STUDIES OF THE ENTERIC BACTERIAL FLORA OF RAW SEWAGE AT EAST LANSING, MICHIGAN

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Irving Le Roy Dahljelm

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STUDIES OF THE ENTERIC BACTERIAL FLORA OF RAW SEWAGE AT EAST LANSING, MICHIGAN

by

Irving LeRoy Dahljelm

A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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INTRODUCTION

The isolation of enteric pathogens from sewage has been reported in the literature on a number of occasions in recent years. Most, if not all, of the pathogens isolated were obtained from sewage originating in areas where the occurrence of enteric infections was either endemic or epidemic. Furthermore, the methods used in the identification of the organisms isolated were often incomplete and render the results reported of questionable value.

A review of the literature revealed that no study has been reported on attempts to isolate enteric pathogens from sewage originating in an area where the occurrence of enteric infections may be considered to be rare or non-existent.

Because of the present tendency to use sewage and sewage solids for the purpose of soil conditioning, it appeared advisable to make a study of raw sewage from such a source using various media which are currently advocated as being superior for the isolation of enteric pathogens. This thesis presents the results of this study.

HISTORICAL REVIEW

The isolation of Salmonella typhosa from water was recorded in the literature as early as 1895. Willson (1) in 1905 summarised the instances in which S. typhosa had been isolated from contaminated drinking water up to that date. According to him, Losener reported that up to 1895 there were 65 cases in which it was claimed that S. typhosa had been isolated from water. Willson states that, "It is possible that in some of these the bacillus was really that of typhoid, but the evidence of identity is now known to be insufficient and none of these cases can be accepted as conclusive." The 1895 isolation was from the Berlin water supply. The isolated organisms exhibited all the then-known characters of S. typhosa and were afterwards confirmed as such by Pfeiffer's reaction.

From 1895 until 1905, various workers have reported the isolation of the typhoid organism from 11 water supply sources. Since 1905 (2), there are 13 instances in which S. typhosa or related enteric pathogens are reported to have been isolated from water or sewage.

Special mention should be made of the work of the London Metropolitan Water Board Laboratories which is, perhaps, the most complete and carefully controlled. Inaugurated by the late Sir Alexander Houston, the 1914 report and each report from 1927 to 1938 (3 to 15) carries some reference to isolations of enteric pathogens. In the 1914 report, 23,353 non-lactose fermenting colonies developing on plates planted mainly with samples of London (Barking) or Hendon sewage and also individual samples from Dublin, Belfast, Edinburgh

and Aberdeen were examined. He was unsuccessful in isolating a single S. typhosa, and concluded that the typhoid organism is not uniformly present in 0.00066 ml. of crude sewage, and that this amount of sewage contains 70⁴ excretal bacteria per ml. Houston, in calling attention to the difficulties of the task, writes:

> "Sewage contains such an enormous number of bacteria that it is quite impracticable to examine more than a very small amount of this material. For example, to examine one cubic centimeter of crude sewage for typhoid bacilli by the direct plating method would mean making at least 1,000 special plate cultures and at least 20,000 primary subcultures, not to speak of the secondary and other cultures for the purpose of differentiation. It is, however, quite practicable to work with as much as 0.01 c.cm. of sewage spread over from 10 to 20 special plates, and therefore to make 200 to 400 primary cultures."

Wilson (16) states, "Non-lactose fermenting organisms are so common in sewage that many thousands or hundreds of thousands of such colonies would require to be examined in order to isolate a single typhoid bacillus. For example, in Belfast sewage I find that on an average 400,000 to 500,000 organisms develop from 1 c.cm. on MacConkey lactose bile salt agar plates, and that of these more than one-half are non-lactose fermenters. In such sewage I have found about one typhoid bacillus in each cubic centimetre, so that, using

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the MacConkey medium, there would have been a chance of isolating the bacillus if 250,000 non-lactose fermenting colonies had been tested. In all probability, even after such a Herculean effort, failure would have resulted, as the chances are that the typhoid bacillus would not have a clear space on the plate to develop, and its growth would have been obscured and inhibited by the coliform colonies. The addition of brilliant green to the medium would doubtless render the isolation of B. typhosus (S. typhosa) from sewage not quite so difficult, but even so the chances against a non-lactose fermenting colony being composed of typhoid bacilli would be many thousands to one."

