COMPARISON OF METHODS FOR DETECTION OF POLLUTION BASED ON STUDIES ON THE SANITARY QUALITY OF RURAL DRINKING WATERS

by

MILTON EDWARD LARKINS

A. B., Washburn Municipal University, 1942

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE COLLEGE
OF AGRICULTURE AND APPLIED SCIENCE

1950

TABLE OF CONTENTS

PREFACE

It has been realized for a long time that consumption of water polluted with human fecal bacteria often is responsible for the spread of human diseases. The purpose of bacteriological examination of water is to detect the presence of bacteria which indicate human pollution. It is impractical to test water for the presence of pathogenic bacteria as these bacteria are comparatively few in polluted water and are difficult to grow by ordinary bacteriological methods. The routine test is the detection of coliform bacteria which are indicators of fecal pollution. It is assumed that water containing non-pathogenic, fecal bacteria may also contain pathogenic bacteria.

Many bacteriologists and sanitarians believe that there are other bacteria in water from intestinal origin which also can be used as indicators of pollution. These bacteria are, namely, fecal streptococci and anaerobic, spore-forming bacteria, particularly Clostridium perfringens. However, it is a general opinion that these bacteria are not so numerous as the coliform bacteria.

This thesis concerns the detection of anaerobic, spore-forming bacilli in relation to the coliform bacilli found in drinking waters from rural areas of Riley County, Kansas.

REVIEW OF LITERATURE

The main purpose of bacteriological examination of water is to determine the presence of bacteria which indicate pollution from fecal wastes of man or animals. For many years in the United States the standard procedure has been to detect coliform bacteria which are used as indicators of pollution.

There are a number of investigators in the United States and in Europe who believe that there are other bacteria which can also be used as indicators of pollution. This is especially true of Streptococcus fecalis* and Clostridium perfringens (Cl. welchii). Greer (1928a) considered Strept. fecalis to be very important in this respect. He felt that Cl. perfringens, the Friedlander's group, and Pseudomonas pyocyaneus may also be of some sanitary significance. He believed that Strept. fecalis probably is of as great sanitary significance as Escherichia coli. There are other lactose-fermenting bacteria found in water, particularly aerobic spore-formers, but it is generally felt that these are of little or no sanitary significance.

Since Strept. fecalis disappears more readily than E. coli from treated waters, it is generally felt that the presence of fecal streptococci indicates recent pollution. Savage and Wood (1917) believed that the presence of streptococci is a better indication of recent pollution than E. coli.

---

*All names of organisms are in accordance with Bergey's Manual of Determinative Bacteriology, 6th Edition.

Houston (1910), in England, was one of the first to attach practical significance to fecal streptococci in water; but he believed that this test could not compete with the coliform test. Savage and Read (1916) were among the first in this country seriously to consider and study the streptococci as indicators of pollution. They stated that absence of these bacteria in a water supply is to be considered of less significance than their presence, and even their absence from a considerable bulk of water is not accepted to the same extent as the absence of coliform organisms as reliable evidence of freedom from contamination. All workers believe that fecal streptococci in large quantities in water supplies indicate an unsatisfactory condition.

It is believed by many investigators that the presence of anaerobic, spore-forming bacilli, particularly Cl. perfringens, indicates pollution from animal sources. As early as 1907 Vincent suggested the enumeration of Cl. perfringens as an index of pollution.

Cl. perfringens, known formerly as Cl. welchii, Bac. perfringens, Bac. welchii, Bac. aerogenes-capsulatus, and the "gas bacillus", was first isolated by Welch and Nuttall (1892) from a cadaver with foamy organs. They named the organism Bacillus aerogenes-capsulatus. Several years later Klein (1897) found an organism, which he named Bac. enteritidis sporogenes, in water from the Thames River. Jackson made a comparative study of a strain of Bac. enteritidis sporogenes obtained from Klein and of the original strain of Bac. aerogenes-capsulatus isolated by Welch, and found

them identical for all tests applied by him. Klein and Houston (1898-1899) found the organism in water and sewage from many parts of England and Scotland. Winslow and Belcher (1904), Larner (1922), Greer (1925, 1926), and Simonds (1915) had found Cl. perfringens in water and sewage. Later the organism was found by others. Meader and Bliss (1923) did not find Cl. perfringens in waters, but did find other anaerobic spore-formers. In 1909 Jackson demonstrated Cl. perfringens in considerable numbers in the Jersey City, N. J., water supply.

One of the main questions, even today, is whether the finding of Cl. perfringens in drinking waters has any sanitary significance. That Cl. perfringens may have sanitary significance was the opinion of well known engineers and sanitarians such as Ellms, Horton, Prescott, Dittoe, Rosenau, Jackson, Greer, Noble, Adams, Wilson, Blair, Thresh, Beale, and Suckling. They all believed the organism had sanitary significance, and if found in water indicated the water was unsafe to drink. Greer and Noble (1928) and others found the organism to be a member of the fecal flora of warm-blooded animals and that it is not found in an actively growing state in nature, except in the animal body, and then it was not present in as large numbers as E. coli. Thresh, Beale, and Suckling (1933) stated that spores are often absent or only sparcely present in the excreta of birds.

Opposed to the above idea are the views of Smith and Gardner (1949), Topley and Wilson (1929), and Skinner and Baskin (1932). Meader and Bliss (1923) claimed that Greer ignored the possibili-

ties of wide distribution of lactose-fermenting anaerobes in soil. Topley and Wilson (1929) believed that the normal habitat of anaerobes is soil, and that their presence in sewage and feces is incidental. Skinner and Baskin (1932) supported this same view. They used a modification of the Wilson and Blair (1924) method for isolation and quantitative counts of these bacilli in soils. They isolated seven separate strains of lactose-fermenting anaerobes. They claimed the isolates were either Cl. perfringens or closely related anaerobes, because the isolates caused "stormy fermentation" of milk and fermented glucose, sucrose, lactose, and maltose with copious gas. They believed that even if heated suspensions in milk caused "stormy fermentation" or blackening of Wilson and Blair's agar with typical colonies of Cl. perfringens, these organisms can hardly be taken to indicate fecal pollution. They claimed that such ubiquitous organisms could get into water supplies. A sudden increase in their numbers may indicate surface drainage due to rain, or agitation of the mud in streams, or other factors not necessarily due to sewage pollution.

Smith and Gardner (1949) obtained information concerning the varieties of pathogenic clostridia, particularly Cl. perfringens, existing in the soil. Soil samples which had not been exposed to known fecal contamination for some years were used for the tests. The research indicated multiplication of this species takes place in the soil, that the soil is a natural habitat of this species, and that one does not need to postulate fecal contamination to account for the presence of Cl. perfringens in soil.

Suckling (1944) found Clostridium species widely distributed in nature, but stated that they were for the most part essentially associated with the intestines of man and animals, although a few strains may have their natural habitat in soil and decaying animal and vegetable matter. He claimed that it is the intestinal association which is of significance to the water bacteriologist. Although the presence of these organisms had been recorded in virgin soils, they were infrequent and in small number.

