PATHOGENIC COLIFORM BACTERIA IN THE ADULT HUMAN MOUTH

by

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INTRODUCTION

For more than half a century, coliform bacteria have been considered as a group of non-pathogenic microorganisms to human beings. It has been known that this group of bacteria is widely spread throughout nature and universally found in the human intestinal tract, and also in the intestinal tract of many of the higher animals.

By using the term "Coliform", one is taking into consideration a great number of different types of Gram-negative, non-spore-forming aerobic and facultative bacilli which are usually good lactose fermenters. This usually involves the members of the genus *Escherichia*, especially *Escherichia coli*. However, in this study the members of the genus *Aerobacter*, *Aerobacter aerogenes* in particular, is included because of its close relationship to the *Escherichia* species, and it is also found in the human intestinal tract. These two genera of bacteria are abundantly found in the colon, especially *Escherichia coli*, and are such characteristic inhabitants of this region of the intestine as fully to deserve the name that has been given to them.

This group of bacteria was first described by Escherich in 1886 under the names *Bacterium coli commune*, which was a short motile rod, and *Bacterium lactis aerogenes*, which was somewhat shorter and plumper, non-motile, and also clotted milk more rapidly than the former one. Some closely related enteric bacilli which were also found seemed to be intermediate between the two extremes of the biochemically defined "colon-aerogenes" group of bacteria. Of course there were immunological varieties, and also certain fermentative types were encountered with varying frequency.

Attention was not called until about 50 years ago that occasionally

Escherichia coli was isolated from cases of infantile gastroenteritis and cases of diarrhea of new born infants, in which no recognizable pathogens such as the members of Salmonella or Shigella group could be found. For a long time, it had been questioned by many workers whether the coliform bacteria could cause outbreaks of infantile diarrhea. The answer remained inconclusive because most of the earlier workers used only biochemical methods in attempts to differentiate between the strains of Escherichia coli isolated from infants with diarrheal diseases and cultures from normal individuals. It has been established that certain serotypes of this group of bacteria are definitely responsible for infant diarrheal infection. It has also been proven that biochemical tests alone are inadequate for the differentiation between pathogenic and normal strains; it is now known that the different serotypes of Escherichia coli usually give identical biochemical reactions.

The interest and the purpose of this study are mainly centered on the isolation and identification of these infantile diarrhea-causing Escherichia serotypes and also the related coliform bacteria from the human mouth of possible adult carriers as a suggested source. It is also within the interest of this study to determine the pathogenicity and the presence of particular types of antigens which are commonly encountered in the etiologic agents of outbreaks of infantile diarrheal diseases.

REVIEW OF LITERATURE

For a long time, the question whether coliform bacteria can cause infection of infantile diarrheal diseases and enteritis has been asked. From past investigations, it has been made clear that diarrheal disease of infants may be caused by a variety of microorganisms. It was found that epidemic and sporadic cases of infantile or newborn diarrhea might be caused by Salmonella and Shigella groups. Occasionally, other groups of bacteria and viral agents were thought to be the etiological agents in a particular outbreak of the diseases. In many other outbreaks, certain Escherichia coli serotypes have been recovered. It is now known that this group of bacteria is definitely responsible for outbreaks of the disease, and the name "Enteropathogenic E. coli" has been suggested to designate these particular serotypes.

The concept that certain Escherichia serotypes might cause infantile diarrhea is not a new one. For more than 50 years, workers have investigated the role of coliform bacteria in outbreaks of infantile enteritis or diarrhea of newborn. Unfortunately, most of the investigators were not too sure about their results, and the answer to the question whether coliform bacteria could cause diarrhea in infants was not conclusive. Investigators were hampered by using only biochemical tests in their attempt to differentiate these particular Escherichia coli serotypes. Consequently, even some suspicion on this concept arose, since this group of bacteria was found to be of human intestinal origin and even believed to possess a role in maintaining the normal intestinal flora.

The only earlier investigator known was probably Goldschmidt (1933), who employed a slide agglutination technique to differentiate the microorganisms recovered from cases of infantile diarrhea in Germany. He identified them as

"Dyspepsiekoli" type A IV. Unfortunately, his work did not receive the attention it deserved.

Dulaney and Michelson (1935) and Michelson and Dulaney (1936) reported on a number of cases of infantile diarrhea that occurred in a hospital in Memphis, Tennessee, during the winter of 1933-1934. During the outbreak 27 infants were affected, and among these a 47 per cent death rate was reported. The only significant microorganisms recovered from stools were identified as Bacterium coli mutabile, which has been classified as E. coli O group 18a and 18c recently by Ewing, et al (1956). The bacteria were so itentified, because they produced white colonies on Endo agar, and those colonies subsequently developed red papillae in the manner of the classic "mutabile" strain. The bacteria were found to predominate in the intestinal tract of the sick infants and from 18 of them these cultures were isolated. Dulaney and Michelson (1935) also used these organisms to prepare antiserum in rabbits with which they were able to show agglutination with heated antigen preparations of all the B. coli mutabile strains recovered from the sick infants. Furthermore, they also found that there were no marked antigenic differences between red and white colony-variants obtained from the "mutabile" cultures.

Bray (1945) and Bray and Beavan (1948) were probably the first ones to emphasize the association of particular E. coli serotypes with outbreaks of infantile diarrhea. They isolated 42 cultures from 44 patients, and the bacteria were the same type as the strain identified by Kauffmann and Dupont (1950) and which is now known as the E. coli O 111:B4 serotype.

Varela, et al (1946), in Mexico City, isolated a bacterium from an infant

who died of diarrhea, and they named the organism: Escherichia coli-gomez. Later, they isolated bacteria of the same serotype from other patients, and Varela found that the somatic antigen of E. coli-gomez was identical with that of Salmonella adelaide O antigen 35, and he was able to employ Salmonella O 35 antiserum to identify this particular E. coli serotype. Further studies on the antigenic relationship to S. adelaide as well as the proof of the E. coli-gomez with E. coli O 111:B4 was made by Olarte and Varela (1952), and the confirmatory investigations were made by Kauffmann (1952) and also by Edwards and Ewing (unpublished).

