A NEW MEDIUM FOR THE DETECTION OF ENTERIC ORGANISMS INDICATIVE OF SEWAGE POLLUTION IN WATER

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by

HOWARD D. MCCURDY, JR.

AN ABSTRACT

Submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE

In Michigan State University
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and
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A new medium was developed, the formula of which was as follows:

Tryptose	2.0%
Dextrose	0.5%
Potassium dihydrogen phosphate	0.275%
Potassium hydrogen phosphate	0.275%
Sodium lauryl sulfate	0.01%
Bile salts (Difco)	1.0%
Sodium chloride	0.5%
Promcresol purple	0.0015%

When tested with laboratory strains of bacteria it was found that this medium permitted maximum growth and detection of typical coliform bacteria and other enteric bacteria of sanitary significance in water while inhibiting non enteric forms.

The specificity of this medium was also demonstrated in comparisons with Standard Method's procedure. It had a higher percentage confirmation for the presence of typical coliforms than lactose broth. Enteric bacteria were found to be responsible for almost all of the positive tubes obtained.

Acid production from dextrose was found to be an adequate indication of the presence of coliform bacteria when used in a selective medium. The use of an indicator (Brom cresol purple) permits the omission of gas vials, thus making the new enteric medium easily adaptable to field work.

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INTRODUCTION

Escherich (1) first isolated Bacterium coli commune from the feces of a cholera patient. Subsequently, this and related organisms, collectively known as the coliform group, were found to be natural inhabitants of the intestinal tract of both man and animals. The significance of this was quickly realized and these organisms became a valuable yardstick of sewage pollution in water.

The litmus lactose agar plate introduced by Sedgwick and Mathews in 1893 (2) was the first device developed for their detection and enumeration. On this medium coliform organisms formed red colonies because of their ability to ferment lactose. However, since no inhibitory agent was used, the plates were frequently overgrown by other forms, some of which also fermented lactose; thus colonies had to be picked for further examination.

Smith (2), in 1893, showed the value of a preliminary enrichment medium and introduced the use of the glucose fermentation tube. This method was quickly accepted. It was simple and permitted the examination of larger quantities of water. In addition, it could be made quantitative by planting a series of tubes with measured quantities of the sample. However, it was found that many organisms, not belonging to the coliform group, could produce gas from glucose.

As a result, the more specific lactose broth was substituted. Although many attempts were made to include inhibitory agents in that medium to prevent growth of non-coliform types, none was successful and lactose broth is still in use in essentially the same form.

The standard procedure of the American Public Health Association (3) is substantially the same now as it was in 1920. It consists in planting a series of standard lactose broth fermentation tubes with equal quantities

of water to be tested. The tubes are then incubated at 35°C and examined for the production of gas within 48 hours. Tubes showing gas at the end of that time are confirmed for the presence of coliforms by transferring to an inhibitory diagnostic medium such as eosin methylene blue agar or brilliant green bile lactose broth. In some cases, this is not sufficient and the "completed test" is necessary. This involves transferring the organisms to an agar slant and a lactose fermentation tube. If microscopic examination of the organisms from the slant reveals the presence of non-sporing gram negative bacilli, and there is gas fermentation in lactose broth, then it is a positive, completed test.

The method has its limitations. It is costly and requires much time and materials. Four days are usually required before the results can be considered significant and sometimes longer. This difficulty has been recognized for many years as evidenced by the many attempts that have been made to devise a one tube procedure. However, none of the host presumptive and enrichment media, which have been proposed to replace lactose broth, has proved satisfactory as a presumptive medium without confirmation. Only one, lauryl tryptose broth (4), has been included in Standard Methods as an alternative to lactose broth.

Standard Methods is almost wholly unadaptable to use in the field. Even if a one tube presumptive medium could be found in which a positive test is based on gas production the inclusion of fermentation vials would cancel much of its practicability in that area.

Another limitation which has been given increasing attention in recent years is the failure of Standard Methods to detect many atypical coliform organisms. These include many slow and even non lactose fermenting

bacteria some of which do not produce gas from any sugar. This group is a heterogeneous one that defies classification and is usually referred to as the paracolon group or as aberrant coliforms.

