A STUDY OF THE BACTERIAL FLORA OF THE CROP OF NORMAL CHICKENS

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THESIS

This is to certify that the

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INTRODUCTION

A preliminary study relating to the value of a certain agent, to which the manufacturer attributes intestinal antiseptic properties, raised the question as to what is the normal bacterial flora of the crop of healthy chickens. In the first study, feed and water were not removed previous to obtaining the crop contents for bacteriological examination so, naturally, some of the organisms isolated from those chickens could have been traced directly to such sources. In this study feed and water were removed, eighteen to twenty-four hours prior to the removal of the crop contents, for the purpose of preventing the introduction of organisms which could not very well be considered a part of the normal flora. However, one cannot disregard the initial effect of feed, water, etc., in the establishment of a bacterial flora of the digestive tract. The digestive tract of young vertebrates is sterile at birth but becomes contaminated in a few hours. Tanner (1) gives an extensive review of the work done by numerous investigators of the intestinal bacterial flora of various vertebrates and of the factors which influence its establishment.

The chickens on which this study was made had been on a standard Michigan Farm Bureau laying mash. Each bird was placed in an individual clean wire cage, but no precautions were taken to prevent it from picking at the fragments of fecal matter which adhered to the wire flooring of its own cage.

REVIEW OF LITERATURE

There have been no reports on the bacterial flora of the crop, but that of the intestinal tract has been the subject of much discussion. Several authors, also, have given some information concerning the bacterial flora of the respiratory tract of fowls ill with respiratory diseases, and one author described the bacterial flora of the respiratory tract of normal healthy fowls.

Gibbs (2) found Staphylococcus albus, Spirocheta, Micrococcus, Escherichia communior, Alcaligenes bronchisepticus and Sarcina lutea in the respiratory tract of normal healthy fowls. He also found a considerable variety of non-pathogenic organisms (probably secondary invaders) in the respiratory tract in conjunction with various diseases. Organisms encountered in cases of infectious tracheitis were: Esch. communior, Escherichia coli, Escherichia gastrica, Escherichia alkalescens, Escherichia pseudocoloides, Micrococci, Alcaligenes bronchisepticus, Pasteurella avicida, Eberthella septicemia, Torula and a yeast. Chickens ill with pullorem disease had Esch. communior, Esch. coli and Sarcina in their respiratory tracts. Staph. albus was found in the respiratory tract of chickens ill with chronic laryngitis and avian paralysis.

Kernoham (3) found, in association with laryngotracheitis of fowls, Pasteurella avicida-like organisms, diplococci, Pseudomonas

pyocaneus and other unidentified bacteria, none of which produced laryngotracheitis by intratracheal instillation.

Graham, Thorp and James (4) found a pleomorphic, gram-positive, non-spore-forming, hemolytic organism in laryngeal and tracheal exudates of fowls suffering from acute infectious laryngotracheitis and subacute or chronic avian laryngotracheitis.

Beach (5) also found gram-negative rods of the Pasteurella type, gram-positive cocci and diphtheroids in association with laryngotracheitis lesions.

The results of Eliot and Lewis (6) were essentially the same as those reported by other investigators of the nasal and tracheal secretions of chickens in health and disease. They found certain staphylococci, streptococci and diphtheroids commonly occurring on mucous membranes of man and animals. There were also a number of ovoid bacteria, gram-positive and gram-negative and several species of micrococci. For a time particular interest was centered on the strains of Pasteurella isolated. These were present in a number of chickens suffering with infectious coryza although fowl cholera did not appear in the flock during the laboratory investigation.

A number of authors have described organisms of the intestinal tract and feces of fowls. Emmel (7) gives the most complete and comprehensive list of bacteria found in the feces of healthy adult birds: Escherichia coli, Escherichia communior,

Escherichia neapolitana, Escherichia acidi lacti, Escherichia alcalescens, Micrococcus aurantiacus, Micrococcus subflavus, Micrococcus percitreus, Staphylococcus albus, Staphylococcus citreus, Bacillus mycoides, Bacillus subtilis, Bacillus cereus, Bacillus cytaceus, Bacillus tritus, Bacillus circulans, Bacillus megatherium, Bacillus petastites, Bacillus ramosus, Bacillus cohaerens, Bacillus vulgatus, Aerobacter aerogenes, Bacteroides bifidus, Clostridium sporogenes, Achromobacter liquefaciens, Salmonella icteroides, and Actinomyces microflavus.