Kauffmann (1947) published an antigenic schema for the E. coli group which was based upon his own earlier work and that of his collaborators, Knipschildt (1945a, 1945b, 1946) and Vahlne (1945). The schema consisted of 25 O antigen groups, 55 K antigens, and 20 H antigens. This was an extension of an earlier schema devised by Kauffman in 1944. He also pointed out that the term "K antigen" was merely a symbol which designated a class of antigens composed of several varieties, and all of them were either envelope, sheath, or capsular antigens which could inhibit agglutination of living cultures in O antisera. The K antigens were further divided into three varieties as L, A, and B antigens, based upon their physical behavior. Table 1 presents the characteristics of the various antigens.

The specificities and characteristics of L and B antigens have been well studied recently, because these antigens are likely to be more often encountered in pathogenic forms of coliform bacteria. The most striking difference between L and B antigens is the fact that the antibody binding power of the former is inactivated by heat at 100°C. for one hour. Thus, it is possible to prepare L antiserum by absorption of an OL antiserum with a heated suspension of homologous strain which will remove O agglutinin but

Table 1. Antigenic characteristics of coliform bacteria.

O antigens	1.	Thermostable, agglutinability with O antiserum not inactivated by heat at 100°C. for 1 hour.
	2.	O-inagglutinability may be caused in living culture by K antigens.
	3.	Antibody binding power not inactivated by heating at 100°C. 1 hour.
	4.	Occur as somatic antigen.
K antigens		Somatic surface antigens designated as envelope or capsular antigen, interfere with agglutination with O antisera. They are either thermostable or thermolabile.
L	1.	Agglutination in L antisera inactivated by heat at 100°C. for 1 hour. Thermolabile.
	2.	Suspensions rendered agglutinable in O antiserum by heating at 100°C. for 1 hour.
	3.	Antibody binding power inactivated by heating at 100°C. for 1 hour.
	4.	Antigenicity inactivated by heating at 100°C. for 1 hour.
	5.	Occur as envelope or sheath, occasionally as a capsule.
A	1.	Agglutination in A antiserum inactivated by heat at 121°C. for 2½ hours. Usually considered to be thermostable.
	2.	Suspensions rendered agglutinable in O antiserum by heating at 121°C. for 2½ hours.
	3.	Antibody binding power not inactivated by heat at 100°C. for 2½ hours or at 121°C. for 2 hours.
	4.	Antigenicity inactivated by heating at 121°C. for 2½ hours.
	5.	Occur as capsules.
B	11.	Agglutination in B antiserum inactivated by heating at 100°C. for 1 hour. Thermo-labile.
	2.	Suspension rendered agglutinable in O antiserum by heating at 100°C. for 1 hour.
	3.	Antibody binding power not inactivated by heating at 100°C. for 1 or 2 hours, or at 121°C. for 2 hours.
	4.	Antigenicity inactivated by heating at 100°C. for 1 hour.
	5.	Occur as envelopes or sheaths.
H antigens	1.	Thermolabile, also known as flagellar antigen, agglutination in H antiserum inactivated by heating at 100°C. for 1 hour.
	2.	May cause erroneous result in K agglutination due to the presence of H agglutinin in K antiserum.
	3.	Antibody binding power inactivated by heat 100°C. for 1 hour.
	4.	Occur as flagella, usually poorly developed in freshly isolated culture, sometimes presented in flagellated non-motile culture.

will leave the L agglutinin. This cannot be done with B antiserum, since the antibody binding power of B antigen is not inactivated by heat at 100°C. for one hour, so if OB antiserum is absorbed with a heated suspension of the homologous strain, the agglutinins for both O and B antigens will be absorbed from the antiserum.

Giles, et al. (1949) described another type of E. coli serotype which was thought to be important with the outbreak of epidemic infantile diarrhea. Kauffmann and Dupont (1950) found that the beta serotype of the bacterium isolated by Giles, et al. (1949) belonged to E. coli O group 55 and contained a new K antigen B5 which has been added to the antigenic schema of coliform bacteria devised by Kauffmann. Since 1947, many investigators have studied the E. coli serotypes, especially those which have been associated with cases of infantile diarrhea, and as a result of their efforts, the E. coli antigenic schema has been extended to 135 O antigens, 77 K antigens and 40 H antigens, as shown in Table 2. (Ewing, 1956).

Table 2. Antigens of coliform bacteria.

O group antigens	K antigens			H group antigens
	L	A	B	
	30	26	21	
135	Total, 77			40

Smith (1949) gave a detailed description of the *E. coli* alpha serotype, which is now known as O 111:B4, and beta serotype, or O 55:B5, and the antigens were added to the schema of *E. coli*. Since 1945, *E. coli* of these two serotypes have been isolated in many cases during epidemics and from sporadic cases of infantile diarrhea in nearly all parts of the world. Also it has been reported that in some cases of outbreaks of the disease, these two strains of *E. coli* were not found, but some other serotypes common to the epidemic were described from the examination of the *E. coli* flora of many patients.

More recently, some new *E. coli* serotypes have been described which have been associated with infantile diarrheal disease: Types O 127:B8 and O 128:B12 are thought to be very important. The isolation of the former serotype from many parts of the United States, Canada, and Mexico has been reported by Ewing, et al. (1955). Taylor and Charter (1955) in the same year reported the latter serotype in similar circumstances in the United Kingdom, and Edwards and Ewing (unpublished) studied four strains of this serotype and stated that this was the only significant organism isolated in connection with a small outbreak in the United States. Kauffmann (1950), De Assis (1948) and later Ewing studied other serotypes as *E. coli* O 112a and 112c which have been also isolated from cases of diarrheal disease in young children, adults and from newborns. Recently, a new strain of *E. coli* O 124:B17 has been repeatedly isolated from individual cases and outbreaks of gastroenteritis and also from acute diarrhea in both children and adults. In addition, Oerskov (1954) and Braun (1954) also described several serotypes of *E. coli* O group 25 two of which had been known to be associated with diarrheal disease. Ewing, et al. (1956) and Ewing (1956) also made studies on this group. Oerskov (1954) also discussed the possible role of the serotypes belonging

to this O group and the difficulties in identification of this group of *E. coli*.

