B. COLI AS AN INDEX OF FAECAL POLLUTION OF WATER SUPPLIES.

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The quantitative determination of *B. coli* in a given sample of water has long been accepted as affording a good measurement of the probability of recent contamination with excreta.

The systematist uses the term *B. coli* to define a single definite type; but the sanitary bacteriologist, who is not concerned with botanical classifications, places a much broader interpretation upon it and includes a whole group of organisms under this heading; yet most workers agree that a bacillus of this class should have the following characteristics—a short bacillus with rounded ends, Gram negative, non-sporing and a facultative anaerobe. It should ferment lactose and dextrose with the production of acid and gas, give a clot in milk, fail to liquefy gelatine, and produce indol in a medium containing peptone.

It has been known for some time that organisms of the *B. coli* group may be present in soil and on grain where there has been little apparent opportunity for faecal pollution, and recent investigations have yielded evidence indicating that certain cultural reactions are highly correlated with different natural habitats. It has therefore become possible, by the use of laboratory tests, to assign an excretal or non-excretal origin to a given strain of *B. coli* with a reasonable degree of probability.

Early conceptions of B. coli.

Bacilli of the colon group were first isolated in 1885 by Escherich in the course of his investigations on the organisms in the faeces of young children. He described *B. coli* as a Gram negative bacillus producing characteristic colonies on gelatine, agar and potato, coagulating milk, and forming gas in glucose.

Several bacteriologists, including Dyar and his co-workers (1894)—who used the cultural characteristics on agar, gelatine and potato, the reduction of nitrates and the clotting of milk as differential tests—have shown that *B. coli* is normally present in the intestines of goats, pigs, rabbits and other animals; and Kruse (1894) pointed out that the term "*B. coli*" applies not to a single type but to a whole group of organisms which is very widely distributed.

Laurent (1899), while investigating certain diseases of potato, noted the occurrence of a bacillus which on testing proved to be of the colon type—a facultative anaerobe, incapable of liquefying gelatine, but able to reduce nitrates, produce a clot in milk and give acid and gas in glucose and in lactose broth. He cultivated these organisms on plants which had received large amounts of chalk, and also on potatoes which had been immersed in alkaline solution, and he found that *B. coli* tended to become parasitic when the resistance of the tubers had been artificially reduced in this way.

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Klein and Houston (1899–1900) made an examination of several different cereals and other foods. The tests used were the type of growth in phenol broth, the absence of liquefaction of gelatine, the clotting of milk, the production of indol and the pathogenicity. They found that in 24 cultures from 12 different samples, 10 gave bacteria which resembled *B. coli*, but of these only 3 proved to be typical, the rest failed in the milk test, or were gelatine liquefiers. In 12 cultures from rice, flour, oatmeal, etc. the typical *B. coli* was present in 3 cases.

Various investigators gave such conflicting evidence regarding the habitat of the colon bacillus, that it was questioned whether this organism were of any real value as a test for faecal contamination. Thus it became important to distinguish the various kinds of coliform organisms and to correlate these types with their habitats, in order to determine whether special sanitary significance could be attached to the occurrence of any particular group.

Classification of B. coli *on a basis of certain fermentation reactions.*

Refik (1896) divided *B. coli* into a number of groups according to the fermentation of lactose and glucose, the clotting of milk, and the production of indol. He considered that there is no correlation between sugar fermentations and the clotting of milk.

Durham (1901) investigated the various reactions characteristic of intestinal bacilli including the serological (agglutination) and sugar tests. He considered that the former are of no differential value, but he devised the first real classification of this group on the basis of certain fermentations. He recognised three principal divisions:

Division 1. Includes the typhoid bacilli.

Division 2. Motile bacilli of colon-like morphology.

Order 1. "Dextrose-non-lactoso fractors." This group includes organisms which are colon-like in the rate of growth, but which only produce acid and gas in dextrose.