Kehr and Butterfield (17) in 1943 presented a review of the results of some recent attempts to isolate pathogens from sewage and an interesting discussion of the indicated relations among the S. typhosa density of sewage and water, the coliform density and the typhoid fever morbidity in the community.

Dunlop (18) reported on the isolation of typhoid, dysentery and salmonella organisms in the final effluent of the Denver sewage disposal plant. More recently, Dunlop (19) has reported on the quantitative isolation of pathogens from sewage. This work, however, cannot be accepted as conclusive because the evidence of identity is insufficient. For example, he assumes that turbidity in an enrichment medium is presumptive evidence of the presence of S. typhosa, and the appearance of non-lactose fermenters on solid differential media and characteristic reactions of the Salmonella or Shigella in Kligler's iron agar results in a completed test.

Methods of Isolating Typhoid and Related Pathogens from Water or Sewage

Various methods have been used or suggested for the isolation of pathogens from water or sewage. All of the methods fall into three main groups: (a) Physical concentration produced by agglutination, chemical precipitation, centrifugation or filtration followed by isolation on differential solid media with or without enrichment; (b) isolation on differential solid media after preliminary cultivation in selective enrichment medium; (c) direct isolation on differential solid media.

The various early differential and enrichment media have been adequately reviewed and discussed by Prescott (2) and Wilson (16). No good purpose could be served by enumerating and discussing these media here. Suffice it to say that the media more recently developed have proved to be far superior but are not all that is to be desired.

Laine (20) in her work reviews the development of more recent media and discusses the author's reasons for recommending each medium.

Some of the media used in this study have presented problems not ordinarily encountered when the media were used for the isolation of enteric organisms from other sources, such as feces or urine. For this reason, the media used will be considered in some detail, even at the risk of presenting material adequately covered elsewhere. The specific problems encountered will be considered in the discussion.

Modifications of some media were attempted. A discussion of these is not included as it could serve no purpose other than add to already existing confusion.

For the purposes of enrichment the media most favored today are sodium tetrathionate broth and selenite broth. Sodium tetrathionate broth was first found to be useful as an enrichment medium by Mueller (21) and reported by him in 1923. The usefulness of the medium is based on the regulation and inhibition of the physiological activities of contaminating organisms by sodium tetrathionate. This is formed by the reaction of sodium thiosulfate and iodine. Iodine is inhibitory to gram-positive organisms and the bile salts contained in the medium inhibit the non-intestinal types. Proteose peptone aids as a buffer against too great a change in the reaction of the medium and serves as a ready source of energy for the bacteria. Calcium carbonate serves to maintain an alkaline reaction.

Liefson (22) in 1936 described selenite F broth. The development of this medium was based on the observations of Handel and Theodorascu who, according to Guth (23), observed that Escherichia coli was much more susceptible to the toxicity of sodium selenite than was S. typhosa. Liefson showed that the selenite broth was not sufficiently toxic to inhibit fecal coli and enterococci completely. However, he found that the colon bacilli were reduced in numbers during the first 8-12 hours and thereafter increased rapidly. The typhoid bacilli multiplied fairly rapidly from the start. Proteus and pseudomonas were not inhibited, but dysentery and alcaligenes were inhibited.

Primary plating media are used for initial isolation of organisms. The composition of each medium is usually such that it will readily differentiate groups of organisms, or be selective for a particular group of organisms.