Attention should be called also to the isolation of the A-D O2 strain (also known as *S. tiete'* or *Alkalescens* II) by De Assis (1939) from cases of diarrhea, and that the A-D O2 strain is in fact the anaerogenic, non-motile variety of *E. coli* O group 25. It has been felt that this organism must be considered to be associated with diarrheal disease, because of its similarity in biochemical tests to *E. coli*, and the frequent recovering of this organism from cases of diarrheal disease reported from many parts of the world.

MATERIALS AND METHODS

Organisms

The organisms used throughout this study were 22 cultures of coliform bacilli isolated from adult human mouths. Among them three cultures were identified as *Escherichia coli*, and nineteen were *Aerobacter aerogenes*, bases upon their reactions on biochemical tests. In addition, four strains of known pathogenic *E. coli* serotypes were studied. These serotypes were *E. coli*: O 26:B6, O 55:B5, O 111:B4, and O 127:B8 which were obtained from the Kansas State Board of Health Laboratories, Topeka, Kansas.

Media for the Primary Isolation and Identification of Coliform Bacteria

For the primary isolation the following described medium was used as a washing fluid for the rinsing of bacteria from adult mouths. Mouth washing samples were taken in 250 ml Erlenmeyer flasks which contained 25 ml of lactose broth which was composed of:

Lactose	5 g.
Proteose peptone No. 3 (Difco)	10 g.

Sodium chloride	5 g.
Distilled water	1000 ml.

Indicator was not added. The medium was adjusted to pH7.0 and sterilized by autoclaving at 121°C. for 15 minutes. All other media were prepared from Difco dehydrated formulations according to manufacturer's recommendations.

Plate preparations of Bacto Levine E. M. B. Agar, Dehydrated, (Difco), composed of:

Bacto peptone	10 g.
Bacto lactose	10 g.
Dipotassium phosphate	2 g.
Bacto agar	15 g.
Bacto eosin Y	0.4 g.
Bacto methylene blue	0.065 g.
Distilled water	1000 ml.

and Bacto MacConkey Agar, Dehydrated, (Difco), composed of:

Bacto peptone	17 g.
Proteose peptone, Difco	3 g.
Bacto lactose	10 g.
Bacto bile salts No. 3	1.5 g.
Sodium chloride	5 g.
Bacto agar	13.5 g.
Bacto neutral red	0.03 g.
Bacto crystal violet	0.001 g.
Distilled water	1000 ml.

were used for primary differentiation of the coliform organisms in these mouth samples.

Biochemical tube media were also used in addition for the identification of the cultures. These media were lactose, glucose and sucrose fermentation tubes which were composed of the same fermentation broth base as in the lactose broth used for the primary isolation, in which 0.5 per cent of the desired fermentable substrate was substituted for the lactose, and 1 ml of 1.6 per cent alcoholic solution of brom thymol blue indicator was added per liter.

Bacto Litmus Milk, Dehydrated, (Difco), composed of:

Bacto skim milk	100 g.

Bacto litmus	0.75 g.
Distilled water	1000 ml.

was used to test action on milk.

For confirmatory taxonomic work in differentiation of E. coli from A. aerogenes, the following media and tests were used:

Bacto Koser Citrate Medium, Dehydrated, (Difco), composed of:

Sodium ammonium phosphate	1.5 g.
Monopotassium phosphate	1 g.
Magnesium sulfate	0.2 g.
Sodium citrate	3 g.
Distilled water	1000 ml.

for the test of citrate utilization.

Bacto M.R.-V.P. Medium, Dehydrated, (Difco), composed of:

Buffered peptone	7 g.
Bacto dextrose	5 g.
Dipotassium phosphate	5 g.
Distilled water	1000 ml.

which was used for methyl red and Voges-Proskauer tests, and one per cent Bacto tryptone, Dehydrated, (Difco) solution was prepared for the indole test.

For the methyl red test, five drops of methyl red indicator were added to about 5 ml of M.R.-V.P. broth culture in a small tube. The indicator was composed of:

Methyl red	0.1 g.
95% ethyl alcohol	300 ml.
Distilled water	200 ml.

Prepare by dissolving the methyl red in the alcohol and diluting with the distilled water. The positive tests showed red, and negative tests appeared yellow. The questionable tests were either orange or weak pink. These cultures were further incubated and retested.

In the Voges-Proskauer test for the presence of acetyl methyl carbinol,

one ml of M.R.-V.P. broth culture was taken in a small tube and 0.6 ml of an alcoholic 5 per cent alpha naphthol solution were added. The tube was shaken to mix it, then 0.4 ml of a 40 per cent aqueous potassium hydroxide solution were added. The tube was shaken again enough to mix and let stand for 15 to 30 minutes. The positive tests showed a bright red layer on top of the mixture.

The presence of indole was tested by the Gore' test in which Ehrlich's reagents were used. The Ehrlich's reagent I was composed of:

95% ethyl alcohol	95 ml.
Para-dimethyl amino benzaldehyde	1 g.
Hydrochloric acid, concentrated	20 ml.

The Ehrlich's reagent II was composed of:

Potassium persulphate ($K_2S_2O_8$)	5 g.
Distilled water	100 ml.

This was made as a saturated solution.

For the test, the cotton stopper from the tryptone broth culture was removed and the bottom part of it was moistened with several drops of Ehrlich's reagent I, then with the same amount of Ehrlich's reagent II. The cotton stopper then was replaced and pushed down into the tube until it was within one inch of the medium. The tube was then placed in a boiling water bath for 10 minutes. The positive test for the presence of indole showed a reddening of the cotton stopper.

Materials and Methods used for Antigenic Identification and Studies on Pathogenicity

Commercially prepared E. coli diagnostic sera were used for identification of the presence of O and B antigens of the four strains of known E. coli serotypes mentioned before. These antisera were: O 26:B6, O 55:B5, O 111:B4, and O 127:B8, (Lederle Labs., Division, American Cyanamid Co.).

