A STUDY OF THE COLIFORM GROUP OF BACTERIA FROM THE INTESTINE OF DISEASED CHICKENS

by

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B. V. M., Kansas State College
of Agriculture and Applied Science, 1919

A THESIS

submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1933

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INTRODUCTION

The fact that chickens are usually permitted "open range" in the agricultural districts makes it possible for them to be a potential source of fecal pollution of the water and food supplies in these localities. Consequently, it is of interest to determine the varieties of the coliform group of bacteria which may be present in the intestinal tract of chickens.

The 1925 Standard Methods of Water Analysis defines the colon group as follows: "It is recommended that the coli-aerogenes group be considered as including all non-spore forming bacilli which ferment lactose with gas formation and grow aerobically on standard solid media".

This grouping may be still further subdivided into "fecal" and "non-fecal" types on the basis of the appearance on eosin-methylene blue agar, acid in a buffered broth, and formation of acetyl-methyl-carbinol. This subdivision has not been accepted and included in the Standard Methods of the American Public Health Association.

Since the Standard Methods makes no distinction between the fecal and non-fecal types of the group, the question arises; should water be considered potable when only the non-fecal type is present? Many sanitarians, especially

in the eastern states, consider the presence in water of either type as a criterian of pollution. Whereas, some investigators in the middle west base their decision as to the potability of water upon the presence or absence of the fecal type only.

It is the purpose of this study to attempt to determine the types of colon organisms which may inhabit the intestinal tract of chickens and their possible significance in a sanitary survey of these localities.

The first member of the colon group was isolated by Escherich (1884) from the intestinal tract of a patient suffering from Asiatic cholera. Again in 1886 Escherich isolated a similar organism from the feces of a normal, milk-fed infant and designated it Bacterium coli commune. This marked the beginning of a very exhaustive study of the bacteria inhabiting the intestinal tract of man and the lower vertebrates, and led to the discovery that members of the genus were normally found in the intestinal lumen of animals of various species.

Further studies of the group, however, led to the discovery that similar organisms were widely distributed in nature outside the intestinal tract. Thus the presence of these organisms elsewhere in nature has usually been considered as an index of fecal pollution. As a result, this group of bacteria has been of first interest to the bacter-

iologist and sanitarian. However, many investigators report the finding of the coliform type on plants and grain. Preston (1906), Rogers, Clark, and Evans (1915) noted the presence of such organisms in flour, bran, corn-meal, oats and barley.

Konrich (1910) studied 547 samples of soil and found that 65 per cent of them showed E. coli in portions of between 0.1 and 0.5 grams. These organisms were also present in samples of soils taken from waste lands as well as from cultivated fields.

A part of the early confusion resulting from the study of this group of bacteria was due to the use of but a single test to prove their presence. The simple test usually applied was the dextrose fermentation tube. All products containing dextrose fermenting organisms were considered as indicating the fecal type of the colon organism. The fact that this proved not to be the case led to considerable confusion in the interpretation of the results of such a test.

The variation in character and the wide distribution, together with the sanitary importance of the colon group has stimulated numerous attempts to devise an acceptable system of classification. Many of those proposed in the past have, with few exceptions, failed to supply the desired information because they did not rest on a sound basis of biological relationships. Previous attempts at classifica-

tion were unsuccessful, due to some extent at least, to a lack of delicate and exact methods, neglect of a consideration of the natural habitat, and faulty interpretation of results obtained. Some of the methods of the earlier investigators have been replaced by more modern procedures based on broader scientific foundations as, for example: Differentiation of types by exact methods of determining the hydrogen-ion concentration in media of known compositions; and the study of quantitative relationships of carbon dioxide to the total gas volume. Again, older methods which had been practically discarded have been reapplied and made to serve as important differential tests, one of which is the Voges-Proskauer reaction.

In this study an attempt has been made to apply the more modern technic to a classification of those species of coliform organisms isolated from feces taken from various levels of the intestinal tract of chickens.

REVIEW OF LITERATURE

Normally colon bacilli, as well as other types of bacteria, are taken into the alimentary tract with the first feeding. Carpenter and Woods (1924) found that the environment of the animal determined the number and types of organisms from the Coli-aerogenes group taken in with its food. They also observed that these organisms were dis-

tributed throughout the intestinal tract and that wherever they lodged they established themselves.

Emmel (1931) in a study of the intestinal flora of chicks, found the average distribution of the colon organisms in various levels of the intestine as follows: Duodenum 32.35 per cent, middle portion 35.02 per cent, and cloaca 63.35 per cent.

Rogers, Clark, and Evans (1914) using special methods, found that in bovine feces the E. coli group greatly predominated over the A. aerogenes group. They were able to isolate only one species of the latter group from this source.

Tonney and Noble (1930) examined numerous samples of feces from various sources and found that E. coli represented 99.3 per cent and A. aerogenes 0.7 per cent of the colon group in the cases observed.

The first biologic division of the colon group was made by Smith in 1895. He divided all lactose-fermenting-non-liquefying organisms into groups according to their power of fermenting sucrose. Thus indicating at least two groups and the possibility of others.

Using Smith's data as a starting point, MacConkey in 1905 suggested a further sub-division of these two groups based on the power of the organisms to ferment dulcite. However, not satisfied that this classification properly

distinguished the different types of organisms belonging to the colon-group, he proposed a further sub-division based on a number of biochemical differences, such as fermentation of certain other sugars, indol production and Voges-Proskauer reaction.