Gilbert and Lion (5) appear to have been the first to describe paracolon bacilli. Ledingham encountered such types in human feces and reported them as numerous in cases of diarrhea but regarded them as of little pathogenic importance. On the other hand, Gyorgy suggested that they played a considerable part in diarrhea in both man and animals.

Stuart, Mickle and Norman (6) proposed the separation of atypical coliforms into four groups based on a study of more than 10,000 strains isolated from water, soil, milk and other sources. They proposed the term "aberrant coliforms" for all gram negative non-spore-forming rods which ferment lactose slowly or weakly at 37°C and these were subdivided as follows:

I Microaerogenic Coliforms - aberrant coliforms producing gas from lactose slowly or in small amounts at 37°C or 20°C.

II Pseudomicroaerogenic Coliforms aberrant coliforms having the characteristics of the true microaerogenic strains at 37°C but showing normal lactose splitting activity at 20°C.

III Papillae-forming coliforms: aberrant coliforms showing the type of dissociation evidenced by Escherichia coli mutabile but not restricted to the genus Escherichia.

IV Anaerogenic coliforms aberrant coliforms producing acid but no gas from lactose with or without gas on other sugar.

These authors also recognize the existance of non-lactose fermenting coliforms.

In a later paper Stuart, Wheeler and Zimmerman (7) presented a study

of the biochemical and antigenic relationships of paracolon bacteria. They suggested a single, coliform genus which would include three species: freundii, aerogenes and coli. Within this group would be included all aberrant coliforms and paracolons. Non-gas producing cultures fermenting lactose rapidly, slowly or not at all would be grouped as anaerogenic aerogenes, freundii or coli or collectively as anaerogenic paracolons.

Stuart, Mickle and Borman (6) stated that the microaerogenic coliforms probably have a significance in water analysis similar to that of Aerobacter and Escherichia strains showing typical lactose fermentation. They noted that the papillae forming coliforms are only infrequently detected in water analysis in which gas production is used as a criterion for their presence and the anaerogenic coliforms would not be detected at all. However, they thought that these two groups were highly important because of their frequent association with gastroenteritis and genitourinary infections.

Reports of isolation of these organisms from such diseases are many. Pless (8) investigated an outbreak of diarrhea which had its source in fricasseed chicken. He was able to isolate paracolon bacteria both from the chicken container and the largest proportion of the cases.

Barnes and Cherry (9) 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 (10) studied the comparative distribution and possible pathogenicity of Paracolobactrum species. These organisms were isolated from 60 percent of the gastroenteritis cases as compared to 20 percent of the healthy individuals examined. These findings were considered to be, at least, indicative of pathogenicity. Paracolobactrum species were found much more

frequently in cases of gastroenteritis than either Salmonella or Shigella types.

Ziegler (11) studied the bacteriology of an epidemic of diarrhea. He isolated late lactose fermenting and non-lactose fermenting organisms from polluted water supplies and from diarrhea patients. The agglutination of at least two strains of these occurred in high dilutions of patients' serum, indicating that they were the probable cause of the infections. During the later part of the epidemic no typical Escherichia coli was isolated but these disease organisms were.

Kriebel (12) isolated many nonlactose fermenters which she was able to make from typical E.B colonies by rapid transfer. She also obtained non-lactose fermenters from typical E. coli, thus establishing a relationship. Such late lactose fermenters or non-lactose fermenters must therefore be regarded as atypical coliforms as suggested by Kriebel and certainly are entitled to equal consideration with typical forms in determining the potability of water.

Most of the information which has been obtained concerning aberrant coliforms in water has been obtained by the use of procedures supplementing those used in routine analysis, and frequently, these are quite time consuming and tedious. In certain cases many of these organisms can be detected by prolonged incubation of the standard methods media but such a procedure aggravates the inherent faults of Standard Methods, and, even then, not all coliforms are detected.

It would seem, from these considerations, that some new, more practicable procedure is needed which will permit the detection of all coliforms quickly and dependably whether typical or otherwise. Such a procedure should, as much as possible, correct the difficulties inherent

in old methods.

The purpose of this thesis is to present the results of studies which were made on a new enteric medium which, it is hoped, will prove a valuable tool in water analysis. Its formulation is based on the need for a practicable single step procedure for bacteriological analysis of water and for a sensitive and specific medium for all coliform organisms.

