the one now stated in the 11th edition of Standard Methods for the Examination of Water and Wastewater (APHA 1960), so the coliform group would include all of the aerobic and facultative anaerobic gram-negative, nonspore-forming, rod-shaped bacteria which ferment a selective carbohydrate (lactose) with acid formation within 48 hr at 35 C.

## LITERATURE REVIEW

Escherichia coli was first isolated from a cholera patient and characterized by Escherich (1885). Subsequently, it was found to be a normal inhabitant of the intestinal tract of man and many other animals and occurred regularly in their excreta. Later, Aerobacter aerogenes, a soil organism, was also demonstrated in feces. Subsequently, they were found to be normal inhabitants of the intestinal tract of man and many animals, and occurred regularly in their excreta.

These two microorganisms along with a closely related group were designated in Standard Methods for the Examination of Water and Sewage, ninth edition, (APHA 1946) as the coliform group. "The coliform group includes all of the aerobic and facultative anaerobic, Gram-negative, nonspore-forming, rod-shaped bacteria which ferment lactose with gas formation within 48 hr at 35 C" (APHA 1960).

The methods of estimation of the density of these bacteria have had a long slow progressive evolution from earlier methods to the standard methods of today.

Mathews (1893) introduced litmus lactose agar pour plates as a method of estimating the number of coliform bacteria in water. The coliform bacteria fermented the lactose with the production of acid, causing the colonies to develop as red spots in a blue field. It was necessary to pick the isolated colonies for identification as there

was no inhibitor included to exclude non-coliform bacteria which fermented lactose.

Smith (1893) suggested the use of a favorable liquid glucose medium for preliminary cultivation for a period of 24 to 48 hr at 37 C. Using this means a comparatively large quantity of sample could be conveniently examined. This method could be easily adapted to quantitative procedures by inoculation of tubes with measured quantities of water.

Irons (1901) in a comparative study of various methods for the isolation of coliform bacteria, reported that the preliminary broth enrichment method frequently yielded positive isolations when results from direct plating in agar were negative.

Phelps and Hammond (1909) and others reported that organisms fermenting glucose, but not lactose, are fairly abundant in relatively pure water. It was then obvious that many bacteria of little or no sanitary significance were able to ferment glucose, but not lactose. Consequently, the more selective lactose was gradually substituted for glucose in media employed for presumptive testing of water. The 2nd edition of Standard Methods for the Examination of Water and Sewage (APHA 1913) recommended the use of one per cent lactose in a medium containing peptone and ten per cent oxgall.

The use of inhibitory agents in the presumptive medium was first introduced by MacConkey (1900) when he used bile salts to differentiate E. coli and Salmonella typhosa.

MacConkey (1901) and MacConkey and Hill (1901) employed similar media for detection of fecal contamination. Since then, bile salts have remained an essential ingredient of the enrichment, or primary medium used in Great Britain for detection of coliform bacteria in water, sewage and other materials. Jackson (1906) recommended the use of ox bile in nutrient medium in this country. He suggested the inclusion of the medium containing oxgall in the 2nd edition of Standard Methods for the Examination of Water and Sewage (APHA 1913). Jackson (1906) thought that all E. coli giving a positive test in lactose bile broth represented recent pollution. However, Jordan (1913) found that lactose bile broth inhibited a large proportion of the culturable cells and freshly isolated cultures were inhibited as well as the old cultures. Cumming (1916) reported that with sewage, lactose bile broth yielded only 25 per cent as many positive results as lactose broth, whereas with river water the proportion was increased to 50 to 70 per cent. In the 3rd edition of Standard Methods for the Examination of Water and Sewage (APHA 1923) lactose broth without oxgall was the recommended presumptive medium.

A further change in the Standard Methods for the Examination of Water and Sewage (APHA 1923) was the reduction of lactose from 1 per cent to 0.5 per cent as recommended by Burling and Levine (1918).

Levine (1922) found that 1 to 2 per cent dried bile inhibited most spore-formers, both aerobic and anerobic,

and accelerated the growth of coliform bacteria. He found that higher concentrations were distinctly inhibitory to the coliform group. He also emphasized that much of the confusion that existed in the earlier years regarding the value of bile enrichment media was no doubt due to the variation in the proportion of bile used by different observers.

Muer and Harris (1920) recommended the use of a brilliant green bile lactose broth. They claimed that the bile would almost completely inhibit the aerobic spore-formers and brilliant green would inhibit the anerobic spore-formers. Dunham, McCrady and Jordan (1925) confirmed their claims, although the medium appeared to inhibit the growth of some coliform organisms.

When brilliant green lactose bile broth was compared with lactose broth (Butterfield 1933 and Parr and Caldwell 1933) a decrease of from 23 to 66 per cent in the coliform isolations was noted. It was finally realized that the brilliant green lactose bile was not generally acceptable as a substitute for lactose broth as a standard presumptive medium. Cowles (1938) recommended the use of standard lactose broth with 0.2 g sodium lauryl sulfate per liter. This material prevented the growth of gram-positive bacteria including lactose fermenting aerobes and anerobes, while the gram-negative organisms are in general able to develop in the presence of relatively high concentrations.