Following to some extent MacConkey's proposed system of classification, Bergey and Deehan (1908) grouped the organisms of the colon group according to their powers of fermenting cuerose, dulcite, adonite and inulin; and with regard to the presence or absence of motility, indol production, the Voges-Proskauer reaction and the liquefaction of gelatin. Using the four latter characters, they demonstrated sixteen different possibilities as to the presence or absence and combinations of these reactions, and using the fermentive characters employing four different sugars, they indicated sixteen still different possibilities with regard to these powers. From this data they conceived the possibility of two hundred fifty-six different combinations of these eight characters. Thus as the number of fermentable substances and characters observed increased, the number of combinations would increase geometrically thereby approaching infinity. Therefore, it would be impossible to regard each character observed as of similar and equal differential value.

Jackson (1910) also following MacConkey's system,

separated the group into sixteen distinct types. He believed that a classification based first, on form and grouping of cells; second, on the relation of their growth to air; third, on their fermentive characters; and finally, on general cultural and morphological and biochemical reaction, would bring allied species and varieties into closely related groups. He also observed that the different members of the colon group could not be used indiscriminately for the production of vaccines, but that the variety causing the infection should be determined and chosen for this purpose. This suggested the possibility of a differentiation of types within the group based upon the specificity of the serological reaction.

This system of classification proposed by Jackson was accepted by the American Public Health Association and included in the standards of methods for 1912.

The classifications of MacConkey, Bergey and Deahan, and Jackson have given rise to serious objections because of their extreme flexibility. However, they were forerunners of a system of differential classification based on the correlation of characters and other cultural features and reactions.

In 1912, Howe made the first attempt to formulate a biologic classification based on the biometric principles.

He studied motility, quantity of gas, fermentation of mannite, dulcite and starch, and concluded that they did not correlate with any other properties, and were, therefore, of little value in classifying this group. However, he confirmed the basic division made by Smith, and claimed that dextrose, lactose, sucrose and raffinose constituted a true "metabolic gradient", and that fermentation of any one sugar implied fermentation of those preceding it in series.

Klighler (1914) sub-divided the sucrose groups on the basis of dulcite and salicin fermentation respectively, and found that the sucrose-salicin groups gave a better correlation with indol production, Voges-Proskauer reaction and gelatin liquefaction, than did the sucrose-dulcite groups. The following is a summary of his groupings based only on carbohydrate fermentation:

The sucrose-positive (+) salicin-positive (+) group, usually dulcite-negative (-) correspond to A. aerogenes.

The sucrose-positive (+) salicin-negative (-) group, usually dulcite-positive (+) correspond to E. communior.

The sucrose-negative (-) salicin-positive (+) group, usually dulcite-positive (+) correspond to E. communis.

The sucrose-negative (-) salicin-negative (-) group, usually dulcite-negative (-) correspond to E. acidi lactici.

He also observed that most of the sucrose-positive (+)

salicin-positive (+) and glycerin-negative (-) group of bacteria were gelatin liquefiers.

Kligler's work (1913) on indol production of organisms of the colon-typhoid group led him to conclude that the indol reaction was sufficiently constant to be of differential value. Employing Ehrlich's method of testing for indol he found that the test should be made on the fourth or sixth day, and that the tubes should always be shaken up with chloroform as a confirmatory test. He found that when this test was applied that E. coli was generally indol positive (+); A. aerogenes and B. proteus variable (±); and A. cloacae and S. paratyphi always negative (-).

Rogers, Clark and Davies (1914) found two distinct groups of coliform bacteria occurring in market milk. One of these groups was characterized by a low and the other by a high gas ratio; i.e. the ratio of carbon dioxide to hydrogen produced in dextrose media. They found E. coli belonged to the low ratio group as it always fermented dextrose with the formation of carbon dioxide and hydrogen in nearly equal parts. They also observed certain very striking correlations between the gas ratio and the source of the culture.

Clark, and Clark and Lubs (1915), found that those coliform organisms which produced a low ratio attained a greater hydrogen-ion concentration when grown in a standard medium than did those which produced a high gas ratio.

Moreover, they observed that the limiting hydrogen-ion concentration of the two groups could be distinguished by an indicator such as paranitrophenol or methyl red.

Brown (1914) showed that the optimum temperature was 37°C. for the maximum production of acid in 24 hours by members of the E. coli groups when grown in a medium containing a fermentable carbohydrate.

Levine (1915), Johnson and Levine (1916), and Rogers and his coworkers (1917) correlated the limiting hydrogen-ion concentration with the Voges-Proskauer reaction and showed that those organisms which gave a high concentration of hydrogen-ions generally failed to give the Voges-Proskauer reaction.

Levine (1917) suggested that the coefficients of correlation be used as a basis for a differential classification of coli-like bacteria.

ORIGIN OF CULTURES STUDIED

All of the 187 cultures studied in this work were isolated from the intestinal tract of twenty-two diseased chickens. These, representing various sections of the State of Kansas, were received for diagnostic purposes by the Department of Bacteriology of the Kansas State College. The material for isolation purposes was obtained at the autopsy of the chicken.

The procedure followed in collecting the cultures will be discussed under methods.

METHODS AND RESULTS

Isolation

The methods herein applied are for the following purposes:

First, to determine the cultural and morphological features of organisms belonging to the group isolated from the intestinal tract of diseased chickens.

Second, to determine the biochemical reaction of members of the group.

Third, to determine the serological relationship of organisms within the group.

Cultures were obtained from four different levels of the intestinal tract, i.e. at the first flexure, the cecum and at two different levels about an equal distance apart. The outer wall of the intestine was seared at the place to be cultured, and an incision made with a sterile scalpel through the wall into the lumen. A small amount of feces was removed with a sterile inoculating loop and placed in a tube of sterile nutrient broth. After 24 hours incubation at 37°C., a loopful of this material was streaked on either Endo's agar, or Levine's eosin-methylene blue agar, and the

plates incubated 24 hours at 37°C.