In formulating any bacterial diagnostic medium the first requirement is that it must stimulate the earliest possible development of all dormant and viable cells. After growth and development have begun, the bacteria should reproduce in such numbers that their presence will be readily recognized by their physiological action on the medium.

Mallmann and Darby (4) presented a new medium, lauryl tryptose broth, for the isolation of coliform organisms from water supplies. They reported that the use of a medium containing tryptose, sodium chloride and phosphate buffers grew out more coliforms present in water as indicated by the higher colon indices obtained by this medium. They also found that the addition of sodium lauryl sulfate in 1:10,000 dilution inhibited the growth of gram positive organisms while permitting the maximum development of coliform bacteria.

The formula of lauryl tryptose broth is as follows:

Tryptose	2.0%
Lactose	0.5%
Dipotassium phosphate	0.275%
Monopotassium phosphate	0.275%
Sodium chloride	0.5%
Sodium lauryl sulfate	0.01%

McCrady (13), Levine (14) and Perry and Hajna (15) all reported the superiority of this medium over lactose broth as a presumptive enrichment medium. Fewer false presumptives were obtained and more slow lactose fermenters were detected than with the older medium.

Lauryl tryptose broth, though permitting the rapid development of coliform types, is limited in its use for the detection of those organisms that give acid and visible gas from lactose in 48 hrs.

The most constant characteristic of enteric organisms indicative of pollution is their ability to ferment dextrose with the formation of acid or acid and gas. If dextrose rather than lactose was used and acid rather than gas production was used as the criterion of fermentation, more enteric organisms would be detected, since bacteria attacking lactose slowly or not at all will attack dextrose with comparative ease. Such a procedure would have the additional advantage of not requiring inserts in the fermentation tubes.

For this reason, in devising a tentative formula for the enteric medium, dextrose is used as the fermentable sugar in a medium essentially the same as lauryl tryptose broth. In addition, brom cresol purple is used as an indicator of acid production.

Because of the wider spectrum of bacteria capable of utilizing dextrose as compared to lactose, an additional selective agent was thought necessary to supplement the action of the sodium lauryl sulfate.

As a result of their habitat in the intestinal tract, enteric bacteria are capable of withstanding relatively high concentrations of bile. For this reason, in seeking an agent that will curb the growth of non enteric gram negative bacteria, the use of bile salts was considered.

The use of bile as an inhibitory agent in media for water analysis has had a long and, heretofore, somewhat dubious history.

Jackson (16) first proposed the use of ten percent bile as a presumptive medium with lactose. He found that it inhibited the growth of non coliform, lactose fermenting strains. The original medium consisted of undiluted ox bile to which was added one percent lactose and one percent

peptone. Lactose bile broth was enthusiastically received and in 1912 it was adopted by the Committee on Standard Methods of Water Analysis (3a).

The new medium supposedly did not permit the growth of weakened or attenuated forms. The 1912 edition of Standard Methods considered this to be an advantage for it stated that, "attenuated Bacterium coli does not represent recent contamination and all Bacterium coli not attenuated grow readily on lactose bile."

Jordan (17), however, found that the bile inhibited from one third to fifty percent of the coliforms. He also concluded that attenuation did not have any bearing on the results obtained, so that it could not be said that bile eliminates attenuated or weakened forms.

Obst (18) and Cumming (19) obtained similar results in comparisons of lactose bouillon and lactose bile.

Because of these and other similar results the 1917 Standard Methods (36) adopted lactose broth for preliminary enrichment and the presumptive test.

Somewhat different results were obtained by some workers. Hale (20) found that by the use of five percent bile in lactose broth, gas production was obtained much more quickly and fewer clostridia were able to develop.

Levine (22) carried out investigations using Difco evaporated bile and sodium taurocholate in tests with E. coli and A. aerogenes. In a basal medium containing one percent peptone and various concentrations of bile and sodium taurocholate he found growth of these bacteria was actually accelerated by concentrations of from one to two percent. In addition, he found that few anaerobic spore formers developed and sporing lactose fermenters growing aerogically did not grow on bile.