EXPERIMENTAL

Procedure for Obtaining Crop Sample

To remove the crop contents, the technique devised by Stafseth (8) was employed. Ten ml. pipettes were cut off near the distal end and flamed so that the glass wall around the opening became somewhat rounded and smooth. Short pieces of rubber tubing (about three inches in length) were attached to the proximal end. The free ends of the rubber tubes were then plugged with cotton. The pipettes fitted with plugged rubber tubes were then placed in metal cylinders and were sterilized by autoclaving. After completion of the sterilization process, the pipettes were allowed to cool and the distal ends were dipped in sterile five per cent agar so as to form an agar plug about one cm. long in the end of the pipette. When the agar plug had become cool and firm, the pipettes were returned to the sterile cylinder in which they had been autoclaved.

A sample of crop contents was taken by inserting a pipette into the crop, forcing out the agar plug by injecting 20 ml. of sterile saline by means of a 20 ml. Luer glass syringe attached to the rubber tube after removal of the cotton plug. The syringe and the rubber tube were then detached from the pipette, the crop was gently massaged for a few moments and by holding the bird head down, while the pipette

was still in the crop, a sample of crop contents could easily be obtained, collecting it directly into sterile test tubes.

Examination of Sample

The samples were examined microscopically for organisms that might not grow on culture media and were then cultured. The microscopic examinations failed to show any protozoa or spirochetes; however, numerous gram-positive and gram-negative rods and cocci were present. In the preliminary study, by cultural methods the following genera were found: Alcaligenes, Cellumonas, Corynebacterium, Escherichia, Lactobacillus, Micrococcus, Shigella, and Streptococcus.

Four healthy white Leghorn cockerels were used for further study. The material obtained from the crop was shaken for fifteen minutes. Streak plates on five per cent blood agar (see Appendix for list of media and reagents used) were made directly from the undiluted sample. Further dilution was found unnecessary as discrete colony formation was obtained.

The plates were incubated at 37° C. aerobically and under 10 per cent carbon dioxide. All the organisms grew well aerobically at 37° C. and grew even better under 10 per cent carbon dioxide. The sample from the first chicken was also streaked on anaerobic media, covered with Brewer's anaerobic Petri dish cover and incubated at 37° C. A streptococcus was isolated, but no biochemical tests were employed and it was not identified. A blood

plate from the third chicken was also incubated at room temperature. One organism (No. 35) grew well at room temperature, crowding out other organisms. It was identified as Escherichia coli var. communior. Blood plates from the fourth chicken were incubated at 45° C. aerobically and at 37° C. under anaerobic condition produced by replacing air in a pressure cooker with illuminating gas. It was thought that illuminating gas might furnish anaerobic conditions under which organisms, which had not been isolated aerobically, might be obtained. However, no organisms were secured which had not grown under aerobic conditions. Only one organism (No. 41) grew well at 45° C. and it was identified as Escherichia coli var. acidilacti.

After the organisms were obtained in pure culture, smears were made and stained by Burke's (9) modification of Gram's stain. Acid fast, methylene blue and Anjeszky's spore stains were also made of the organisms obtained from the second chicken. Since none were acid fast or revealed metachromatic granules or spores, all further cultures were stained by Gram's stain only.

Bergey's Manual, Fifth Edition, was followed in so far as possible in the identifica tion of the organisms although frequently it was necessary to resort to other sources for a more detailed description of specific organisms.

The numbering of the cultures was done in sequence corresponding to the number of the chicken from which they

were isolated. The cultures numbering from one through nine were isolated from the first chicken. Those numbering 20 through 29 were from the second chicken. Those numbering 30 through 39 were from the third chicken. Those numbering 40 through 50 were from the fourth chicken.

