THE OCCURRENCE OF GRAM-NEGATIVE BACILLI IN THE INTESTINAL TRACT OF NEWBORNS DURING THE FIRST FIVE DAYS OF LIFE

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PREFACE

Perhaps the detection of gram-negative bacilli in feces is one of the most complicated bacteriological problems which a clinical laboratory encounters. The isolation of these organisms, whether they are pathogenic or non-pathogenic, is attended by many technical pitfalls. Intelligent and careful work is required in the identification of species of medical significance. From a clinical point of view, the rapid grouping of gram-negative bacilli as to genus is important, so that the clinician will be able to institute therapy as soon as possible. A statement, made by a pediatrician, that too much time was consumed in the culturing of stools, presented the challenge which led to the present investigation.

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I INTRODUCTION

The study of the occurrence and the succession of gram-negative bacilli in the intestinal tract of the newborn during the first five days of life was undertaken from two points of view. The first was to ascertain what constitutes normalcy of the intestinal flora in newborns. The second aspect of the study was to determine simplified methods whereby reports on stool cultures could be given more quickly, yet accurately. The dilemma of the bacteriologist is a real one. If he insists on accuracy at all costs, his reports may be several days too late to be of any practical value. If he attempts to give a rapid report, he may find the so-called "short-cuts" disastrous. While the author was interested in the statistical data, the greater concern was to establish a few criteria whereby a tentative report could be given to the physician so that proper treatment could be instituted, and in the meantime further identification of the organisms involved could be made.

This problem grew out of a challenge made by a medical man to a laboratory technician that cultures of feces on infants and younger children were not requested more frequently because of the length of time required to complete the identification. Stool cultures are time consuming. Practices adopted must give assurance of thoroughness and accuracy. This has necessitated the sifting of known methods, which are legion, and the selection of those methods which will help in this rapid identification.

The subject matter of this paper makes no pretense of presenting new methods. The aim was to use common culture media available to any medical laboratory, and to employ such tests which would require only simple laboratory equipment.

II REVIEW OF LITERATURE

Breslau (1866) first observed microscopically the early invasion of the intestinal tract of newborn infants. A few years later Billroth (1874) and Nothnagel (1881) confirmed his work. Nothnagel, using iodine as a stain, tried to identify Saccharomyces ellipsoideus, Bacillus subtilis, and the so-called Clostridium of Prazmowski. Bienstock (1884) severely criticized Nothnagel for his failure to use "modern" staining and cultural methods of Koch. With these methods Bienstock made beautifully colored plates of fecal bacilli with red spores, but one wonders why he failed to discover Bacillus coli (now Escherichia coli), for, if he never had it in pure culture, his records certainly failed to prove it. Had he known Gram's method of staining, which was reported in the same year, he might have discovered the bacterium.

However, the first systematic study of intestinal bacteria is attributed to Escherich (1886), who made an extensive investigation of the microorganisms in infants' stools. Escherich utilized Gram's method of staining and Koch's method for the isolation of bacteria on solid media, and thus laid the real foundation for the modern concept of normal intestinal bacteriology. He showed that, even if no organisms can be demonstrated microscopically, their presence can be shown culturally as early as the fourth hour. He likewise demonstrated that whereas the flora of the first day was usually simple, that of the succeeding days became more complex. He reported finding Bacillus

coli, Bacillus aerogenes, Bacillus subtilis, other sporulating bacilli, and yeasts. Moreover, he observed that this flora changed about the fourth day when the meconial stool had been replaced by the yellow, mucoid, acid-milk stool. In direct examination of the milk-stool he found that slender, granular-staining, gram-positive, non-sporulating rods greatly predominated. However, when agar plates were inoculated with these milk-stools, the gram-positive bacteria rarely appeared, but only the gram-negative bacilli, Bacillus coli and Bacillus aerogenes. This enigma Escherich attempted to solve by assuming that the staining reactions of these organisms were altered in the intestinal tract. Although Escherich had observed the predominance of gram-positive rods, probably lactobacilli, in the feces of infants, he failed to isolate two which later aroused considerable interest, Bacillus bifidus and Bacillus acidophilus, and are now known as Lactobacillus bifidus and Lactobacillus acidophilus. Schmidt (1892), under the guidance of Escherich, attempted to show that the difference in staining abilities was due to the fat in the feces; however, the bacteria grown on butter agar and butter gelatin were still gram-negative. He concluded that, despite the staining differences, the two types were growth forms of the one and the same species, although he knew that Bienstock in 1884 had grown fecal gram-positive organisms anaerobically.

The tinctorial discrepancy between bacteria in milk-stools stained directly and those from aerobic cultures, found its true explanation in the isolation of Bacillus bifidus by Tissier (1899), and of Bacillus acidophilus by Moro (1900). Tissier (1905) noted the presence of the following organisms in a five-year old child: Bacillus bifidus, Bacillus acidophilus, the enterococci, Bacillus coli, Bacillus exilis,

and Bacillus III of Rodella. These he regarded as the constant fundamental flora. Besides these, he observed the following variable, secondary flora which he reported to be: Bacillus perfringens, Staphylococcus parvulus, Bacillus fundiliformis, Bacillus capilosus, Bacillus ventriosus, Coccobacillus praecutus, Coccobacillus oviformis, Diplococcus orbiculus, and different yeasts.

Tissier (1905), in a review of his work on the newborn's intestinal flora, discussed the development of the infant fecal flora in three phases: (1) aseptic, (2) increasing infection, and (3) transformation. In his dissertation, Escherich (1886) had stated that the meconium of the infant was sterile, and bacteria did not appear until 4 hours after birth in the summer and after 17 hours in the winter months. This period is probably what Tissier termed as the "aseptic phase".

In their investigation, Hymanson and Hertz (1917) reported that 9 out of 39 meconia, collected by rectal swabs shortly after birth and streaked out on serum glucose agar, contained bacteria.

Burrage (1927) reported that the examination of nearly 100 samples of meconium showed the presence of bacteria in about 38 per cent of the specimens. In the positive cases, which were called such only if a large number of organisms was present, Bacillus coli was found in pure culture in about 50 per cent. The other positive cases usually contained a staphylococcus in pure culture, but there were a few mixed cultures containing two or three species.

Hall and O'Toole (1934) made a study of the bacterial flora of first specimens of meconium passed by 50 newborn infants. They showed that, upon microscopic examination, bacteria were absent in 94 per cent of the first specimens of meconium. In about 38 per cent of the

first specimens of meconium, aerobic bacteria were demonstrated culturally. Although only 13 per cent of the specimens passed during the first five hours contained bacteria, the percentage rose rapidly to 100 per cent during the first 24 hours of life. According to this study the predominating bacteria were white micrococci and Bacillus coli. Sporulating bacilli and streptococci were rare. Of considerable interest and note was the fact that Bacillus paratyphosum B and Clostridium welchii were reported once in 50 first specimens.

Snyder (1936) made a similar study of the bacterial flora of meconial specimens collected from 64 infants within 4 hours after delivery. The species isolated were: Lactobacillus acidophilus, Bacillus coli, Streptococcus pyogenes, Streptococcus mitis, Bacteroides, Micrococcus epidermidis, and Streptococcus faecalis. He concluded that prenatal bacterial invasion of the intestinal tract does occur in a small percentage of cases.

The organisms appearing in breast-fed infants in the phase of increasing infection, i.e., the first two or three days of life were found by Tissier (1905) to be: white staphylococci, Bacillus perfringens, Bacillus III of Rodella, and other spore-bearing anaerobes. This diversified flora reached its height on the third day and was then mostly replaced by Bacillus bifidus. In bottle-fed infants the phase of increasing infection and transformation was greatly prolonged, and the flora of the latter period presented a variable picture composed of the same organisms listed above, but with none predominant. The most significant fact in Tissier's work was his conclusion that the diet of the infant, rather than the age, controlled the fecal flora. A similar extensive review by Moro in 1905 stated that Bacillus bifidus, Bacillus

coli, Bacillus aerogenes, Bacillus acidophilus, the non-motile butyric-acid bacillus, and the intestinal streptococci were the constant inhabitants of the breast-stool. At the same time Moro described the aerobic acid-tolerant form, Bacillus acidophilus, which he believed was the predominant gram-positive organism in the stool of the weaned baby.