In 1920 Muer and Harris (23) proposed the use of a brilliant green

lactose bile medium for water analysis. They found that the bile appeared to inhibit completely the aerobic and the brilliant green bile to inhibit almost completely the anaerobic spore formers. Although the new medium was found to inhibit coliforms to some degree results obtained in other laboratories were considered favorable.

Dunham and Schoenlein (2) made a careful study to determine the optimum bile: brilliant green ratio and recommended the formula of brilliant green bile as it is used now.

Jordan (24) Butterfield (21) and Parr and Caldwell (25) found that brilliant green bile broth inhibited from ten percent to one third of the coliform organisms and was, therefore, not satisfactory, as a presumptive or enrichment medium.

Little more was done with media containing bile for water analysis until Perry and Hajna (26) developed their E. C. medium which is essentially the same as lauryl tryptose broth except that they used bile instead of sodium lauryl sulfate. They found that their medium was much superior to standard lactose broth. It inhibited almost completely fecal streptococci and other gram positive organisms with no apparent inhibition of coliform bacteria.

Levine used a more controlled medium, than employed by earlier authors, to which peptone had been added and obtained good results with lower bile concentrations. Perry and Hajna (26) used a base medium which had been proved to be more conducive to bacterial growth and obtained very favorable results.

It is our thesis that much of the difficulty encountered through the use of bile was due to the use of base media which were not of themselves as favorable as possible for the development of bacteria.

Early investigators used crude undiluted fresh ox bile. Hence, the concentration of bile was extremely high (about 10%) and variable and the nutritive value of the medium questionable.

EXPERIMENTAL WORK

The experimental work of this thesis was divided in two parts. First, an attempt was made to determine the effect of adding bile salts to a modified lauryl tryptose broth on the growth of enteric organisms and to determine its selective properties in such a medium. Secondly, the results obtained with the new medium were compared to those obtained using Standard Methods in testing routine water samples.

Procedure

Part A.

The rate of growth of Escherichia coli was studied on a base medium to which various combinations of bile salts and sodium lauryl sulfate had been added. The base medium had the following formula:

Tryptose	2.0%
Dextrose	0.5%
Monopotassium phosphate	.275%
Dipotassium phosphate	.275%
Sodium chloride	0.5%

Particular attention was given to the lag and early logarithmic phases of growth when minimal inocula were used. Huntington and Winslow (27) showed that during these stages the young cells exhibit all the characteristics of physiological ~~growth~~ youth comparable to those exhibited by multicellular organisms. It is, therefore, considered more important to know whether an organism can adjust to new environment to the extent that fission will occur, and after fission that the two physiologically young cells will survive, than to know the total number at the end of the complete growth cycle.

In the first set of experiments a pure culture of E. coli was used. Before seeding in the various media it was transferred from a nutrient agar slant every twenty-four hours for three days to insure uniformity.

In order that minimal numbers would be used in the inocula, the organisms were transferred from a slant to a tube of sterile saline (28). They were added until the first turbidity visible to the naked eye appeared. At that density the number of organisms in suspension approximated 50,000,000 per milliliter (26). This suspension was diluted in sterile saline until such concentration was obtained that the test medium would contain one organism or less per milliliter when one milliliter was used as the quantity for inoculation.

Two hundred and fifty milliliter portions of the medium were used and incubated at 35°C.

Growth of the pure culture of E. coli was studied on the following media.

I. The base medium
II. The base medium to which was added 0.01% sodium lauryl sulfate.
III. The base medium plus 1% bile salts (Difco)
IV. The base medium plus 0.01% sodium lauryl sulfate and 1% bile salts.

The numbers of organisms at 0, 2, 4, 6 and 24 hours were determined by plating portions of the test medium. Tryptone glucose extract agar was used as the plating medium and the plates were incubated at 35°C for 48 hours before counting.

In order to supplement the findings obtained in the first experiment, a somewhat different procedure was tried. While minimal numbers were used in the first set of determinations to ascertain the effect of the two inhibitory agents, it may be argued that this does not indicate that the more weakened or attenuated forms, found in water will react in the same way.

Mallmann and Darby (4), in their original studies on lauryl tryptose broth, used organisms which had been subjected to refrigeration in order to simulate the condition of attenuated forms. Such a process cannot duplicate

the many factors which affect the organisms in water.