According to Thresh, Beale, and Suckling (1933) pure waters are free from vegetative cells and spores of Cl. perfringens, and no naturally pure water should give a positive milk test in 100 ml, although artificially purified waters may often yield such results. These investigators regard the "stormy fermentation" reaction as a valuable confirmation to E. coli findings. Occasionally pure, untreated waters gave a positive reaction in 100 ml with absence of E. coli, and such results may be a valuable warning. The spores are quite resistant; hence, this would point to old or intermittent contamination. However, the water at the present time might not have been unwholesome, because the pathogenic organisms reacting like E. coli, may have previously perished. Since the spores of Cl. perfringens are quite resistant, their presence gives no idea of the date of pollution.

Several investigators have found that many spores of Cl. perfringens can withstand chlorination; however, very few can pass through fine sand filters. In fact, Ginter (1927) found that gas production in standard lactose broth was more rapid in chlorine-

treated waters than untreated. This indicates that organisms in untreated water which are killed by chlorine inhibit to a certain extent the microorganism in question. It was reported by Larner (1922) that the spores of Cl. perfrinmens survived the chlorination treatment and some had passed through the rapid sand filters at Montclair, N. J., and caused an outbreak of gastro-intestinal disease.

Greer and Noble (1928) claimed that not so many Cl. perfringens may survive as long in water as common impression appeared to indicate. The fact that the organism is a spore-former did not justify the assumption that it should survive for long periods of time in water. However, they also added that it probably survives a little longer than E. coli. They stated that factors such as hydrogen ion concentration, temperature, sunlight, and sedimentation can influence longevity of these organisms in water.

Many investigators have found many aerobic and facultative anaerobic, spore-forming bacilli in waters. However, these organisms have generally been considered to have very little sanitary significance. They have been found in most all natural conditions and are commonly washed into waters. They have been considered to be nonpathogenic. Koser and Shinn (1927), Norton and Weight (1924), and others have stated that these organisms, especially facultative anaerobes, have caused much confusion, particularly in interpreting results in standard lactose tests.

The question next arises as to whether the above mentioned microorganisms may cause pathogenic conditions in man when taken

by mouth in drinking waters. Greer, Tonney, and Wyhan (1928) stated that according to a survey of literature only two common lactose-fermenting organisms, other than E. coli, are found in association with pathological conditions in man. These are Cl. perfringens and the Friedlander's group. They believed that experimental evidence showed that Cl. perfringens may cause diarrhea when taken by mouth.

Klein (1901) reported finding an anaerobic bacillus, Bac. enteritidis sporogenes, in water contaminated from such sources as sewage, excreta from horses, dust, cultivated soil, etc. He believed this organism was responsible for severe intestinal disturbances, particularly in children.

Larner (1922) reported finding Cl. perfringens in the drinking water at Montclair, N. J., and he blamed this organism for a rather serious outbreak of gastro-intestinal disorders. Bacterial examination of feces from the patients revealed only a few E. coli, they having been almost completely displaced by an organism which resembled the bacterium that had been isolated from the drinking water. This organism produced a "stormy fermentation" in sterile milk, caused gas formation in lactose broth, and was accompanied by foaming and a very pronounced odor of butyric acid. There were no deaths in about 190 cases, and most cases cleared up without additional treatment in two or three days after they were placed on boiled water. It was decided that the spores of this organism had survived the chlorination treatment and some had passed through the rapid sand filters. Larner (1922) and other officials recalled

that this outbreak had many points in common with approximately 2,000 cases of intestinal disease which occurred in Montclair in January, 1918. At that time much of the water was not filtered. Many waterworks men believed that this organism was not responsible, but medical men recalled that over a period of years there were many reports of gastro-intestinal outbreaks from various parts of the country, the cause of which was in doubt, although the evidence frequently pointed to water.

Suckling (1944), Thresh, Beale, and Suckling (1933), Skinner and Baskin (1932) and many others believed that the presence of Cl. perfringens in the intestines is not injurious or that it does not cause gastro-intestinal upsets. Skinner and Baskin (1932) stated that if these organisms are dangerous when taken into the alimentary tract, such uncooked foods as carrots, tomatoes, lettuce, etc., grown in soil could also be viewed with suspicion; and evidence that these organisms from soil are dangerous in food and water is far from conclusive.

Simonds (1915) stated that Cl. perfringens is constantly present in stools of healthy adults and may be present in small numbers in the normal stools of healthy infants. The number of spores of Cl. perfringens varies a great deal, depending mainly upon diet. He claimed that variations in individual susceptibility explains the failure to cause diarrhea in some cases; variations in pathogenicity of different strains of the organisms explain the harmlessness in others; while absence of those conditions of diet, etc., which favor its assuming a pathogenic role explains the

failure to produce diarrhea in other cases.

Tenbroeck and Bauer (1922), and Noble (1915) demonstrated that tetanus bacilli are present in the intestines of man and that they may multiply in the intestines. The Cl. tetani is taken into the intestinal tract from fecal contamination from herbivora. However, these workers do not attach any sanitary or pathological significance to Cl. tetani in water.

Aerobic, spore-forming bacilli are also widely distributed in water supplies. They have been found by: Meyer (1918) in the water supply of Newport, Ky.; Ewing (1919) in Baltimore drinking water; Hinman and Levine (1922) in water in Iowa; Lisk (1923) in a milk sample in Florida; Schreiner (1927) in chlorinated waters of Kansas; Greer (1928b, 1928c) in water from Lake Michigan; Raab (1923) in treated waters of Minneapolis; Ginter (1927) in water at Tulsa, Oklahoma; Norton and Weight (1924) in the water distribution system in the vicinity of Chicago. Koser and Shinn (1927) examined soil samples. Aerobic, spore-forming, lactose-fermenters were encountered in 23 of the 52 soil samples examined. Distribution appeared to be rather irregular.

Several methods have been proposed by various investigators for the isolation of Clostridium species from water and sewage. However, the aerobic spore-formers are often encountered and may even outgrow the anaerobes. Also, many aerobes are facultative anaerobes and may cause trouble in isolation methods. Greer (1926) and Thresh, Beale, and Suckling (1933) recommended the "stormy fermentation" test in addition to the E. coli test for water. In

the "stormy fermentation" test, samples of water are heated to kill vegetative forms and are mixed with sterile milk, and then the surface is covered with a layer of sterile vaseline or vaseline-paraffin mixture. If anaerobes are present, gas should form after 24 to 48 hours incubation. In unheated samples the demonstration of the presence of a particular organism is not always a simple task, especially in mixtures containing other organisms more prevalent or tending to outgrow the organism sought. Meader and Bliss (1923), Greer and Nyhan (1928), Sears and Putnam (1923), and Holman and Meekison (1926) suggested that bacterial association, especially synergism and symbiosis, may be responsible for the production of gas rather than any one organism in a water sample. Meader and Bliss (1923) isolated anaerobes from 16 (or 21 percent) of 76 tubes of lactose broth inoculated with samples of water. No E. coli could be isolated, but tubes showed gas production. A total of 25 strains of anaerobic bacilli were tested, of which only two fermented lactose with gas. Anaerobic organisms by themselves appeared to have been an unimportant factor in fermentation of lactose in the samples investigated. Meader and Bliss (1923) gave the possibility that symbiotic activity may have been responsible for the gas production.