Order 2. "Dextrose-lactoso-non-sucroso fractors." Organisms which produce acid and gas from dextrose and lactose but not sucrose were called *B. coli communis verus*. Members of this group grow vigorously in milk and produce a great deal of acid. No reversion to alkalinity takes place.

Order 3. "Dextroso-lactoso-sucroso fractors." This is the *B. coli communior* group, similar to the *B. coli communis verus* except in the fermentation of sucrose. According to Durham this is the commonest intestinal organism.

Division 3. "Polysaccharide fractors," which include *B. lactis aerogenes*. The bacilli are shorter and stouter than *B. coli*, and are non-motile. They not only produce acid and gas in dextrose, lactose and sucrose, but in certain of the polysaccharides also, namely starch and inulin.

Ford (1901) made agar plate cultures from different parts of the intestine. He subcultured his organisms in media containing the sugars used by Durham and distinguished (*a*) the *B. coli*, (*b*) the *B. lactis aerogenes*, and (*c*) the *B. cloacae* groups. He found that the commonest of these is the *B. coli communior* (Durham) and that the *B. coli communis verus* (Durham) is also very frequently present.

Jordan (1903) gives a very similar classification.

A distinct advance was made in the differentiation of colon bacilli as a result of the work of MacConkey (1905) who made an extensive study of all possible reactions of coliform organisms from various sources. He divided them into four main groups according to the sucrose and dulcite fermentations (Table I).

In addition these organisms should all be of typical morphology, Gram negative, non-gelatine liquefying, fermenting glucose, lactose and mannite, clotting milk and forming indol.

Table I.

MacConkey's classification of the colon group.

Group	Sucrose	Dulcite	Type
A	0	0	*B. acidi lactici*
B	0	+	*B. coli communis*
C	+	+	*B. pneumoniae, B. neapolitanus*
D	+	0	Group D includes four types, one of which is *B. lactis aerogenes.*

This method of classification was later revised by Kligler (1914) who substituted salicin for dulcite, on the ground that the saccharose-salicin fermentations give a better biological correlation with the other characters than the saccharose-dulcite (Table II).

Table II.

Kligler's classification of the B. coli *group.*

Group	Saccharose	Salicin	Dulcite	Type
A	0	0	0	*B. acidi lactici*
B	0	+	+	*B. coli communis*
C	+	0	+	*B. coli communior*
D	+	+	0	*B. lactis aerogenes*

Certain authors (Jordan 1901) have suggested that the reactions of the ordinary *B. coli* may be modified by prolonged aquatic life. Horrocks (1903) and Savage (1905) contended that *B. coli* never becomes atypical; MacConkey brought forward presumptive evidence in support of the views of these two investigators, and he is emphatic in his statement that the sugar fermentations are a reliable basis of classification. He also lays special stress on the comparatively small numbers of the *B. lactis aerogenes* type in faeces, maintaining that in 241 samples of human stools the sucrose + dulcite 0 type was only found in 4 cases.

Winslow and Walker (1907) isolated 52 strains of dextrose fermenting organisms from human faeces, 31 of which proved to be typical *B. coli* as determined by the reaction in milk and in nitrates, the production of indol and the failure to liquefy gelatine. 25 of these strains were used for comparative tests in the various sugar media, and only 4 per cent. belonged to the saccharose + dulcite 0 (*B. lactis aerogenes*) type. These authors also attempted to isolate the same type of organisms from 178 samples of grain and 40 samples of grass. None of the cultures from grass and only 50 of those from grain were dextrose fermenters. The 50 positive strains were replated on litmus lactose agar and only 3 of them gave acid colonies, all of which were gelatine liquefiers.

The same investigators noticed that bacteria which ferment sucrose usually ferment raffinose also, and later W. W. Browne (1914) showed that both the configuration and the complexity of the molecule are important, and that organisms of the *B. coli* group are able to produce more acid in solutions containing carbohydrates of simple chemical structure than in those of more complex structure.