It is our idea that in order to obtain information concerning the growth of such bacteria in a medium which is to be used for their detection, no more practical way of attempting to solve this problem can be found, than to study the behavior of these organisms under conditions in which they will actually be encountered.

For this reason, a water sample was obtained from a source known to be heavily polluted. The sample was then diluted to such an extent that the final concentration of the coliforms in the sample would be 250 per milliliter. This procedure was based, by approximation, on the estimated coliform density of the polluted water. One milliliter of the suspension was then inoculated into 250 milliliter quantities of the same media used in the first set of experiments. Growth was then determined by making plate counts using tryptone glucose extract agar at 0 and 6 hours. In order to check the identity of the cultures obtained, several colonies were picked from each plate and inoculated in tubes of lauryl tryptose broth.

In the third set of experiments the effect of altering the concentration of bile was determined on a base medium to which sodium lauryl sulfate had been added. The procedure was the same as in the first set of experiments except that growth was determined only at 0 and 6 hours. Concentrations of 0.5%, 1.0%, 2.0%, and 5.0% bile were used.

Since there are many enteric organisms which have sanitary significance it was considered desirable to obtain some information concerning the behavior of other enteric organisms in the new medium. There being so much interrelationship among members of the Enterobacteriaceae and so much diversification in the coliform group, it was thought that a study of the behavior of many related species might indicate the reaction of many of the

coliforms that may be encountered.

For this purpose single growth rate determinations were made using stock cultures of Paracolobactrum arizona, Salmonella typhimurium, Aerobacter aerogenes, Shigella sonnei, Salmonella typhosa, Salmonella enteritidis, Salmonella paratyphi and Salmonella schotmuelleri. Minimal numbers were used in the inocula again and plate counts were made at 0, and 6 hours. Three media were tested and their composition was as follows:

I The base medium containing .275% each of dipotassium and monopotassium phosphate, .5% glucose and .5% sodium chloride.
II The base medium plus .01% sodium lauryl sulfate.
III The base medium plus .01% sodium lauryl sulfate and 1% bile salts.

Having determined the effect of the various agents in the enteric medium on the growth of enteric organisms, the next step was a determination of its selective ability. For this purpose a number of known stock cultures of gram negative and gram positive organisms were used as well as several unknown gram negative organisms not belonging to the coliform group which had been isolated from water examined in this laboratory. All cultures were transferred every 24 hours for three days before use. The inhibitive properties of various percentages of bile in the enteric medium with sodium lauryl sulfate 0.01% concentration was compared to a non inhibitory base medium containing tryptose. Ten milliliter amounts of the various media were seeded with standard loopfuls of broth culture and growth recorded on the basis of turbidity.

Results

In the first series of experiments, in which growth on the base medium containing tryptose, sodium chloride, glucose and phosphate buffers was compared to that in the various combinations of the base medium and the selective agents, favorable results were obtained. The results of these runs are presented in Table I. A critical examination of the data reveals

very little difference between the various media at the end of six hours. In most cases the counts at the 24-hour interval gave the same picture but the 6-hour counts were considered more significant.

The series of experiments in which a diluted water sample was used as an inoculum gave results which are considered excellent. All the colonies which were picked produced gas when seeded into lauryl tryptose broth, an indication that most, if not all, of the colonies were those of coliform organisms. In terms of growth, these runs gave a picture somewhat the same as the first experiment. The data obtained are presented in Table II. Consistently higher counts were obtained in media III and IV which might indicate a slight stimulatory effect on the part of the lauryl sulfate either alone or in combination with the bile salts.

In the next series of experiments (Table III) the effect of various concentrations of bile salts in a basal medium of sodium lauryl sulfate glucose broth was noted. The bile salts exerted a very slight, if any, inhibitive action in concentration up to 2%. However, the counts obtained from the 5% concentration was consistently lower in all cases and in one case quite significantly so, indicating that the bile did inhibit E. coli in that concentration.

The results of the fourth group of experiments added further support to the results already obtained, showing that the use of bile salts and sodium lauryl sulfate in the medium had no effect on the growth of any of the enteric organisms studied.