Other investigators, such as, Cahn (1901), Sittler (1908), Logan (1914), Adam (1921, 1922, 1922b), Rettger and Cheplin (1921), Brown and Bosworth (1922), Cruickshank and Berry (1924), Cruickshank (1925), Kopeloff (1926), and many others have attempted with more or less success to clarify the picture established by the earlier workers.

Naujoks (1921) showed that the majority of pregnant women harbor Bacillus acidophilus in the vaginal secretions, hence practically every infant acquires this organism at birth.

In 1933 Eggerth and Gagnon (1933) reopened a neglected field of bacteriology by showing that non-sporulating anaerobes, the Bacteroides, were probably the most frequent bacteria in the feces.

Following their first work on the bacterial flora of meconia, Hall and O'Toole (1935) made an investigation of the daily microbial changes in the feces of normal breast-fed infants during the first 10 days of life. While 7 babies passed sterile specimens on the first day, bacteria were present in all specimens by the second day. The following species were represented: Micrococcus albus, Micrococcus candidus, Micrococcus epidermidis, Streptococcus faecalis, Streptococcus mitis, Bacillus coli, Bacillus aerogenes, "Kopfenbacterien" and the lactobacilli. All these appeared at some time during the first 10 days in more than 50 per cent of the infants studied.

Snyder (1940) reported a study he made of the development of the

normal fecal flora of infants between two weeks and one year of age. In this work it was noted that the following species occurred in more than 25 per cent of the stools examined: (1) Bacillus coli, 93.4 per cent; (2) Streptococcus faecalis, 78.5 per cent; (3) Clostridium welchii, 68.7 per cent; (4) Bacillus tertius, 51.6 per cent; (5) Bacteroides, Group I, 47 per cent; (6) Bacillus aerogenes, 44 per cent; (7) Streptococcus mitis, 29.7 per cent; (8) Micrococcus epidermidis, 26.4 per cent; (9) Lactobacillus bifidus, 24.2 per cent. It is the common finding that the Lactobacillus bifidus is uniformly present in stools of nurslings and is predominant in supplemented breast-fed babies. Snyder concluded that the expectancy of finding any bacterial species in the feces, except possibly the streptococci, would depend not so much on the child as on the diet. There is no question but that the breast milk is inhibitory to the growth of sporulating anaerobes in the intestines of the infant. Snyder recovered only four strains of Clostridium welchii from the stools of breast-fed infants. However, whenever the infants received supplemental feedings in addition to breast-feedings, then a variety of bacteria appeared in their stools. The saccharolytic forms were far more common than the proteolytic varieties. The gram-negative, non sporulating anaerobes were isolated with far more regularity after weaning then before. The micrococci seemed to be encountered more frequently in the early period of supplemented breast-feeding than in later months.

In an investigation of the initial aerobic flora of premature infants, Torrey and Reese (1944) found that of the 85 infants from whom nasopharyngeal cultures were obtained, 3.5 per cent showed the presence of some member of the colon group on the first day of life,

and 13.5 per cent were positive for coliform organisms within 3 days. The 31 strains which were classified were divided as follows: Escherichia coli communis, 75 per cent; Escherichia coli communior, 20 per cent; and Aerobacter aerogenes, 6 per cent. The authors showed that the primary implantation of the intestinal tract of newborns with Escherichia coli was not of oral origin as might be supposed. Specimens from the oral and rectal regions were cultured daily from the time of birth. The rectal specimens were taken on swabs and plated on Levine's EMB lactose agar. They showed that in 19 infants the first rectal cultures which were positive for Escherichia coli antedated the positive oral cultures. They likewise found that in 8 of the 19 infants or 42 per cent, alpha streptococci with proteolytic action on milk and gelatin were encountered with surprising frequency in the initial rectal flora. In 7 of the 8 cases in which they were obtained, these organisms were present within 3 days after birth. The strains produced no hemolysis on blood agar, but rather a green discoloration. Sherman, Stark, and Mauer (1937) recommended that this hemolytic, proteolytic streptococcus be designated as Streptococcus faecalis, var. liquefaciens.

In a more recent study, Ross (1950) found by direct microscopical examination that a gram-positive bacillus comprises almost 100 per cent of the flora of the breast-fed infant. This is probably the organism which Tissier first described and named Bacillus bifidus communis. On the other hand, the microscopic picture of a smear made from the feces of an artificially-fed infant presents a greater variety of bacteria including gram-positive and gram-negative cocci and bacilli.

III EXPERIMENTAL

A. METHOD AND PROCEDURE

The specimens were obtained by means of rectal swabs. Rubber tubing with an inside diameter of 3/16 inch and a wall thickness of 1/16 inch was cut to a 6-inch length. One end of the tubing was beveled to an angle of approximately 30°. An applicator stick with a cotton swab just large enough to pass through the tubing was inserted into the tube so that the swab-end was just back of the beveled edge. One other swab was placed into the test tube, together with the aforementioned swab, and both were autoclaved at 15 lbs. pressure for 20 minutes.

The rectal swab method was used because the technique is simple, easy to perform and readily adaptable for use in the home, office, and hospital. There is less danger of contamination than in the use of the diaper method. Individual swabs in sterile test tubes assure immediate collection of material and eliminate many of the cumbersome and unpleasant phases of the collection of the conventional fecal specimen. Fischer (1947) made a comparative study of the value of the rectal swab and the conventional method, and concluded that the two appear to be of comparable value for bacteriological studies.

In order to obtain the specimen the baby was placed in the knee-chest position. Sterile K-Y jelly was applied to the outer surface of the rubber tubing. The skin around the anus was cleansed with a cotton pledget soaked in a 1:1000 solution of Phemerol. After ascertaining that the swab was back in the tube, the pointed end of the tube was

gently inserted into the anal canal to a depth of about two inches. Holding the rubber tubing in place, the swab was gently forced through the beveled end so that it came in contact with the mucosa. After making three or four turns with the applicator and still holding the tube in place, the swab was withdrawn and a direct smear was made. The second swab was inserted and another specimen was obtained.The swab was then brought back into the rubber tubing, and both tube and swab were withdrawn. With this last swab, brain-heart-infusion broth was inoculated.

After three to four hours' incubation, streak plates were made on nutrient agar, blood agar, and eosin-methylene-blue (EMB) agar, from the brain-heart infusion broth. All plates were incubated at 37° C. for 24 hours. Discrete colonies were picked from the nutrient, blood, and EMB agar plates after 24 hours' incubation and were transferred to nutrient agar slopes. These slopes were incubated at 37° C. for 24 hours. After this period, direct smears were made from the agar slants and stained by the Gram's method to determine the purity of the culture and to indicate further steps in the identification process. All gram-negative bacilli were inoculated into lactose broth, dextrose broth, and sucrose broth (phenol-red broth base [Difco] plus 0.5 per cent carbohydrate). The carbohydrate broth tubes were examined at the end of 24 hours and again at the end of 48 hours. If at the end of 48 hours no fermentation was evident, the carbohydrate cultures were re-incubated for one week before a negative fermentation reaction was recorded. Also from these nutrient agar slopes, Kligler's iron agar, as well as tryptone broth, MR-VP broth, and Simmons' citrate agar were inoculated and all tubes were incubated at 37° C. for 24 hours,

except the MR-VP broth, which was incubated for 48 hours. The Kligler's iron agar slopes were examined at the end of 24 hours' incubation. The organisms that had produced acid and gas in the butt, but showed neutral or alkaline reaction in the slant, were inoculated into urea broth. All urease-splitters were transferred into maltose and mannite broth (phenol-red broth base plus 0.5 per cent carbohydrate). The organism was considered negative for urease production, if no change in the urea broth was evident after 24 hours.