Greer and Nyhan (1928) recalled the observations of several earlier workers in connection with war wounds. The early workers had described methods of growing Cl. perfringens and other anaerobes without any special anaerobic technic, in symbiosis with Bac. subtilis and other aerobes. They found that all the common

aerobes found in wounds have the power to stimulate growth of all common anaerobes. They also found that Cl. perfringens, grown in association with streptococci and staphylococci in milk, grows more rapidly and in higher dilutions than in tubes containing only the anaerobe. Greer and Nyhan (1928) found that in tubes of milk inoculated with mixtures in which Cl. perfringens was present, Cl. perfringens usually outgrew all other organisms up to dilutions in which the anaerobe was present in very small numbers.

Holman and Meekison (1926) stated that when the phenomenon of gas production is used as the index of changes, it may be a source of error in identifying bacteria. They also stated that the process of fermentation of carbohydrates and alcohols is quite involved. There are many involved problems and confusing results when two or more organisms are growing together. Gas production can be inhibited as well as stimulated, and these two phenomena are closely related through the various factors affecting the metabolism of bacteria. All grades of these phenomena can be obtained, depending on the characters of the bacteria involved, their age, and use of different media.

EXPERIMENT I

THE DETECTION OF ANAEROBIC, SPORE-FORMING BACILLI AND COLIFORM ORGANISMS IN DRINKING WATERS OF RILEY COUNTY (KANSAS) SCHOOLS

Procedure

Samples of drinking water were obtained from 30 Riley County (Kansas) Schools. These samples were obtained directly from the wells used by the pupils to obtain drinking water. Various types of well mechanisms were encountered. Most of the wells were located outside the school buildings and the pumps were hand operated. A few pumps were electrically operated. The water supplies for two schools were wells located on nearby farms, because the school supplies were considered polluted. Several pumps and outlets were located in the school buildings.

In all cases the mouth of the outlet was sterilized by washing with 95 percent ethyl alcohol, and then igniting with an alcohol swab used as a torch. The outlet was thoroughly flamed for about one-half minute. Approximately 2 buckets of water were pumped out and discarded. One 100 ml portion of each sample of water was inoculated directly into gas fermentation bottles containing 25 ml of quintuple strength, sterile, lactose broth for the coliform test. Also, one 100 ml portion was obtained in a sterile bottle and placed in an ice chest to keep it cold. The latter portion was later used for the detection and isolation of

anaerobic spore-forming bacilli. The procedure used for detection of coliform bacteria was that outlined in Standard Methods for the Examination of Water and Sewage, 9th edition, 1946.

Samples showing gas production in the lactose broth within 24 hours at 35° C. were considered as positive presumptive tests. If no gas was produced in 24 hours, or gas production doubtful, the samples were incubated an additional 24 hours. If gas was produced during this second 24 hour period, the samples were considered as doubtful presumptive tests. If no gas was produced within the 48 hours, the samples were considered negative for coliform bacteria. All positive and doubtful presumptive reactions were confirmed on Eosin Methylene Blue Agar. Typical colonies of the coliform group were inoculated into lactose broth tubes and onto nutrient agar slants and incubated for 24 hours at 35° C. If gas was produced within this time, or within 48 hours, portions of nutrient agar slant growths were stained by Gram's method, and if Gram-negative bacilli with no spores were present, the test was called positive for coliform bacteria. If no gas was produced, the test was considered negative for coliform bacteria.

One hundred milliliters of each water sample was inoculated into a large bottle containing 200 ml of sterile skim milk and the surface covered with a layer of vaseline-paraffin mixture. The bottles had cotton stoppers. These bottles were heated in a water bath at 80° C. for 20 minutes to kill any vegetative cells present. At first these bottles were cooled by placing them in cool water, but since they were not Pyrex, breakage troubles were encountered. It was found best to allow them to air cool. When cool, these

inoculated bottles were incubated at 35° C. and were checked within 18 to 24 hours for "stormy fermentation". Negative tests were checked at two, three, four, five, and six days.

From each "stormy fermentation" bottle a loopful of liquid whey was streaked upon the surface of Anaerobic Agar (BBL) in Petri plates with Brewer anaerobic tops and incubated at 35° C. for 24 to 48 hours. Transfers were made from colonies into secondary tubes of milk, into tubes of Brewer Thioglycollate Broth, and onto a Nutrient Agar slant. All were incubated at 35° C. The milk tubes were observed for "stormy fermentation" and the agar slants observed for growth upon the surface. If growth appeared upon an agar slant, a stain by Gram's method was made. Then a test was made for the presence of the catalase enzyme. A positive catalase test on a bacillus form indicated that the organism was an aerobic Bacillus species and not a member of the genus Clostridium. If no growth appeared upon the Nutrient Agar slant, the Thioglycollate Broth was observed for growth and a test made for presence of catalase. No growth or very scant growth on the Nutrient Agar slant, and a negative catalase test indicated that the organism was a micro-aerophilic or anaerobic, spore-forming bacillus.

Results

From a total of 30 samples, 5 were positive for coliform bacteria, but negative for "stormy fermentation". Five samples were positive for "stormy fermentation" but negative for coliform

bacteria. Six were positive for both coliform bacteria and "stormy fermentation", and 14 samples were negative for both.

Results of the coliform, "stormy fermentation", and catalase tests will be found in Table 1.

Discussion and Conclusions

There appeared to be no definite correlation of results concerning the presence of coliform organisms and organisms which caused "stormy fermentation". Except for 3 samples, all samples showing "stormy fermentation" in the original milk tubes were found to contain aerobic spore-forming bacilli. The organism isolated from one sample (Winkler) appeared to be a true, anaerobic, spore-forming bacillus. It would not grow on a nutrient agar slant, and it produced "stormy-fermentation" each time it was inoculated into sterile milk. It was found to be motile; so it could not have been Cl. perfringens.

It is very possible that the "stormy fermentation" in all positive milk tubes was produced by aerobic spore-forming bacilli or bacterial association, particularly synergism.

Table 1. Results of the coliform tests, "stormy fermentation" tests, and catalase tests.

School	Coliform test	Stormy fermentation	Sec. milk tubes	Catalase test
Zeandale	≠	≠	G	≠
Tabor Valley*	-	≠	-	≠
McDowell Creek	≠	-	-	-
Oak Grove	-	≠	G	≠
Ashland	-	≠	-	≠
Hunters Island	-	-	-	-
Ober**	≠	≠	G	≠
Columbus	-	≠	G	≠
Winkler	≠	≠	Stormy	-
Star	-	-	-	-
Swede Creek	-	-	-	-
May Day	≠	-	-	-
Rose Hill***	≠	≠	-	?
Cleburne	≠	≠	G	≠
Peach Grove	-	-	-	-
Strong	-	-	-	-
Stockdale	≠	≠	-	?
Grandview	-	-	-	-
Sherman	-	-	-	-
Walsburg	≠	-	-	-
Alert	≠	-	-	-
Pleasant Hill	-	-	-	-
Laurel Hill	-	-	-	-
Bala	-	-	-	-
Myersdale	-	≠	-	≠
Magic	-	-	-	-
Keats Grade	-	-	-	-
Keats High	≠	-	-	-
Moehlman Bottom	-	-	-	-
College Hill	-	-	-	-

≠ Positive test.
- Negative test.
G Gas produced, but no "stormy fermentation".
? Results uncertain (could not isolate organism on agar slant).
* Water from school well not used for drinking purposes.
** Water obtained from well on farm ¼ mile north of school.
***Water obtained from well on farm 3/4 mile west of school.