The selective properties of the medium was studied next using pure laboratory cultures of various bacteria. Table V gives the results of this study. Most of the non enteric gram negative organisms were completely inhibited by all concentrations of bile salts with sodium lauryl sulfate.

Table I.

Growth of *E. coli* on Media Containing Various Combinations of the Selective Agents.

Trial	Time (hours)	I	II	III	IV
A	0	1*	0	0	0
	2	1	0	1	1
	4	15	10	1	1
	6	196	254	111	242
	24	517M	631M	483M	538M
B	0	0	0	0	0
	2	2	2	0	0
	4	10	10	170	10
	6	260	295	254	261
	24	447M	798M	474M	506M
C	0	1	1	1	0
	2	1	0	1	1
	4	11	0	100	0
	6	314	212	240	241
	24	563M	425M	338M	302M
D	0	0	1	0	0
	2	2	0	0	0
	4	20	0	20	40
	6	580	637	444	619
	24	620M	1120M	304M	960M

M = Million
I = Base medium
II = Base medium and sodium lauryl sulfate
III = Base medium and bile salts
IV = Base medium with sodium lauryl sulfate and bile salts
* = Number of bacteria per milliliter.

Table II.

Growth of Coliforms Planted Directly from Polluted Sources on Various Media.

Trial	Time	I*	II	III	IV
A	0 hrs.	1	0	0	2
	6 "	115	156	179	158
B	0 hrs.	0	3	1	0
	6 "	132	141	162	147
C	0 hrs.	0	0	0	1
	6 "	129	113	143	182
D	0 hrs.	0	0	0	0
	6 hrs.	-	120	149	163

I* = Base medium
II = Base medium with bile salts
III = Base medium and sodium sulfate
IV = Base medium with bile salts and sodium lauryl sulfate.

Table III.

Growth of E. Coli on a Basal Medium Containing Sodium lauryl sulfate and Various Concentrations of Bile Salts.

Trial	Time	0% Bile	0.5% Bile	1.0% Bile	2% Bile	5% Bile
A	0 hrs.	0*	1	1	0	1
	6 "	506	502	416	356	257
B	0 hrs.	0	0	2	1	4
	6 "	513	345	395	396	278
C	0 hrs.	1	0	0	1	5
	6 "	312	267	275	212	190
D	0 hrs.	0	0	0	0	0
	6 "	364	293	230	-	87

* No. of bacteria per ml.

Table IV.

Growth of Various Enterics on an Enteric Medium.

Organism	Time	Medium I	Medium II	Medium III
Paracolobactrum Arizona	0 hrs.	0	1	0
	6 "	215*	172	188
Salmonella typhimurium	0 "	0	2	0
	6 "	1568	1226	1470
Aerobacter aerogenes	0 "	0	0	1
	6 "	3551	4859	5320
Shigella sonnei	0 "	0	0	0
	6 "	402	536	430
Salmonella typhosa	0"	0	0	0
	6 "	51	52	41
Salmonella enteritidis	0 "	0	1	0
	6 "	384	487	368
Salmonella paratyphi	0 "	0	5	0
	6 "	453	526	671
Salmonella schottmuelleri	0 "	1	0	2
	6 "	113	152	171

* All counts are the average of two plates (per 1 ml.)

Medium I - Base medium of 2.75 gm of monopotassium phosphate, 2.75 gm dipotassium phosphate, 20 gm tryptose (Difco) 5 gms. sodium chloride, 5 gms dextrose, water-one liter.

Medium II - Base medium + 0.1 gm sodium lauryl sulfate

Medium III - Base medium + 0.1 gm sodium lauryl sulfate + 10 gm bile salts.

Table V.

Selectivity of Bile - Sodium Lauryl Sulfate.