Jackson (1911) suggested a further differentiation of the lactose fermenting bacilli—an elaboration of MacConkey's original scheme—in which he used the fermentation of raffinose and mannite, as well as the sucrose-dulcite tests. He believed that the so-called typical *B. coli* does not exist as such, but that the entire group indicates faecal contamination when water or milk examinations are to be considered.

On the other hand Rivas (1908) considered that many organisms said to belong to the colon group have no connection with *B. coli*, and he wished to narrow the conception of this bacillus as applied to the organisms present in milk, water, etc. He suggested three new tests which have not been generally accepted for routine purposes.

Houston (1913) believes that true *B. coli* should develop fluorescence in neutral red

broth and give acid and gas in dextrose after 2 days incubation at 37° C., it should form indol in peptone medium, and produce acid and clot in milk after an incubation period of 5 days at the same temperature. He called this series of standards the "flaginac" reaction. The formation of gas in glucose gelatine and acid and gas in sucrose—after 2 days at 37° C.—may be applied as extra tests.

The data obtained by the application of certain tests not depending upon the selective fermentation of carbohydrates, have, however, made it clear that the correlation between habitat and these special reactions is far closer than that between habitat and any of these series of fermentation reactions considered above.

The correlation of the fermentation tests with certain special reactions.

A. *The Voges and Proskauer reaction.*

Voges and Proskauer (1898) while investigating the bacteria which cause haemorrhagic septicaemia, discovered that when a solution of caustic potash is added to a glucose broth culture of certain intestinal bacilli and the tube is allowed to stand at room temperature for 24 hours or longer, a red fluorescence is developed. This colour, which is not unlike an alcoholic solution of eosin, forms particularly well in the upper part of the solution which is exposed to the air. It is insoluble in ether or amylalcohol and is not influenced by prolonged boiling. Voges and Proskauer tried the reaction with several different kinds of bacilli, and found that some organisms, *e.g. B. coli*, fail to give the test.

Freeland Howe (1904) isolated strains of colon-like bacilli from water, all of which gave acid and gas in glucose, red colonies on litmus lactose agar, indol in a medium containing peptone, clot in milk, and reduction of nitrates. He observed that the Voges and Proskauer reaction is only obtained with cultures that give a large amount of gas in glucose broth, and also that the quantity of sugar which remains in the medium is greater in those tubes which fail to give the reaction. He stated that the development of this colour is a perfectly definite distinguishing feature of the bacteria by which it is produced, and suggested that the test be applied to coliform organisms from various sources.

Harden and his colleagues (1906) succeeded in working out the chemistry of the reaction. They examined the products of the fermentation of glucose by *B. coli* and *B. lactis aerogenes*. Both organisms yield lactic, acetic, succinic and formic acids, together with ethyl alcohol, carbon dioxide, and hydrogen, but when a quantitative estimation was made, it was found that in the case of *B. lactis aerogenes* only about two-thirds of the carbon from the glucose could be accounted for in this way. They showed that the remainder of the carbon is used in the formation of a certain amount of 2 : 3 butylene glycol, $CH_3 . CH(OH)CH(OH)CH_3$ a substance which on oxidation produces acetyl methyl carbinol $CH_3 . CH(OH)CO . CH_3$. In the presence of alkali and atmospheric oxygen the carbinol is further oxidised to diacetyl, $CH_3 . CO . CO . CH_3$, which gives a pink colour in the presence of peptone.

MacConkey (1909) made a further study of the bacilli occurring in human and animal faeces, in which he used the acetyl methyl carbinol test as well as the sugar fermentations. In 178 strains from human faeces 11 were Voges and Proskauer positive; in 67 strains from horse faeces 8 were Voges and Proskauer positive, and in 87 strains from the faeces of calf, goat, pig and goose none were Voges and Proskauer positive. All the strains produced acid and gas in lactose and clotted milk.