The results of the biochemical test employed are recorded in Tables I, II and III.

Results

On all blood plates incubated at 37° C. a beta-hemolytic organism was the predominating one. Subcultures from these were numbered 1, 21a, 30 and 40.

The colony in all instances was circular in form, convex, with smooth surface, translucent to transmitted light, grayish in color and had an entire edge.

The agar streak culture was similar on all subcultures: the growth was moderate, the form of growth was filiform, glistening, butyrous in consistency and the color was a grayish-cream.

The tryptose broth cultures showed moderate clouding with considerable clearing in five days. There was a moderate amount of grayish viscid sediment.

All these organisms were gram-negative, pleomorphic rods which occurred singly, in pairs and in filaments. The average size of the rods was 0.6 x 1μ and of the coccoid forms 0.4 x 0.6μ. Some of the filaments were as long as 50μ. Smears from the colonies on the isolation plates showed bipolar staining organisms.

Biochemically these four cultures showed some variation. Culture No. 1 gave an acid reaction on litmus milk in one day, had reduced litmus in one day and had formed a curd by the fourth day. No. 21a did not produce any change in litmus milk. No. 30 did not change the pH but did reduce the litmus of litmus

milk. No. 40 gave a slightly acid reaction in litmus milk in seven days.

The morphological and biochemical reactions of culture No. 1, especially the hemolytic property, the coagulation of milk and the failure to produce indol, indicate that it was closely related to Pasteurella hemolytica described by Rosenbusch and Merchant (10) and Merchant (11).

Culture No. 21a showed a similarity to Pasteurella avicida (Gamaleia) Trevisan except that it was hemolytic, failed to form indol and produced no H_2S. Patton (12) gives the results of studies on strains of P. avicida by nine investigators, and several found rare hemolytic strains, also strains that failed to form indol within a week.

Cultures No. 30 and No. 40 showed characteristics resembling those of P. hemolytica and P. avicida.

Because a beta-hemolytic organism, which showed a morphological, cultural and biochemical relationship to the Pasteurellae, was consistently isolated from the crop of the chickens studied, it was decided that the pathogenicity of the latest culture isolated (No. 40) should be tested. A young Rhode Island Red was given an intravenous injection of 0.25 ml. of a heavy suspension of an eighteen-hour-old culture grown on a tryptose agar slant. The suspension was made by adding, aseptically, several ml. of sterile saline. The tube was gently rotated, thereby creating a suspension

of organisms free from clumps large enough to produce embolism upon intravenous injection. After two days, blood was withdrawn aseptically and cultured by making a pour plate (one ml. blood to fifteen ml. sterile nutrient agar at 45° C.) and broth culture(two ml. blood to fifty ml. sterile broth.) A hemolytic organism was recovered in almost pure culture. Morphologically it was like Culture 40 and exhibited a definite capsule. It produced strong acid reaction in dextrose, mannitol and sucrose, weak acid reaction in lactose and maltose and no acid in salicin. In lactose broth with a peptone base there was no reaction after a week. Blood smears were made five days after inoculation but no organisms were found. The bird was still alive at the end of three weeks and apparently in good health. It is of course obvious that a test on one bird is misleading since it is known that individual birds vary greatly as to their susceptibility. Time was too limited for further study of the pathogenicity of this organism.

The second most predominant colony on the blood plates was a small non-hemolytic one. Subcultures from this type of colony were numbered 29c, 36 and 48.

On agar slants a scant grayish film was formed along the needle tract with discrete colony formation along the

edge of the film.

Tryptose broth cultures were clear with scant, slightly flaky grayish sediment.

Litmus milk remained unchanged in each instance.

Microscopic examination of smears revealed small, pleomorphic gram-positive rods. There were straight and curved rods, some were club-shaped and others were coccoid. They occurred singly, in pairs and short chains. Some short chains showed branching-like arrangement. The size ranged from .5 x 1μ to .5 x 2.5μ. They were non-spore-forming, did not have capsules and were nonmotile.