Darby and Mallmann (1939) reported the results of studies on enrichment media for coliform bacteria from water and other sources, using organisms that were attenuated or appeared in minimal numbers. Previous to this time little effort had been made to determine the efficiency of media or to develop enrichment media. They suggested the following medium for use in the detection of coliform organisms:

| | | |
|---|---|---|
| Tryptose | 2.00 | per cent |
| Lactose | 0.50 | per cent |
| $K_2HPO_4$ | 0.40 | per cent |
| $KH_2PO_4$ | 0.15 | per cent |
| NaCl | 0.50 | per cent |

Mallmann and Darby (1941) incorporated sodium lauryl sulfate in the above medium. This medium, designated lauryl tryptose broth (LTB), is used as a presumptive medium for the isolation of coliform bacteria. They reported that the medium inhibited gram-positive bacteria, while allowing the coliform bacteria to grow uninhibited. McCrady(1944), Levine (1941), and Hajna and Perry (1943) reported that LTB was superior to the standard lactose broth as a presumptive enrichment medium, fewer false positive cultures were obtained and more coliform bacteria that fermented lactose slowly were detected.

Perry and Hajna (1944) used a base medium proposed by Darby and Mallmann (1939) in which they included bile salts no. 3. It proved to be an excellent enrichment medium for the growth of coliform bacteria. It inhibited fecal streptococci and other gram-positive bacteria almost completely

without any apparent inhibition of coliform bacteria.

McCurdy (1955) used a medium similar to LTB, except that dextrose was substituted for lactose with one per cent Bacto bile salts. He reported that the medium permitted the detection of more coliform bacteria than the standard lactose broth, and that nothing but coliform bacteria gave a positive test. A positive test was the production of acid as detected by the use of an acid-base indicator, brom cresol purple.

Many workers have reported the existence of coliform organisms called paracolon bacilli which ferment lactose without gas formation or produce it slowly. Gilbert and Lion (1893) were first to report paracolon bacilli. Morgan and Ledingham (1909) encountered such types in human feces. These organisms were isolated from numerous cases of diarrhea. Gyorgy (1920) suggested that they played a large role in diarrhea in both man and animals.

Stuart, Mickle and Borman (1940) proposed the separation of atypical coliform bacteria into four groups based on a study of more than 10,000 cultures isolated from water, soil, milk and other sources. They proposed the term "aberrant coliforms" for all gram-negative nonspore-forming rods which fermented lactose slowly or weakly at 37 C. Stuart, Wheeler and Zimmerman (1943) presented a study of the biochemical and antigenic relationships of coliform bacteria. They suggested a single coliform genus which would include

three species; E. freundii, A. aerogenes and E. coli. Within this group would be included all aberrant coliform and paracolon bacteria. Non-gas-producing cultures fermenting lactose rapidly, slowly or not at all would be grouped as anaerogenic A. aerogenes, E. freundii, E. coli or collectively as anaerogenic paracolon.

Stuart, Mickle and Borman (1940) stated that the micro-aerogenic coliform bacteria probably have significance in water analysis similar to that of Aerobacter and Escherichia organisms showing typical lactose fermentation.

Plass (1947) investigated an outbreak of diarrhea attributed to fricasseed chicken. He was able to isolate paracolon bacteria broth from the fricasseed chicken containers (the fricasseed chicken was unavailable) and from the patients.

Barnes and Cherry (1946) reported paracolon bacteria as the cause of an epidemic of diarrhea in a United States Naval Hospital.

In an area highly endemic for enteric infections, Christensen (1947) studied the distribution and possible pathogenicity of Paracolobacterum species. These organisms were isolated from 60 per cent of the gastroenteritis cases as compared to 20 per cent of the healthy individuals examined. These findings were considered to be at least indicative of pathogenicity. Paracolobacterum species were found much more frequently in cases of gasterenteritis than salmonellae or shigellae.

Ziegler (1937) studied the bacteriology of an epidemic of diarrhea. He isolated disease producing late lactose and non-lactose fermenting organisms from polluted water supplies and from diarrheal patients. During the latter part of the epidemic no typical E. coli were isolated, but late and non-lactose fermenting disease organisms were present.

Kriebel (1936) isolated 60 different cultures of non-lactose fermenting organisms which she was able to develop into lactose fermenters, or they fermented sucrose, gave positive indol tests, or did both. Based on these results she included them as coliform bacteria. She also described the dissociation of 19 different cultures of E. coli into permanently non-lactose fermenting variants of which 13 were cultures originating from single cells. Kriebel suggested that the late lactose or non-lactose fermenters must be regarded as atypical coliform bacteria.

Recently it has been suggested by Schiavone and Passerini (1957) that Pseudomonas aeruginosa, be included as indicators of pollution. Schiavone and Passerini (1957) reported that pseudomonads may have an important part in water-borne epidemics of gasteroenteritis. They may also cause an inhibitory influence on the coliform group which might lead to errors in reporting the safety of a water supply.

Caldwell and Parr (1933) reported a comparison of the recovery of colon group bacteria from iced and uniced samples examined at varying periods. In bottled samples of contaminated

water the death of *E. coli* occurred and became progressively greater as the period of holding increased. Icing during storage offered a distinct advantage. Direct inoculation of the water was found to be practical and showed marked superiority over taking the water to a laboratory for inoculation of a medium.