B. CULTURE MEDIA USED IN THIS STUDY

Nutrient agar is a general culture medium used for the cultivation of the majority of the less fastidious microorganisms. It is also added as a base to a variety of materials to give selective, differential, or enriched media. It is recommended by the American Public Health Association and the American Water Works Association for the examination of coliform organisms in water supply. It is used for the ordinary routine examination of water, sewage, and food products; and for the carrying of stock cultures.

Blood agar plates were utilized to study the hemolytic characteristics of colonies. The blood agar base (Difco) employed was slightly acid in reaction. It is believed that the slightly acid reaction (pH 6.8) permits the development of clearer zones of hemolysis than does an alkaline reaction (Norton 1932). Approximately 0.5 ml. of sterile whole human blood was added for every 10 cc. of melted blood agar base.

Nutrient gelatin was inoculated for the detection of proteolysis as evidenced by the liquefaction of gelatin.

Simmons' citrate agar is a medium used in the differentiation between members of the coli and the aerogenes groups on the basis of citrate utilization. Strains of Escherichia coli either do not grow at all upon this medium, or else grow so scantily that no change in reaction is apparent. The medium usually prepared as slopes, is inoculated by either stab or streak, and is incubated at 37° C. Growth is shown by colony formation and is usually accompanied by a color change of the indicator, brom thymol blue. Strains of Aerobacter aerogenes grow luxuriantly upon this medium, producing alkali and changing the medium from its original green to a deep blue within 24 to 48 hours (approximate pH change from 6.8 to 7.4 or more).

To brain-heart infusion broth a small amount (0.1 per cent) of agar, was added to enhance the growth of bacteria. Common forms ordinarily considered easy to cultivate appear to be aided by the presence of a small quantity of agar. Hitchens (1921) and Simmons (1939) showed that growth is initiated in a much shorter incubation period in such media.

Urea broth was used as a differential medium for the detection of members of the Proteus group, of Paracolobactrum aerogenoides, and of Paracolobactrum intermedium.

For the study of carbohydrate fermentation, phenol-red broth base to which had been added the desired carbohydrate, was used. The concentration of the carbohydrates employed for testing the fermentation reactions of bacteria was 0.5 per cent. The fermentative properties of bacteria are valuable criteria in their identification. Phenol-red broth base is a basic medium prepared with phenol-red as an indicator of the changes in the hydrogen ion concentration (pH).

For the indol tests, Bacto-tryptone in a 1 per cent concentration as specified in Standard Methods for the Examination of Water and Sewage (1946), was used.

The commonly accepted buffered glucose peptone broth (MR-VP broth) was used in the differentiation of bacteria by means of the Methyl Red test and the Voges-Proskauer reaction.

Levine (1943) states that the eosin-methylene-blue agar (EMB) was designed primarily for the purpose of differentiation of Escherichia coli from Aerobacter aerogenes. He later found this medium equally serviceable for the differentiation of the Salmonella-typhoid-dysentery group from the coliform group. Colonies of enteric pathogens possess a transparent amber color, or are sometimes colorless, while typical colonies of the colon bacillus are blue-black and have a metallic sheen when viewed by reflected light. Colonies of members of the Aerobacter group are pink. The medium is strongly inhibitory to gram-positive bacteria.

Frobisher (1950) says that the selection of coliform colonies on EMB plates is facilitated by three factors, namely:

(1) plates contain lactose, readily fermented and acidified by these organisms.
(2) plates contain dyes, eosin and methylene blue, which inhibit many other bacteria.
(3) acidification of the lactose under the lactose- fermenting coliform colonies turns the dyes in the agar a distinctive red, blue or purple color in and around each colony so that it is easily recognized.

Kligler's iron agar is a modification of the lead acetate medium described by Kligler (1918). This medium gives fermentative reactions similar to Russell's double sugar agar together with sulfide reactions. The inoculation is made by streaking the slant and stabbing the butt of the agar. Fermentation of dextrose is indicated by a change of the butt

to a yellow color (acid reaction of phenol red). Coliforms generally attack lactose and produce an acid reaction in the slant as well as in the butt of the tube. Blackening of the medium due to liberation of sulfide is characteristic of Proteus vulgaris, and Proteus mirabilis. On the basis of hydrogen sulfide production this medium is useful in the differentiation of Salmonella typhosa from the other Salmonella and the Shigella groups. It differentiates Salmonella paratyphi from Salmonella schottmuelleri and Salmonella enteritidis. Time may be saved by using Kligler's iron agar since this medium combines the principles of Russell double sugar agar and lead acetate agar. This one medium, Kligler's iron agar, permits a differentiation of the gram-negative rods on the basis of their ability to ferment lactose or dextrose and to produce hydrogen sulfide.

C. ADDITIONAL BIOCHEMICAL TESTS

1. INDOL PRODUCTION

Indol is a substance resulting from the attack of bacteria upon the amino acid, tryptophane. In this study, a 1 per cent concentration of Bacto-tryptone broth was inoculated with the organism and incubated for 24 hours. After this period of incubation, the culture to be tested was shaken with a few drops of xylol. Indol is soluble in xylol and is concentrated in it. After a few minutes of standing the indol is carried to the surface by the solvent. A few drops of Erhlich's reagent, were gently added to the culture from a pipette, with the tip of the pipette at the junction of the broth and xylol layer. This interposed a layer of reagent between the broth and xylol. If indol was present a pink color formed in a few minutes as a ring at the junction

of the xylol and the reagent.

Ehrlich's reagent:

Para dimethylaminobenzaldehyde.....2 grams
Alcohol, ethyl, 95%.....190 cc.
Hydrochloric acid, concentrated.....40 cc.

2. METHYL RED TEST

The Methyl Red test has been used and probably still is the most popular and widely used differential test in the study of coliform organisms. The test was introduced by Clark and Lubs (1915) and is practically the same today as when originated. This test determines the pH of a glucose broth culture after 2 to 4 days' incubation. The acid-alkali indicator used in this test is methyl red(pH range 4.4-6).

Members of the Escherichia group produce acids in the buffered glucose broth used for the Methyl Red test. These cultures remain strongly acid for several days. When methyl red is added to the culture after 2 to 3 days' incubation a definite red color results. This is a positive Methyl Red test. On the other hand, strains of the Aerobacter group possess the ability to attack the acids produced in the primary fermentation of the glucose broth and convert them into carbonates or other non-acid substances. The medium then reverts to a neutral or alkaline reaction and gives a yellow color when methyl red is added, thus indicating a negative Methyl Red test.

Approximately 10 ml. of MR-VP broth (BBL), in a 150 X 20 mm. test tube, were inoculated and incubated for 48 hours at 37° C. After this period of incubation, about 2 ml. of the culture were transferred into a smaller test tube. Three drops of methyl red solution were added. A positive reaction was indicated by a distinct red color, showing the

presence of acid. A negative reaction was indicated by a yellow color. The indicator solution was prepared by dissolving 0.1 gram of Bacto-methyl red in 300 ml. of 95 per cent of ethyl alcohol and by diluting to 500 ml. with distilled water.

3. VOGES-PROSKAUER REACTION

Voges and Proskauer (1898) introduced a test that can be used to distinguish between Aerobacter aerogenes and Escherichia coli. This reaction tests for the presence of acetyl-methyl carbinol, which is one of the intermediate products of dextrose fermentation. Escherichia coli does not produce this substance, whereas Aerobacter aerogenes does. The test as performed by Voges and Proskauer consisted in growing the organisms to be tested in dextrose peptone broth for 48 hours or more at 37° C. At the end of this time a strong solution of KOH or NaOH was added to the medium and the test tube well shaken. The appearance of a pink color after 6 hours was considered a positive Voges - Proskauer reaction whereas, the absence of a pink color was a negative test. Later this technique was found to be unsatisfactory in many ways. Considerable time was apparently necessary for the appearance of the pink color. In many cases after the pink color finally did appear, it was so indistinct that the results were difficult to interpret. In 1934 the Ministry of Health of Great Britain, reported that the test as originally carried out gave faint and indefinite reactions. The Standard Methods Committee (1946) likewise, declared the test, as originally carried out, unsatisfactory.