These appear to be diphtheroids and may possibly be related to Bacillus maculatus described by Graham-Smith (13).

Culture No. 6, an alpha-hemolytic, gram-positive pleomorphic rod, closely resembled Corynebacterium enzymicum (Mellon) Bergey et al. and the pleomorphic organism of Graham, Thorp and James (4). There was no perceptible growth in tryptone broth after one week and the test for indol formation was negative. According to Bergey C. enzymicum produces slight indol formation. Loeffler's blood serum, potato, dextrin and glycerol media were not employed.

Culture No. 37 was a small alpha-hemolytic colony on blood agar. The cells were gram-positive, non-spore-forming,

motile rods of irregular shape. Some cells were straight, some coccoid, others were dumb-bell-shaped and some were club-shaped.

The range of size was from 1 x 1.5μ to .8 x 15μ. On an agar slant the growth was a scant, grayish film with discrete colonies along the edge. The tryptose broth culture was slightly turbid, clearing in five days, with a moderate amount of grayish granular sediment. It was not identified for lack of information in available literature.

Culture No. 27c was a punctiform, convex, alpha-hemolytic colony on blood agar. The growth on an agar slant was scant, with discrete small colonies. Tryptose broth did not show any visible growth. Morphologically the culture consisted of short, plump, gram-positive pleomorphic rods which occurred singly and in pairs. Identification was not possible due to lack of sufficient information in available literature.

Culturally and morphologically Culture No. 39 was related to Lactobacillus brevis (Orla-Jensen) Bergey et al. There was an especially close relationship with regards to the fermentation of xylose, levulose, galactose and the vigorous fermentation of arabinose. There was only slight fermentation of dextrose, which was also in agreement with the preference of

some strains of L. brevis for levulose over dextrose. No tests were made to determine the products of fermentation of the hexoses and the pentoses. No acid was produced in litmus milk or maltose. Calcium lactate, dextrin, glycerol and starch fermentation media were not employed.

Cultures No. 5, 25, 38 and 50 were similar to Lactobacillus fermenti Beijerinck morphologically, culturally and biochemically in so far as the tests employed corresponded to those enumerated by Bergey. Yeast extract-dextrose gelatin, dextrin and starch fermentation media were not used; nor were tests made to determine the products of fermentation of the hexoses and the pentoses. There was no reduction of litmus in litmus milk.

Cultures No. 9, 22a, 31, and 49 were identified as Streptococcus lactis (Lister) Lohnis from Sherman's (14) description. Potato, glycerol, sodium hippurate and esculin media were not used. Chemical tolerance tests were not employed. Temperature tolerance was not determined. Neither antigenic analysis nor serological identification was attempted.

Cultures No. 27, 32 and 46 were identified as Streptococcus equinus var. ignavus Holman as described by Sherman (14). Sodium hippurate, esculin and glycerol media were not employed.

Temperature relations and chemical tolerance were not determined.

Culture No. 33 corresponded closely to Streptococcus liquefaciens Sternberg emend. Orla-Jensen. It varied from the description in that the curd which formed in litmus milk was not peptonized, however caseolysis fails in variants which do not liquefy gelatin. Sodium hippurate, esculin and glycerol were not determined.

According to the tests employed cultures No. 3, 23c, 34 and 45 were similar to Neisseria catarrhalis (Frosch and Kolle) Holland.

The biochemical, cultural and morphological characteristics of Culture No. 24c were similar to Flavobacterium proteus Shimwell and Grimes.

Culture No. 35 was identified as Escherichia coli var. communior (Topley and Wilson). Culture No. 41 was identified as Escherichia coli var. acidilacti (Topley and Wilson). Culture No. 42 was identified as Escherichia coli var. neapolitana (Topley and Wilson).

Two micrococci were isolated: Culture No. 43 was

identified as Micrococcus percitreus Bergey, et al and Culture No. 47 as Micrococcus epidermidis (Kligler) Hucker.