Noble and Gullans (1955) reported a decrease of 35 to 97 per cent in the number of culturable organisms in water samples that were held at 5-6 C for 18 hr. Hedrick *et al.* (1960) reported that there was a marked decrease in the number of coliform bacteria surviving for 6 to 24 hr in samples of lake water at 21 C. Mallmann (1962) stated that the number of coliform organisms recoverable from samples of Red Cedar River water stored for 6 to 24 hr at 4.5 C was considerably lower than the number originally in the sample.

Standard Methods for the Examination of Water and Wastewater (1960) states, "The bacteriologic examination of water samples ought to be initiated immediately after collection. However, such a requirement is seldom practical, and more realistic ones must be established. It is therefore recommended that the technical procedures should be started preferably within 1 hr after collection; the time elapsing between collection and examination should in no case exceed 24 hr. When local conditions necessitate delays in receipt of sample longer than 24 hr, consideration should be given

to providing for a field examination of such samples, by making use, for example, of the membrane filter technique or of temporary laboratory facilities at the site".

Mallmann and Peabody (1961) stated that the membrane filter technique yielded lower coliform indices than the multiple tube dilution procedure. They also stated that when the filter procedure was used for testing marginal water, results were particularly poor in that the membrane filter technique failed to detect many of the coliform organisms.

## MATERIALS AND METHODS

For Experiment I dextrose lauryl bile broth (DLBB) and lauryl tryptose broth (LTB) were compared to determine which gave the higher MPN and per cent confirmation. The MPN for each medium was determined for each sample. They were compared on the basis of 95 per cent confidence limits. DLBB in a dehydrated form was inoculated at the site of the water to be tested. The LTB was inoculated in the laboratory using water collected at the site in standard water sample bottles.

DLBB contained the following ingredients:

| | | |
|---|---|---|
| Tryptose | 20.000 | g/l |
| Dextrose | 5.000 | g/l |
| Sodium lauryl sulfate | 0.100 | g/l |
| $K_2HPO_4$ | 2.750 | g/l |
| $KH_2PO_4$ | 2.750 | g/l |
| Bile salts | 10.000 | g/l |
| NaCl | 5.000 | g/l |
| Brom cresol purple | 0.015 | g/l |

final pH was 6.8

For Experiment II a surface plating technique (drop-plate) was used to compare lactose and dextrose as selective agents for acid production to detect coliform bacteria. The total number of colonies, number of yellow colonies and the per cent of yellow colonies transferred to LTB that fermented lactose with acid or acid and gas formation were determined.

The drop-plates were inoculated with 0.2 ml pipettes graduated in 0.01 ml volume. The amount of inoculum varies from 0.01 to 0.2 ml and one to ten inocula were placed on each plate. The inoculum was delivered by touching the surface of the agar lightly.

The media for the surface plating technique contained the following ingredients:

| | | |
|---|---|---|
| Dextrose or lactose | 5.000 | g/l |
| Tryptose | 20.000 | g/l |
| Bile salts no. 3 | 1.500 | g/l |
| NaCl | 5.000 | g/l |
| Sodium lauryl sulfate | 0.100 | g/l |
| Brom cresol purple | 0.015 | g/l |
| Agar | 15.000 | g/l |

pH adjusted to 6.8

For Experiment III lactose lauryl bile broth (LLBB) and LTB were compared to determine which gave higher MPN and percentage confirmation for coliform bacteria. The MPN for each medium was determined for each sample. LLBB in a dehydrated form was inoculated at the site of the water to be tested. The LTB was inoculated in the laboratory with water collected at the site in standard water sample bottles.

LLBB contained the following ingredients:

| | | |
|---|---|---|
| Tryptose | 20.000 | g/l |
| Lactose | 5.000 | g/l |
| Sodium lauryl sulfate | 0.100 | g/l |
| $K_2HPO_4$ | 2.750 | g/l |
| $KH_2PO_4$ | 2.750 | g/l |
| Bile salts no. 3 | 1.500 | g/l |
| NaCl | 5.000 | g/l |
| Brom cresol purple | 0.015 | g/l |

final pH was 6.8

For Experiments IV and V, LTB was used with the addition of an acid-base indicator. Acid and gas production versus acid production only were compared for the determination of the presence of coliform bacteria. The medium was in liquid form when it was inoculated in the laboratory with the water sample.

LTB used in these experiments had the following ingredients:

| | | |
|---|---|---|
| Tryptose | 20.000 | g/l |
| Lactose | 5.000 | g/l |
| $K_2HPO_4$ | 2.750 | g/l |
| $KH_2PO_4$ | 2.750 | g/l |
| NaCl | 5.000 | g/l |
| Sodium lauryl sulfate | 0.100 | g/l |
| Brom cresol purple or brom thymol blue | 0.015 | g/l |

In Experiments I and III the dehydrated media were prepared by mixing and grinding the proper proportions of each of the dry ingredients with a mortar and pestle. Each batch consisted of at least enough of the dry ingredients to make five liters of single strength medium in order that the resulting mixture would be uniform. The powder was then passed through a cheese cloth to remove any lumps that remained. These lumps were ground and mixed with the entire batch of medium.