Harden (1906) worked out the chemistry of the Voges-Proskauer reaction. The acetyl-methyl carbinol in the presence of a strong base and

air becomes oxidized to diacetyl which in turn probably reacts with the arginine in the peptone producing a pink color. The original technique was modified by O'Meara (1931) by increasing the amount of the guanidine group reacting with the diacetyl. This was accomplished by the addition of creatine to the culture together with potassium hydroxide. This was a definite improvement, as the addition of the creatine considerably intensified the color change. However, the results obtained were still very inconsistent.

Barritt (1936) suggested the use of alpha-naphthol in the reaction. His test consisted in growing the organisms in dextrose peptone broth for 48 hours at 37° C. At the end of this time 0.6 ml. of a 5 per cent solution of alpha-naphthol in absolute alcohol, and 0.2 ml. of a 40 per cent solution of KOH were added to 1 ml. of the culture medium. A positive reaction was indicated by the presence of a crimson to a ruby color within 2 to 10 minutes. The method used in the present investigation was very similar to the one followed by Barritt.

To the remaining portion of the MR-VP broth, which had not been used for the Methyl Red test, 1 ml. of a 40 per cent solution of potassium hydroxide containing 0.3 per cent creatine was added and the tube was shaken. Then 0.3 ml. of alpha-naphthol solution was added. The contents of the tube were mixed and shaken vigorously on a Kahn shaker for 5 minutes. It was found that it was necessary to aerate the mixture thoroughly before a definite clean-cut reaction could be noticed. After a 5-minute shaking the cultures were examined. A positive reaction was characterized by an intense pink-rose color, developing in a few seconds to ten minutes after the reagents had been added. Those tubes that showed a doubtful reaction were shaken for an additional

5 minutes before the final reading was made.

4. CITRATE UTILIZATION

Koser (1923) showed that the ability of an organism to use citrate as its sole source of carbon, is a constant and reliable characteristic of the organism. In his study, Koser pointed out that Escherichia coli fails to develop while Aerobacter aerogenes and Aerobacter cloacae multiply rapidly and produce a luxuriant growth. Slopes of Simmons' citrate agar were made and inoculated by streaking the slant and stabbing the butt. Cultures were incubated at 37° C. and were examined daily for one week. At the end of this time, if no growth was visible, the cultures were read as negative.

D. CLASSIFICATION AND IDENTIFICATION

Bergey's classification was followed in this study. The family, Enterobacteriaceae, includes a large number of gram-negative, non-sporulating rods whose natural habitat in most cases is the intestinal tract of man and other animals. Bergey (1948) lists five tribes and nine genera as belonging to this family. An abridged outline of Bergey's classification is given in Chart I.

CHART I: MEMBERS OF THE FAMILY ENTEROBACTERIACEAE

Family	Tribe	Genus
Enterobacteriaceae	I. Escherichieae	Escherichia Aerobacter Klebsiella Paracolobactrum
	II. Erwineae	Erwinia
	III. Serrateae	Serratia
	IV. Proteae	Proteus
	V. Salmonelleae	Salmonella and Shigella

Some members of this family possess definite pathogenicity for man and are the cause of various types of gastro-intestinal diseases, such as typhoid (Salmonella) or dysentery (Shigella). The Escherichia appear to lead a saprophytic life in the intestinal tract, but may cause pathologic conditions in various parts of the body, such as the genito-urinary tract and the respiratory system. The Aerobacter group is believed to occur most comonly in nature, although they may likewise be found in the human body. Thus far the science of bacteriology has not found simple differential criteria for these organisms, and classification is based on morphological characteristics, biochemical reactions, antigenic properties, and ecological considerations. Frequently organisms fail to exhibit all the characteristics of a single group; appearing to occupy an intermediate position between the Escherichia and the Aerobacter groups. These are known as the Intermediates.

A primary differentiation of the Enterobacteriaceae is on the basis of lactose-fermentation. The coliform bacteria which include the coli, aerogenes, and intermediate types ferment lactose rapidly with the production of acid and gas in 24 hours. Bacteria of the Salmonella and Shigella do not ferment lactose. Chart II presents a key to the more common species of the Escherichia and Aerobacter groups.

Differential tests used for the identification of these gram-negative, lactose-fermenting bacilli were: (1) indol production, (2) Methyl Red test, (3) Voges-Proskauer reaction, (4) citrate utilization. Parr (1936) referred to these four most commonly used tests by the mnemonic IMViC. This mnemonic fixes in order the four tests so that it is possible to write a formula for the organism described. Thus, " + + - - " is the symbol for the colon bacillus and " - - + + "

CHART II

KEY TO THE ESCHERICHIA AND AEROBACTER GROUPS

A. Voges-Proskauer reaction negative, methly-red positive, indol usually positive, citrate not utilized.

Escherichia Group

I. Sucrose Positive

E. coli communior

II. Sucrose Negative

E. coli communis

B. Voges-Proskauer reaction positive; methyl-red negative; indol usually negative, citrate utilized as sole source of carbon.

Aerobacter Group

I. Gelatin not liquefied

A. aerogenes

II. Gelatin liquefied

A. cloacae

for the aerogenes bacterium. Chart III shows the IMViC grouping of coliform organisms. Members of the coliform groups were termed as Escherichia, Aerobacter, or Intermediates. Those strains which produced indol, did not form acetyl-methyl carbinol, gave a positive methyl red reaction, and were incapable of utilizing citrate as the sole source of carbon, were designated as Escherichia. Gram-negative bacilli which did not produce indol, formed acetyl-methyl carbinol, gave a negative methyl red reaction, and were capable of utilizing citrate as the sole source of carbon were classified as Aerobacter. A group with reactions resembling in part Escherichia and in part Aerobacter were called Intermediates.

For the purpose of this survey, paracolon bacilli were defined, according to Bergey (1948), as gram-negative, non-sporulating aerobic bacilli of the coliform type which are characterized by consistently delayed fermentation of lactose. Glucose is fermented with formation of gas. Borman et al. (1944) suggest that those organisms that are Voges-Proskauer positive and have other characteristics as for the genus are termed Paracolobactrum aerogenoides. Those bacilli which are Voges-Proskauer negative, utilize citrate as sole source of carbon and possess other characteristics as for the genus should be designated as Paracolobactrum intermedium. Finally, the gram-negative, lactose-fermenting bacteria that are Voges-Proskauer negative, but are not capable of utilizing citrate as sole source of carbon, and have other characteristics as for genus are classified as Paracolobactrum coliforme.

Final identification of all lactose-fermenting bacilli was made from biochemical characteristics and carbohydrate fermentations as shown in Chart IV.