Culture No. 44 corresponded closely to Shigella minutissima (Migula) Bergey, et al.

TABLE NO. I

Biochemical Characteristics and Motility

Culture Number	mot. med. / motile	mot. med. / L. ferm.	gel. liquef.	nitrites	prod. of NH_3	catalase	Voges Prosk.	meth. red	indol	citrate	H_2S	litmus milk	dextrose	lactose	maltose	mannitol	sucrose	starch hydrol.
1	-	+	-	+	-	+			-		-	ARC	+	+	+	+	+	-
3	-	-	-	-	-	+			-		-	N	-	-	-	-	-	-
5	-	+	-	-	-	-			-		-	sl A	+	+	+	-	+	-
6	-	+	-	±	-	-			-		-	ARC	+	+	+	-	+	-
9	-	+	-	±	±	-			-		-	RAC	+	+	+	-	+	-
21a	-	+	-	+	-	+	-	+	-	-	-	N	+	-	-	+	+	-
22a	-	+	-	+	±	+	-	+	-	-	-	RAC	+	+	+	+	+	-
23c	-	-	-	±	-	+	-	-	-	-	-	N	-	-	-	-	-	-
24	-	-	-	±	±	+	-	-	-	-	-	N	(+)	-	(+)	-	(+)	-
25	-	+	-	-	-	-	-	-	-	-	-	sl A	+	+	+	±	+	-
27c	-	-	-	±	+	-	-	+	-	-	-	N	-	-	-	-	-	-
29c	-	-	-	-	-	+	-	-	-	-	-	N	-	-	-	-	-	-

A - Acid
R - Reduction
C - Curd
± - slight
N - Neutral
(+)- Acid and slight amount of gas
Blank space - test not run

TABLE NO. II

BIOCHEMICAL CHARACTERISTICS AND MOTILITY

Culture Number	mot med. mot.	mot med. L. ferm	gel. liquef.	nitrites	prod. of NH_3	catalase	Voges Prosk.	meth. red	indol	citrate	H_2S	litmus milk	dextrose	lactose	maltose	mannitol	sucrose	salicin	l-xylose	dulcitol	d-sorbitol	i-inositol	l-arabinose	l-rhamnose	d-xylose	d-galactose	d-mannose	d-levulose	trehalose	raffinose	inulin	starch hydr.
27	-	+	-	-	-	-	-	-	-	-	-	N	+	-	+	-	+	-	-	-	-	-	-	-	-	+	±	+	+	+	-	+
30	-	+	-	-	-	+	-	-	-		-	N R	+	+	±	+	+	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-
31	-	+	-	+	+	-	-	+	-		-	ARC	+	+	+	+	+	+	-	-	-	-	+	-	-	+	+	+	+	±	-	-
32	-	+	-	-	-	-	-	+	-		-	sl A	+	-	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+
33	-	+	±	+	+	+	-	+	-		-	ARC	+	+	+	+	+	+	+	-	±	-	+	-	+	+	+	+	+	-	-	-
34	-	-	-	-	-	+	-	-	-		-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	+	+	-	+	+	+	-	+	+		-	ACR	⊕	⊕	⊕	⊕	-	⊕	⊕	⊕	⊕	-	⊕	⊕	⊕	⊕	⊕	⊕	⊕	-	-	+
36	-	-	-	-	-	+	-	-	-		-	N	-	-	-	-	-	-	-	-	-	-	±	-	-	±	±	+	-	-	-	-
37	+	+	-	-	-	+	-	-	-		-	sl A	+	-	±	-	±	±	-	-	-	-	-	-	-	+	+	+	+	+	-	-
38	-	+	-	-	-	-	-	+	-		-	sl A	+	+	+	±	+	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-
39	-	-	-	-	-	±	-	-	-		-	N	±	-	-	-	-	-	+	-	-	-	+	-	+	+	-	+	-	+	-	+