EXPERIMENT II

ISOLATION OF ANAEROBIC, SPORE-FORMING BACTERIA FROM SEWAGE AND RIVER WATER BY THE WILSON AND BLAIR METHOD

Wilson (1922) stated that in a medium containing sodium sulphite, glucose, and iron salts, the sulphite is reduced to sulphide by certain members of the Salmonella group. Wilson and Blair (1924) found that many anaerobic, spore-forming bacteria are strong reducers of sulphites even in the absence of a fermentable carbohydrate. They found that Salmonella typhosa, Sal. enteritidis, certain members of the paratyphoid group, and the obligatory, anaerobic bacteria of the intestinal tract produce dark, or almost black, colonies in a sulphite medium. They found that Cl. tetani and Cl. histolyticum caused the sulphite medium to turn greenish-black.

Wilson and Blair (1924) believed that the above mentioned test, when applied to water, offered a useful supplement to the presumptive E. coli test. They claimed that the great majority of strains of E. coli were unable to bring about a reduction of sulphite. They stated that only on one occasion had they encountered a reducing organism of the coliform group in water supplies. The black colonies were composed of Gram-positive, anaerobic bacteria. The black colonies could be classified according to size, as large (5 mm or more), medium (3 to 4 mm), small (1 to 3 mm), and tiny (1 mm or under). Colonies of Cl. perfringens nearly always were medium or large sized. These colonies could be verified by

transferring them to milk tubes and noting if "stormy fermentation" developed. They claimed that colonies of Cl. perfringens may be larger if the suspension is heated to 80° C. for 10 minutes as it kills the E. coli organisms which can interfere with growth of Cl. perfringens. Wilson and Blair (1924) expressed the opinion that, of all the dark colonies encountered, half of them would be large or medium, and of these about 50 percent would be found to be Cl. perfringens. Probably one in every four of the dark colonies would be classified as Cl. perfringens. They believed that by this method a good quantitative estimation of Cl. perfringens and other sulphite-reducing bacteria in water supplies could be obtained. They also believed that this test is a good indication of pollution from the decomposition of animal remains. They claimed that bacteria responsible for decay of vegetable matter are not sulphite-reducers.

Lewis, Green, and Hamilton (1930) stated that the sulphite reduction test appears to have little or no value as a supplement to the standard test for coliform bacteria. They tested waters from wells, springs, and rivers using the standard established by the United States Treasury Department for coliform bacteria in drinking water and the standard proposed by Wilson and Blair (1924) for sulphite-reducing bacteria. They claimed the tests failed to correlate.

It is generally known that anaerobic bacteria are found in sewage. It was decided to try the Wilson and Blair (1924) method for the detection and isolation of anaerobic bacteria in sewage and

river water to determine if this test is of value for use in the testing of drinking waters.

Wilson and Blair (1924) recommended the use of 3 percent nutrient glucose agar to every 100 ml of which when melted are added 10 ml of a freshly prepared 20 percent solution of sodium sulphite in distilled water and 1 ml of an 8 percent solution of ferric chloride. To 20 ml of melted medium cooled to about 60° C. and contained in a large test tube, 20 ml of water to be examined are rapidly mixed, and the contents poured into a Petri dish. After the agar has set the same procedure is carried out with another 20 ml of the water, and finally the surface of the sulphite agar is covered with 20 ml of the medium mixed with 20 ml of sterile water. In highly contaminated water there may be difficulty in counting the black colonies. In such cases it is well to use only 10 ml of water sample. Inoculated medium may be kept in tubes, but microscopic examination and counts of colonies are more easily made in plates. Wilson and Blair (1924), (1925) recommended incubating the mixture of water sample and medium in a water bath at 56° C. for one-half hour or longer, or heating the samples to 80° C. for 10 minutes, to kill the bacteria in the vegetative stage.

Procedure

Approximately 1 liter of sewage was obtained a few feet below the Manhattan, Kansas, raw sewage outlet on the Kansas River. This sample was carried immediately to the laboratory. Approximately

1 pint of the sample was thoroughly mixed in an electric beater device (Osterizer) for about one-half minute. As it was felt that the number of anaerobic organisms would be very high unless diluted, dilutions of 1:10 to 1:1,000,000 were made. Sterile, distilled water was used for the dilutions. As the standard 90 mm Petri dish was used in this experiment, the amount of sample and agar in each dish recommended by Wilson and Blair (1924) was too large. Ten ml of the sulphite agar, which had previously been heated between 70° to 80° C., were poured into each tube containing 10 ml of the diluted sample and thoroughly mixed. As fast as the agar medium could be poured and mixed, the tubes were placed in a 56° C. water bath to keep them in a liquid condition.

Temperatures of 80° C. and 56° C. and various time intervals at each temperature were used to determine if a particular temperature and time would not destroy the spores of anaerobic, spore-forming organisms, which cause blackened areas in the sulphite medium, but would destroy vegetative forms of other organisms. Four tubes of each dilution were placed in an 80° C. water bath and the remaining four placed in the 56° C. water bath. At intervals of 5, 10, 15, and 20 minutes one tube of each dilution with medium was removed from the 80° C. water bath and poured into a Petri dish. At intervals of 30, 40, 50, and 60 minutes one tube of each dilution plus medium was removed from the 56° C. water bath and poured into a Petri dish. When all the agar plates were solidified, more sulphite medium was layered on top of the solidified medium to obtain anaerobic conditions in the inoculated layer. Ten

ml of sterile water at room temperature were mixed with 10 ml of sulphite medium heated between 70° and 80° C. for this purpose.

A second and third sample of sewage and one sample of river water were obtained. The river water was obtained at a point approximately 200 yards upstream from the sewer outlet. The river water was not diluted.

The procedure for these latter samples of sewage and river water was the same as for the first sewage sample with the exceptions that only 1:10 dilutions of sewage were made and the method of heating in the water baths was changed. (It was found by the procedure of the first sewage sample that it was not necessary to make dilutions greater than 1:10.) The 10 ml samples without medium were heated for the specified times. Ten ml of warm liquid sulphite agar were added and mixed with the heated samples and poured immediately into Petri dishes.

All plated samples were inverted and incubated at 35° C. The plates were examined at the end of 24 hours for the presence of black colonies. These colonies were counted by means of a Quebec Colony Counter. The number counted in each plate was multiplied by the dilution factor to determine the number of colonies there would have been in 10 ml of undiluted sample. These black colonies were counted again in 48 hours.

Some of the black colonies were removed from the sulphite agar plates and inoculated into tubes of sterile milk under paraffin-vaseline seals. These tubes were observed for evidence of "stormy fermentation". The whey from positive tubes was streaked

upon a Nutrient Agar slant and inoculated into liquid sodium sulphite-iron-agar and plated and conditions made anaerobic. The agar slants were observed for growth and the agar plates observed for appearance of black colonies.

Results

Very few bacteria other than those producing black colonies appeared in the anaerobic agar when the samples were heated at 80° C. for at least 5 minutes; but many more colonies appeared from samples heated at 56° C., even when heated for 60 minutes.

The majority of black colonies produced "stormy fermentation" in sterile milk tubes.

Aerobic bacteria grew on some of the agar slants from the second sewage sample and river water sample, but these were found on the anaerobic plates containing colonies producing gas bubbles in the anaerobic agar. In the river water the bacteria often grew as a film on the surface of the medium.

Results of this experiment will be found in Tables 2 and 3.