These results confirm those of Ferreira, Horta and Paredes (1908), who isolated 117 strains of colon bacilli from human faeces and found that only 8 of these gave a positive Voges and Proskauer test.

Bergey and Deehan (1908) introduced a very extensive classification of colon bacilli based on MacConkey's initial method, but including the Voges and Proskauer reaction together with motility, indol production, gelatine liquefaction and the fermentation of

inulin and adonite as well as saccharose and dulcite. They formed a separate sub-group for each of the possible 256 combinations given by these 8 reactions.

B. *The consideration of the gas ratios.*

Many investigators working on the sugar fermentations have given particular attention to gas production.

Theobald Smith (1895) was the first to notice that the differences in the production of hydrogen and carbon dioxide are of value in separating certain coliform bacilli. His researches were followed by those of Pammel and Pammel (1896), Grimbert (1896), Harden (1899 onwards), Howe (1904) and Keyes (1909) who succeeded in developing an accurate method for estimating the volumes of gases evolved.

Rogers and his co-workers (1914 onwards) isolated organisms of the *B. coli* type from milk, and by using a modification of Keyes's method, estimated the gases which they produce when grown in 5 c.c. of 1 per cent. dextrose broth at 30° C. These authors regard sugar fermentation as a reliable criterion. They believe, however, that acid production cannot be accurately measured on account of the frequency of secondary alkaline fermentations, and attach great importance to determinations of gas ratios because these are constant under uniform conditions.

They showed that coliform bacilli yield a mixture of hydrogen and carbon dioxide, and the ratio of the two gases affords a basis for classification of these bacteria. The total volume of gas which is liberated varies very considerably; the difference is almost entirely due to an increase in the amount of CO_2, the hydrogen volume remains remarkably constant. These investigators recognised three more or less well-defined groups among the 124 cultures which they examined.

Group I. 65 cultures had a CO_2H_2 ratio of approximately 1·1.

Group II. 24 cultures had a CO_2H_2 ratio falling between 1·5 and 2·0.

Group III. 35 cultures had a gas ratio CO_2H_2 of more than 2·0.

These organisms were also classified according to the saccharose-dulcite fermentations, *i.e.* on MacConkey's system, together with the gas ratio, the fermentation of adonite, starch and glycerine, the production of indol and the reduction of nitrates.

The low ratio group, which has a constant relation of $CO_2/H_2 = 1$ included all the strains which could be identified as *B. coli communis* according to MacConkey's classification, all those which were identified as *B. acidi lactici*, 50 per cent. of the *B. coli communior* and 21 per cent. of the *B. lactis aerogenes*. The group as a whole shows slightly higher activity in the production of indol and the reduction of nitrates than the high ratio group.

These investigators suggest that the low ratio organisms be called *B. coli*. This includes two sub-groups; the first type ferments saccharose, and raffinose, and not starch, inulin, or adonite (*B. coli communior*); the second ferments none of these carbohydrates (*B. coli communis*).

The second group (which includes Groups II and III previously mentioned) shows a less constant relation between the volumes of gases produced, which is approximately $CO_2/H_2 = 2$. 79 per cent. of the strains which were identified as *B. lactis aerogenes* (MacConkey), and 50 per cent. of the *B. coli communior* strains belonged to the high ratio type.

Some time later 150 cultures from bovine faeces were studied by the same investigators. None of these liquefied gelatine and all but one gave indol from tryptophane; only one of them proved to belong to the high ratio group, the remaining 149 cultures gave gas ratios varying between 0·98 and 1·20. The same authors also isolated 166 strains of colon bacilli from 33 samples of dried grass and two samples of green oats, and found that 151 belonged to the high ratio group giving values between 1·9 and 3·00, and only eight cultures giving a gas ratio of 1·06, agreed with the bovine faecal types. Seven strains produced CO_2 alone and gave an infinity ratio.