MacConkey's agar (23) described in 1905 continues to enjoy popularity as a differential medium. The present modification not only serves to differentiate strains of S. typhosa from members of the coliform group, but has the added advantage of supporting excellent growth of all Shigella and Salmonella strains. The differential action of this medium is clear and distinct. Isolated colonies of coliform bacteria are described as being brick red in color and may be surrounded by a zone of precipitated bile. The reaction is due to the action of acids, produced by fermentation of lactose, upon bile salts and the subsequent absorption of neutral red. Typhoid, paratyphoid and dysentery bacilli have little effect on the appearance of the medium. Gram-positive organisms are inhibited by the selective bacteriostatic action of crystal violet.

S. S. (Shigella-Salmonella) agar, developed by Difco Laboratories, was devised to provide differentiation of lactose fermenters from lactose non-fermenters, and to give maximum inhibition of coliform organisms without restriction of the growth of pathogenic gram-negative bacilli. Shigella, Salmonella and other organisms not fermenting lactose form opaque, transparent and translucent uncolored colonies which generally are smooth. Lactose fermenting organisms which may not be inhibited are generally recognized by the formation of a red color in the colony. Some coliform colonies do not show a

definite red color, being pink or nearly colorless with a pink center. Some Proteus and Salmonella types produce black-centered colonies; a characteristic large white or cream-colored opaque and mucoid colony may be developed by some types of aerogenes.

Bismuth sulfite agar as modified by Wilson and Blair (24) is a selective medium used by almost all investigators in recent years. The composition of this medium is based on the fact that the Salmonella are able to reduce sulfites to sulfides. In the presence of a fermentable carbohydrate and metallic salts, this reduction results in the medium surrounding the colonies becoming blackened. The acids produced by the organisms from the carbohydrate, which serves as an indispensable source of energy, facilitate the characteristic changes by bringing the metallic salts into solution. Brilliant green promotes the blackening of the Salmonella colonies and aids in the inhibition of the colon group. Bismuth in combination with sodium sulfite causes a suppression of Escherichia coli without reparably suppressing the development of the Salmonella colonies. Sodium phosphate is used as a buffer to absorb excess acids produced.

Laine (20) used Hoyle's brilliant green acid fuchsin agar which was recommended by Cruickshank (25). She confirmed the finding that the medium was particularly inhibitory to Proteus. The medium had the advantage of simplicity in preparation, and good colonial differentiation was obtained. Sodium taurocholate enters into the composition of this medium and seems to stimulate the growth of enteric pathogens when present. At the same time, it is inhibitory to non-intestinal organisms. Brilliant green serves to inhibit the

growth of gram-positive organisms, while the decolorized acid fuchsin indicates the production of acid resulting from the fermentation of lactose.

Jeter and Wynne (26) developed and described acid fuchsin methylene blue agar. This is a medium which was formulated to provide a more satisfactory differential medium essentially free from objections described as common to other media of primarily differential nature. The objections listed for other media are: (1) Generally, only colonies of lactose fermenters are colored, whereas the colorless colonies of non-fermenters tend to be masked by the diffusion of dye from fermenting colonies. (2) The medium may be too toxic for the growth of delicate enteric species. (3) Deterioration may occur on exposure to light. (4) Weak fermenters may not be differentiated from non-fermenters. Acid fuchsin methylene blue agar contains, in addition to peptone, lactose and agar, a combination of acid fuchsin and methylene blue buffered at an optimum concentration of 0.3 per cent. Colonies of lactose fermenters take on the red color of the acid dye, and colonies of non-fermenters take on the blue color of the basic dye. This is possible because the dyes do not combine chemically. The optimum buffer concentration of 0.3 per cent prevents the masking of non-fermenting colonies by those producing acid which occurs at lower concentrations, while higher concentrations tend to prevent ready differentiation of weak fermenters. The recommended pH is 6.6, since at appreciably higher values the increase in adsorption, and therefore in toxicity, of methylene blue results in dye-sensitive species, such as S. dysenteriae Shiga and A. faecalis failing to develop.

EXPERIMENTAL STUDIES

A study of the organisms occurring in raw sewage, with particular reference to the incidence of enteric pathogens, should be a useful contribution to knowledge.