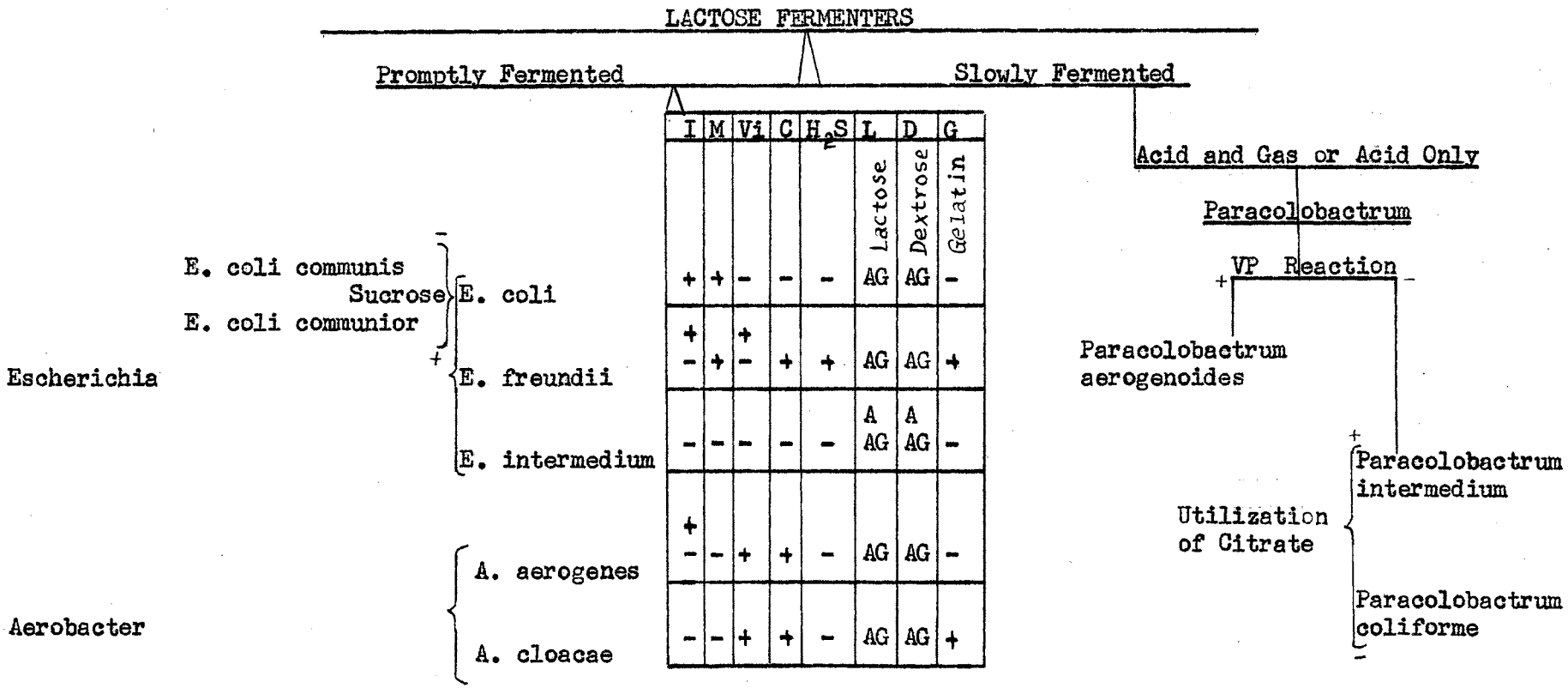
CHART III

IMViC GROUPING OF COLIFORM ORGANISMS

I	M	Vi	C	
+	+	-	-	Typical Escherichia
-	+	-	-	Atypical Escherichia
+	-	-	-	Atypical Escherichia
+	+	+	-	Intermediate
+	+	-	+	Intermediate
-	+	-	+	Intermediate
+	-	+	-	Intermediate
-	-	-	-	Intermediate
+	-	-	+	Intermediate
-	+	+	-	Intermediate
+	+	+	+	Intermediate
+	-	+	+	Intermediate
-	+	+	+	Intermediate
-	-	-	+	Atypical Aerobacter
-	-	+	-	Atypical Aerobacter
-	-	+	+	Typical Aerobacter

BIOCHEMICAL DIFFERENTIATION OF GRAM-NEGATIVE LACTOSE-FERMENTING BACILLI

CHART IV



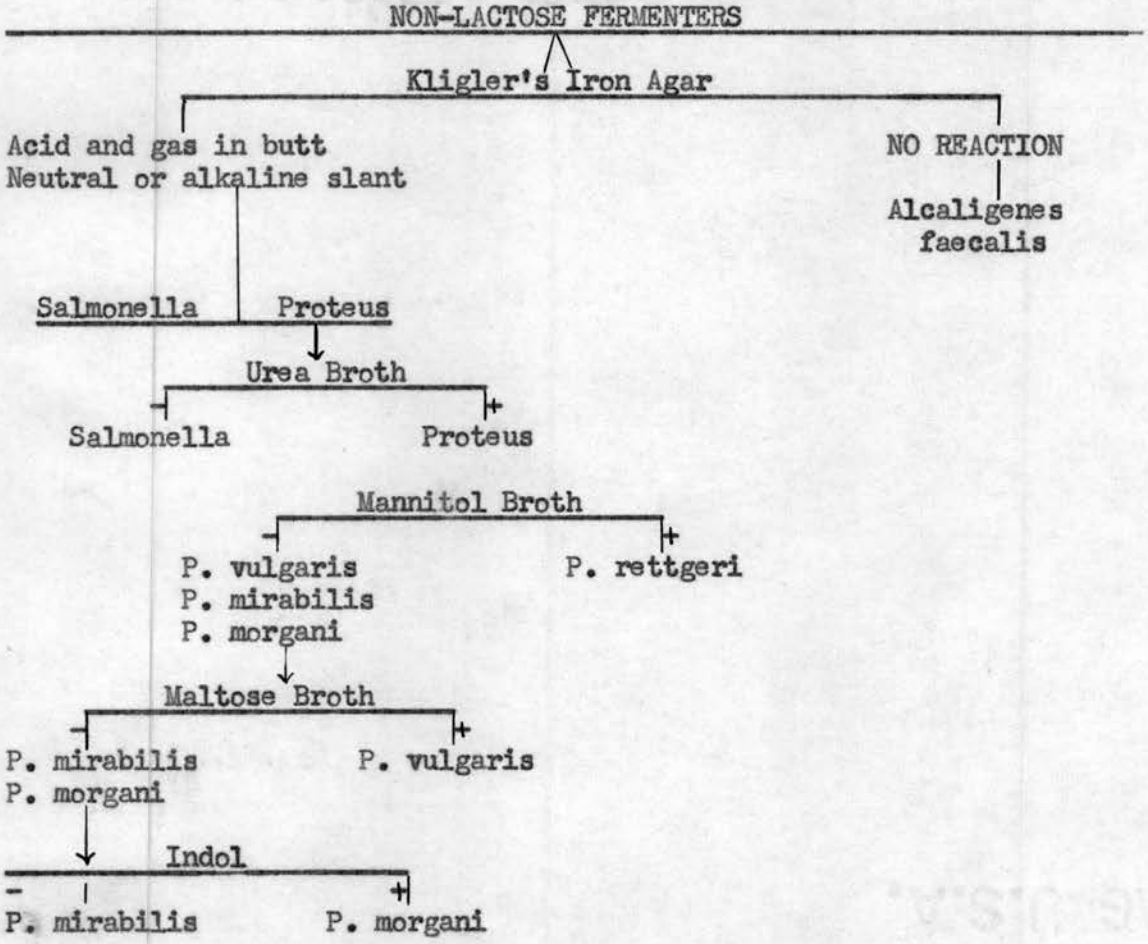
	I	M	Vi	C	H_2S	L Lactose	D Dextrose	G Gelatin
E. coli	+	+	−	−	−	AG	AG	−
E. freundii	+ or −	+	+ or −	+	+	AG	AG	+
E. intermedium	−	−	−	−	−	A or AG	A or AG	−
A. aerogenes	+ or −	−	+	+	−	AG	AG	−
A. cloacae	−	−	+	+	−	AG	AG	+

Strains of *Proteus* ferment dextrose but not lactose and possess the distinctive characteristic of hydrolyzing urea. Members of the genus were classified according to their ability to ferment various carbohydrates and to produce indol. Chart V gives an outline for the identification of the various species.

Other members of the family *Enterobacteriaceae* are the *Salmonella* and the *Shigella*. According to Bergey (1948), lactose, sucrose, and salicin ordinarily are not attacked. No strains of these organisms were encountered in this study.

CHART V

SCHEMATIC OUTLINE FOR THE IDENTIFICATION OF THE GENUS PROTEUS



III RESULTS AND DISCUSSION

The first fecal specimen for this study was received for analysis on September 14,1950, and the last one on January 2, 1952. This investigation was made on babies born at the Stillwater Municipal Hospital, Stillwater, Oklahoma. A pilot experiment was undertaken on 25 babies. Three specimens were obtained from each newborn. The first specimen was taken as soon after birth as time permitted, before the baby had received anything by mouth. Additional cultures were made on the third and fifth days of life. A direct smear for microscopic examination was likewise made at the same time the specimens were obtained. A somewhat more detailed study of gram-negative bacilli in the fecal flora of newborns was carried out on an additional 50 infants. The present report comprises the data collected from the study of the fecal specimens from the latter group of 50 babies.