Typical well isolated colonies were picked from the plates with a sterile inoculating needle, and lactose fermentation tubes were inoculated. After 24 hours growth at 37°C., material from those tubes showing 10 per cent or more gas development with acid production was placed on nutrient agar slants and incubated at 37°C. for 24 hours. These served as the stock cultures for the study, and were kept at room temperature.

The nutrient broth was prepared as follows:

Peptone	10 grams
Beef extract (Liebig's)	3 grams
Sodium chloride (C.P.)	0.5 grams
Distilled water	1000 cc.

The reaction of the medium was adjusted to pH 7.0 and sterilized in the autoclave at 15 pounds pressure for 20 minutes.

The nutrient agar was prepared by adding 1.5 per cent shredded agar to the nutrient broth before sterilizing. Endo's agar and Levine's eosin-methylene blue agar were prepared from the "Difco" brand of dehydrated media. Uric acid medium was prepared according to the formula of Koser (1918). The distilled water used in the preparation of this medium was distilled two or more times in order to remove traces of ammonia which may have been present. The glass ware used

for the experiment was chemically cleaned.

The effect on the growth of the organisms by various solid differential media was distinctly different. The Escherichia group when grown on Endo's agar possessed a distinct metallic sheen, the colonies were flat and button-like, and about 2 or 3 mm. in diameter. The internal structure of the colony was obscured due to a dark-red coloration of the colonies and the surrounding medium.

When cultivated on Levine's eosin-methylene blue agar, the group possessed a metallic sheen when examined by reflected light. The colonies were flat or slightly concave, often concentrically ringed, and about 1 or 2 mm. in diameter. In the central portion there was a light to dark-brown or brownish-black area which extended about three-fourths the distance across the diameter of the colony. The internal structure of this portion of the colony could not be clearly decerned.

When grown on the uric acid medium there was a marked inhibitory effect on the growth of E. coli. Whereas a pure culture of A. aerogenes developed a luxuriant growth.

Morphology and Staining Reactions

The morphological characteristics, such as shape and arrangement, were observed by means of the compound microscope.

The organisms were short, somewhat plump, rods having rounded ends. The average measured about 0.4 to 0.6 microns in width and from 1.0 to 2.0 microns in length. They were non-spore bearing, aerobic and facultative anaerobic microorganisms which grew luxuriantly at an incubation temperature of 37°C.

The usual staining procedures were used for making the Gram's stain and the simple stains.

The organisms stained readily with the common anilin dyes, and were Gram negative.

Motility of the organisms was determined by the hanging drop method. Young broth cultures, sixteen to eighteen hours old, were used for this purpose. However, in a number of instances the same culture was examined for motility after 72 hours.

It was found that motility varied with the different strains and with the age of the cultures, it being more evident in young cultures than in old cultures. Some of the strains were quite actively motile, while others were sluggish and exhibited a motility hardly distinguishable from Brownian movement. Table I shows the per cent of motile and non-motile strains isolated from the intestinal tract of chickens.

Table I. Motility of Fecal Type Colon Cultures

Number of cultures	Motile	Non-motile	Per cent motile
127	72	55	56.6

Summary. The colon group develop characteristic colonies when cultivated on either Levine's eosin-methylene blue agar or Endo's agar. On these media the fecal type produce colonies possessing a metallic sheen, whereas in the non-fecal type the metallic sheen is replaced by a pinkish red color.

The uric acid medium markedly inhibits the growth of the fecal type.

These organisms stain readily with the common anilin dyes, and were Gram negative.

Motility of the strains isolated was variable.

Table II summarizes the morphological and cultural features of the fecal type colon organisms isolated from the intestinal tract of chickens.

Table II. Morphological and Cultural Features of the Fecal Type Colon Organisms From Chickens

Total number of cultures	Variety	Form	Arrangement	Ends	Motile	Non-motile	Capsules	Gram stain	Spores	Agar streak		Nutrient broth *		
										Growth	Form	Pellicle	Cloudy	Sediment
39	E. coli	Short rods	Usually single	Rounded	23	16	-	-	-	A	F	+	+	+
89	E. communior	Short rods	Usually single	Rounded	50	39	-	-	-	A	F	+	+	+
20	E. formica	Short rods	Usually single	Rounded	11	9	-	-	-	A	F	+	+	+
31	E. anindolica	Short rods	Usually single	Rounded	14	17	-	-	-	A	F	+	+	+

\- = Negative for Gram's stain, capsules and spores.
A = Abundant growth.
F = Filiform.
* = Pellicle usually present, slightly cloudy, sediment granular-like.

Biochemical Reactions

Carbohydrate fermentation. The fermentation reaction of various carbohydrates such as lactose, dextrose, sucrose, salicin, dulcite, raffinose, mannite, maltose and glycerol was observed, as well as those of the methyl-red test and Voges-Proskauer reaction.

The sugars were prepared in 10 per cent aqueous solutions, and sterilized in the autoclave at 10 pounds pressure for 15 minutes.

The Durham type fermentation tube was used. This type of tube was made by inverting a small vial about two inches long in an ordinary test tube. About 10 cc. of nutrient broth containing brom-thymol-blue as an indicator was placed in the tubes then sterilized in the usual manner. One cc. of the ten per cent sugar solution was added to each fermentation tube and incubated 24 hours at 37°C. They were then inoculated from a 24 hour old broth culture and incubated five days at 37°C. A control tube was included with each series inoculated. Observations were made every 24 hours during the incubation period and the progress of the fermentation recorded.