Considering the conclusions drawn by Houston (3) as a result of his failure to isolate S. typhosa, and the statement of Wilson (16) regarding the incidence of non-lactose fermenting organisms in sewage, it at once becomes apparent that it would be desirable to concentrate the bacteria present in the sewage into a small volume in order to increase the probability of isolating any enteric pathogens which might be present. It would also be desirable, if not absolutely necessary, to use some form of selective enrichment media to increase the number of pathogenic organisms and, at the same time, inhibit the growth of non-pathogens. Various methods of concentration were tried. As a result of these trials, it was concluded that precipitation of the sewage using alum would best serve the purposes of this investigation. Other workers usually add a constant volume of a 10 per cent solution of aluminum sulfate to each liter of water or sewage. Wilson (27), for instance, added 2.5 ml. of a 10 per cent solution of aluminum sulfate to each liter of water and then adjusted the pH to about 7. In the present investigation, this method did not give satisfactory results.

Aluminum hydroxide is amphoteric in nature; the varying kinds and concentrations of mineral salts present in water or sewage definitely affect the solubility of the precipitate. Experience in

water treatment has demonstrated that each water has a definite optimum pH value at which the aluminum hydroxide precipitate is least soluble, and that the optimum pH value for a given water may vary from day to day, or hour to hour.

The composition of sewage varies considerably, and it was decided to carry out all precipitation at the optimum pH as determined by jar tests.

Growth and the production of enzymes by bacteria are closely associated with or dependent upon the pH of the medium in which the bacteria are seeded. To subject enteric pathogens, already in an environment not conducive to their growth, to additional adversity is not likely to enhance the chances of recovering such organisms. Porter (28) gives the minimum, optimum and maximum pH for the growth of several bacteria. The minimum pH values for all of the enteric forms listed are above 4.0. When aluminum sulfate was used as the precipitating agent, the optimum pH value was often found to be as low as 3.5. The effect of such a low pH on the organism, which may have been adversely affected from the conditions of its environment, is likely to be death. Aluminum ammonium sulfate was found to give optimum pH values ranging from 4.6 to 6.6, on East Lansing sewage, and for this reason was selected as the precipitating agent to be used.

A number of enrichment media was tried; and in addition, experimental enrichment broths were devised. None, however, gave entirely satisfactory results. Of the enrichment media used,

Bacto-Tetrathionate Broth and Bacto-Selenite Broth showed the greatest inhibition of the coliform organisms and were selected for enrichment purposes as representing the most satisfactory of the media available.

For primary plating purposes, a large number of media was used. Coliform organisms grew abundantly in all media. On Desoxycholate Agar there was a tendency for the coliforms to overgrow or mask the non-lactose fermenters. It was found that best colonial differentiation could be obtained on Bacto-Bismuth Sulfite Agar, Bacto-SS Agar, Bacto-MacConkey Agar, Brilliant Green Acid Fuchsin Agar and Acid Fuchsin Methylene Blue Agar. Brilliant Green Acid Fuchsin Agar was particularly useful in suppressing the growth of Proteus, although a few strains were found to grow on this medium.

For primary differential screening, Kligler's Iron Agar, Triple Sugar Iron Agar and Urea Broth were used. Kligler's Iron Agar gave the best differentiation as far as the Paracolobactrum was concerned. Urea Broth was ideal for the differentiation of Proteus.

Secondary differentiation was accomplished using dextrose, lactose, saccharose, maltose, mannite and xylose in Bacto-Purple Broth Base. The IMViC reactions were also determined. When it was considered desirable, motility tests were also used.

For final identification of suspected pathogens, agglutination tests, using specific antisera, and bacteriophage typing were used.

The general treatment of the sewage samples is shown in

outline form as Table I.