Table 2. Effects of temperature and time of heating samples on plate counts of sewage and river water.

Sample used	80° C. Minutes				56° C. Minutes			
	5	10	15	20	30	40	50	60
Sewage sample #2 (1:10)								
Black colonies	200	380	330	250	100	120	270	120
Other colonies	0	3	0	0	650	850	960	150
Sewage sample #3 (1:10)								
Black colonies	200	180	150	350	100	100	140	90
Other colonies	0	20	30	30	300	500	200	140
River water (not diluted)								
Black colonies	33	7	9	7	52	60	57	80
Other colonies	60	90	300	560	500	450	600	560

Note: Number of colonies from sewage samples multiplied by 10.

Table 3. Results of cultural confirmation of organisms isolated from black colonies from sodium sulphite agar.

Sample used	Black colonies producing "stormy ferm."	Black colonies : sodium sulphite agar	Growth on nutrient agar slant
Sewage sample #2 (1:10)	7 out of 11 (24 hrs.)	Black colonies. Several plates contained organisms producing gas bubbles. (Especially true for samples heated at 56° C.)	Aerobic organisms grew on agar slants corresponding to plates with gas bubbles. Remaining colonies did not grow on agar slants
Sewage sample #3 (1:10)	2 out of 2 (24 hrs.)	Black colonies	No growth
River water (not diluted)	3 out of 3 (24 hrs.)	Black colonies	Growth

Discussion and Conclusion

Apparently there was no decrease in the number of bacteria which produced black colonies in the sodium sulphite-iron-agar when heated for 30 minutes at 56° C. or 20 minutes at 80° C. However, it was found that a temperature of 56° C. was not high enough to kill bacteria which produced colonies other than black ones. It was not determined what organisms produced these colonies. It was decided that it would be best to heat future water samples or sewage at 80° C. for 10 to 20 minutes to kill the vegetative cells.

Practically every plate with a sample of river water produced a bacterial film on the surface of the agar, which in some cases practically covered all of the surface area. When transferring black colonies to sterile milk tubes, an effort was made to keep the wire loop from becoming contaminated with this surface organism, but apparently this effort was wasted. Evidently the river water portions contained an aerobic or facultative anaerobic organism which was not killed when heated. No trouble like this was encountered with the sewage samples. The river water was quite muddy after recent rains, which may have accounted for these results.

EXPERIMENT III

THE DETECTION OF ANAEROBIC, SPORE-FORMING BACILLI AND COLIFORM ORGANISMS IN FARMSTEAD DRINKING WATERS NEAR THE KANSAS RIVER IN RILEY COUNTY, KANSAS

Procedure

One hundred and one samples of drinking water were obtained from farm units near the Kansas River in Riley County, Kansas. These samples were obtained along the course of the river from where it enters Riley County near Ogden to the eastern border of the county. These samples were obtained from wells near the river, because it was felt that it was very possible for these well waters to be polluted from this source. Raw sewage is dumped into the Kansas River from most of the nearby small towns, as well as Manhattan. Most of the wells were located outside the dwelling, but many were located under the dwelling and operated by electric pumps.

In all cases the mouth of the outlet was sterilized by flaming with an alcohol torch for approximately one-half minute. Approximately 2 buckets of water were pumped out and discarded. One 100 ml portion of each sample of water was obtained in a sterile bottle. These samples were placed in an ice chest to keep them cold and were carried back to the laboratory and tested for the presence of coliform organisms and for anaerobic spore-forming bacilli. The method used for the detection of coliform bacteria

was that outlined in Standard Methods of Water Analysis, (1936), 8th edition, for use with the Table of Most Probable Numbers per 100 ml water.

For this procedure five 10 ml portions of water were inoculated into 10 ml of double strength lactose broth fermentation tubes and one 1 ml portion inoculated into 10 ml of single strength lactose broth. All positive lactose tubes were confirmed in "Brilliant Green Bile 2% (Difco)". The method used for the detection and isolation of anaerobic, spore-forming bacilli was that used by Wilson and Blair (1924) as outlined in Experiment II. All inoculated fermentation tubes and anaerobic plates were incubated at 35° C.

Wilson and Blair (1924) believed that water in shallow wells and springs containing more than 2 reducing colonies in 10 ml should be regarded with suspicion. They also believed that deep pure waters should contain no reducing bacteria in 40 ml of water.

A 10 ml portion of each water sample was placed in a sterile large test tube and heated at 80° C. for 15 to 20 minutes to kill vegetative cells. At the end of this heating period 10 ml of warm sodium sulphite-iron-agar were poured into each tube and mixed with the heated water sample. The mixture was poured immediately into Petri dishes and allowed to cool until gelled. A few minutes later 20 to 30 ml of warm diluted sodium sulphite-iron-agar (diluted 1:1 with sterile distilled water) were poured over the inoculated layer of medium and allowed to gel.

All agar plates were inverted and incubated at 35° C. for 24

hours to several days. These plates were observed each 24 hours for appearance of black colonies. A count of these black colonies was made.

The black colonies were removed from the agar plates with a stiff, sterile, inoculating needle and inoculated into previously heated (to drive out any absorbed oxygen) and cooled, sterile, skimmed milk tubes under paraffin-vaseline seals. These milk tubes were incubated at 35° C. and observed each 24 hours for the "stormy fermentation" reaction. The whey from each "stormy fermentation" tube was streaked on the surface of a Nutrient Agar slant and an inoculating needle wetted in the culture was then stirred in a small test tube containing 10 ml of warm sodium sulphite-iron-agar and poured into a sterile Petri dish. The plates were covered by another layer of sodium sulphite-iron-agar to obtain anaerobic conditions. The agar slants were observed for growth upon the surface, and the anaerobic agar plates were observed for appearance of black colonies.

Results

It was found that 25 out of 101 water samples contained 39 or more coliform bacteria in 100 ml. One sample contained 21, and one contained 15 coliform bacteria in 100 ml. Seventeen samples contained 2 to 8.9 coliform bacteria in 100 ml. Fifty-seven samples contained no coliform bacteria in 100 ml.

Black colonies appeared upon sodium sulphite-iron-agar medium

from 11 samples. Nine of these samples produced 1 to 9 black colonies; one produced 167, and one produced 257 colonies in 48 hours. Practically all of these colonies were small or tiny (none more than 3 mm in diameter). The colonies from 2 samples produced "stormy fermentation" in anaerobic, sterile milk tubes. The whey from these "stormy" milk tubes contained anaerobic bacteria as no growth was observed on Nutrient Agar slants, but black colonies were produced upon inoculation into secondary sodium sulphite-iron-agar medium.

The locations of farmsteads from which samples of drinking waters were obtained and the types of bacteria found are shown on Maps 1 and 2.

Results of the coliform (expressed as Most Probable Numbers), sulphite reduction, and "stormy fermentation" tests will be found in Table 4.

Table 4. Results of coliform tests, sulphite reduction tests, and "stormy fermentation" tests from farmstead drinking waters.

Sample No.	No. positive lactose ferm. tubes 10 ml	1 ml	MPN per 100 ml	No. black colonies in Na_2SO_3-iron-agar in 10 ml	Stormy ferm. milk tubes
1	0	0	0	0	
2	0	0	0	0	
3	5	1	over 39.0	0	
4	5	1	over 39.0	0	
5	5	0	39.0	2 (2 days)	--
6	5	1	over 39.0	6 (2 days)	--
7	2	0	5.0	1*	1 (2 days)
8	0	0	0	0	
9	0	0	0	0	
10	0	0	0	0	

Table 4. (cont.).