Only those cultures which fermented lactose with acid production and gas formation were tentatively considered as

belonging to the colon group of bacteria.

The data for the fermentation reactions were qualitative and were based on the changes produced in the culture media after 72 hours incubation. No attempt was made during the course of this experiment to accurately measure the volume of gas developed, or to determine the amount of acid produced by this process.

Although the methods employed for this study were somewhat crude, some quite interesting data were obtained.

Lactose fermentation was found to be more reliable as a presumptive test for the true colon group than dextrose. This was evidenced by the fact that 23 strains of 155 dextrose fermenting strains failed to attack lactose.

Dulcite, mannite, maltose and glycerol were fermented by all of our strains belonging to the E. coli group. Whereas only two strains failed to decompose raffinose.

By the fermentation reaction of these organisms in sucrose the colon group could be divided into two distinct groups; one of which was sucrose-positive (+) and the other sucrose-negative (-). Some of the sucrose-negative group possessed the power to ferment salicin, thus making possible another subdivision of the group. Tables III and IV show a grouping of the organisms isolated based upon these fermentation reactions; and classified according to Bergey's (1930) classification.

Table III. Grouping of Fecal Type Colon Organisms Isolated from the Intestinal Tract of Chickens

Lactose	Sucrose	Salicin	Variety	Groups
+	-	+	E. coli	Group I
+	+	+	E. communior	Group II
+	-	-	E. formica	Group III
+	+	-	E. anindolica *	Group IV

\+ Acid and gas production.
\- No acid or gas produced.
* Cultures studied correspond to this species in all features observed except indol production.

Although the fermentation of sucrose and salicin, by these organisms, definitely subdivides the colon group into four subgroups, there still remains the possibility of further distinguishing closely related varieties within each of these subgroups.

Table IV. Fermentation Reactions of Fecal Type Colon Cultures from Chickens

Type cultures *	Total number of cultures	Per cent of cultures	Variety	Lactose	Dextrose	Sucrose	Salicin	Mannite	Dulcite	Raffinose	Maltose	Glycerol
18	39	21.78	E. coli	+	+	-		+	+	+		
27				+	+	-	+	+	+		+	+
43	89	49.72	E. communior	+	+	+		+	+	+		
44				+	+	+	+	+	+		+	+
2				+	+	+		+	+	-		
20	20	11.17	E. anindolica	+	+	+	-	+	+		+	+
31	31	17.31	E. formica	+	+	-	-	+	+		+	+

\+ = Gas and acid production.
\- = No gas or acid produced.
* = Cultures are representative of the various species studied.

Acetyl-methyl-carbinol production. "Difco" brand dehydrated glucose medium prepared according to Clark and Lub's formula was used for this reaction. The medium was inoculated from a 24 hour old broth culture and incubated 48 hours at 37°C.

The presence of acetyl-methyl-carbinol was determined in the following manner: To a given volume of the culture was added an equal volume of a solution of copper sulphate and ammonium hydroxide, then incubated 24 hours at 37°C. A positive test was indicated by the formation of an eosin-like red coloration in the culture.

The test solutions were prepared as follows: One gram of copper sulphate ($CuSO_4$ $5H_2O$) was dissolved in 40 cc. of concentrated ammonium hydroxide (specific gravity 0.90). This solution was then added to 960 cc. of a 10 per cent solution of sodium hydroxide.

The test was applied to 152 strains of the group. Of this number 149 reacted negatively. Whereas in three of the cultures, the reaction was doubtful.

Final hydrogen-ion concentration. "Difco" brand dehydrated Clark and Lub's medium was inoculated with a 24 hour old broth culture, then incubated 48 hours at 37°C. However, the method which we usually employed was to inocu-

late one tube of the medium and after the incubation period, about one cubic centimeter of the culture was poured into a clean test tube and the balance reserved for the Voges-Proskauer reaction. Ten drops of a 0.04 per cent aqueous solution of methyl-red was added to the culture. The formation of a red color was indicative of a positive reaction. Whereas the formation of a yellow color indicated a negative test. The test as shown by Clark (1915), and Clark and Lubs (1915) was dependent upon the final hydrogen-ion concentration.

The cultures tested by this method gave 152 positive reactions. These results showed a marked correlation with the Voges-Proskauer reaction and the source of the culture as shown in Table VI.

Liquefying of gelatin. Nutrient gelatin was inoculated from a 24 hour old broth culture by touching the inoculating needle to the culture and then stabbing deeply into the gelatin. The tubes were incubated 48 hours at 37°C. A control tube was used with each series of cultures. After 48 hours incubation the cultures were placed in the refrigerator until the control tubes were solidified. To confirm the results here obtained the tubes were again incubated at 37°C. for a period of 14 days. They were then placed in the refrigerator and checked as before.

There were 152 fecal type colon cultures tested in this manner, all of which failed to liquefy gelatin.

Action on milk. The coagulation of milk was determined by inoculating brom-cresol-purple milk with a 24 hour old broth culture and incubating 48 hours at 37°C.

The medium was prepared by adding 8 cc. of a 0.04 per cent aqueous solution of brom-cresol-purple to 1000 cc. of separated milk. It was sterilized in the steamer by the intermittent method of sterilization.

Of the strains isolated, 152 possessed the power to coagulate milk within 48 hours. The change in the milk was evidenced by the reduction of the brom-cresol-purple, the production of acid with the occasional development of gas, and in the majority of instances by coagulation which was usually complete after 48 hours incubation. There was no digestion of the curd, and the whey, when present, was clear.