For convenience the media were made into tablets with an Eureka tablet machine[1]. Enough tablets were added to each tube to be equivalent of the dehydrated medium required for one tube of single strength medium after hydration. It was necessary to prepare, press, package and store the tablets in an atmosphere of low relative humidity as they were very hygroscopic.

The packaging material was 450 MSBO cellophane[2]. The cellophane was sealed with a heat sealing iron. The tablets

[1] Manufactured by F.U. Stokes Machine Company, Philadelphia, Pennsylvania.

[2] Manufactured by Sylvania Division, American Viscose Corporation, Philadelphia, Pennsylvania.

were then sterilized by beta irradiation at the level of 200,000 rep on each side using a General Electric one million volt resonance transformer electron beam machine[3]. The tablets were aseptically removed from the cellophane packages and placed into the calibrated sterile screw-cap test tubes.

The water for the studies with the dehydrated media was collected directly in the test tubes (25 mm x 150 mm screw-cap with teflon liners) calibrated for 10 ml volume. Water (from the same source as above) was collected in standard water sample bottles (125 ml wide-mouth bottles with ground glass stoppers) for the multiple tube dilution (MTD) and surface-plating procedure. The liquid media were inoculated in the laboratory as soon as possible after collection (one to four hr).

All media, in test tubes or Petri dishes, were incubated at 35 ± 0.5 C. The pH reaction of the liquid media and gas production were observed at 24 and 48 hr ± 2 hr. The number of colonies on the drop-plates was counted at the end of 16 to 18 hr.

Cultures from the test tubes with acid or acid and gas production were confirmed as coliform bacteria by streaking the culture on eosin methylene blue agar (EMB) or inoculating brilliant green lactose bile broth (BGB). Colonies that did not produce sheen on EMB were transferred to LTB to determine if they would ferment lactose. Inocula from the test tubes

[3] Manufactured by General Electric Company, Syracuse, New York.

of BGB that did not confirm (no gas production) were streaked onto EMB and tergitol-7 agar. Both typical and atypical colonies were transferred to LTB. Those cultures that did not ferment lactose with the formation of gas were streaked onto nutrient agar in test tubes for storage. At a later date the cultures were transferred every 24 hr for ten days, using purple broth base plus 0.5 per cent lactose to determine if lactose fermentation would occur.

For Experiments IV and V acid production and gas production were compared for the detection of coliform bacteria. Cultures that did not confirm as coliform bacteria by the above procedures were tested for oxidase using the method of Kovacs (1956). He recommended the use of a 1 per cent tetramethyl-p-phenylenediamine. Most of the pseudomonads have a high concentration of cytochrome oxidase within the cell (Gaby and Hadley 1957), thereby causing them to give a positive test. Flagella stains were made of the cultures giving a positive oxidase reaction to determine the flagella arrangement.

In each experiment using two liquid media the results were compared with the use of 95 per cent confidence limits. The 95 per cent confidence limits allows for the discrepancies which result when five tubes are inoculated per dilution for each medium. For example, in water containing one bacterium per ml 63 per cent of the tubes, inoculated with 1 ml, could

be expected to yield positive results due to the irregularity in distribution of the bacteria in the sample. The same phenomonon can be expected with the 10 ml sample when there is one bacterium in this volume. In other words, the chances of a negative culture which should be positive or visa versa for the purposes of comparing different media or a series of samples are taken into consideration. The range included in the 95 per cent confidence limits for a five-tube multiple dilution test is approximately 24 to 324 per cent of the indicated MPN (Woodward 1957).

# RESULTS

The results of a comparison of dextrose lauryl bile broth (DLBB) and LTB are listed in Table 1. Of 105 samples of water, 62 were positive for coliform organisms on the presumptive tests and seven failed to confirm on eosin methylene blue agar. Only one positive presumptive test on LTB failed to confirm whereas nine positive presumptive tests on DLBB failed. Thus the acid produced from dextrose in the presence of bile salts was due to organisms other than coliform bacteria in these nine samples. Actually five of these samples showed the presence of coliform organisms in LTB, indicating that the coliform organisms had been inhibited from growing, presumably by the bile salts. The inhibitory effect of the DLBB was demonstrated also by the lower indices of coliform organisms obtained. Four samples of water with higher indices of coliform organisms were obtained with DLBB whereas eight were higher with LTB.

A comparison was also made of the DLBB and the control medium (LTB) on an individual tube basis. In Table 2 is listed the number of tubes that indicated the presence of coliform bacteria for the presumptive tests, the number of tubes that confirmed (coliform organisms present) and the per cent confirmation. In 99.1 per cent of the cases the cultures in the tubes of LTB confirmed while only 81.3 per cent of the cultures in the tubes of DLBB confirmed for the presence of coliform organisms.