The bottles containing 1.0 ml of OB antisera each were diluted 1:3 by adding two ml of 0.85 per cent sodium chloride solution. This was important for avoiding the appearance of non-specific or heterologous antigen-antibody reactions that might mask the specific reactions of the antisera.

A portion of the growth of an organism to be tested was taken directly from the differential media or from the subculture on nutrient agar slants with a wire loop and mixed with 0.5 ml of saline in a little tube to make a smooth, fairly dense suspension of about one to two billion bacteria per ml or a density of No. 3 on the MacFarland scale. Culture suspensions prepared in this manner were used for the slide agglutination test. Four drops from each culture suspension were put on a clean slide. A satisfactory size of drop is about 0.05 ml. A drop of approximately this volume can be obtained by use of Kahn serological pipettes of 0.2 ml capacity. This is important because the erroneous result might be read from the excess or inadequate volume of the drop or the density of the suspension. A drop of each diluted antisera was then added to each drop of the suspension on the slide separately. A control was set up by adding a drop of saline in place of diluted serum. These drops were mixed well with a sterile loop, and the slide was tilted.

The agglutination tests were read within 30 to 60 seconds, and if feeble or no agglutination appeared during this period, or delayed reaction, the sample was considered to have no significance for the presence of the B antigens checked. The suspensions were then heated in a boiling water bath or steamed for one hour, and then cooled to room temperature. The same procedure was repeated on a microscopic slide by using the same antisera. In this way the presence of the O antigens was checked.

Instead of the drop technique, a specially prepared bacteriological

wire loop (Figure 1.) was also used for the preparation of bacterial suspensions. The wire loop was made by folding two continuous loops about 5 mm in diameter, with the two loops about 3 to 3.5 mm apart from each other as shown in the figure.

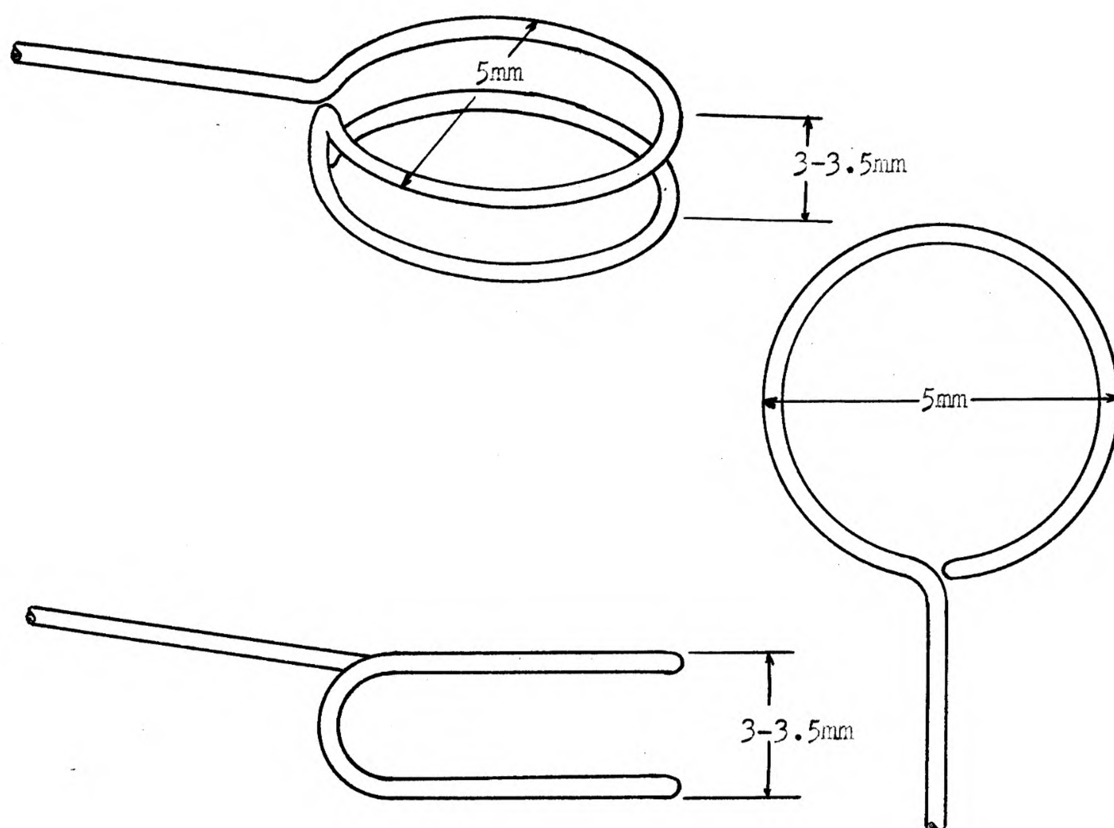

Fig. 1. Design of special loop for agglutination test.

A dip with this loop into the prepared suspensions of the testing organisms picked up approximately 0.05 ml for the slide agglutination test. The same type of loop was also found to be useful in scraping a freshly grown

culture from a nutrient agar slant.

Instead of making suspensions of organisms in tubes, it is also possible to make drop suspensions on slides by placing a drop of saline on a slide and a portion of growth is taken with a loop and mixed on the slide in the drop of saline directly. When this method is applied it is more important to watch the density and the volume of the suspension to avoid the erroneous results mentioned before.

Young chicks were used as the experimental animals for the study on pathogenicity. Fairly dense saline suspensions of testing organisms were prepared and were given orally to one-day old chicks and later injected intravenously into two-week old chicks.