Microscopical examination of direct smears of feces has one great advantage over the study of feces by cultural methods in that it affords a representative picture of the entire intestinal flora. Examination of direct smears from the fecal samples obtained on the first day showed the presence of gram-negative bacilli and cocci in 4 out of 50 or 8 per cent of the cases studied. Gram-positive bacilli and cocci were seen in 6 or 12 per cent of the specimens. Two of the 4 specimens that revealed gram-negative organisms likewise showed gram-positive bacteria. The cultures on these same specimens were positive for gram-negative

bacilli in 3 out of the 50 cases or 6 per cent. The organisms isolated from these 3 specimens were: Escherichia coli communis, Intermediates, and Proteus rettgeri. Forty-seven specimens were free from gram-negative aerobic bacilli; however, 12 out of these 47 contained gram-positive bacilli and cocci, with the coccal forms predominating. The shortest period that elapsed from the time of birth and the obtaining of the first specimen was 11 minutes and the longest period was 610 minutes. Gram-negative bacilli were absent from both specimens although, Case No.40, the specimen which was obtained 11 minutes after birth showed gram-positive cocci microscopically and culturally. Case No. 2, specimen obtained 610 minutes after birth was sterile both microscopically and culturally. Table I shows the findings in the fecal samples obtained from newborns the first day of life.

The direct smear made from fecal material collected on the third and fifth days revealed a mixed bacterial flora in which were seen large and small gram-positive rods, gram-positive cocci, occasional yeast cells, bacterial spores, and gram-negative bacilli. The gram-positive bacteria are more difficult to cultivate, often requiring nutrient media and anaerobic methods. The large gram-positive rods were probably sporulating organisms common to the environment like Bacillus subtilis and Bacillus mesentericus. According to Escherich (1886), putrefactive bacteria are uncommon in the stools of newborns possibly because they are inhibited by such species as Bacillus bifidus and Bacillus acidophilus. The fecal films from the specimens collected on the third day showed that 38 specimens or 76 per cent contained gram-negative bacilli; the remaining 12 specimens or 24 per cent showed gram-

Table I: Results of First Fecal Specimen - First Day

Case No. of Baby	Minutes After Birth	Smear	Culture	Case No. of Baby	Minutes After Birth	Smear	Culture
1	153	Neg.	Neg.	26	85	Neg.	Neg.
2	610	Neg.	Neg.	27	270	Neg.	Neg.*
3	415	Neg.	Neg.	28	65	Neg.	Neg.
4	67	Neg.	Neg.	29	101	Neg.	Neg.
5	442	Neg.	Neg.*	30	176	Neg.*	Pos.*
6	600	Neg.	Neg.	31	99	Neg.	Neg.
7	250	Neg.	Neg.	32	287	Neg.*	Neg.*
8	126	Neg.	Neg.*	33	163	Neg.	Neg.
9	321	Neg.	Neg.	34	226	Pos.*	Neg.
10	100	Neg.	Neg.	35	269	Pos.	Neg.
11	376	Neg.	Pos.	36	82	Neg.	Neg.
12	537	Pos.	Neg.*	37	340	Neg.	Neg.*
13	117	Neg.	Neg.*	38	18	Neg.	Neg.
14	88	Neg.	Neg.*	39	107	Neg.	Neg.
15	265	Neg.	Neg.	40	11	Neg.*	Neg.*
16	62	Neg.	Neg.	41	25	Neg.	Neg.*
17	19	Neg.	Neg.	42	400	Neg.	Neg.
18	45	Neg.	Neg.	43	44	Neg.	Neg.
19	46	Neg.	Neg.	44	75	Neg.	Neg.
20	210	Neg.	Neg.	45	254	Neg.	Neg.
21	28	Neg.	Pos.*	46	189	Neg.	Neg.
22	33	Neg.	Neg.	47	120	Neg.*	Neg.*
23	76	Neg.	Neg.	48	76	Neg.	Neg.*
24	42	Pos.*	Neg.	49	16	Neg.	Neg.
25	586	Neg.	Neg.	50	142	Neg.	Neg.

Percentage of Cases Positive for Gram-negative Bacilli: Smear 8% Culture 6%

*Cultures contained Gram-positive organisms

positive bacilli and cocci, with a predominance of bacilli. The percentage of positive cultures for gram-negative bacilli had increased from 6 per cent on the first day to 66 per cent by the third day; the other 34 per cent of the specimens contained gram-positive bacilli or cocci. Table II presents the results of the study of the specimens obtained on the third day.

Four of the 50 babies studied went home before the fifth day and consequently no specimens were obtained from them. Only 46 samples were studied on that day. By the fifth day gram-negative bacilli were seen microscopically in 42 out of the 46 cases or 91.3 per cent. Gram-negative bacilli were absent from the remaining 4 specimens, but gram-positive cocci did appear on the smear. When these specimens were brought to cultivation, 38 samples or 82.6 per cent showed gram-negative bacilli. The results obtained from the study of fecal material for the fifth day are shown in Table III.

Tissier (1905), Rettger (1915), Gerstley et al. (1932), and other workers have shown that diet has a definite and important bearing upon the intestinal flora and that definite changes may be brought about in the bacterial contents of the intestines by modifying the diet.

Theoretically, at birth the intestinal canal of the child is sterile. Smears and cultures from the meconium have failed to show any organisms or at most very few. However, within a few hours after birth infection takes place (Escherich 1886), and it may well be said that the "intestines of man, and lower animals may be likened to a veritable culture tube in which definite bacterial types appear to be struggling constantly to gain supremacy." (Rettger 1921).

Table II: Results of Second Fecal Specimen - Third Day

Case No. of Baby	Smear	Culture	Feeding			Case No. of Baby	Smear	Culture	Feeding		
			Breast	Bottle	Supplemental				Breast	Bottle	Supplemental
1	Pos.	Pos.		Carnation		26	Pos.	Pos.	✓		SMA
2	Neg.*	Neg.*		SMA		27	Pos.	Pos.	✓		SMA
3	Pos.	Pos.		Formulac		28	Pos.	Pos.		Formulac	
4	Neg.*	Neg.*	✓		SMA	29	Neg.*	Neg.*	✓		Carnation
5	Pos.	Pos.	✓		SMA	30	Pos.	Pos.	✓		SMA
6	Pos.	Pos.	✓		SMA	31	Neg.*	Neg.*	✓		SMA
7	Pos.	Pos.		Formulac		32	Pos.	Pos.	✓		Carnation
8	Pos.	Pos.		Carnation		33	Pos.	Pos.	✓		SMA
9	Pos.	Pos.		Formulac		34	Pos.	Pos.	✓		SMA
10	Pos.	Pos.	✓		SMA	35	Neg.*	Neg.*	✓		SMA
11	Pos.	Pos.		Carnation		36	Neg.*	Neg.*		Carnation	
12	Pos.	Neg.*		Carnation		37	Pos.	Pos.	✓		SMA
13	Neg.*	Neg.*	✓		SMA	38	Neg.*	Neg.*	✓		Carnation
14	Pos.	Pos.		Formulac		39	Neg.*	Neg.*		Formulac	
15	Pos.	Neg.*		Formulac		40	Pos.	Pos.	✓		SMA
16	Neg.*	Neg.*	✓		SMA	41	Pos.	Pos.	✓		SMA
17	Pos.	Pos.	✓		Carnation	42	Pos.	Neg.*		SMA	
18	Pos.	Pos.	✓		SMA	43	Pos.	Pos.		Carnation	
19	Pos.	Pos.	✓		SMA	44	Pos.	Pos.		Carnation	
20	Pos.	Pos.		Carnation		45	Pos.	Pos.	✓		SMA
21	Pos.	Pos.		Carnation		46	Neg.*	Neg.*	✓		SMA
22	Pos.	Neg.*		SMA		47	Pos.	Pos.		Formulac	
23	Pos.	Neg.*		Carnation		48	Pos.	Pos.		Formulac	
24	Pos.	Pos.	✓		SMA	49	Neg.*	Neg.*	✓		SMA
25	Pos.	Pos.		Formulac		50	Pos.	Pos.	✓		SMA