N - Neutral
R - Reduction

A - Acid
C - Curd

± - slight
⊕ - acid and gas

sl A - slight acid reaction
Blank space - test not run

TABLE NO. III

Biochemical Characteristics and Motility

Culture Number	mot. med.		gel. liquef.	nitrites	prod. of NH_3	catalase	Voges Prosk.	meth. red	indol	litmus milk	dextrose	lactose	maltose	mannitol	sucrose	salicin	l-xylose	dulcitol	d-sorbitol	i-inositol	l-arabinose	l-rhamnose	d-xylose	d-galactose	d-mannose	d-levulose	trehalose	raffinose	inulin
	mot.	L. ferm.																											
40	-	+	-	+	-	+	-	-	-	sl A	+	±	±	+	+	-													
41										45°C-	+	-	-	-	-	-													
										37°C-	⊕	⊕	⊕	⊕	-	-													
42	+	+	-	±	+	+	-	+	+	AC	⊕	⊕	⊕	⊕	⊕	⊕													
43	-	-	+	+	+	+	-	+	-	Alk R P	+	-	+	+	+	-													
44	-	-	-	+	-	±	-	-	-	sl ARC	+	-	+	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	-
45	-	-	-	-	-	+	-	-	-	N	-	-	-	-	-	-													
46	-	+	-	-	±	-	-	+	-	A	+	±	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	-
47	-	+	-	+	+	+	-	±	-	sl A	+	+	+	+	+	+													
48	-	-	-	-	-	+	-	-	-	N	-	-	-	-	-	-													
49	-	+	-	-	-	-	-	+	-	ARC	+	+	+	+	+	+													
50	-	+	-	-	-	-	-	-	-	A	+	+	+	±	+	-													

sl A - slightly acid
± - slight
⊕ - acid and gas
C - curd
Alk - alkaline
R - Reduction
P - Peptonization
N - Neutral
Blank space - test not run

DISCUSSION

The flora of the crop of healthy chickens appears to be subject to some variation, however some organisms seem to be constant inhabitants. The Pasteurella-like organism was consistently isolated as was the Neisseria sp., Lactobacillus fermenti and Streptococcus lactis. Streptococcus equinus var. ignavus and Corynebacterium sp. were isolated from the crop of three of the chickens. Escherichia coli was identified from only two chickens. The Micrococcus, Shigella, Flavobacterium, Lactobacillus brevis, Corynebacterium enzymicum and Streptococcus liquefaciens were each isolated from only one bird. This apparent variation might be due to inadequacies of bacteriological procedures. It is quite possible that these organisms might not actually have been absent.

The colonies that appeared to be alike were picked in duplicate. But this did not guarantee that other colonies, apparently identical, might not represent other organisms. In subculturing colonies from the blood plates from the crop contents of the third and fourth chickens this was taken into consideration and a greater number, but not all the alpha-hemolytic and non-hemolytic punctiform colonies were subcultured in semi-solid agar. This was effective in securing more species and more abundant growth.

In regards to possible sources of contamination, the

following should be considered:

1. The possible contamination of the agar when plugging the distal ends of the pipettes. The pipettes had to be used soon after they were plugged since the agar dried in a few hours and broke the seal. Hence there was no opportunity for detection of contamination. However, if the agar was practically solid when the pipettes were plugged, the time interval was reduced to only a few minutes before the pipettes were returned to the sterile container. Since the table had been wiped-down well and the windows and the doors were closed to prevent cross currents of air, the amount of contamination was practically nil. The important area of the pipette, the inside, still remained completely sterile.

2. In inserting the pipette into the crop of the chicken, it is obviously possible that organisms may be carried down from the mouth and esophagus. However, this is not serious because of the fact that the bacterial flora of the crop is constantly conditioned by organisms from the upper respiratory tract and oral cavities.

A considerable amount of difficulty was encountered in attempting to identify organisms which were isolated because of incompleteness of the available literature dealing with the genera concerned with respect to description of various characteristics of the organisms and also with respect to the exact nature of the media used.

In this study no antigenic analyses or serological identifications were attempted, the identifications were based solely upon cultural, biochemical and morphological features. Most of the organisms isolated were closely related to previously described organisms but might nevertheless be different species.