Table 1 Quantitative comparison of dextrose lauryl bile broth and lauryl tryptose broth for the detection of coliform bacteria using 95 per cent confidence limits.

| | Number presumptive samples | Number confirm samples | Number failing to confirm |
|---|---|---|---|
| Total tested | 105 | 105 | - |
| Total positive | 62 | 55 | - |
| DLBB with higher MPN | 13 | 4 | - |
| LTB with higher MPN | 5 | 8 | - |
| DLBB failed to confirm | -- | -- | 9 |
| LTB failed to confirm | -- | -- | 1 |

Table 2 The comparison of the number of positive tubes for dextrose lauryl bile broth and lauryl tryptose broth.

| | MEDIA | |
|---|---|---|
| | LTB | DLBB |
| Total tubes planted | 515 | 515 |
| Presumptive tubes positive | 220 | 230 |
| Number of tubes confirming | 218 | 187 |
| Per cent confirmation | 99.1 | 81.3 |

The results of Experiment I indicate two possible sources of error, namely the non-specificity of dextrose and the inhibitory action of bile salts on coliform organisms. In Experiment II tests were made to evaluate the specificity of dextrose and lactose in the presence of varying concentrations of bile salts no. 3 using a surface plating technique. Brom cresol purple was included as an indicator so the coliform bacteria colonies on these media would be yellow due to the acid formed.

When river water was used only half as many colonies fermented lactose with acid production as colonies fermented dextrose with acid production (Table 3). However, when the colonies were transferred to LTB for the determination of acid and gas production from lactose (at this time it was believed that the colonies producing acid only when transferred to lactose fermentation tubes were attenuated coliform bacteria), 24 to 34 per cent of the colonies from dextrose agar failed to confirm as coliform bacteria whereas only one to four per cent of the colonies from lactose agar failed. The 0.50 per cent concentration of bile salts failed to inhibit many more non-coliform bacteria than the lower concentration (0.15 per cent). Also the higher concentration of bile salts inhibited some of the coliform present. For this reason comparisons were made in a second series of tests using dextrose and lactose for four successive weeks with only 0.15 per cent bile salts with river water (Table 4).

Table 3 Quantitative comparison of dextrose and lactose in agar with varying concentrations of bile salts no. 3 using surface plating technique for river water.

Dextrose

| | Bile concentration (per cent) | | |
|---|---|---|---|
| | 0.00 | 0.15 | 0.50 |
| Total colonies | 956* | 877 | 609 |
| Total yellow colonies | --- | 460 | 253 |
| Reaction in LTB | | | |
| Acid and gas | | 32** | 56 |
| Acid only | | 34 | 20 |
| Growth only | | 34 | 23 |
| No growth | | 0 | 1 |

Lactose

| | Bile concentration (per cent) | | |
|---|---|---|---|
| | 0.00 | 0.15 | 0.50 |
| Total colonies | 1283* | 1074 | 848 |
| Total yellow colonies | ---- | 221 | 182 |
| Reaction in LTB | | | |
| Acid and gas | | 53** | 55 |
| Acid only | | 46 | 41 |
| Growth only | | 1 | 3 |
| No growth | | 0 | 1 |

* Number of organisms per ml of sample.

** Per cent of colonies giving reaction based on 100 colonies per medium.

The dextrose medium did not inhibit the non-coliform bacteria as 26 to 42 per cent of the colonies did not confirm. However with lactose only one to five per cent of the colonies did not confirm as coliform bacteria.

A third series of tests was made using sewage to determine whether the acid non-gas producing colonies from river water were found in sewage too (Table 5). If they were found in sewage they were not necessarily attenuated coliform bacteria. The tests were made also to determine whether colonies from dextrose agar would have a better percentage confirmation. If the colonies that produced acid only on lactose were found in sewage as well as the contaminated river water they could be indicative of pollution. When sewage was used the total number of bacteria per ml was similar for both lactose and dextrose media in which bile salts was included, but the number of yellow colonies was greater for the dextrose medium. However, it must be noted that there was a greater per cent confirmation of yellow colonies with the lactose agar and that all of the colonies transferred to LTB produced acid or acid and gas. The studies with sewage and river water gave similar results. Attenuated-like coliform bacteria were found in sewage, but a lesser percentage of these colonies were found on lactose agar. Also some acid producing colonies from dextrose agar would not ferment lactose with acid production. Thus it was apparent that the lactose would be more selective. The 0.50 per cent bile

Table 5 Quantitative comparison of dextrose and lactose in agar with varying concentrations of bile salts no. 3 using surface plating technique with sewage.

Dextrose

| | Bile concentration (per cent) | | |
|---|---|---|---|
| | 0.00 | 0.15 | 0.50 |
| Total colonies | $1.4 \times 10^6$* | $4.1 \times 10^5$ | $1.8 \times 10^5$ |
| Total yellow colonies | ----- | $1.5 \times 10^5$ | $9.0 \times 10^4$ |
| Reaction in LTB | | | |
| Acid and gas | | 80** | 84 |
| Acid only | | 8 | 7 |
| Growth only | | 12 | 9 |
| No growth | | 0 | 0 |

Lactose

| | Bile concentration (per cent) | | |
|---|---|---|---|
| | 0.00 | 0.15 | 0.50 |
| Total colonies | $2.7 \times 10^5$* | $3.3 \times 10^5$ | $1.7 \times 10^5$ |
| Total yellow colonies | ----- | $7.6 \times 10^4$ | $4.3 \times 10^4$ |
| Reaction in LTB | | | |
| Acid and gas | | 90** | 96 |
| Acid only | | 10 | 4 |
| Growth only | | 0 | 0 |
| No growth | | 0 | 0 |

* Numberof organisms per ml of sample.