EXPERIMENTAL

Isolation and Primary Identification of Coliform Organisms from Adult Human Mouths

Mouth samples were taken in lactose broth and were incubated for 20 to 24 hours at 37°C. Throughout the entire work all incubations were carried out at 37°C. unless otherwise stated. After incubation, the samples were streaked on two differential media: Levine E. M. B. agar and MacConkey agar plates. These plates were incubated for 24 hours. If members of coliform group of bacteria were present, small dark colonies with typical, green, metallic sheen might be observed on E. M. B. agar plates, specially if the organisms were *Escherichia coli*. If they were *Aerobacter aerogenes*, the colonies on this medium would be larger and with or without brownish centers, the colonies would tend to run together and there would be no green, metallic sheen present. On MacConkey agar plates, well isolated colonies of *E. coli* would appear brick

red and might be surrounded by a zone of precipitated bile. Colonies of *A. aerogenes* might also appear red but usually not as bright red as *E. coli* colonies. They were usually much larger and tended to appear whitish red.

The organisms from these colonies were transferred aseptically to nutrient agar slants which was composed of:

Bacto beef extract	3 g.
Bacto peptone	5 g.
Bacto agar	15 g.
Distilled water	1000 ml.

These slants were incubated for 24 hours. The confirmatory identification was made with biochemical media, and also the presence of the antigens of the aforementioned *E. coli* strains was sought by the agglutination technique.

For the further determination of the organisms, the cultures were inoculated into: Lactose, glucose, and sucrose fermentation tubes, litmus milk, Koser's citrate medium, M. R.-V.P. broth medium and tryptone broth. These media were incubated for 24 hours and examined. Acid and gas production were checked on fermentation tubes. Litmus milk tubes were checked for acid or rennet curd production, peptonization, and the reduction of the litmus indicator. Growth was checked on the Koser's citrate medium for the ability to utilize citrate. The methyl red test and Voges-Proskauer test for acetyl methyl carbinol were conducted from the M.R.-V.P. medium. Indole production was tested for in tryptone broth.

Usually, the differentiation of coliform bacteria can be done satisfactorily with: Lactose, and glucose fermentation tube media, Koser's citrate medium M.R.-V.P., and tryptone broth media. The typical reactions of *E. coli* and *A. aerogenes* on these media are shown in Table 3.

Table 3. The typical reactions of E. coli and A. aerogenes on certain biochemical media.

Tests / Organisms	Lactose	Glucose	Koser's citrate	M.R.	V.P.	Indole
E. coli	⊕	⊕	−	+	−	+
A. aerogenes	⊕	⊕	+	−	+	−

⊕ : for acid and gas production

From these tests, the cultures found to be coliform, that is either E. coli or A. aerogenes, were saved for later experiments.

Detection of the Presence of Certain Antigens Related to Some Pathogenic E. coli in the Coliform Organisms Isolated from Adult Human Mouths and the Study on their Pathogenicity.

The organisms from adult human mouths and identified as coliform members were further tested for the presence of antigens of the four known pathogenic E. coli strains; O 26:B6, O 55:B5, O 111:B4, and O 127:B8.

For the detection of the presence of pathogenic E. coli antigens, fresh-grown cultures were used to make fairly dense suspensions of about one to two billion bacteria per ml, a density corresponding to tube No. 3 on the MacFarland scale. By slide agglutination test, the suspension of each culture was tested for the presence of thermolabile B antigens of the known strains of E. coli serotypes with the specific known OB antisera. The suspensions were then placed in a boiling water bath or steamed for one hour to inactivate the B antigens, and then with the same technique and the same antisera, the presence of O antigens of the known pathogenic E. coli strains were tested.

The study for the pathogenicity was then conducted for those cultures which were found to possess the antigens of the known pathogenic strains of

E. coli. One-day old baby chicks were used as experimental animals. Saline suspensions of the test cultures were made as had been done previously, and by oral administration, five chicks for each culture were given one ml. of the suspension per chick, and 20 chicks were held as controls. The pathogenicity for the four known pathogenic E. coli serotypes were also studied in the same manner in chicks along with this experiment.

A study of the pathogenicity of these cultures by intravenous injection in chicks was also conducted. The suspensions of the organisms were prepared as before, and 0.5 ml. dose per chick was injected into two-week-old chicks. Five chicks per culture were injected. In addition, the pathogenicity of the four known E. coli serotypes was also studied in the same manner. Thirty chicks were held as a control group, among which one group of five chicks was injected with the same dose of a suspension of ordinary A. aerogenes, and another group of five was injected with an ordinary strain of E. coli known to have none of the antigens of the known E. coli serotypes. The other twenty chicks were held uninjected.

Following the injection of the organisms, all chicks that died or exhibited illness and also some unaffected chicks were autopsied, and internal organs, such as heart, liver, and intestines were cultured on E. M. B. agar and blood agar plates. Inoculation was also made into the other biochemical media which had been used in the identification of coliform organisms in an effort to recover the injected organisms. The organisms recovered were also tested for the presence of the antigens they contained prior to injection.

The Adaptation of the Antigens of Certain
Pathogenic E. coli Serotypes by Non-Pathogenic Coliform Bacteria

It was thought there might be a possibility of non-pathogenic coliform organisms becoming pathogenic by acquiring through some genetic mechanism, antigens comparable to those possessed by pathogenic strains of E. coli.

The 19 A. aerogenes cultures isolated from adult human mouths and the four known strains of pathogenic E. coli: O 26:B6, O 55:B5, O 111:B4, and O 127:B8 used in the previous experiments, were used for this study. The general procedure was to grow each strain of A. aerogenes and each of the four pathogenic strains of E. coli together in nutrient broth. The medium was composed of:

Sodium chloride	5 g.
Bacto peptone	10 g.
Beef extract	3 g.
Distilled water	1000 ml.

After a preliminary incubation of 24 hours in broth, one drop of each strain of A. aerogenes was placed in each of four tubes of sterile broth by adding a drop of the culture with a 1 ml pipette. Then a drop of the broth culture of each of the four known strains of pathogenic E. coli was added to these newly inoculated broth tubes separately. These tubes were incubated for 24 hours, after which each broth tube of these mixed cultures was streaked on E. M. B. and MacConkey agar as in the primary identification of coliform organisms. These plates were incubated for another 24 hours. Growth and colonial morphology were examined on each plate, and the colonies which appeared to be different from either typical E. coli or A. aerogenes were picked and subcultured on nutrient agar slants. These slants were incubated for 24 hours and then transplanted into biochemical media for the confirmatory identification of the cultures. Saline suspensions of these organisms

were made, and by slide agglutination were tested for the presence of the O and B antigens of the four known strains of E. coli serotypes.