Sample No.	No. positive lactose ferm. tubes 10 ml	1 ml	MPN per 100 ml	No. black colonies in Na_2SO_3-iron-agar in 10 ml	Stormy ferm. milk tubes
11	5	0	39.0	0	
12	0	0	0	0	
13	5	1	over 39.0	257* (2 days)	1 (2 days)
14	5	1	over 39.0	0	
15	0	0	0	0	
16	0	0	0	0	
17	5	1	over 39.0	0	
18	5	1	over 39.0	0	
19	2	0	5.0	0	
20	0	0	0	0	
21	0	0	0	0	
22	0	0	0	0	
23	0	0	0	0	
24	5	1	over 39.0	0	
25	4	0	15.0	0	
26	0	0	0	0	
27	0	0	0	0	
28	0	0	0	0	
29	0	0	0	0	
30	0	0	0	0	
31	3	0	8.9	0	
32	5	0	39.0	1 (4 days)	--
33	0	0	0	0	
34	0	0	0	0	
35	1	0	2.2	0	
36	0	0	0	1 (4 days)	--
37	0	0	0	0	
38	1	0	2.2	0	
39	1	0	2.2	0	
40	5	1	over 39.0	0	
41	4	1	21.0	0	
42	0	0	0	0	
43	0	0	0	0	
44	5	0	39.0	0	
45	0	0	0	0	
46	5	1	over 39.0	0	
47	5	0	39.0	0	
48	5	1	over 39.0	0	
49	3	0	8.9	0	
50	0	0	0	0	
51	1	0	2.2	9 (4 days)	--
52	0	0	0	0	
53	0	0	0	8 (4 days)	--
54	0	0	0	0	

Table 4. (cont.).

Sample No.	No. positive lactose ferm. tubes 10 ml	1 ml	MPN per 100 ml	No. black colonies in Na_2SO_3-iron-agar in 10 ml	Stormy ferm. milk tubes
55	0	0	0	0	
56	0	0	0	0	
57	1	0	2.2	0	
58	0	0	0	0	
59	0	0	0	0	
60	0	0	0	0	
61	0	0	0	0	
62	0	0	0	0	
63	0	0	0	0	
64	0	0	0	0	
65	0	0	0	0	
66	2	1	7.6	167 (2 days)	--
67	5	0	39.0	4 (5 days)	--
68	1	0	2.2	0	
69	0	0	0	0	
70	1	0	2.2	0	
71	0	0	0	0	
72	0	0	0	0	
73	0	0	0	0	
74	3	0	8.9	0	
75	0	0	0	0	
76	0	0	0	0	
77	0	0	0	0	
78	0	0	0	0	
79	0	0	0	0	
80	0	0	0	0	
81	0	0	0	0	
82	5	1	over 39.0	0	
83	5	1	over 39.0	5 (2 days)	--
84	5	0	39.0	0	
85	0	0	0	0	
86	0	0	0	0	
87	5	1	over 39.0	0	
88	3	0	8.9	0	
89	1	0	2.2	0	
90	0	0	0	0	
91	2	0	5.0	0	
92	0	0	0	0	
93	0	0	0	0	
94	0	0	0	0	
95	0	0	0	0	
96	5	1	over 39.0	0	
97	5	0	39.0	0	
98	5	1	over 39.0	0	

Table 4. (concl.).

Sample No.	No. positive lactose ferm. tubes 10 ml	1 ml	MPN per 100 ml	No. black colonies in Na_2SO_3-iron-agar in 10 ml	Stormy ferm. milk tubes
99	5	1	over 39.0	0	
100	0	0	0	0	
101	1	0	2.2	0	

*Did not produce growth on agar slant but did produce black colonies in secondary sodium sulphite-iron-agar.

Discussion and Conclusion

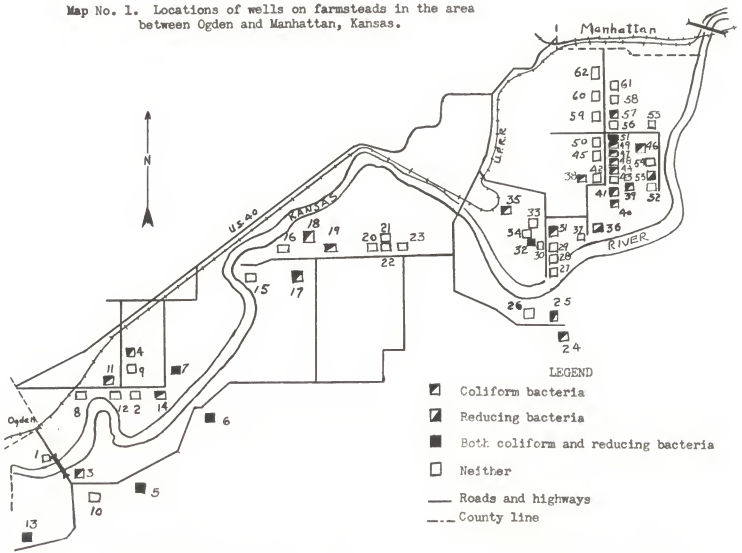
It has been shown by use of the technics employed in this experiment that there is little or no correlation of the sulphite reduction test and the standard tests for coliform bacteria as suggested by Wilson and Blair (1924, 1925). It is felt that the sulphite reduction test has no value as a supplement to the standard test for coliform organisms.

It can be seen by observation of Maps 1 and 2 that the location of farmsteads quite near the Kansas River probably has no relation to pollution. It is felt that the pollution is from a purely local condition and not from the river.

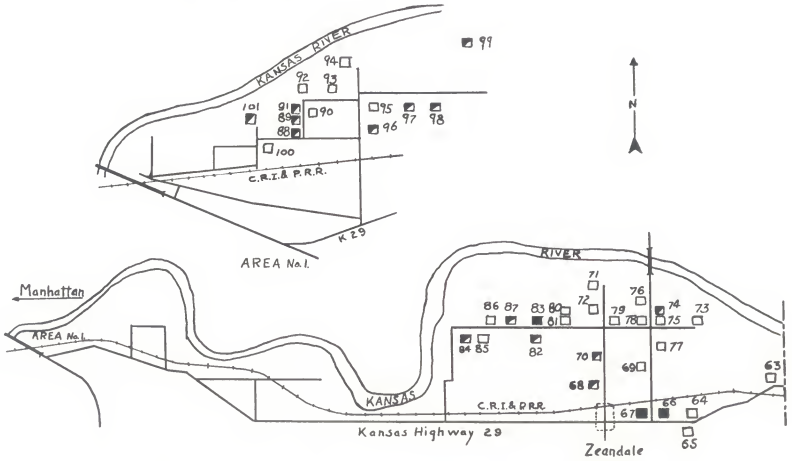
According to the Public Health Service Drinking Water Standards, 1946, 44 out of 101 samples would be considered not safe for human consumption.

According to the standard set by Wilson and Blair (1924) for

Map No. 1. Locations of wells on farmsteads in the area between Ogden and Manhattan, Kansas.