Three 2-liter portions of sewage were collected at the sewage plant in sterile flasks for each sample. One portion was further divided into ten 200 ml. portions in 400 ml. beakers, and varying amounts of aluminum ammonium sulfate solution were added. After thorough rapid stirring, the beakers were allowed to stand and observations were made to determine in which beaker the precipitate formed first, and settled most rapidly, leaving the clearest supernatant fluid above the precipitate. That portion was chosen having the optimum conditions for concentration of the organisms, and the pH value of the supernatant fluid was determined. This pH value was designated as the optimum.

The proper amount of aluminum ammonium sulfate solution was then measured into the remaining 2-liter portions, the flasks were thoroughly shaken and the precipitate was allowed to settle by gravity for a period of four hours. The supernatant fluid was decanted until the remaining volume was about 200 ml. The entire amount of concentrate was planted. The procedure generally followed was to plant 1 ml. of the precipitated sewage directly onto each of the various primary isolation media without prior enrichment. The remaining precipitated sewage was planted into the various enrichment broths in ten-fold serial dilutions ranging from 10 ml. in the first tube to 10^{-9} ml. in the eleventh tube. After incubation at 37° C. for 8-12 hours in the case of Selenite Broth, and 12-24 hours in the case of Tetrathionate Broth, material from each tube was streaked onto the various primary isolation media regardless of whether the tube showed visible

TABLE I

General Outline Showing Treatment of Raw Sewage

I. Concentration
   1) Precipitation using ammonium aluminum sulfate at the optimum pH value for flocculation
      a) Centrifugation of precipitate and sediment
      b) Gravity settling of precipitate and sediment

II. Enrichment
   1) Sodium Tetrathionate Broth
   2) Selenite Broth
      a) Primary Isolation
         1. Bismuth Sulfite Agar
         2. S.S. Agar
         3. MacConkey's Agar
         4. Brilliant Green Acid Fuchsin Agar
         5. Acid Fuchsin Methylene Blue Agar

III. Primary Isolation as in II-a without enrichment

IV. Primary Differential Screening
   1) Kligler's Iron Agar
   2) Triple Sugar Iron Agar
   3) Urea Broth

V. Secondary Differentiation
   1) Dextrose Broth
   2) Lactose Broth
   3) Saccharose Broth
   4) Maltose Broth
   5) Mannitol Broth
   6) Xylose Broth
   7) IMViC Reaction

VI. Identification
   1) Agglutination in specific antisera
   2) Bacteriophage typing

evidence of growth. After incubation at 37° C., all plates were examined at the end of 15, 24 and 48 hours. Only those plates showing isolated colonies were retained for further study.

An effort was made to secure cultures of all different types of organisms growing on the plates, although colonies which showed the typical appearance described for E. coli and A. aerogenes were not taken.

After purification, the cultures were planted in Kligler's Iron Agar slants, Triple Sugar Iron Agar slants, Urea Broth and Carbohydrate Broths. The IMViC reactions were also determined.

Broth cultures of S. typhosa and S. schottmuelleri were added to raw sewage and used as controls. The results of this study are given in Table II.

TABLE II

Cultures Isolated from Raw Sewage at East Lansing, Michigan

Number of sewage samples examined	28
Number of colonies studied	656
Number of Pseudomonas	323
Number of Proteus	110
Number of Paracolobactrum	103
Number of Alcaligenes	53
Number of *E. coli*	53
Number of *A. aerogenes*	13
Number of *S. typhosa*	1
Number of other Salmonella	0
Number of Shigella	0

DISCUSSION

The greatest concentration of organisms in water or sewage, when using alum as the precipitating agent, was found to occur when the precipitation took place at the optimum pH value. In the present investigation, it was found that the optimum pH value when using aluminum ammonium sulfate was higher than when aluminum sulfate was used as the precipitating agent. When a constant amount of the precipitating agent was added to a given volume of sewage, poor settling took place and the supernatant fluid was turbid, having the appearance of colloidal material in suspension. Gravity settling for a four-hour period, when precipitation was accomplished at the optimum pH value, was superior to centrifugation at 3,400 r.p.m. (3,600 x G.) for a period of one-half hour.