** Per cent of colonies giving reaction based on 100 colonies per medium.

salts in the lactose agar was inhibiting some of the coliform bacteria present, since only about 50 per cent as many coliform bacteria were able to grow in it.

Thus in Experiment III lactose lauryl bile broth with 0.15 per cent bile salts no. 3 (LLBB) and LTB were compared. The results of this experiment are listed in Table 6. Of 133 samples of water, 97 were positive for coliform organisms on the presumptive tests and five failed to confirm on eosin methylene blue agar. All of the positive presumptive tests for LTB confirmed whereas five positive presumptive tests on LLBB failed. Thus organisms other than coliform bacteria produced acid from lactose in the presence of bile salts in these five samples. Actually four of these samples showed the presence of coliform organisms in LTB, indicating that the coliform organisms were inhibited from growing, presumably by the bile salts. The inhibitory effect of LLBB was demonstrated also by the lower indices of coliform organisms obtained. Eleven samples of water gave higher indices of coliform organisms with LLBB whereas 37 were higher for LTB.

Table 7 lists the number of tubes inoculated for each of the two media, the number of presumptive positive cultures for coliform bacteria and the percentage of cultures confirming. There was a considerably greater percentage of the cultures from LTB (92 per cent) confirming than with LLBB (73 per cent).

Table 6 Quantitative comparison of lactose lauryl bile broth and lauryl tryptose broth for the detection of coliform bacteria using 95 per cent confidence limits.

| | Number presumptive samples | Number confirmed samples | Number samples failing to confirm |
|---|---|---|---|
| Total tested | 133 | 133 | - |
| Total positive | 97 | 94 | - |
| LLBB with higher MPN | 13 | 11 | - |
| LTB with higher MPN | 34 | 37 | - |
| LLBB failed to confirm | -- | -- | 5 |
| LTB failed to confirm | -- | -- | 0 |

Table 7 Comparison of the number of positive tubes for lactose lauryl bile broth and lauryl tryptose broth.

| | Media | |
|---|---|---|
| | LTB | LLBB |
| Total tubes planted | 990 | 990 |
| Total presumptive positive | 547 | 570 |
| Total number of tubes confirming | 503 | 416 |
| Per cent confirmation | 92 | 73 |

From the results of Experiment III it can be determined that bile salts no. 3 was not inhibiting the gram-negative non-coliform bacteria that fermented lactose with acid formation. Also the bile salts was inhibitory to the coliform bacteria. Therefore in Experiments IV and V tests were made to evaluate LTB with an acid-base indicator for determination of acid production and Durham tubes were placed in the tubes to determine gas production.

The results of Experiment IV are listed in Tables 8 and 9. The comparisons were made using LTB with brom cresol purple (BCP) as an indicator for the determination of acid production. Of the 181 samples of water tested, 110 samples were positive for coliform organisms on the presumptive tests and six failed to confirm. Three positive presumptive tests failed to confirm as coliform bacteria with gas production whereas five positive presumptive tests failed with acid production. Using gas production, 5 samples failed to detect coliform bacteria when acid production was positive. For only one sample did acid production fail and the gas production detected coliform bacteria. However, when the tubes that contained oxidase positive pseudomonas-like bacteria were included as confirmed cultures as well as the coliform organisms, 90 per cent of the individual cultures of acid production confirmed. Using acid production for testing water, with coliform and pseudomonas-like bacteria as indicators, 110 samples were positive for presumptive tests

Table 8 Quantitative comparison of acid versus gas production in lauryl tryptose broth with brom cresol purple for the determination of coliform bacteria.

| | Number presumptive samples | Number confirmed samples | Number samples failing |
|---|---|---|---|
| Total tested | 181 | 181 | - |
| Total positive | 110 | 104 | - |
| Gas production with higher MPN | 4 | 6 | - |
| Acid production with higher MPN | 46 | 22 | - |
| Gas production failing to confirm | -- | -- | 3 |
| Acid production failing to confirm | -- | -- | 5 |

Table 9 Quantitative comparison of acid versus gas production in lauryl tryptose broth with brom cresol purple for the determination of gram-negative lactose fermenting bacteria.

| | Number presumptive samples | Number confirmed samples | Number samples failing to confirm |
|---|---|---|---|
| Total tested | 181 | 181 | - |
| Total positive | 110 | 105 | - |
| Gas production with higher MPN | 4 | 4 | - |
| Acid production with higher MPN | 46 | 52 | - |
| Gas production failing to confirm | -- | -- | 3 |
| Acid production failing to confirm | -- | -- | 2 |

and five failed to confirm. Three positive presumptive tests failed to confirm for gas production whereas five samples failed for acid production.

Six samples had higher MPN for gas production than acid production at the end of 48 hr with BCP indicator. This phenomenon could be explained by the fact that either not enough acid was produced to change the color of the indicator or the acid was no longer present. For this reason brom thymol blue (BTB) was tried in Experiment V to detect the acid produced during the fermentation of lactose as it has a higher pK value.