Percentage of Cases Positive for Gram-negative Bacilli: Smear 76% Culture: 66%

*Cultures contained Gram-positive organisms

Table III: Results of Third Fecal Specimen - Fifth Day

Case No. of Baby	Smear	Culture	Feeding			Case No. of Baby	Smear	Culture	Feeding		
			Breast	Bottle	Supplemental				Breast	Bottle	Supplemental
1	Pos.	Pos.		Carnation		26	Pos.	Pos.	✓		SMA
2	Pos.	Pos.		SMA		27	Pos.	Pos.	✓		SMA
3	Pos.	Pos.		Formulac		28	Pos.	Pos.		Formulac	
4	Neg.*	Neg.*	✓		SMA	29	Pos.	Pos.	✓		Carnation
5	Pos.	Pos.	✓		SMA	30	Pos.	Pos.	✓		SMA
6	Pos.	Pos.	✓		SMA	31	Pos.	Pos.	✓		SMA
7	Pos.	Neg.*		Formulac		32	Pos.	Pos.	✓		Carnation
8	Pos.	Pos.		Carnation		33	Pos.	Pos.	✓		SMA
9	Pos.	Pos.		Formulac		34	---	---			---
10	Neg.*	Pos.	✓		SMA	35	Pos.	Pos.	✓		SMA
11	Pos.	Pos.		Carnation		36	Neg.*	Pos.		Carnation	
12	Pos.	Neg.*		Carnation		37	---	---			---
13	Pos.	Neg.*	✓		SMA	38	Pos.	Pos.	✓		Carnation
14	Pos.	Pos.		Formulac		39	Pos.	Pos.		Formulac	
15	Pos.	Pos.		Formulac		40	Neg.*	Neg.*	✓		SMA
16	Pos.	Pos.	✓		SMA	41	Pos.	Pos.	✓		SMA
17	Pos.	Pos.	✓		Carnation	42	Pos.	Pos.		SMA	
18	Pos.	Pos.	✓		SMA	43	Pos.	Pos.		Carnation	
19	Pos.	Pos.	✓		SMA	44	Pos.	Pos.		Carnation	
20	---	---		---		45	Pos.	Pos.	✓		SMA
21	Pos.	Pos.		Carnation		46	Pos.	Neg.*	✓		SMA
22	Pos.	Neg.*		SMA		47	Pos.	Pos.		Formulac	
23	Pos.	Neg.*		Carnation		48	Pos.	Pos.		Formulac	
24	Pos.	Pos.	✓		SMA	49	---	---		---	
25	Pos.	Pos.		Formulac		50	Pos.	Pos.	✓		SMA

Percentage of Cases Positive for Gram-negative Bacilli: Smear 91.3% Culture 82.6%

*Cultures contained Gram-positive organisms

According to the above-named investigators as well as many others, the predominating organism present in the lower intestines in the breast-fed babies is Lactobacillus bifidus, a gram-positive acid-producing organism. Escherichia coli and Lactobacillus acidophilus are found in small numbers, and occasionally Clostridium perfringens is present. Streptococcus faecalis may be present in large numbers.

In the colon of the bottle-fed infants the flora is more complex, with a predominance of Escherichia coli and Aerobacter aerogenes. In the larger intestines the Bacillus bifidus types are largely replaced by colon bacilli, Bacillus acidophilus, and other similar organisms.

In this study the author found that in a film of feces from a breast-fed infant the gram-positive bacilli seemed to predominate. In the direct smear of fecal material from the bottle-fed baby there was no predominance of any organism. About 75 per cent of the flora consisted of gram-positive organisms, the majority of these being lanceolate, cocco-bacilli arranged singly or in pairs. In the smears of the breast-fed infant that received supplemental feeding there was still a predominance of gram-positive bacteria.

Twenty-seven of the babies studied were breast-fed, but all received supplemental feedings of S-M-A, Carnation Formula with Dextri-Maltose, or Formulac. Since these breast-fed babies received the same formula of supplemental feedings as the bottle-fed babies the results are probably not comparable to the investigations of early workers who studied infants that were completely breast-fed or completely bottle-fed. However, it was of interest to note that the supplemental feedings did not change the flora considerably, due to the fact that the products used are similar in chemical content to human milk.

The Carnation Formula consisted of evaporated cow's milk to which had been added Dextri-Maltose (Mead and Johnson). Dextri-Maltose is a dried product manufactured especially for infants' feedings. Formulas made with cow's milk and Dextri-Maltose contain three carbohydrates, namely: lactose, dextrins, and maltose. S-M-A (Wyeth) is an infant food derived from the milk of tuberculin-tested cows. It is essentially the same as human milk in percentages of protein, fat, carbohydrates, ash, in chemical constants of the fat and in physical properties. Formulac (McCollum 1944) is the trade name for a reduced milk, sufficiently supplemented with vitamins C and D, as well as the B complex vitamins, iron, copper, and manganese. Formulac is comprised of sterilized evaporated milk to which has been added fish liver oils and various vitamins.

A summary of the various strains of gram-negative bacilli that were encountered in this study is shown in Table IV.

The gram-negative bacilli, isolated, studied, and identified were: Aerobacter aerogenes, Aerobacter cloacae, Escherichia coli communis, Escherichia coli communior, Intermediates, Proteus mirabilis, Proteus rettgeri, and Paracolobactrum aerogenoides.

TABLE V: SUMMARY OF DATA BY SUBJECTS

		% of cases
Total number of babies studied	50	—
Number showing Escherichia coli communis	23	46
Number showing Escherichia coli communior	15	30
Number showing Aerobacter aerogenes	5	10
Number showing Aerobacter cloacae	1	2
Number showing Intermediates	21	42
Number showing Proteus mirabilis	3	6
Number showing Proteus rettgeri	3	6
Number showing Paracolobactrum aerogenoides	1	2

Table IV: Summary of Specimens by Babies

	Case No. of Baby			% of Cases Positive		
	First Day	Third Day	Fifth Day	1st	3rd	5th
Culturally sterile for Gram-negative Bacilli	1,2,3,4,5,6,7,8,9, 10,12,13,14,15,16, 17,18,19,20,22,23, 24,25,26,27,28,29, 31,32,33,34,35,36, 37,38,39,40,41,42, 43,44,45,46,47, 48,49,50.	2,4,12,13,15,16, 22,23,29,31,35, 36,38,39,42,46, 49.	4,7,12,13,22, 23,40,46.			
Escherichia coli communis	21	3,6,8,10,17,19,21, 24,25,26,27,30,32, 33,34,37,44,45,47, 50.	2,3,10,16,19,24, 25,27,28,31,32, 36,44,45,47,50.	2	40	34.8
Escherichia coli communior	0	6,8,9,10,14,21,24, 27,30,43,47,48.	6,8,9,10,14,15,21, 24,38,39,47,48.	0	24	26.1
Intermediates	11	6,7,11,18,19,24, 34,40,41,43,50.	1,3,11,14,17,18,21, 25,29,33,41, 42,43,48,50.	2	22	32.6
Aerobacter aerogenes	0	1,5,20,50.	5,17,50.	0	8	6.5
Aerobacter cloacae	0	0	17	0	0	2.1
Proteus rettgeri	30	1,28,30	28,30	2	6	4.3
Proteus mirabilis	0	1	9,43	0	2	4.3
Paracolobactrum aerogenoides	0	0	17	0	0	2.1
Number of Babies Studied	50	50	46	6	66	82.6

Escherich (1886) described Escherichia coli under the name of Bacterium coli communis. He isolated it from the dejecta of a breast-fed infant. Bergey (1948) defines the bacterium as a gram-negative rod, varying from almost coccoid forms to a long rod, occurring singly, in pairs, or in chains. It is usually not encapsulated. Motility is variable. The organism produces acid and gas from glucose and lactose. Sucrose may or may not be fermented. The strains of Escherichia coli that ferment sucrose are termed Escherichia coli communior and those which do not ferment this disaccharide are termed Escherichia coli communis. One specimen or 2 per cent contained this gram-negative bacillus on the first day. By the third and fifth days the percentages of occurrence of these organisms were 64 and 60.9 per cent respectively.