C. *The methyl red test, and its relation to the gas ratio and to the Voges and Proskauer test.*

Unfortunately there is no convenient routine method for determining gas ratios, but Clark and Lubs (1915) have discovered that there is a definite correlation between the hydrogen ion concentration and the ratio of the gases evolved. They used a synthetic medium containing 0·5 per cent. K_2HPO_4, 0·5 per cent. glucose, and 0·5 per cent. Witte's peptone, incubated 5 days—or as a minimum 3 days—at 30° C. and estimated the hydrogen ion concentration by the use of methyl red or paranitrophenol as an indicator. The low ratio organisms ($CO_2/H_2 = 1$) soon reach the limit of acidity and all growth ceases, leaving a large proportion of the sugar still unattacked; the solution is now acid to methyl red. The high ratio organisms do not produce enough acid to inhibit their growth and activity; they continue the dextrose fermentation until all the sugar is exhausted; the *p*H then increases and the medium becomes alkaline to methyl red.

Levine (1916) examined a number of colon bacilli from several different sources in which he employed the Voges and Proskauer as well as the methyl red reaction, and found that an almost perfect correlation exists between high acidity (methyl red positive) and a negative Voges and Proskauer, and low acidity (methyl red negative) and a positive Voges and Proskauer.

He also pointed out that there is a relationship between these reactions and the source of the organisms. In 117 strains from human and animal faeces all were methyl red positive and Voges and Proskauer negative, but in 39 cultures from sewage nine were found which produced acetyl carbinol and gave a negative methyl red reaction.

In another paper (1916) Levine states that out of 187 bacilli of the colon type, 31 of which were obtained from other investigators, and 156 from sewage and human and animal faeces, only organisms which gave the Voges and Proskauer reaction were methyl red negative; 159 cultures were Voges and Proskauer negative, and 28 were Voges and Proskauer positive. No bacilli isolated from faeces produced acetyl methyl carbinol (Voges and Proskauer positive) but 23 per cent. of the sewage strains gave this reaction.

Johnson (1916) examined 363 coli-like organisms from 42 samples of soil and found that the prevailing type is the *B. lactis aerogenes*. She failed to find perfect correlation between the Voges and Proskauer and the methyl red tests; out of the 261 strains which were methyl red negative, only 84 per cent. (219) were Voges and Proskauer positive.

Hulton (1916) found perfect correlation between the methyl red and the Voges and Proskauer reactions among 45 strains of coliform bacilli derived from various sources. Twelve strains from human and rabbit faeces were all Voges and Proskauer negative and methyl red positive, but of 33 strains from other and various sources—milk, water, sewage, urine, and egg powder—11 gave positive and 22 gave negative methyl red reactions.

Rogers, Clark and Lubs (1918) made a study of 177 strains of lactose-fermenting bacilli isolated from human faeces. 131 strains gave an approximate gas ratio of $CO_2/H_2 = 1{\cdot}06$; 127 of these were indol producers, all were non-gelatine liquefiers, and all were methyl red positive and Voges and Proskauer negative; 46 strains gave a gas ratio varying between 1·5 and 2·7; 10 of these were indol positive; all failed to liquefy gelatine, and all were methyl red negative and Voges and Proskauer positive.

In view of the fact that certain authors have been unable to report a perfect correlation between the methyl red and the Voges and Proskauer tests, and also because cases have been found (Levine 1916 and others) where it has been difficult to decide whether the latter reaction was positive or negative, the acetyl methyl carbinol test has recently been the subject of much intensive research.

Levine (1916) used several carbohydrates and found that those organisms which gave a positive Voges and Proskauer test in glucose gave a positive in all other sugars. Similarly those giving a negative in glucose give a negative in other carbohydrates, except maltose, in which most organisms give a trace of acetyl methyl carbinol.

D. *The Koser reaction.*

Koser (1918) investigated the growth of bacilli of the colon group in a medium containing uric acid as the only source of nitrogen. He found that *B. lactis aerogenes* is able to use the combined nitrogen of certain organic substances while *B. coli* and its allied forms cannot make use of these substances and will not grow in a medium containing uric acid unless some other source of nitrogen is available.