When precipitated sewage was planted in enrichment media, there was little inhibition of the coliform organisms. This may have been due to the massive plantings of coliforms. It is possible that the great numbers of coliforms were able to overcome the inhibitory effect of the medium, thus allowing more rapid growth. In the examination of sewage, there is a definite need for an enrichment medium which will be capable of greater coliform inhibition and at the same time allow rapid development of enteric pathogens which might be present.

Of the plating media, Bismuth Sulfite Agar showed the greatest inhibition of coliforms.

The development of numerous black colonies during July,

August and September by organisms having the ability to reduce sulfites confirmed the observations of Wilson and Blair (16). The chief characters of this organism to which Wilson gave the name B. effluviei are as follows: A gram-negative actively motile bacillus with growth on agar resembling B. coli; it liquefies gelatin, is methyl red negative and gives a positive Voges-Proskauer test; it resembles B. cloacae, from which it differs in its reduction of sulfites and in being a non-lactose fermenter; it grows in Koser's citrate solution; it ferments glucose, maltose, mannite, saccharose (and starch) with the production of acid and gas, and has no action on lactose, dulcite and salicin, and it forms indol and does not decompose urea.

Acid Fuchsin Brilliant Green Agar was excellent for eliminating the troublesome Proteus organisms. It did, however, allow the more-or-less unrestricted development of coliforms. The plates were usually overcrowded with lactose fermenters, making the recognition of lactose non-fermenters difficult. It should be mentioned that difficulty may be expected in using this medium if the somewhat alkaline pH value of 7.4 is not strictly observed in its preparation. Salmonellas have a tendency to be inhibited at lower pH values.

Acid Fuchsin Methylene Blue Agar had no advantage over the other plate media in the examination of sewage.

Motile and non-motile, indol-positive cultures were isolated which produced no gas from carbohydrates when originally isolated. Many such cultures could easily be classed as Eberthella or Shigella, but after varying periods of laboratory cultivation, many

of the strains produced gas. Stuart, Wheeler, Rustigian and Zimmerman (29) include these in the Paracolobactrum group as anaerogenic variants of coliforms. They found from serological and physiological properties that the Paracolobactrum group is intermediate between normal coliforms and Salmonella. There appears to be no sharp distinction between the groups, and a more-or-less continuous series of types exist. This group of organisms was the cause of considerable concern as they were often mistaken for Salmonellas on original isolation.

During the early part of the study, Hollenbach and Dahljelm (30) isolated an organism on Bismuth Sulfite Agar which gave the typical colonial appearance of S. typhosa. When first cultured for biochemical reactions, the results in lactose and sucrose were atypical in that acid was produced. Continued incubation resulted in these two media becoming alkaline and thus giving the typical reactions expected of this organism. Subcultures from the original isolation all gave biochemical reactions typical of those described for S. typhosa. Agglutination tests were positive in a titre of 1-320, and a culture sent to the Michigan Department of Health Salmonella Typing Station was reported as having the somatic antigens IX and XII and the flagellar antigen d. The organism was sluggishly motile and the presence of Vi antigen could not be demonstrated.

Agglutination tests using the control S. typhosa organism were positive in a titre of 1-5,120 using the same antisera as that used for the organism isolated from sewage. Wilson and Blair (27)

when discussing the identification of organisms isolated from sewage state that, "The identification was completed by seeing whether the organism was agglutinated to full titre by a typhoid agglutinating serum."

CONCLUSIONS

The results of this study indicate that enrichment media, which are quite satisfactory for fecal samples and urine, will not adequately suppress the growth of coliforms found in precipitated raw sewage.

Enteric pathogens, if present in raw sewage at East Lansing, Michigan, were not in numbers sufficiently large to permit their ready isolation by the methods used in this investigation.

The use of aluminum ammonium sulfate for precipitation of sewage at the optimum pH value is more satisfactory than the use of aluminum sulfate. A greater concentration of the organisms was obtained with aluminum ammonium sulfate.