Organisms	Control	1% Bile	2% Bile	3% bile	5% Bile
Paracolobactrum (Arizona)	4	4	3	3	2
Paracolobactrum II	4	4	4	3	2
Paracolobactrum sp. IV	4	4	4	3	2
Anaerogenic paracolon III	4	4	4	3	2
Anaerogenic paracolon VIII	4	4	4	3	2
Salmonella typhimurium	4	4	4	3	2
Salmonella paratyphi	4	4	4	3	2
Salmonella typhosa	4	4	3	3	1
Salmonella enteritidis	4	4	4	3	2
Aerobacter aerogenes	4	4	4	4	3
Shigella sonnei	4	4	4	3	2
Salmonella choleresuis	4	4	4	3	2
Bacillus subtilis	4	0	0	0	0
Pseudomonas aeruginosa	4*	1	1	1	1
Bacillus cereus	4	0	0	0	0
Streptococcus fecalis	4	4	3	3	2
Micrococcus agilis	4	0	0	0	0
Micrococcus aureus	4	0	0	0	0
Flavobacterium sp. (1)	4	0	0	0	0
Flavobacterium sp. (2)	4	0	0	0	0

* No acid
4 - same amount of growth as control
3 - less growth than control
2 - medium amount of growth
1 - little growth
0 - no growth.

The only exception was Pseudomonas aeruginosa which gave limited growth on all of the bile concentrations. Of the gram positive organisms tried only Streptococcus fecalis grew on any of the inhibitory media. Since it is an indicator of fecal pollution itself and would be outnumbered by enteric medium.

Part Two

The results of the experiments carried out in part one, indicates that a bile concentration of one percent in an enteric medium containing sodium lauryl sulfate, permits maximum growth of enteric organism while inhibiting non intestinal forms.

In order to check the value of the enteric medium under actual laboratory conditions a study was initiated in which it could be compared to standard procedures. The formula for the enteric medium which was used was as follows:

Tryptose	2.0%
Dextrose	0.5%
K_2HPO_4	0.275%
KH_2PO_4	0.275%
Sodium lauryl sulfate	0.01%
Bile salts (Difco)	1.0%
Sodium chloride	0.5%
Brom cresol purple	0.0015%

Water samples which had been brought to the Michigan Department of Health from private wells for routine testing were used. There, they were tested according to Standard Methods procedure using Standard lactose broth as the presumptive medium with confirmation on brilliant green bile broth.

The remainder of the samples were then sent to our laboratory where parallel tests were run using the enteric medium. All positive tubes were confirmed by streaking on eosin methylene blue agar plates and transfer to lauryl tryptose and brilliant green broths. During the later part of the study Tergitol-7 and tryptone glucose extract agars were added in order to insure

early isolation of organisms giving acid reactions on the enteric medium.

Tubes which gave positive results on all three or any two of the confirmation media were recorded as confirmed. In all cases when confirmation was obtained on less than two, an attempt was made to isolate the organisms responsible for the positive result and subject them to identification procedures.

The method used was adapted from that of Cope et al (29) and was as follows: 1. Colonies were picked from plates showing growth and transferred to dextrose and lactose fermentation tubes containing brom cresol purple as an indicator and nutrient agar slants. Care was taken to pick only from the surfaces of the colonies and to use only one fishing to avoid transferring a mixed culture. The tubes were then incubated at 35°C. At the end of 48 hours a gram stain preparation was made from the slant culture and examined microscopically. The sugars swere incubated for ten days until acid and gas were produced.

2. If the slant culture was found to be a pure culture of gram negative non-sporing rods and no fermentation of lactose was obtained, transfers were made directly to other diagnostic media. If a mixed culture was found, the organisms were transferred to nutrient broth and pure culture obtained by streaking on either MacConkey agar plates or Tryptone glucose extract agar plates. In such cases the identification procedure was begun again as in (1).

3. Other diagnostic tests were run as follows:

a. Fermentation of sucrose
b. Growth on Simmon's citrate agar
c. Motility, hydrogen sulfide production and indole using S.I.M. medium (Difco)
d. Voges-Proskauer test for acetyl-methyl carbinol production using M.R.-V.P. medium (Difco).
e. Decomposition of urea broth.

Organisms fermenting dextrose and lactose with the production of acid

and gas in 48 hours that gave positive results on any of the three confirmation media were classed as typical coliforms and the tubes from which they came were considered as confirmed. Those that did not confirm on any of those media were classed as atypical. Those fermenting lactose slowly with the production of gas were also considered atypical coliforms.

Strains producing acid and gas on dextrose but not lactose in ten days and which decomposed urea were classed as Proteus. Those fermenting dextrose with no gas, were non motile and did not utilize citrate or urea were considered as Shigella-like.