The results of Experiment V are listed in tables 10 and 11. The comparisons were made using LTB with BTB for determination of acid production and Durham tubes were placed into the medium for the collection of gas produced. Of the 157 samples tested, 112 samples were positive for coliform organisms on the presumptive tests and 22 failed to confirm. Eight positive presumptive tests failed to confirm as coliform bacteria with gas production whereas 19 positive presumptive tests failed with acid production. Using gas production, five samples failed to detect coliform bacteria when acid was positive. For one sample acid production failed and the gas production detected coliform organisms. Acid production was compared with gas production on an individual tube basis (Table 12). Only 83 per cent of the cultures for acid production that were positive confirmed while 90 per cent

Table 10 Quantitative comparison of acid versus gas production in lauryl tryptose broth with brom thymol blue for determination of coliform bacteria.

| | Number presumptive samples | Number confirmed samples | Number samples failing to confirm |
|---|---|---|---|
| Total tested | 157 | 157 | - |
| Total positive | 112 | 90 | - |
| Gas production with higher MPN | 5 | 3 | - |
| Acid production with higher MPN | 42 | 17 | - |
| Gas production failing to confirm | -- | -- | 8 |
| Acid production failing to confirm | -- | -- | 19 |

Table 11 Quantitative comparison of acid versus gas production in lauryl tryptose broth with brom thymol blue for the determination of gram-negative lactose fermenting bacteria.

| | Number presumptive samples | Number confirmed samples | Number samples failing to confirm |
|---|---|---|---|
| Total tested | 157 | 157 | - |
| Total positive | 112 | 103 | - |
| Gas production with higher MPN | 5 | 3 | - |
| Acid production with higher MPN | 42 | 50 | - |
| Gas production failing to confirm | -- | -- | 8 |
| Acid production failing to confirm | -- | -- | 5 |

Table 12 Comparison of the positive cultures for acid versus gas production using lauryl tryptose broth.

| Indicator | | Acid | Gas |
|---|---|---|---|
| Brom cresol purple | Total tubes | 2526 | 2526 |
| | Presumptive positive tubes | 776 | 624 |
| | Tubes with confirmed coliform bacteria | 628 | 594 |
| | Tubes with confirmed gram-negative lactose fermenting bacteria | 702 | --- |
| | Per cent of total tubes positive presumptive | 31 | 25 |
| | confirmed coliform bacteria | 25 | 24 |
| | confirmed gram-negative lactose fermenting bacteria | 28 | -- |
| | Per cent of positive tubes confirming coliform bacteria | 81 | 95 |
| | confirming gram-negative lactose fermenting bacteria | 90 | -- |
| Brom thymol blue | Total tubes | 2276 | 2276 |
| | Presumptive positive tubes | 694 | 570 |
| | Tubes with confirmed coliform bacteria | 573 | 513 |
| | Tubes with confirmed gram-negative lactose fermenting bacteria | 661 | --- |
| | Per cent of total tubes positive presumptive | 31 | 25 |
| | confirmed coliform bacteria | 25 | 23 |
| | confirmed gram-negative lactose fermenting bacteria | 29 | -- |
| | Per cent of positive tubes confirming coliform bacteria | 83 | 90 |
| | confirming gram-negative lactose fermenting bacteria | 95 | -- |

of the cultures that were positive for gas production confirmed as coliform bacteria. However, when the tubes that contained oxidase positive pseudomonas-like organisms were included as indicator organisms, as well as the coliform organisms, 95 per cent of the individual tubes for acid production confirmed. Using acid production for the indication of the presence of coliform and pseudomonas-like bacteria in water tested, 112 samples were positive for the presumptive tests and nine failed to confirm. Eight positive presumptive tests failed to confirm as coliform bacteria for the gas production whereas five failed to confirm as coliform or pseudomonas-like bacteria for acid production.

# DISCUSSION

The purpose of this research was to test a medium reported by McCurdy (1955) as a dehydrated medium in which the water to be tested was used for hydration.

Lauryl tryptose broth (LTB), using gas production as the indication of fermentation of lactose, and dextrose lauryl bile broth (DLBB), using acid production as the indication of fermentation, were compared to determine their properties to select and detect coliform bacteria from well water.

LTB gave higher indices for more samples than DLBB did after confirmation. Also nine of the samples for which DLBB gave an indication of the presence of coliform bacteria did not confirm whereas only one LTB sample failed. Comparisons of the number of tubes from which coliform bacteria were isolated showed that more coliform bacteria were detected by LTB and that it had a higher percentage confirmation than DLBB.

Suspecting that the carbohydrate was the cause for a lack of selectivity, comparative tests were made using lactose and dextrose in agar media. In these experiments acid production was used as the indicator for both sugars. Bile salts no. 3 in varying quantities was added in an attempt to eliminate growth of non-coliform organisms. The medium containing lactose had a higher percentage of confirmation for coliform bacteria, by showing acid or acid and gas production from lactose (at this time it was believed that

the colonies that would produce acid only when transferred to lactose fermentation tubes were attenuated coliform). Among the colonies from the medium containing dextrose there were many that did not produce acid or gas from lactose. These colonies were not considered to be coliform bacteria.

Tests were made using sewage to determine whether the acid non-gas producing colonies like the ones found in contaminated river water were present. If the acid producing colonies were found in sewage as well as the contaminated river water, they would be indicative of pollution.