The Paracolobactrum group of organisms often give biochemical and serological reactions similar to the Salmonellas upon first being isolated from sewage. This is especially true of the anaerogenic strains.

APPENDIX

1. Aluminum ammonium sulfate solution

Aluminum ammonium sulfate	34.2 grams
Distilled water	1000.0 ml.

One ml. of this solution, when added to a 200 ml. sample equals one grain per gallon.

2. Tetrathionate Broth (Bacto)

Broth base

Proteose-peptone No. 2 (Difco)	5.0 grams
Bacto-bile salts	1.0 grams
Calcium carbonate	10.0 grams
Sodium thiosulfate	30.0 grams
Distilled water	1000.0 ml.

Iodine Solution

Iodine crystals	6.0 grams
Potassium iodide	5.0 grams
Distilled water	20.0 ml.

To prepare 1000 ml. of medium, to 1000 ml. of broth base which has been boiled and then cooled to below 45° C., add 20 ml. of the iodine solution. Shake well to mix and dispense in 10 ml. quantities in test tubes, taking care to obtain an even distribution of the insoluble material. The medium was found to give better results when freshly prepared and used on the same day.

3. Selenite Broth (Bacto)

Bacto-Tryptone	4.0 grams
Bacto-Lactose	4.0 grams
Disodium phosphate	10.0 grams
Sodium Acid Selenite	5.0 grams

To prepare the medium, 23 grams of the dehydrated medium are suspended in 1000 ml. of distilled water and heated to boiling. It is then dispensed into sterile culture tubes to give a depth of medium of at least 2 inches. Excessive heating is to be avoided. Do not sterilize in the autoclave. The final reaction of the medium will be pH 7.0.

4. MacConkey's Agar (Bacto)
(Dehydrated - Difco)

Bacto-Peptone	17.0 grams
Proteose-Peptone	3.0 grams
Bacto-Lactose	10.0 grams
Bacto Bile Salts No. 3	1.5 grams
Sodium chloride	5.0 grams
Bacto-agar	17.0 grams
Bacto-neutral red	0.03 grams
Bacto-crystal violet (D.C.-2)	0.001 gram
Distilled water	1000.0 ml.

To prepare the medium, 53.5 grams of the dehydrated medium are suspended in 1000 ml. distilled water. Boil for one or two minutes to dissolve the medium. Sterilize for 20 minutes at 15 pounds pressure.

5. Bismuth Sulfite Agar (Bacto)
(Dehydrated - Difco)

Peptone	10.0 grams
Beef extract	5.0 grams
Dextrose	5.0 grams
Disodium phosphate	4.0 grams
Ferrous sulfate	0.3 grams
Bismuth sulfite indicator	8.0 grams
Agar	20.0 grams
Brilliant green	0.025 grams
Distilled water	1000.0 ml.

Suspend all dry ingredients in the distilled water and heat to boil as rapidly as possible; then allow to simmer for a minute. Into sterile Petri plates pour 15 to 20 ml. of the medium. This medium should not be autoclaved as prolonged heating destroys its selectivity. Final pH 7.6 ±.

6. Salmonella and Shigella Medium (Bacto)
(Dehydrated - Difco)

Beef extract	5.0 grams
Proteose-peptone	5.0 grams
Lactose	10.0 grams
Bile salts	8.5 grams
Sodium citrate	8.5 grams
Sodium thiosulfate	8.5 grams
Ferric citrate	1.0 grams
Agar	13.5 grams
Brilliant green	0.33 grams
Neutral red	0.025 grams
Distilled water	1000.0 ml.

Dissolve ingredients in distilled water. Steam for 15 to 20 minutes or bring to the boiling point. Do not allow to boil or do not sterilize. Pour about 20 ml. medium in each sterile Petri plate. Allow to dry with covers partially removed.

7. Brilliant Green Acid Fuchsin Agar

Base medium

Agar	20.0 grams
Peptone	20.0 grams
Sodium taurocholate	5.0 grams
Sodium chloride	5.0 grams
Distilled water	1000.0 ml.