The Intermediates are groups of organisms that are still poorly defined. They are intergrading forms which possess qualities characteristic of the Escherichia group and some qualities which belong to the Aerobacter group. Parr (1938) defined intermediates as coliform organisms which have one or more coli characteristics and one or more of those attributed to aerogenes. An intermediate form was present in 1 specimen or 2 per cent on the first day, in 11 specimens or 22 per cent on the third day, and in 15 specimens or 32.6 per cent on the fifth day.

Aerobacter aerogenes was likewise first described by Escherich (1886). The organism is a gram-negative rod, producing acid and gas from glucose and lactose. This bacillus is commonly found in nature on the surface of grains (Burrows 1949). There is a close resemblance in the morphology of Escherichia coli and Aerobacter aerogenes and this trait cannot be used as a differential character. As has been

pointed out earlier in this paper, the two species are differentiated on the basis of the IMViC tests. A relatively small percentage, none on the first day, 8 per cent on the third day, and 6 per cent on the fifth day, of this organism occurred in the specimens used in this study.

Aerobacter cloacae was originally found by Jordan (1890) in sewage. This is a short, rather thin bacillus; it is gram-negative, ferments lactose and dextrose with the production of acid and gas. Bergey (1948) uses its ability to liquefy gelatin as a point of differentiation from the other members of the colon-aerogenes group. Burrows (1949) believes that this organism may be regarded as an intermediate form connecting the colon bacilli with the Proteus group, which do not ferment lactose but actively liquefy gelatin. This organism was found in 1 out of 146 or 0.7 per cent.

Members of the Proteus group ferment glucose but not lactose. Acid is produced and usually gas is visible; sucrose is usually fermented. These microorganisms were first described by Hauser (Burrows 1949). Urea is hydrolyzed by strains of Proteus. It is frequently isolated from the dejecta. Four species are recognized: Proteus mirabilis, Proteus vulgaris, Proteus rettgeri, and Proteus morgani. These various species are distinguished one from another on the basis of the fermentation of various carbohydrates, mannitol and maltose. The species, Proteus rettgeri was found in 6 out of the 146 specimens or 4.1 per cent. Another member of the groups, Proteus mirabilis occurred in 3 out of the 146 specimens or 2.1 per cent.

The paracolon organisms are characterized by their consistently delayed fermentation of lactose. Bergey (1948) mentions 3 species,

namely: Paracolobactrum coliforme, Paracolobactrum aerogenoides, Paracolobactrum intermedium. The group is a heterogenous one and is not to be regarded as a well-defined type of enteric bacteria(Burrows 1949). Parr(1938) states that the occurrence of small numbers of slow-lactose fermenting bacteria in normal feces is of considerable interest. He suggests that these bacteria arise in the body as expressions of a variation or succession in coliform flora induced by changes in the body physiology and biochemistry resulting from disease. Only 1 out of the 146 specimens or 0.7 per cent contained a species of paracolon, Paracolobactrum aerogenoides.

Table VI presents a summary of data by specimens. The total number of specimens studied was 146. From these specimens 424 strains of bacteria were isolated. The number of colonies selected from each specimen for detailed pure-culture study varied from 4 to 12. The number of strains of gram-negative bacilli that were isolated, purified and studied in pure culture was 253 or 59.7 per cent of the total number of strains isolated. Strains of gram-positive organisms that were isolated, but not identified, included bacilli, cocci, and tetrads. The number of these organisms equaled 171 or 40.6 per cent of the total 424 strains. Seventeen colonies failed to grow and 1 colony yielded a yeast. Jordan and Falk (1929) state that yeasts and molds are found frequently, but it is doubtful if they multiply in the digestive tract. Ordinary yeasts may resist the antagonistic forces of the digestive tract and pass through more or less unharmed.

Table VI: Summary of Data by Specimens

	No.	% of Cases
Total number of specimens studied	146	
Total number of strains isolated	424	
Strains of gram-negative bacilli isolated, purified, and studied in pure culture	253	
Aerobacter aerogenes	20	
Aerobacter cloacae	3	
Escherichia coli communis	103	
Escherichia coli communior	53	
Intermediates	51	
Proteus mirabilis	9	
Proteus rettgeri	12	
Paracolobactrum aerogenoides	1	
Strains of gram-positive organisms isolated but not identified	171	
Strains of cocci	139	
Strains of bacilli	25	
Strains of tetrads	7	
Specimens which yielded coli only	36	24.6
Specimens which yielded coli and aerogenes	2	1.4
Specimens which yielded coli and intermediates	11	7.5
Specimens which yielded coli, aerogenes, and intermediates	2	1.4
Specimens which yielded aerogenes and intermediates	3	2.1
Specimens which yielded aerogenes only	4	2.7
Specimens which yielded intermediates only	14	9.6
Specimens which yielded Proteus mirabilis	3	2.1
Specimens which yielded Proteus rettgeri	6	4.1
Specimens which yielded gelatin-liquefying cloacae	1	0.7
Specimens which yielded slow-lactose coli	0	0.0
Specimens which yielded slow-lactose aerogenes	1	0.7
Specimens which yielded slow-lactose intermediates	0	0.0
Specimens which yielded no coliform organisms on plating	25	17.1

Every isolated strain was subjected to each of the different tests four to six times to determine whether there would be any variation in these tests at different intervals. At first a small percentage of the coli strains were rather slow in their fermentative reactions of lactose, but after two or three transfers, they fermented lactose rapidly and bred true.

IV SUMMARY AND CONCLUSION

In this study the author tried to show (1) which strains of aerobic gram-negative bacilli are present in the intestinal tract in newborns, and (2) what bacteriological studies of the feces are of practical importance from the standpoint of both promptness and accuracy.

An intensive study of the occurrence of gram-negative bacilli in 146 specimens of feces secured from 50 newborns during the first five days of life, showed, that while 94 per cent of the newborn infants passed specimens free from aerobic gram-negative bacilli on the first day, gram-negative bacilli were present in 66 per cent of the specimens by the third day and in 82.6 per cent by the fifth day. Five genera and eight species were represented, namely: Escherichia coli communis, Escherichia coli communior, Aerobacter aerogenes, Aerobacter cloacae, Intermediates, Proteus mirabilis, Proteus rettgeri, and Paracolobactrum aerogenoides. Strains of the Escherichia group occurred in 76 per cent of the cases and of the Intermediates in 42 per cent of the cases studied. From this investigation all of the above-named organisms may be regarded as the most common gram-negative bacterial invaders of the intestinal tract of the newborn.

The first problem in the bacteriological examination of any intestinal infection is the assurance of a dependable specimen with which to work. The method of obtaining the specimen by means of a rectal swab was considered a technique that is easy to perform and is adaptable for use in the home, hospital, and office.

This report has briefly sketched a plan of classification of the different species of intestinal bacteria which is based upon definite bacteriological reactions. It is possible to give a report to the physician within 24 to 48 hours after primary isolation of an organism concerning the pathogenicity or non-pathogenicity of the gram-negative bacilli involved on the basis of lactose fermentation and a battery of other biochemical tests. After the primary isolation has been made on some selective media, such as SS agar or EMB agar, individual colonies can be picked for study. Various culture media, such as Kligler's iron agar, urea broth (or agar), 1 per cent tryptone broth, MR-VP broth, and Simmons' citrate agar can be inoculated for further study and identification. Within a period of 24 to 48 hours a tentative report indicating the genus can be given to the physician. This will enable him to institute proper therapy early. Sufficient time will be available for further identification and confirmation of the species.

No pathogenic gram-negative bacilli were encountered in this study. However, with the use of proper selective media, primary isolations can be made; and the organisms identified by biochemical and serological tests.

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