The studies using sewage gave results somewhat comparable to the river in that the attenuated-like coliform organisms were found, as well as some non-acid producing colonies from the dextrose medium. Since the dextrose medium was detecting the non-acid producing colonies it was apparent that the lactose medium would be more selective. The lactose medium with the higher concentration of bile salts was inhibiting some of the coliform bacteria present. Thus lactose lauryl bile broth (LLBB) with 0.15 per cent bile salts no. 3 and LTB were the next media compared.

LTB gave higher indices for more samples than did LLBB after confirmation. LLBB failed to confirm for five samples that coliform bacteria were indicated by the presumptive test. Comparisons of the number of tubes from which coliform bacteria were isolated, showed that more coliform bacteria were detected by LTB and a greater percentage of the

cultures confirmed than did cultures from LLBB. Thus, bile salts no. 3 failed to inhibit non-coliform organisms, was inhibitory to the coliform bacteria present, and did not increase the confirmation percentage, even when lactose was used. For this reason it was decided that the next medium to be tried would be LTB with an acid-base indicator. A comparison of the results of acid and gas with acid production only was made.

It was noted in the experiments with brom cresol purple indicator (BCP) that some tubes showed gas production, but that not enough acid remained at the end of 48 hr to cause the indicator to remain yellow. Hence brom thymol blue indicator (BTB) was used for the next set of experiments.

The medium with either indicator gave similar results. Acid production detected more coliform bacteria than gas production. However with BTB, 17 per cent of the positive samples as determined by acid production did not confirm while only 4.5 per cent of the positive samples for BCP did not confirm. Using acid production for determining the presence of coliform bacteria showed the highest presumptive results but some of the cultures did not confirm as coliform bacteria present.

Organisms isolated from the tubes in which the cultures did not confirm as coliform bacteria gave a positive oxidase test. Polar flagella were demonstrated by staining organisms

from cultures that were oxidase positive. It has been the experience of the author that pseudomonas-like organisms mentioned above were present in water in which pollution was indicated by standard methods. When Red Cedar River water was tested, 15 to 25 per cent of the colonies on drop plates of selective media were oxidase positive and fermented lactose with acid formation at 35 C. These organisms were also found in sewage, but the percentage of these colonies was less.

It is probable that the pseudomonas-like bacteria have the same origin as the coliform bacteria, from sewage, and should be included in the test of sanitary quality of drinking water. Recently some authors (Reitler and Seligmann 1957 and Schiavone and Passerini 1957) suggested that Pseudomonas aeruginosa be used to supplement the existing test for the detection of polluted water. When the pseudomonas-like organisms constitute 15 to 25 per cent of the colonies isolated repeatedly from polluted river water, and grow at 35 C, it could mean that these organisms were originating from the same place as the coliform bacteria. In sewage these organisms made up about 10 per cent of the population. These organisms were probably not growing in the polluted river water, at least in the winter, but their numbers keep abreast with the changes in the amount of sewage that entered the river, as measured by coliform organisms indices.

Hence, the tubes that did not confirm were not really losses. The gram-negative bacteria that fermented lactose with acid production as the indication of pollution gave equal or higher indices than the standard methods test. Using the oxidase positive organisms as well as coliform bacteria for the indication of sewage, less samples failed to confirm. Only 1.8 per cent of the samples with BCP failed to confirm and 4.5 per cent of the samples for BTB failed to confirm.

Furthermore, the pseudomonas-like organisms are already detected by the millipore filter technique, an approved test for pollution of water and wastewater. The membrane filter technique is outlined in Standard Methods for the Examination of Water and Wastewater (APHA 1960). The membrane filter procedure detects an equal number of the pseudomonas-like organisms and they give the characteristic sheen producing colonies as do coliform bacteria using M-Endo-MF broth. The membrane filter technique does not give comparable coliform indices to those obtained by the multiple tube dilution method for marginal and potable waters. For these waters the membrane filter gives lower results or does not detect any coliform bacteria (Mallmann and Peabody 1961).

It was demonstrated that a dehydrated medium could be compressed into tablets and water to be tested used to hydrate the medium. This would overcome the problem of shipping of samples to a central laboratory or treating the sample with extreme care to preserve the coliform bacteria

that may be present and obtain an accurate estimation of the sanitary quality of the water. The sanitarian could carry the medium in sterile calibrated tubes in his car. When the need arose, a series of tubes could be inoculated with the water in question and taken to his office for incubation. At the end of 48 hr he would determine the MPN by the number of tubes that had changed to yellow from the original blue color.

## SUMMARY

A new field method for detection of bacteria indicative of sewage pollution has been proposed.

The medium lauryl tryptose broth, with either brom cresol purple or brom thymol blue for indication of acid production, was found to be an adequate method of testing water for pollution. The presence of acid at the end of 48 hr incubation at 35 C was a positive test.

Lactose was more specific than dextrose for detection and selection of coliform bacteria. Acid rather than gas production was a better method for detection of more coliform bacteria.

Pseudomonas-like bacteria that ferment lactose with acid production were found in waters polluted with sewage. The colonies of the pseudomonas-like bacteria could not be distinguished from the coliform organisms. The pseudomonas-like bacteria appear to be indicative of sewage-pollution.

The method includes the use of a dehydrated medium which can be incorporated into tablets for convenience. The tablets are placed in sterile tubes calibrated for specific amounts of water to be collected at the site.

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