Autoclave at 10 pounds pressure for 20 minutes, adjust pH to 7.4 and filter. Add 10 grams lactose, mix and distribute in 200 ml. quantities in flasks and sterilize at 12 pounds pressure for 15 minutes.

To prepare plates, to 200 ml. of base medium, melted and cooled to 50° C., add 2 ml. of Andrade's indicator and mix. Add 0.8 ml. of freshly prepared brilliant green (1 per cent) solution. Mix and pour plates.

8. Acid Fuchsin Methylene Blue Agar

Difco peptone	10.0 grams
Lactose	10.0 grams
Dipotassium phosphate	3.0 grams
Acid fuchsin	0.5 grams
Methylene blue	0.05 grams
Agar	15.0 grams
Distilled water	1000.0 ml.

Dissolve ingredients in distilled water by heating. Adjust pH to 6.6. Sterilize at 15 pounds pressure for 20 minutes.

Dissolve ingredients in distilled water. Steam for 15 to 20 minutes or bring to the boiling point. Do not allow to boil or do not sterilize. Pour about 20 ml. medium in each sterile Petri plate. Allow to dry with covers partially removed.

7. Brilliant Green Acid Fuchsin Agar

Base medium

Agar	20.0 grams
Peptone	20.0 grams
Sodium taurocholate	5.0 grams
Sodium chloride	5.0 grams
Distilled water	1000.0 ml.

Autoclave at 10 pounds pressure for 20 minutes, adjust pH to 7.4 and filter. Add 10 grams lactose, mix and distribute in 200 ml. quantities in flasks and sterilize at 12 pounds pressure for 15 minutes.

To prepare plates, to 200 ml. of base medium, melted and cooled to 50° C., add 2 ml. of Andrade's indicator and mix. Add 0.8 ml. of freshly prepared brilliant green (1 per cent) solution. Mix and pour plates.

8. Acid Fuchsin Methylene Blue Agar

Difco peptone	10.0 grams
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Dipotassium phosphate	3.0 grams
Acid fuchsin	0.5 grams
Methylene blue	0.05 grams
Agar	15.0 grams
Distilled water	1000.0 ml.

Dissolve ingredients in distilled water by heating. Adjust pH to 6.6. Sterilize at 15 pounds pressure for 20 minutes.

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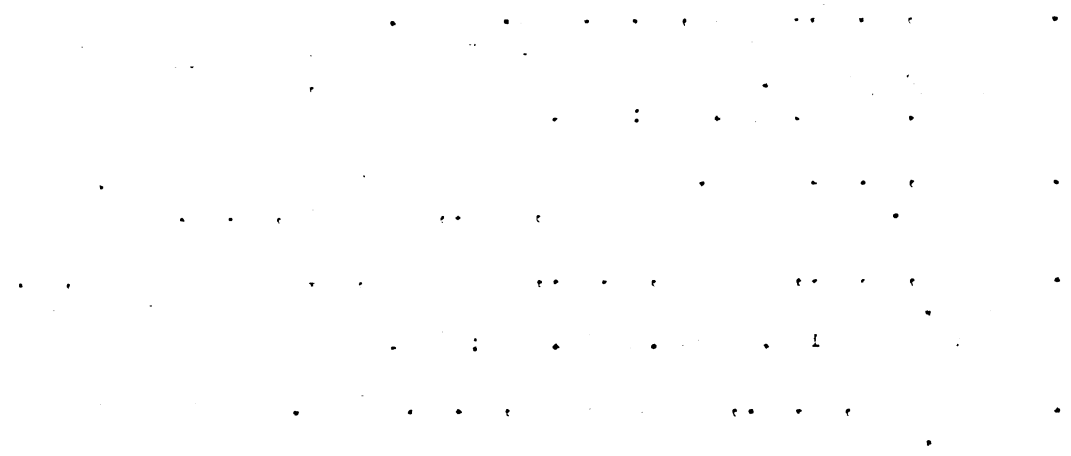

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