RESULTS

In the isolation of the coliform organisms from adult human mouths, 254 samples were taken, and from 22 samples organisms having coliform-like colonies were found on the differential plating media used. Several of these coliform-like colonies were picked from each plate and subcultured on nutrient agar slants for further studies. Three samples out of these 22 were found to contain E. coli and the remaining 19 contained A aerogenes. Several coliform-like colonies from each mouth sample were streaked on the differential media for taxonomic study. The organisms from the different colonies of a given mouth sample appeared to be culturally and biochemically identical. These organisms seemed to play some role in the mouth flora of human beings, and the population of these organisms in human mouths appeared to be different from person to person.

When an antigenic study was made by applying the OB antisera of the four known strains of pathogenic E. coli: O 26:B6, O 55:B5, O 111:B4, and O 127:B8 which are known to be associated with outbreaks of infantile diarrhea, none of the three E. coli cultures isolated from human mouths was found to contain any antigens of the mentioned serotypes. However, six cultures of the identified A. aerogenes strains isolated from human mouths were found to contain the thermolabile envelope antigen (B4) of the known E. coli serotype O 111:B4, but not the thermostable somatic antigen (O 111) of the strain. The thermolabile antigen of E. coli O 111:B4 serotype seemed to be more widely spread and also more often found in coliform organisms from other sources. It may be also present in organisms other than the coliform group.

In comparison with an ordinary A. aerogenes, the A. aerogenes which was found to possess the thermolabile envelope antigen of E. coli O 111:B4 serotype exhibited some distinguishable differences, such as weaker sugar fermenting ability and production of smaller, less swollen, lighter colonies on differential media. Similar characteristics were also noticed on the four cultures of known E. coli serotypes when compared with an ordinary non-pathogenic E. coli. Yet neither these differences nor biochemical tests were satisfactory in differentiating the antigenic cultures from non-antigenic ones or for the study of their pathogenicity.

When one-day old chicks were inoculated with a one ml oral dose of suspensions of those A. aerogenes which possessed the B antigen of E. coli O 111:B4 serotypes, or with similar preparation of the four known strains of E. coli serotypes, none of the chicks was affected, although the organisms were recovered from the intestines of the chicks. When a 0.5 ml dose of suspensions of the A. aerogenes which had the B antigen of E. coli O 111:B4 serotype were injected intravenously into two-week old chicks, two were killed and eight became sick from the injection. When similar doses of suspensions of the four known E. coli serotypes were also injected, three chicks died on the day following the injection of O 26:B6 serotype, and one died following the injection of O 55:B5 serotype. Two chicks became sick from the injection of O 26:B6 serotype, one from O 55:B5 serotype, and three from O 111:B4 serotype. No chick was killed or became sick from the injection of O 127:B8 E. coli serotype, a non-antigenic E. coli, or A. aerogenes isolated from human mouths. The injected organisms could be recovered from the unaffected chicks as well as the sick or dead chicks, at certain intervals following the injection. The results are shown as in tables 4, 5, and 6.

In the study of the antigen adaptation of the known E. coli serotypes by non-antigenic organisms, the A. aerogenes grown together with the E. coli

serotypes were found to produce colonies of a peculiar appearance on differential media. They were different in appearance from both typical A. aerogenes and typical E. coli. Only a few E. coli-like colonies were found on a few plates of the differential media, and the majority of the colonies appeared to look more like A. aerogenes. When they were inoculated into biochemical media, several of them showed non-typical E. coli or A. aerogenes reactions. These were especially noticed in the methyl red and Voges-Proskauer tests. When these non-typical coliform colonies were tested antigenically, some of them showed the presence of thermolabile B antigens, thermostable O antigens or both of the antigens of the known E. coli serotypes with which they had been grown. Besides, the A. aerogenes cultures which were found to possess the thermolabile B antigen of E. coli O 111:B4 serotype from previous experiments were found to retain their antigenicity when grown together with other known strains of E. coli. Table 7 shows the results on this experiment and the antigenicities of these A. aerogenes cultures tested after grown together with the known strains of E. coli serotypes.

The three non-antigenic E. coli cultures isolated from human mouths were not used for this experiment, because there were no distinct identifying differences between these organisms and the known E. coli in their colonies on differential media when they were grown together.

DISCUSSION

It has been definitely established that coliform organisms are present in human mouths among a variety of other organisms. It is felt that these coliform bacteria may play some role in maintaining the composition of the mouth flora.

Table 4. Oral administration of antigenic A. aerogenes to one-day old chicks.

Culture No. of A. aero. injected \ Tests	After 1 day: Recovery	After 1 day: Test with O 111:B4 antiserum O	After 1 day: Test with O 111:B4 antiserum B	After 5 days: Recovery	After 5 days: Test with O 111:B4 antiserum O	After 5 days: Test with O 111:B4 antiserum B	After 2 weeks: Recovery	After 2 weeks: Test with O 111:B4 antiserum O	After 2 weeks: Test with O 111:B4 antiserum B
A5*	+	-	+	+	-	-	-	-	-
B6*	+	-	+	-	-	-	-	-	-
B11*	+	-	+	-	-	-	-	-	-
D18*	+	-	+	-	-	-	-	-	-
H9*	+	-	+	+	-	+	-	-	-
J15*	+	-	+	-	-	-	-	-	-
E10°	+	-#	-#	-	-#	-#	-	-#	-#

* A. aerogenes cultures possess B antigens of E. coli O 111:B4 serotype.

o Control, non-antigenic A. aerogenes.

-# Negative agglutination with all four antisera.

Intestines were considered to be the best source for recovering the organisms administered.

Table 5. Oral administration of E. coli known serotypes to one-day old chicks.