Chen and Rettger (1920) included this test together with the methyl red and Voges and Proskauer reactions in a comparative study of 467 strains of colon bacilli from soils and 173 strains from the faeces of men and certain animals. All the faecal strains and 20 of the soil strains were of the *B. coli* type, the remaining 447 cultures from soils resembled *B. lactis aerogenes*. All the organisms failed to liquefy gelatine when cultivated at 20° C., but 17 of the *B. lactis aerogenes* type were able to digest the gelatine when grown at 37° C., so that the medium would not become solid when placed in the refrigerator.

As regards the methyl red, the Voges and Proskauer and the Koser reactions, it was found that all cultures of *B. lactis aerogenes* were alkaline to methyl red, acetyl methyl carbinol positive and Koser positive. All cultures of *B. coli* from faeces were acid to methyl red, acetyl methyl carbinol negative and Koser negative; *i.e.* there was no growth in Koser's uric acid medium. The 20 strains of *B. coli* from soil were all methyl red positive and Voges and Proskauer negative, but 10 of them were able to grow in Koser's medium.

Indol was tested by Ehrlich's method. All the methyl red positive strains from faeces, and 15 of those from soil were indol positive, and 141 of the methyl red negative strains gave indol.

The following table gives the results obtained by various investigators who have studied the correlation of the different reactions given by coliform bacilli from various sources.

Table III.

Author	Date	Source of strains	No. of strains	CO_2:H_2		M.R.		V.P.		Indol		Gelatine	
				2	1	+	−	+	−	+	−	+	−
Ferreira, Horta and Paredes	1908	Human faeces	117	.	.	.	.	8	109	116	1	0	117
MacConkey	1909	Human faeces	178	.	.	.	.	11	167	163	15	3	175
		Horse faeces	67	.	.	.	.	8	59	58	9	7	60
		Calf, goat, pig and goose faeces	87	.	.	.	.	0	87	87	0	0	87
Rogers, Clark and Davies	1914	Milk and milk products	124	59	65	.	.	.	.	.	.	.	.
Rogers, Clark and Evans	1914	Bovine faeces	150	1	149	.	.	.	.	149	1	0	150
	1915	Grasses and grain	166*	151	8	.	.	.	.	.	.	.	.
Levine	1916	Faeces, sewage and cultures from other workers	187	.	.	159	28	28	159	.	.	.	.
		Human and animal faeces	117	.	.	117	0	0	117	.	.	.	.
		Sewage	39	.	.	30	9	9	30	.	.	.	.
Johnson	1916	Soil	363	.	.	102	261	219	144	.	.	.	.
Hulton	1916	Human and rabbit faeces	12	.	.	12	0	0	12	11	1	2	10
		Milk, water, sewage urine, egg powder	33	.	.	11	22	22	11	16	17	15	18
Rogers, Clark and Lubs	1918	Human faeces	177	46	131	131	46	46	131	137	40	0	177
Chen and Rettger	1920	Soil	467	.	.	20	447	447	20	156	311	0	467†
		Faeces (men and animals)	173	.	.	173	0	0	173	173	0	0	173

* 7 strains produced CO_2 only, and gave an infinity ratio. † When grown at 20° C.

It becomes evident by consideration of the above results that the lactose fermenting bacilli can be divided into two more or less distinct classes. The first is characterised by the low gas ratio, the acid reaction with methyl red, the negative Voges and Proskauer reaction and the inability to utilise the combined nitrogen of certain organic substances. This is the *B. coli* group which is superabundant in faeces. The presence of these bacilli in water, milk or other foods is of great significance since it indicates the probability of pollution with potentially harmful material.

The second class are the high ratio organisms which give an alkaline reaction with methyl red, a positive Voges and Proskauer test, and are able to grow in Koser's medium. *B. lactis aerogenes* belongs to this group; it has been shown to be the prevailing type in soil, but it seldom occurs in excreta and therefore is obviously of much less significance in the bacteriological examination of water supplies.