Indol production. One per cent "Difco" proteose peptone water was inoculated from a 24 hour old broth culture, and incubated 48 hours at 37°C.

The presence of indol was determined by Ehrlich's test, which was performed in the following manner: To each 48 hour old peptone culture, was added one cc. of Ehrlich's test solution number one and three or four drops of test solution number two. When indol was present a red ring

developed in a few minutes, at the junction of the test solution and the culture. The tubes were then shaken up with chloroform, as suggested by Kligler (1915) for a confirmatory test.

Ehrlich's test solution number one and two were prepared as follows:

Test solution number one.

Para-dimethyl-amino-benzaldehyde ..... 1 gram
Absolute alcohol ..... 95 cc.
Hydrochloric acid (conc.) ..... 20 cc.

Test solution number two.

Potassium persulphate ..... 1 gram
Distilled water ..... 100 cc.

Indol formation appeared to be a constant character of the fecal type colon group inhabiting the intestinal tract of chickens. Since, upon testing 152 strains of these organisms from this source, each gave a positive test for indol.

When chloroform was shaken up with the cultures, the color material was found to be chloroform soluble.

Reduction of nitrates. The reduction of nitrates to nitrites was determined by inoculating nitrate broth from a 24 hour old broth culture, and incubating 48 hours at 37°C. The cultures were tested by adding two or three drops each of a solution of sulphanilic acid and of a solution of naphthylamin hydrochloride. The formation of a red color

in the culture constituted a test for nitrites. Each new batch of medium prepared was tested for nitrites before being used.

The ingredients of the nitrate broth were one gram peptone (Difco), 0.1 gram potassium nitrate, and 1000 cc. distilled water.

Nitrates were reduced to nitrites by all the cultures under observation. The reduction of nitrates to nitrites indicated a close correlation between this character and those of the various other differential tests which have been applied in this work. Tables V and VI summarize the results of these tests.

Summary. Lactose fermentation was found to be a reliable presumptive test for the colon group of bacteria. Mannite, maltose, glycerol and dulcite were fermented by 100 per cent of the cultures tested. Sucrose and raffinose were apparently of equal value as a differential character. Whereas lactose, sucrose, and salicin were found to serve as a metabolic gradient. The fermentation of these sugars by the organisms studied, divided the colon group into four distinct subgroups.

The results of the Voges-Proskauer reaction, methyl-red test, gelatin liquefaction, coagulation of milk, indol production, nitrate reduction and the source of the culture were closely correlated.

Table V. Results of Differential Tests Applied to Fecal Type Colon Cultures from Chickens

Number of cultures	Variety	Methyl-red test	Voges-Proskauer reaction	Indol	Nitrate reduction	H_2S in lead acetate	Gelatin liquefaction	Milk
64	E. coli	+	-	+	+	+	-	A.C.
27	E. communior	+	-	+	+	+	-	A.C.
12	E. formica	+	-	+	+	+	-	A.C.
	E. aninolica	+	-	+	+	+	-	A.C.

\+ = Positive reaction.
\- = Negative reaction.
A.C. = Acid and coagulation.

Table VI. Correlation of Tests for 152 Fecal Type Colon Cultures Isolated from the Intestinal Tract of Chickens

Voges-Proskauer reaction	Methyl-red test	Milk	Liquefaction of gelatin	Indol	Nitrate reduction	Number of strains tested	Source of culture
98 per cent*	100 per cent	100 per cent	100 per cent	100 per cent	100 per cent	152	Intestinal contents
-	+	A.C.	-	+	R		

\- = Negative reaction.
\+ = Positive reaction.
A.C. = Acid and coagulation.
R = Reduced.
* = 3 cultures typical except for Voges-Proskauer reaction.

In the study of any group of bacteria it is essential to employ a medium for isolation purposes which promotes a luxurient and characteristic growth of the particular organism in question. The use of differential solid media, such as Endo's agar and Levine's eosin-methylene blue agar, were especially valuable in the isolation of the colon group. Organisms of this group, when cultivated upon these media, develop quite characteristic colonies. Thus it was possible to identify members of the colon group directly from the media, with, of course, a small probable error, possibly due to the development of atypical colonies.

Medium which contained ingredients that inhibited the growth of the Escherichia group, such as uric acid medium, were obviously of little value for differential purposes.

Other types of media which are just as essential as those suitable for isolation, are those used for the purpose of identification and classification of the organism. Thus the fermentibility of the various carbohydrate media was invaluable for the identification of different species of the colon group. The more important of these were lactose, sucrose, and salicin. The fermentation reaction of these substances made possible the subdivision of the genus Escherichia into four distinct groups.

The methyl-red test and the Voges-Proskauer reaction, when correlated with the lactose fermentation, the appearance of typical colonies upon Endo's agar, or Levine's eosin-methylene blue agar and the source of the culture, were reliable confirmatory tests for the colon group as a whole.

Indol formation and nitrate reduction were constant characters evidenced by these organisms; as also were the coagulation of milk and the non-liquefaction of gelatin. These characters, however, to be of value in a differential classification should be correlated with other important differential tests.

From the above results we may conclude as follows:

1. The Escherichia coli group of bacteria was prevalent at all levels of the intestinal tract of diseased chickens.

2. The colon group may be identified by the use of either Levine's eosin-methylene blue agar or Endo's agar.

3. Lactose as a presumptive test for the colon group was more reliable than dextrose.

4. A subdivision of the colon group can be made on the fermentation reaction of lactose, sucrose, and salicin.

5. The differential tests, which have been devised for the study of the colon group, are usually valuable and efficient means for the identification and classification of

these organisms.