Map No. 2. Locations of wells on farmsteads in the area between Manhattan and the eastern border of Riley County, Kansas.



the number of reducing colonies in 10 ml of water, 7 out of 101 samples would be regarded with suspicion. However, this standard was established for water from shallow wells and springs. It is not known how many of the wells are shallow, but it is felt that most of them are shallow. Sample number 6 was obtained from a spring.

ANALYSIS OF FINDINGS

There are several investigators who believe that the presence of anaerobic, spore-forming bacilli in water supplies indicates pollution and that there is a definite correlation of tests for these bacilli and the standard tests for coliform bacilli. The research that this thesis represents was carried out in order to determine if these above tests do correlate and if tests for the presence of anaerobic, spore-forming bacilli in drinking waters are of value in the testing of these waters.

It is believed by many persons that the production of gas and the breaking up of the curd in milk (the so-called "stormy fermentation") implies that this condition is produced by the anaerobic, spore-forming (Clostridium) species. Results from the "stormy fermentation" tests on samples of drinking waters from the Riley County (Kansas) Schools indicated that most of the bacteria isolated did not produce typical "stormy fermentation". Most of these bacteria were found to be aerobic, spore-forming bacilli. It appeared that the "stormy fermentation" condition was produced either by bacteria which may have been present in the sample but were not isolated by the technic used or by bacterial association, particularly synergism. Holman and Meekison (1926) stated that when the phenomenon of gas production is used as an index of change, it may be a source of error in identifying bacteria. They stated that there may be many involved problems and confusing results when two or more bacteria are growing together. It is felt

that the so-called "stormy fermentation" produced from drinking waters often may have been produced by bacterial synergism and not necessarily by Clostridium species.

One of the main tasks was to find a practical method for the detection and isolation of Clostridium species in drinking waters. Wilson and Blair (1924) recommended the use of sodium sulphite-iron-agar for the isolation of sulphite reducing bacteria, particularly Clostridium perfringens, which is an organism commonly found in the intestinal tract of warm-blooded animals. The method and medium of Wilson and Blair was tried first with raw sewage with favorable results. Then it was used in an effort to isolate the spore-forming, anaerobic bacilli in drinking waters. The few black colonies that appeared in the sulphite-iron-agar were quite small, and failed to produce a "stormy fermentation" in milk. These small colonies may have been composed of bacterial species which would not produce "stormy fermentation".

In conclusion it can be stated, from results of the experiments performed, that there was no correlation of tests for coliform bacilli and for anaerobic, spore-forming bacilli in drinking waters.

SUMMARY

1. From a total of 30 samples of drinking water from the Riley County (Kansas) Schools, 5 samples (16.6 percent) were positive for coliform bacteria but negative for "stormy fermentation"; 5 samples (16.6 percent) were negative for coliform bacteria but positive for "stormy fermentation"; 6 samples (20 percent) were positive for both coliform bacteria and "stormy fermentation"; 14 samples (46.7 percent) were negative for both.

2. From a total of 101 samples of drinking water from farmsteads near the Kansas River in Riley County, Kansas, 44 samples gave positive tests for coliform bacilli. However, only 11 samples produced black colonies upon sodium sulphite-iron-agar medium, and of these black colonies only 2 produced "stormy fermentation" when inoculated into milk tubes with vaseline-paraffin seals. The bacteria in whey did not grow on agar slants, but produced black colonies on secondary sodium sulphite-iron-agar medium.

According to the Public Health Service Drinking Water Standards, 1946, 44 out of 101 samples would be considered not safe for human consumption.

According to the standard set by Wilson and Blair (1924) for the number of reducing colonies in 10 ml of water, 7 out of 101 samples would be regarded with suspicion.

3. It is apparent, by the methods employed, that there is no correlation of results concerning the tests for presence of coliform bacteria and bacteria which produced "stormy fermentation"

from drinking waters, and that the "stormy fermentation" test has little or no value as a supplement to the standard test for coliform bacteria.

4. It is apparent, by the methods employed, that there is no correlation of the sulphite reduction test for anaerobic, spore-forming bacilli and the standard test for coliform bacilli, and that the sulphite reduction test has little or no value as a supplement to the standard test for coliform bacteria.

5. It is felt that the so-called "stormy fermentation" reaction in milk from drinking waters may often be produced by bacterial association, particularly synergism, and not necessarily by anaerobic, spore-forming bacilli.

6. It is shown that the location of farmsteads quite near the Kansas River probably has no relation to pollution. It is felt that the pollution is from a purely local condition and not from the river.

ACKNOWLEDGMENT

Indebtedness is acknowledged to Dr. T. H. Lord, the major instructor, for his counsel, advice, and criticism in the technical procedures and in the preparation of the manuscript. The author also wishes to give special thanks to Dr. Lord for his translations from Les Microbes Anaérobies, by Weinberg, Nativelle, and Prévot. Thanks are also expressed to Dr. P. L. Gainey, Head of the Department of Bacteriology, and to Dr. L. D. Bushnell for their advice and counsel.

BIBLIOGRAPHY

Breed, Robert S., E. G. D. Murray, and A. Parker Hitchens. Bergey's manual of determinative bacteriology. 6th ed. Baltimore: Williams and Wilkins, 1948.

Ewing, C. Leroy. Presence of a spore-bearing aerobic gas-forming bacillus in Baltimore city drinking water. Amer. Jour. Pub. Health. 9:257-258. 1919.

Ginter, R. L. Spore bearers in the Spavinaw water supply. Amer. Waterworks Assoc. Jour. 17:591-594. 1927.

Greer, Frank E. Anaerobes in sewage. Amer. Jour. Pub. Health. 15:860-867. 1925.

Greer, Frank E. Anaerobes in sewage. Amer. Jour. Pub. Health. 16:500-501. 1926.

Greer, Frank E. The sanitary significance of lactose-fermenting bacteria not belonging to the B. coli group. 6. Sanitary considerations. Jour. Infect. Dis. 42:551-555. 1928a.

Greer, Frank E. The sanitary significance of lactose-fermenting bacteria not belonging to the B. coli group. 1. Groups reported in the literature and isolated from water in Chicago. Jour. Infect. Dis. 42:501-513. 1928b.

Greer, Frank E. The sanitary significance of lactose-fermenting bacteria not belonging to the B. coli group. 2. Number of lactose-fermenting organisms found in Chicago sewage and Chicago water supply. Jour. Infect. Dis. 42:514-524. 1928c.

Greer, Frank E. The sanitary significance of lactose-fermenting bacteria not belonging to the B. coli group. 5. Factors influencing the survival of microorganisms in water. Jour. Infect. Dis. 42:545-550. 1928d.

Greer, Frank E., and R. E. Noble.
The sanitary significance of lactose-fermenting bacteria not belonging to the B. coli group.
8. Conclusions. Jour. Infect. Dis. 42:568-574. 1928.

Greer, Frank E., R. E. Noble, F. W. Nyhan, and A. E. O'Neil.
The sanitary significance of lactose-fermenting bacteria not belonging to the B. coli group.
7. Mediums and methods. Jour. Infect. Dis. 42:556-567. 1928.

Greer, Frank E., and Florence V. Nyhan.
The sanitary significance of lactose-fermenting bacteria not belonging to the B. coli group.
3. Bacterial associations in cultures containing lactose-fermenting bacteria. Jour. Infect. Dis. 42:525-536. 1928.