EXPERIMENTAL.

The present investigation extended over a period of 18 months and 525 potable waters were examined.

These waters were samples which came to the Laboratory for ordinary bacteriological analysis. They had been collected from various sources and represented many different types. Some of them were from deep or shallow wells, many were spring or raw river waters, but the majority had been artificially purified by storage or filtration, or disinfected by chemical means. Coliform organisms were frequently found in the untreated waters, but since many of the samples were from purified supplies, there were a number which yielded no *B. coli* in the amounts examined.

The procedure was similar in all cases. The specimen was well shaken and suitable dilutions were prepared in sterile water. Preliminary incubations were made by adding varying amounts of the sample to tubes of MacConkey's bile salt lactose peptone medium, which is a simple primary test for faecal organisms. The cultures were examined for the development of acid and gas after 24 and after 48 hours' incubation at 37° C. and those which gave a negative result were assumed to contain no lactose fermenting bacteria. Organisms of the colon group were isolated by making a plate culture from the highest dilution giving a positive reaction and picking off a number of single acid colonies for further testing. All colonies which proved to be gram negative, non-sporing bacilli, giving acid and gas in lactose, were submitted to the following tests:

1. *Litmus Milk.* Incubations were made at 37° C. for 48 hours and, if no clotting took place by the end of that time, a portion of the milk was boiled in a water bath for a few minutes. If there was still no coagulation the remainder of the culture was returned to the incubator for a further period of 3 weeks.

2. *Indol production.* Böhme's reagent was used to detect indol production in a medium containing 1 per cent. peptone. This reagent consists of two solutions:

(*a*) Paradimethylamido-benzaldehyde in absolute alcohol to which pure hydrochloric acid has been added.

(*b*) A saturated aqueous solution of potassium persulphate.

During the earlier part of the work the Böhme's reagent was added directly to a culture which had been grown at 37° C. for 48 hours, but later a 5 days' growth was used and the

indol was extracted by shaking up with a small quantity of ether before the test substance was added. It was found that in practice the first solution was usually sufficient to produce a pink colour, it was seldom necessary to add the persulphate.

3. *Methyl red and Voges and Proskauer tests.* Both reactions were tested in Clark and Lubs' glucose-phosphate-peptone medium, after incubation at 30° C. for 5 days. At the end of this time a portion of the culture was poured into a small tube, and a few drops of methyl red were added. There were a few organisms which gave doubtful reactions and these were compared with a series of standard solutions in order to determine the exact hydrogen ion concentration. The remainder of the culture was vigorously shaken with an equal volume of a 10 per cent. solution of KoH, and the tubes were returned to the incubator. A positive Voges and Proskauer reaction was apparent in 4 or 5 hours.

4. *Koser's reaction.* The medium was prepared exactly as Koser recommended, all the tubes used were specially cleaned in sodium hypochlorite and then washed in tap water and distilled water in order to exclude extraneous nitrogen. The inoculations were performed very sparingly to limit the chances of adding products of bacterial metabolism. All cultures were incubated at 30° C. for 5 days.

5. *Gelatine.* Stabs were made in nutrient gelatine broth and incubated at 20° C. They were examined after 5 days for liquefaction.

RESULTS OBTAINED.

Of the 525 samples of potable water examined 265 gave a positive presumptive test for the colon bacillus in MacConkey's bile salt medium, and 1441 strains of lactose fermenting bacilli were isolated.

REACTIONS GIVEN BY THE 1441 STRAINS.

Correlation of the methyl red and the Voges and Proskauer tests.

The total number of colonies which were tested in dextrose phosphate culture was 1441. These yielded the results shown in Table IV.

Table IV.