Serological Relationships

Introduction. Many organisms when injected into the animal body, under proper conditions, stimulate the reticulo-endothelial system to produce specific antibodies. The power possessed by various species of bacteria to stimulate antibody formation is variable. As, is also the reaction of the individual to the particular stimuli which certain organisms may exert.

The phenomena of antibody formation may be demonstrated by various methods; such as the agglutination, precipitation, and complement fixation tests.

In this work we have not only attempted to produce and demonstrate specific antibodies in rabbits by injecting them with three species of the Escherichia group, but also to show a correlation of the sucrose-salicin fermentation with the results of the serological tests. Therefore, the choice of the organisms for anticolon serum production was limited to the following: Antigen I was prepared from sucrose-negative (-), salicin-positive (+), cultures corresponding to variety E. coli. Antigen III from cultures of organism sucrose-positive (+) salicin-positive (+), corresponding to variety E. communior. Antigen IV from su-

erose-negative (-) salicin-negative (-), cultures corresponding to variaty E. formica.

Preparation of antigens. About 100 cc. of a 2 per cent nutrient agar medium was placed in several 300 cc. flasks, sterilized and inoubated 24 hours. Then each flask was inoculated with one cc. of a 24 hour old broth culture and incubated 48 hours at 37°C. After which five cc. of a sterile salt solution was pipetted into each flask; the growth gently washed from the medium, and the resulting suspension transferred to sterile flasks.

The organisms in the suspension were killed by the application of heat, or by the addition of a chemical. The former treatment consisted of placing the flask containing the suspension in a water bath, then by applying heat the temperature was raised to 60°C. and maintained for one hour. The latter treatment consistad of adding 0.1 cc. of formalin to each 10 cc. of the suspension. The antigen was incubated 24 hours at 37°C. and the viability of the organisms was tested by inoculating nutrient agar slants with a loopful of the suspension, and incubating several days at 37°C.

Enough antigen was prepared at one time to serve throughout for the immunization of the rabbits. Therefore, the stock antigen was prepared so as to be fifty times the density of tube one McFarland's nephelometer, and was held under refrigeration.

Preparation of anticolon sera. The subcutaneous method of injecting the antigens was employed for the immunization of twelve rabbits. Thus alloting two rabbits for immunization with each type of antigen. The antigenic dose was gradually increased until each of six rabbits were receiving 4 cc. of the suspension which was 50 times tube one, McFarland's nephelometer. Whereas the other six were receiving 1.5 cc. of the same suspension. The animals were injected with the suspension at intervals of four or five days until 10 injections had been made. The titre of the antisera was checked for agglutinins toward the latter period of the immunization process.

Fifteen days after the last injection of the antigen, the antisera was collected by removing 10 cc. of blood from the heart by means of a sterile syringe. The blood was placed in a sterile test tube and allowed to clot. After standing for two hours the clot was rimmed and the tubes centrifugated at moderate speed for ten minutes. After which the antiserum was pipetted into sterile tubes and heated for thirty minutes at 57°C. No preservative was added to the antisera since it was to be used immediately.

Agglutination test. The antigens for this test were prepared in the same manner as those used for injecting the rabbits, with, however, the following exceptions: The

cultures were incubated 24 hours instead of 48 hours; and the suspension of organisms was standardized to tube one McFarland nephelometer. The antigen was preserved by the addition of 0.5 cc. of melted phenol crystals to each 100 cc. of the bacterial suspension.

The dilutions employed in the test ranged from 1:10 to 1:1280 inclusive. The dilutions were made with a physiological salt solution in the usual way. A control tube of the antigen was included with the tests which were incubated 24 hours at 37°C.

As shown by table VII, the anticolon sera apparently possessed a distinct quantitative specific action. Although these sera were found to agglutinate the homologous organisms in dilutions of 1:1280, there was very little agglutinating power evidenced for heterologous organisms of the colon group. The highest non-specific agglutination which occurred among the organisms under observation was 1:320.

There is also an apparent difference, (see table VII), in the power of these organisms to stimulate in the experimental animal the formation of agglutinine. The varieties, E. coli and E. communior of the colon group apparently possessed this power, whereas it was not evidenced by the variety E. formica.

Table VII. Results of Agglutination Tests With Homologous and Heterologous Anticolon Sera

Antigen	Antisera	Dilutions 1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	Control
I	I F (22)*	C	C	C	C	C	C	0	0	0
I	I H (31)*	C	C	0	0	C	C	0	C	0
I	III F (30)	C	C	C	C	C	P	0	0	0
I	III F (32)	0	C	C	P	0	0	0	0	0
I	III H (10)	0	C	C	0	P	0	0	0	0
I	IV F (9)	0	C	C	C	C	P	0	0	0
I	IV F (33)	C	C	P	0	0	0	0	C	0
I	IV H (19)	C	C	C	C	C	P	0	0	0
I	Negative	0	0	C	0	0	0	0	0	0

C = Complete.
P = Partial.
0 = No agglutination.

Antigen I = E. coli
* = Homologous anticolon sera.

Table VII continued.

III	I F (22)	0	C	0	P	0	0	0	0	0
III	I H (31)	0	C	0	P	0	0	0	0	0
III	III H (10)*	C	C	0	C	C	C	0	C	0
III	III F (30)*	C	C	0	0	P	0	0	0	0
III	III F (32)*	C	C	C	P	0	0	0	0	0
III	IV F (9)	C	C	C	P	0	0	0	0	0

III	IV F (33)	C	C	P	0	0	0	0	0	0
III	IV H (19)	C	C	C	P	0	0	0	0	0
III	Negative	0	0	0	0	0	0	0	0	0

C = Complete
P = Partial
0 = No agglutination

Antigen III = E. communior
* = Homologous anticolon sera

Table VII continued.