E. coli injected \ Tests	After 1 day			After 5 days			After 2 weeks		
	Recovery	Agglutination test		Recovery	Agglutination test		Recovery	Agglutination test	
		O	B		O	B		O	B
O 26:B6	+	+	+	+	+	-	+	-	-
O 55:B5	+	+	+	+	+	-	+	-	-
O 111:B4	+	+	+	+	+	+	+	-	+
O 127:B8	+	+	+	+	-	-	+	-	-
D13*	+	-#	-#	+	-#	-#	+	-#	-#

* Control, non-antigenic E. coli.
-# Negative agglutination with all four antisera.

Agglutination test: Tested with the specific antiserum for each known serotype only.
Intestines were considered to be the best source for recovering the organisms administered.

Table 6. Intravenous Injection of antigenic A. aerogenes and E. coli into 2-week old chicks.

Organisms \ Tests	After 1 day			After 5 days			Effect	
	Recovery of organisms	Agglu. test O	Agglu. test B	Recovery of organisms	Agglu. test O	Agglu. test B	Killed	Sick
A5	+	-	+	-	-	-	1	2
B6	+	-	+	-	-	-	0	3
B11	+	-	+	-	-	-	0	0
H9	+	-	+	-	-	-	0	1
J15	+	-	+	-	-	-	1	2
E10*	+	-#	-#	-	-#	-#	0	0
O 26:B6	+	+	+	-	-	-	3	2
O 55:B5	+	+	+	-	-	-	1	1
O 111:B4	+	+	+	-	-	-	0	3
O 127:B8	+	+	+	-	-	-	0	0
D13**	+	-#	-#	-	-#	-#	0	0

* Control culture of A. aerogenes
** Control culture of E. coli
-# Negative agglutination with all four antisera

Agglutination test: A aerogenes were tested with O 111:B4 antiserum.
E. coli tested with antiserum of its own serotype.

Table 7. Biochemical and antigenic test of the organisms obtained from the mixed growth of the known E. coli and A. aerogenes cultures obtained from human mouths.

Tests / Cul. No. of A. aero.	O 26:B6					Agglu		O 55:B5					Agglu		O 111:B4					Agglu		O 127:B8					Agglu	
	Lc	Gl	Ci	MR	VP	O	B	Lc	Gl	Ci	MR	VP	O	B	Lc	Gl	Ci	MR	VP	O	B	Lc	Gl	Ci	MR	VP	O	B
A3	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	–	–	+	–	–
A5*	Ag	Ag	+	+	+	+	–	AG	AG	+	–	+	–	–	ag	aG	+	–	+	–	+	Ag	AG	–	+	+	+	–
B5	Ag	AG	+	+	+	–	–	AG	AG	+	–	+	+	–	AG	AG	+	–	+	–	+	AG	AG	+	–	+	–	–
B6*	Ag	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	+	AG	AG	+	–	+	–	–
B11*	Ag	Ag	+	+	+	+	–	Ag	AG	+	+	+	–	–	AG	AG	+	–	+	–	+	Ag	Ag	+	–	+	–	–
B23	AG	AG	+	–	+	–	–	Ag	AG	+	+	+	–	–	Ag	AG	+	–	+	–	+	AG	AG	+	–	+	–	+
B26	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	+
C18	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	+	+	+	–	–	AG	AG	+	–	+	–	–
D18*	AG	AG	+	+	+	–	–	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	+	AG	AG	–	–	+	–	–
E4	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	–	–	+	–	–	AG	AG	+	–	+	–	–
E10	AG	AG	+	–	+	–	–	AG	AG	+	+	+	+	+	AG	AG	+	–	+	–	–	AG	AG	+	+	+	+	–
E9	AG	AG	+	–	+	–	+	AG	AG	+	–	+	–	–	AG	AG	+	+	+	+	–	AG	AG	–	–	+	–	–
H7	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	–	+	+	–	–	AG	AG	+	–	+	–	–
H9*	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	–	–	+	–	+	AG	AG	+	–	+	–	–
I12	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–
I14	AG	AG	+	+	+	–	–	ag	Ag	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	–	+	+	+	+
I16	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–
J5	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–	AG	AG	+	–	+	–	–
J15*	Ag	AG	+	–	+	–	–	AG	AG	+	+	+	+	+	AG	AG	+	–	+	–	+	Ag	AG	+	–	+	–	–

* A. aerogenes which possessed B antigen of E. coli O 111B4 serotype. Lc: lactose. Gl: glucose. Ci: citrate. MR: methyl red test. VP: Voges-Proskauer test. Agglu: agglutination test with the antiserum for the strain of E. coli.

A: acid.
a: little acid.
G: gas.
g: little gas.

During this study, although no E. coli was found to have the antigens of the known strains of E. coli serotypes which are responsible for outbreaks of infantile diarrhea, there is a possibility that human mouths could be the main source for harbouring the strains of E. coli that have been known to be associated with infantile diarrheal diseases. These pathogenic serotypes may be found subsequently if a larger population of mouth samples are examined.

Because of the limited variety of antisera used, there might be some coliform organisms which belonged to the pathogenic serotypes other than the known strains or which might contain different types of antigens that could not be tested with the antisera employed.

From the works of many investigators in the past, it has been known that these pathogenic E. coli serotypes appear to be less virulent for adults than for infants and young children. This may be due to the better resistance in adults, although some strains of these serotypes (as O 111:B4) have been recovered in a few cases of infection of epidemic diarrheal disease in adults. The outbreaks of these infections have been more frequently reported in summer than in winter which indicates that seasons may play some part in the spread of the organisms.

Some of the A. aerogenes isolated from human mouths during these studies were found to possess the thermolabile B antigen of one of the known E. coli serotypes. They were found to be more virulent than those which had no antigen of this type when tested in vivo in experimental chicks. This appears to support the study by Kauffmann (1947) on the serology of the "coli" group, in which he stated in his summary:

> "Strains containing K antigens are more toxic than strains without K antigens — and particularly toxic when the strains are isolated from pathologic material."

He also stated:

"Strains with K antigens are particularly resistant to the defensive forces of the organism and bacteriophages."