Greer, Frank E., Fred O. Tonney, and Florence V. Nyhan.
The sanitary significance of lactose-fermenting bacteria not belonging to the B. coli group.
4. Pathogenicity. Jour. Infect. Dis. 42:537-544. 1928.

Hinman, Jack J., Jr., and Max Levine.
A facultative spore-forming lactose-fermenting organism from Iowa surface waters, (B. macerans). Amer. Waterworks Assoc. Jour. 9:330-342. 1922.

Holman, W. L., and D. M. Meekison.
Gas production by bacterial synergism. Jour. Infect. Dis. 39:145-172. 1926.

Houston, A. C.
Fifth Rept. on Res., to the Metropolitan Water Bd., London. 1910. Cited by Tanner, Fred W. Microbiology of foods. 2nd ed. Champaign, Ill.: Garrard Press, p. 166, 1944.

Jackson, D. D., and T. C. Muer.
Liver broth: A medium for the determination of gas-forming bacteria in water. Jour. Infect. Dis. 8:289-294. 1911.

Jordan, Edwin O., and William Burrows.
Textbook of bacteriology. 14th ed. Philadelphia and London: Saunders, pp. 218-230, 511-542. 1946.

Klein, E.
Differentiation of the several anaerobes present in the intestinal contents of man and other animals. Local Gov't. Board. Med. Off. Rep't. 404. 1901. Cited by Meader and Bliss. Amer. Jour. Hyg. 3:394-400. 1923.

Klein, E.
Rep. Med. Off. Local Gov. and Board. 210. 1897-1898. Cited by Greer, Frank E. The significance of lactose-fermenting bacteria not belonging to the B. coli group.
1. Groups reported in the literature and isolated from water in Chicago. Jour. Infect. Dis. 42:501-513. 1928.

Klein, E., and A. C. Houston.
Rep. Med. Off. Local Gov. and Board. 318. 1898-1899; 326. 1901-1905; 472. 1903-1904. Cited by Greer, Frank E. The sanitary significance of lactose-fermenting bacteria not belonging to the B. coli group.
1. Groups reported in the literature and isolated from water in Chicago. Jour. Infect. Dis. 42:501-513. 1928.

Koser, Stewart A., and Winifred C. Shinn.
Aerobic spore-forming bacilli which ferment lactose. Amer. Waterworks Assoc. Jour. 18:328-336. 1927.

Larner, Herbert B.
Bacillus welchii in a public water supply. Amer. Med. Assoc. Jour. 78:276-279. 1922.

Lewis, I. M., Thomas C. Green, and Viola Hamilton.
The correlation of sulphite reducing and colon bacteria in water. Amer. Waterworks Assoc. Jour. 22:667-672. 1930.

Lisk, Henrietta.
Gas production by an aerobic spore bearing bacillus. Amer. Waterworks Assoc. Jour. 10:139-144. 1923.

Meader, Percy Davol, and Eleanor A. Bliss.
Observations on anaerobes from water samples. Amer. Jour. Hyg. 3:394-400. 1923.

Meyer, E. M.
An aerobic spore-forming bacillus giving gas in lactose broth isolated in routine water examination. Jour. Bact. 3:9-14. 1918.

Noble, Willis.
Experimental study of the distribution and habitat of the tetanus bacillus. Jour. Infect. Dis. 16:132-141. 1915.

Norton, John F., and J. J. Weight.
Aerobic spore forming lactose fermenting organisms and their significance in water analysis. Amer. Jour. Pub. Health. 14:1019-1021. 1924.

Public health service drinking water standards. Public health reports. 61:371-384. 1946. Reprint No. 2697.

Raab, Frank.
Anaerobic, lactose-fermenting spore-bearers in the city of Minneapolis water supply. Amer. Waterworks Assoc. Jour. 10:1051-1055. 1923.

Savage, William G., and W. J. Read.
Significance of streptococci in water supplies. Jour. Hyg. 15:334-351. 1916.

Savage, William G., and D. R. Wood.
The vitality and viability of streptococci in water. Jour. Hyg. 16:227-239. 1917.

Schreiner, W. R.
Presented at Kansas section, Amer. Waterworks Assoc. 1927. Cited by Koser, Stewart A., and Winifred C. Shinn. Aerobic spore-forming bacilli which ferment lactose. Amer. Waterworks Assoc. Jour. 18:328-336. 1927.

Sears, H. J., and John J. Putnam.
Gas production by bacteria in symbiosis. Jour. Infect. Dis. 32:270-279. 1923.

Simonds, J. P.
Studies in Bacillus welchii, with special reference to classification and to its relation to diarrhea. Monog. Rockefeller Inst. Med. Res., No. 5. September, 1915.

Skinner, C. E., and A. H. Baskin.
Lactose fermenting anaerobes in soil and their relation to sanitary water analysis. Soc. Expt. Biol. and Med. Proc. 29:551-554. 1932.

Smith, Louis DeSpain, and Mary Virginia Gardner.
The occurrence of vegetative cells of Clostridium perfringens in soil. Jour. Bact. 58(3):407. 1949.

Standard methods for the examination of water and sewage. 9th ed. New York: American Public Health Association. 1946.

Standard methods of water analysis. 8th ed. New York: American Public Health Association, p. 220. 1936.

Suckling, E. V.
The examination of waters and water supplies. Philadelphia: Blakiston, p. 508. 1944.

Tanner, Fred Wilbur.
Microbiology of foods. 2nd ed. Champaign, Ill: Garrard Press, pp. 161-192. 1944.

Tenbroeck, Carl, and Johannes H. Bauer.
The tetanus bacillus as an intestinal saprophyte in man. Jour. Expt. Med. 36:261-271. 1922.

Thresh, John C., J. F. Beale, and E. V. Suckling.
The examination of waters and water supplies. 4th ed. Philadelphia: Blakiston, pp. 492-502. 1933.

Topley, W. W. C., and G. S. Wilson.
Principles of bacteriology and immunity. New York: William Wood, pp. 536-537. 1929.

Vincent, M. H.
Recherches sur les microbes anaérobies des eaux. Ann. Inst. Pasteur. 21:62-75. 1907. Cited by Tanner, Fred W. Microbiology of foods. 2nd ed. Champaign, Ill: Garrard Press, p. 170. 1944.

Weinberg, M., R. Nativelle et A. Prévot.
Les microbes anaérobies. Paris: Masson et Cie., 1937.

Welch, W. H., and G. H. F. Nuttall.
Bull. Johns Hopkins Hosp. 3:81. 1892. Cited by Simonds, J. P. Monog. Rockefeller Inst. Med. Res., No. 5, p. 1. September, 1915.

Wilson, W. James.
Reduction of sulphites by certain bacteria in media containing a fermentable carbohydrate and metallic salts. Jour. Hyg. 21:392-398. 1922.

Wilson, W. James, and E. Maud McV. Blair.
The application of a sulphite-glucose-iron agar medium to the quantitative estimation of B. welchii and other reducing bacteria in water supplies. Jour. Path. and Bact. 27:119-121. 1924.

Wilson, W. James, and E. Maud McV. Blair.
Correlation of the sulphite reduction test with other tests in the bacteriological examination of water. Jour. Hyg. 24:111-119. 1925.

Winslow, C.-E.-A., and D. M. Belcher.
Changes in the bacterial flora of sewage during storage. Jour. Infect. Dis. 1:170-192. 1904.