IV	I F (22)	C	C	P	0	0	0	0	0	0
IV	I H (31)	C	C	P	0	0	0	0	0	0
IV	III F (30)	C	C	P	0	0	0	0	0	0
IV	III F (32)	C	C	C	P	0	0	0	0	0
IV	III H (10)	C	C	P	0	0	0	0	0	0
IV	IV F (9)*	C	C	P	0	0	0	0	0	0
IV	IV F (33)*	C	C	P	0	0	0	0	0	0
IV	IV H (19)*	C	C	C	P	0	0	0	0	0
IV	Negative	0	0	0	0	0	0	0	0	0

C = Complete
P = Partial
0 = No agglutination

Antigen IV = E. formica
* = Homologous anticolon sera

I = E. coli antigen
III = E. communior antigen
IV = E. formica
I F (22) = E. coli antiserum
I H (31) = E. coli antiserum
III F (30) = E. communior antiserum

III F (32) = E. communior antiserum
III H (10) = E. communior antiserum
IV F (9) = E. formica antiserum
IV F (33) = E. formica antiserum
IV H (19) = E. formica antiserum

Precipitation test. The ectoantigen, prepared after the method of Bushnell and Hudson (1927) was diluted 1:10, 1:50 and 1:100 with salt solution. This diluted antigen was superimposed on the antisera to be tested.

Several samples of the anticolon sera were tested for the presence of precipitin antibodies. However, in only three instances, see Table VIII, were we able to demonstrate the presence of this type of antibody. Anticolon sera from rabbits number 22, 30 and 31 gave evidence of the presence of precipitins in dilutions of 1:100, with the homologous organism. Apparently the variety E. formica was lacking in the power to stimulate the formation of precipitins, while the varieties E. coli and E. communior distinctly possessed this power.

Complement fixation tests. The usual procedure for running the complement fixation test was used for testing the anticolon sera. However, the antigens employed were clear ectoantigens.

The anticomplementary action of each substance used in the test was determined before testing the anticolon sera. Table IX gives the protocol herein used for the complement fixation tests.

Table VIII. Results of Precipitin Tests with Homologous and Heterologous Anticolon Sera

Antigen	Antisera	Dilutions 1:1	1:10	1:50	1:100	Antisera control	Antigen control
I	I F(22)*	+	+	+	+	-	-
I	I H(31)*	+	+	+	+	-	-
I	III F(30)	+	-	-	-	-	-
I	IV F(9)	-	-	-	-	-	-
III	I F(22)	+	-	-	-	-	-
III	III F(30)*	+	+	+	+	-	-
III	III H(10)*	+	-	-	-	-	-
III	IV F(9)	-	-	-	-	-	-
IV	I F(22)	-	-	-	-	-	-
IV	III F(30)	-	-	-	-	-	-
IV	IV F(9)*	-	-	-	-	-	-
IV	IV H(19)*	-	-	-	-	-	-

\+ = Positive reaction.
\- = Negative reaction.
* = Anticolon sera and homologous antigens

Antigen I = E. coli.
Antigen III = E. communior.
Antigen IV = E. formica.

Table IX. Protocol Used for Complement Fixation Test

Tube	1	2	3	4	5	6	7	8	9	10	11	12
Antigen	0.2	0.2	0.2	0.2	0	0	0	0	0.2	0	0.2	0
Serum	0.3	0.01	0.001	0.005	0.3	0.01	0.001	0.005	0	0	0	0
Complement	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0	0.3
Amboceptor	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0
Cells	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Results	4	4	1	0	0	0	0	0	0	0	0	0

The antisera and complement were diluted 1:10 and a 1:500 dilution of the amboceptor was used.

The complement fixation tests of anticolon sera showed a noticeable difference in the antibody activity of this sera with the homologous antigens and the heterologous antigens. In the former instance apparent fixation of the complement with the antigen and anticolon sera occurred, whereas in the latter instance fixation did not occur to any appreciable extent, as shown in Tables X and Xa.

I F (22) = *E. coli* formalized antigen and *E. coli* antiserum.
I H (31) = *E. coli* heat killed antigen and *E. coli* antiserum.
III F (30) = *E. communior* formalized antigen and *E. communior* antiserum.
III F (32) = *E. communior* formalized antigen and *E. communior* antiserum.
III H (10) = *E. communior* heat killed antigen and *E. communior* antiserum.
IV F (9) = *E. formica* formalized antigen and *E. formica* antiserum.
IV F (33) = *E. formica* formalized antigen and *E. formica* antiserum.
IV H (19) = *E. formica* heat killed antigen and *E. formica* antiserum.
Negative = Sheep serum.
4 = No hemolysis, 100 per cent fixation of complement.
2 = Approximately 50 per cent fixation of complement.
0 = Complete hemolysis, no fixation of complement.

Table X. Results of Complement Fixation Tests with Homologous Sera

	Complement fixation with serum				Anticomplementary action of serum				
Tube	1	2	3	4	5	6	7	8	9
Antigen (1)	0.2	0.2	0.2	0.2	0	0	0	0	0.2
Serum dilution	0.3	0.01	0.001	0.005	0.3	0.01	0.001	0.005	0
I F (22) (2)	4	4	1	0	0	0	0	0	0
I H (31)	4	4	0	0	0	0	0	0	0
III F (30)	4	4	0	0	2	0	0	0	0
III F (32)	4	4	0	0	4	4	-	-	0
III H (10)	4	4	0	0	2	2	-	-	0
IV F (9)	4	2	0	0	2	0	0	0	0
IV F (33)	4	2	0	0	0	0	0	0	0
IV H (19)	4	4	0	0	2	0	0	0	0
Negative	0	0	0	0	0	0	0	0	0

Table Xa. Results of Complement Fixation Tests with Heterologous sera.