A known strain of E. coli serotype (O 26:B6) recovered from a chick following intravenous injection was found to have become hemolytic. More study will be required to determine whether the chick blood system actually contributed the hemolytic characteristics to the organism or whether it simply gained the property when grown in the blood system of the animal. It also appears that the other known strains of E. coli serotypes, as well as the coliform organisms with some antigens of pathogenic E. coli, became more virulent when allowed to infect experimental animals artificially.

The B antigens are similar to Vi antigen of Salmonella species in many respects. When these B antigens are heated for 1 hour at 100°C. their agglutinability is inactivated, but their antibody binding power is not destroyed. From the data of Stuart et al. (1948) it is believed now that the boiling or heating is likely to remove the envelope antigen from the bacteria rather than actually destroy it. In the case of the Vi antigen in Salmonella, it has been shown that alcohol removes the antigen from the bacteria by bringing it into solution.

The instability of B antigens has been recorded by many investigators in the past, and during this study it was also noticed that the organisms which possessed B antigens tended to lose their antigenicities at refrigerator temperature more readily than at room temperature.

It must not be concluded that B inagglutinable coliform organisms are not pathogenic, even though they apparently do not possess the O antigens which are known to be associated with infantile diarrhea, for the B antigens may be detached from the organism rather quickly or their antigenicities may

not be pronounced enough to produce agglutination with antisera. These non-agglutinable strains may be still virulent enough to cause infantile diarrhea.

Organisms other than the *E. coli* serotypes known to be associated with infantile diarrhea may harbour some envelope antigens of the known serotypes, and they may participate in the infection of infants.

There is also a possibility that some organisms may be able to pick up certain antigens of the known strains of pathogenic *E. coli* serotypes and thus may actually become pathogenic. The adaptability of antigens was found in both somatic and envelope antigens. Genetic mechanisms probably were involved more than the ordinary environmental factors. Further genetic studies will be necessary for demonstrating whether this is a true recombination, transduction, or transformation of the organisms. The intermediate forms produced by this process possessed the characteristics of both parental types. Such intermediate forms may be responsible for those cases of infantile diarrhea in which some investigators have reported that no typical *E. coli* were recovered from patients.

It should be realized that difficulty is encountered in studying the pathogenicity of these enteropathogenic coliform organisms. Although young chicks were used in this study, a number of coliform-like organisms with some *E. coli* antigens were isolated from the chick feed. The isolation of *E. coli* serotypes, especially O 111:B4, from fowls has been also pointed out by Edwards (1956) and the isolation of O 111a, O 111c:B4 serotype from monkeys has been studied by Ewing et al. (1955).

Pathogenicity studies should not depend entirely upon the effect on chicks because chicks may develop resistance as they grow.

SUMMARY

From various investigations in the past, it has been definitely established that certain serotypes of coliform bacteria are responsible for outbreaks of epidemic and sporadic cases of infantile diarrheal diseases.

Adult human mouths were thought to be one of the main sources of the infection of these particular serotypes. A study was made in an attempt to isolate some of these pathogenic serotypes from adult human mouths. The organisms isolated were identified, and further studies were carried out for detecting the possession of certain antigens of some known E. coli serotypes, and their pathogenicities were also studied. The adaptability was also studied on these non-antigenic organisms isolated from human mouths.

Coliform bacteria were isolated from human mouths, and some of these organisms were found to possess the thermolabile B antigen of E. coli O 111:B4 serotype. These organisms appeared to be more virulent than those which had no antigen of this type.

When non-antigenic coliform organisms were grown together with some known E. coli serotypes which had the antigens associated with infantile diarrheal diseases, some of the non-antigenic coliform organisms were found to acquire some antigens of the known E. coli serotypes.

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PATHOGENIC COLIFORM BACTERIA IN THE ADULT HUMAN MOUTH

by

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Epidemic and sporadic cases of infantile diarrhea have been found in the past in which no pathogenic organisms such as Salmonella and Shigella species were found. Escherichia coli was isolated occasionally, but most workers did not believe that some particular E. coli serotypes could be responsible for these outbreaks, due to the fact that most investigators were hampered by using only biochemical tests in differentiation of these particular E. coli strains from the ordinary E. coli.

Because of these misleading results, the credible works of certain investigators such as Goldschmidt in 1933 did not receive any attention. Bray (1945) and Bray and Beavan (1948) were probably the first to emphasize the fact that some E. coli serotypes were associated with these infections in infants. Since then many workers have been investigating these bacteria. Antigenic studies were emphasized and Kauffmann in 1947 established an E. coli antigenic schema. Today, it is established that certain E. coli are responsible for outbreaks of infantile diarrhea and gastroenteritis, and more new strains of E. coli serotypes have been reported.

As for the study in tracing the source of these organisms, adult human mouths were examined for these particular strains of E. coli and related coliform organisms. Coliform organisms were actually found in 22 human mouths and the organisms were isolated and identified biochemically; those cultures were identified as E. coli and the remaining 19 as A. aerogenes. An antigenic study was also made and 6 cultures of A. aerogenes besides E. coli were found to possess a thermolabile B antigen of a certain E. coli known serotype O 111:B4. These organisms were also found to be more toxic to experimental animals than those which did not have this antigen. E. coli O 26:B6, one of the known strains of E. coli serotypes studied, was found

to become hemolytic when injected intravenously into chicks. When non-antigenic coliform organisms were grown together with some known strains of E. coli serotypes, isolates from the mixture were found to have different characteristics from both types, and some antigens of the known strains of E. coli serotypes were picked up by these non-antigenic coliform organisms.

It was concluded from these studies that there is a possibility that the E. coli serotype strains related to infantile diarrhea might be present in adult human mouths, and that these adults might serve as carriers to infect infants. There is also the possibility that other pathogenic coliform organisms, or even other group of bacteria, present in human mouths, some of which might contain or might pick up the pathranic E. coli antigens and thus might become responsible for infantile diarrheal diseases.

Adults are usually not affected by these pathogenic coliform organisms because they possess a higher resistance than do infants. Since outbreaks of infections usually occur in summer time, the season may have to be taken into consideration as a factor in the spread of such infections.