SUMMARY

1. A study of the flora of the crop of healthy chickens was made.

2. Organisms which were either identical with or similar to the following were isolated:

Pasteurella sp.

Neisseria catarrhalis

Lactobacillus fermenti

Lactobacillus brevis

Corynebacterium enzymicum

Corynebacterium sp.

Streptococcus lactis

Streptococcus equinus var. *ignavus*

Streptococcus liquefaciens

Flavobacterium proteus

Escherichia coli var. *communior*

Escherichia coli var. *neapolitana*

Escherichia coli var. *acidilacti*

Micrococcus percitreus

Micrococcus epidermidis

Shigella minutissima

3. As indicated in the discussion only morphological, cultural and biochemical procedures were employed in the identi-

fication, therefore it is quite possible that some of the isolated species are not identical with the ones named above.

4. A Pasteurella-like organism was tested for pathogenicity with negative results.

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APPENDIX

The Media Used Were Made As Follows:

Gelatin liquefaction test medium:

120 gm. gelatin
5 gm. sodium chloride
3 gm. beef extract
1000 ml. distilled water

Nitrate-peptone solution:

1 gm. Bacto peptone
1 gm. nitrite-free KNO_3
5 gm. sodium chloride
1000 ml. distilled water

Proteose-peptone medium (V-P):

5 gm. Bacto proteose-peptone
5 gm. dipotassium phosphate
5 gm. dextrose
1000 ml. distilled water

Starch agar:

15 gm. agar
2 gm. peptone
3 gm. beef extradt
1 gm. dipotassium phosphate
1 gm. sodium chloride
5 gm. soluble starch
1000 ml. distilled water

Semi-solid agar:

37 g. Bacto brain heart infusion dehydrated
1.5 gm. agar
1000 ml. distilled water

Nutrient broth:

20 gm. Bacto tryptose
5 gm. sodium chloride
3.5 ml. N. sodium hydroxide
10 gm. dextrose
1000 ml. distilled water

Blood plates were made by adding 15 ml. sterile defibrinated sheep's blood aseptically to 300 ml. sterile chicken infusion agar base at 48° C.

The motility medium was a modification of Bacto* Motility Test Medium to which 1 per cent lactose, 1 per cent Andrade's indicator solution and .05 per cent dipotassium phosphate were added.

Litmus milk was prepared from skimmed milk with sufficient litmus solution added to give good color.

For fermentation studies a 1 per cent sugar solution was made with a tryptose broth base. Only 0.5 per cent of the rare sugars was used.

Agar slants were prepared from Bacto tryptose dextrose agar. Citrate medium was Bacto Simmons citrate agar. Iron agar was prepared from Bacto Kligler's iron agar. Difco tryptone broth was used to detect indol formation. Anaerobic agar prepared by Baltimore Biological Laboratories for use with Brewer's Anaerobic Petri dish cover was used for anaerobic culture.

*See Difco Manual, 7th Edition for all Bacto products.

Test Reagents Employed:

Litmus solution:
40 gm. granular litmus
300 ml. 40% alcohol

Indol Reagent:
75 ml. Iso-Amyl alcohol
25 ml. conc. hydrochloric acid
5 gm. p-dimethylaminobenzaldehyde

Nitrite test solutions:

Number 1.
8 gm. sulfanilic acid
250 ml. glacial acetic acid
750 ml. distilled water

Number 2.
5 gm. a-naphthylamine
250 ml. glacial acetic acid
750 ml. distilled water

Acetyl-methyl-carbinol test (Voges-Proskauer):
1 gm. copper sulphate in 10 ml. water
40 ml. conc. ammonium hydroxide
950 ml. (10% aqueous) sodium hydroxide

For production of NH_3 Nessler's reagent was used.

Andrade's Indicator:
100 ml. 0.2% aqueous solution acid fuchsin
16 ml. N/1 NaOH

For catalase production:
1 ml. of Parke-Davis Hydrogen Peroxide (3%) to broth culture several days old.

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