Organisms fermenting lactose of sucrose without gas or producing indole were classified as anaerogenic paracolons and were considered as typical coliforms.

If gram positive bacterial or non-dextrose fermenters were isolated they were tested for growth on the enteric medium and if no growth was obtained in 48 hours they were discarded.

Results

The enteric medium was compared with those recommended in Standard Methods on the basis of the number of positive samples and the number of positive tubes. The results are given in Tables VI and VII.

Referring to Table VI, it will be noted that, although there were fewer presumptive positive samples with the enteric medium, a higher percentage of these showed typical coliforms than did the lactose broth. All the samples positive in bile broth medium, contained coliform organisms which were either typical or of the paracolon group.

Seventy-three samples were presumptively positive by both methods. Sixty seven of these confirmed in both. The fact that all 73 were found to contain enteric organisms indicates that all of the samples probably contained gas

Table VI.

Comparison of the Enteric Medium with Lactose Broth (A).	Enteric Medium	Standard Lactose
Number of samples tested	324	324
Number of positive presumptive samples	86	97
Number of positive samples confirmed	71	73
Number of positive samples containing enterics	86	-
Number of samples positive on both		
a) Presumptive	73	73
b) Confirmed	67	67
c) Enterics	73	?
Numbers of samples positive on one, not the other		
a) Presumptive	13	24
b) Confirmed	4 (17 tubes)	6 (6 tubes)
c) Enterics	13	-
Percent Confirmed	83%	75%
Percent with lactose fermenters (acid or acid gas)	94%	-

Table VII.

Comparison of the Enteric Medium with Lactose Broth (B).	Enteric Medium	Standard Lactose
Number of tubes planted	1,561	1,565
Number of presumptive positive tubes	361	361
Number of presumptive positive tubes confirmed	294	264
Number of tubes from which enteric organisms were isolated	359	-
Percent confirmed	82	73
Number of tubes not confirmed	67	-
Number of tubes having lactose fermenting enterics present	48	
Number of tubes having sucrose fermenting enterics present	9	
Others-mostly citrate and indole positive organisms	8	
Percent of positive tubes having lactose fermenters present (acid or acid and gas)	95%	

producing coliforms, some of which were either eliminated or altered by passage through the media used in the analysis.

Thirteen samples were positive on the enteric medium and negative by Standard Methods. Four of these samples, represented by 17 positive tubes, confirmed. All contained enteric organisms.

On the other hand, 24 samples gave initial positive reactions on lactose broth and six of these confirmed. None of these samples had more than one positive tube.

In terms of rapidity the enteric medium was found to give on the whole, an earlier indication of the presence of pollution than lactose broth . Considering the latter two thirds of the sampling program, 68 percent of the enteric medium positives were positive in 24 hours while 42 percent of the lactose broth positives were so. There was no correlation between whether the organisms were typical or not and the speed of detection.

During the study, it was found that there was a great deal of variation in the results obtained by the various methods of confirmation used for the enteric medium positives. It was found that lauryl tryptose broth gave a much higher number of confirmed tubes when it was used as the secondary medium. Brilliant green bile broth frequently showed growth but gas production seemed to have been suppressed and in many cases no growth at all was obtained on E.M.B. For this reason, in tabulating the results in Table VI, the lauryl tryptose broth confirmations were used since these were found more dependable. Brilliant green bile did show at least one positive tube in all except one of the confirmed samples.

Table VI gives a picture similar to that of Table V. More enteric tubes confirmed than did lactose broth positives., All except two of the enteric positive tubes showed the presence of typical coliforms or paracolon bacteria. Failure

to isolate enterics from these tubes was attributed to technique since the other tubes of the same sample yielded typical coliforms.

All the paracolon organisms that were isolated were of the microaerogenic or anaerogenic type. These organisms, as noted earlier, are approximately as significant as typical coliforms.

CONCLUSIONS

1. The enteric medium is specific; it does not permit the detection of any but enteric organisms indicative of pollution.

2. More coliform organisms are detected using the bile medium than is possible by standard methods. This includes both typical and atypical types.

3. Acid production is an adequate indication of presence of enteric bacteria when used in a selective medium.

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