	Complement fixation with serum				Anticomplementary action of serum				
Tube	1	2	3	4	5	6	7	8	9
Antigen (1)	0.2	0.2	0.2	0.2	0	0	0	0	0.2
Serum dilution	0.3	0.01	0.001	0.005	0.3	0.01	0.001	0.005	0
I (30) (2)	4	1	0	0	2	0	0	0	0
I (9)	2	2	0	0	2	0	0	0	0
III (22)	2	0	0	0	0	0	0	0	0
III (9)	2	0	0	0	2	0	0	0	0
IV (22)	0	0	0	0	0	0	0	0	0
IV (30)	4	1	0	0	2	0	0	0	0
Negative	0	0	0	0	0	0	0	0	0

(1) The anticomplementary dose of the antigen was 0.5 cc.
(2) These characters explained in foot-note on page 41.

Summary. A survey of the results obtained by the serological tests apparently indicates that the antigenic properties of the Escherichia group of bacteria are not such as to sufficiently stimulate cellular activity in experimental animals to produce a high titre antiserum. This was evidenced in the agglutination test wherein the highest agglutinating titre was observed in dilutions of 1:1280. These organisms also showed a close relationship, in that non-specific, or group action, occurred in dilutions as high as 1:320.

A comparison of the results of the agglutination tests with those of the precipitation tests would indicate that the varieties E. coli and E. communior possess considerably greater antigenic properties than does the variety E. formica. However, the latter was shown to possess the power to stimulate some antibody formation as evidenced by the complement fixation tests. And these antibodies as well as those produced by E. coli and E. communior showed a distinct specificity for its homologous organism. Although the results obtained by these tests would indicate a very close serological relationship between various species and varieties of the colon group, it also indicates that certain of the organisms possess an inherent power to stimulating specific antibody formation, and that this specific action

between antigen and anticolon sera may be used to distinguish and differentiate organisms belonging to the colon group of bacteria.

The results of the serological tests also substantiate and apparently confirm the subdivision of the colon group which was based upon the results of the biochemical reactions.

With the above results in mind, we may conclude as follows:

1. Although the Escherichia group of bacteria probably serve as very weak antigens, they incite the production of specific antibodies when injected subcutaneously into an experimental animal.

2. Apparently E. coli and E. communior possessed a slightly greater antigenic property than did E. formica.

3. The basic subdivision of the Escherichia group, resulting from the carbohydrate fermentation reactions, was apparently confirmed by the results obtained with the serological tests.

GENERAL SUMMARY AND CONCLUSIONS

The use of differential solid media such as Endo agar and Levine's eosin-methylene blue agar were invaluable factors for the isolation and differentiation of members of

the colon group of bacteria. When grown upon these media there was observed a characteristic difference in the appearance of the colonies of the fecal and non-fecal colon types. The metallic sheen being the chief characteristic displayed by the fecal type, whereas colonies of the non-fecal type possessed a pinkish red surface coloration.

The differential tests such as gelatin liquefaction, methyl-red test, Voges-Proskauer reaction, coagulation of milk, indol production and nitrate reduction were apparently valuable and reliable tests which may be used for the identification of the colon group as a whole. These tests, however, will not serve as a possible basis for identifying individual species of the group.

Motility is an inconstant character possessed by organisms of the colon group and is probably of little value in the identification of species belonging to the group.

The fermentation reaction of lactose may be considered a reliable character on which to base a presumptive test for the colon group. Whereas, the fermentation reaction of certain other carbohydrates may serve as a basis for a subdivision of the group. For example, the fermentation of sucrose by these organisms distinctly subdivide the group into two divisions. Again salicin may be fermented by those

organisms which fail to ferment sucrose, thus making possible another well marked subdivision of the group.

The species which were identified as a result of the sugar fermentation were, *E. coli*, *E. communior*, *E. formica*, and *E. anindolica*. This classification of these organisms conforms to that of Bergey's except for the species *E. anindolica*. And it corresponds in all features except the production of indol. The organism isolated in this work produced indol, whereas Bergy classifies it as not producing indol.

As indicated by the serological tests the anticolon sera was specific for the homologous organism. However, close relationships of the colon species was indicated by non-specific or group action as indicated by the results of the agglutination tests.

The specificity manifested by the anticolon sera for the homologous organism tends to confirm the subdivision of the colon group as based on the results obtained by the fermentation of certain carbohydrates.

The fecal type colon bacteria were found to be present in the various levels of the intestinal tract of twenty-two diseased chickens. Whereas the non-fecal type was not once isolated from this source during the course of this work.

The species *E. coli* and *E. communior* were found to be distributed more or less evenly throughout the intestinal

tract. E. anindolica was also present at the various levels examined, though fewer in proportion than either of the other two organisms. E. formica although rarely present in the upper one-half of the intestine, was apparently quite prevalent in the lower portion, especially the cecum.

ACKNOWLEDGMENT

I wish to thank Dr. L. D. Bushnell for the many helpful suggestions offered during this study and for his careful guidance throughout the problem. Also, I thank Dr. C. A. Brandly and Dr. J. P. Scott for their kind interest and assistance.

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