Total number of colonies which gave acid and gas in lactose and which were	V.P. +	V.P. 0
1. M.R. + = 1276	20	1256
2. M.R. 0 = 165	160	5

Of the 1441 strains there were 1256, 87·16 per cent., which gave a positive methyl red reaction and failed to produce acetyl methyl carbinol; 160, 11·10 per cent. gave an alkaline reaction to methyl red and a positive Voges and Proskauer test. This leaves 25 strains, 1·73 per cent., which gave anomalous reactions in the dextrose phosphate medium.

In 25 cases cultures of this kind were repeatedly replated and retested, and by this means 12 strains which had previously given either double negative or double positive results were found to give normal reactions. The 25 strains shown in the table—of which 20 were methyl red positive, Voges and Proskauer positive, and 5 were methyl red negative Voges and Proskauer negative—includes 13 strains where the anomalous reaction had been confirmed on retesting, and 12 strains which had not been further retested.

Previous workers have isolated organisms which have been either methyl red negative, Voges and Proskauer negative, or methyl red positive, Voges and Proskauer positive.

Rogers and his associates reported 16 atypical strains which gave a *p*H falling between 5·6 and 6·0, and 11 of these were Voges and Proskauer positive.

Johnson (1916) found that out of 261 methyl red negative organisms from soil only 219 gave acetyl methyl carbinol.

Burton and Rettger (1917) concluded that the low ratio organisms remain consistently methyl red positive, Voges and Proskauer negative, but that the high ratio types, *i.e.* the *B. lactis aerogenes* types, vary apparently without law as regards the *p*H obtained in any given time, and so become either methyl red positive Voges and Proskauer positive, or methol red negative Voges and Proskauer positive.

Clark and Lubs acknowledge the occurrence of doubtful tints in making the methyl red test, and Winslow and Cohen found the same difficulty.

Koser (1918) investigated 74 strains of *B. coli* and 50 strains of *B. lactis aerogenes*, and two of his organisms of the coli type gave a negative methyl red and a negative Voges and Proskauer reaction.

Chen and Rettger (1920) found 18 strains which gave a double positive reaction, and by continual replating they were able to return some of these cultures to the proper group, although they still had some cultures which repeatedly gave a positive methyl red and a positive Voges and Proskauer test.

The Koser reaction was used in the examination of the first 979 strains, but was afterwards discontinued. The following table gives the correlation of the results obtained with the methyl red and the Voges and Proskauer tests.

Table V.

Total number of colonies which gave acid and gas in lactose, and which were	Koser +	Koser 0
1. M.R. + V.P. 0 = 850	48	802
2. M.R. 0 V.P. + = 106	92	14
3. M.R. + V.P. + = 18	10	8
4. M.R. 0 V.P. 0 = 5	0	5

The correlation of this test with the methyl red and the Voges and Proskauer reaction was by no means perfect, since 48, 5·6 per cent., of the methyl red positive, Voges and Proskauer negative strains were Koser positive; and 14, 13·2 per cent., of the methyl red negative, Voges and Proskauer positive strains were Koser negative.

All the methyl red negative, Voges and Proskauer negative colonies failed to grow in Koser's medium, and therefore were most likely variants from the *B. coli* type (M.R. + V.P. 0); on the other hand the strains which gave double positive reactions were almost equally divided into Koser positive, and Koser negative organisms, and may have belonged to the *B. coli* or to the *B. lactis aerogenes* group.

The correlation of the methyl red and the Voges and Proskauer tests, with the gelatine, milk and indol tests is found in Table VI.

1256 strains were isolated which gave a methyl red positive, Voges and

Proskauer negative reaction, and 1244 of these were non-gelatine liquifiers. These organisms would all be reported as *B. coli* in any routine examination of water on the basis of the fermentation of lactose, the clotting of milk and the non-liquefaction of gelatine, and although the 128 strains which failed to produce indol may possibly be regarded as atypical varieties, yet they would still be included in the *B. coli* group for all practical purposes of diagnosis. 160 strains were found which gave a negative methyl red and a positive Voges and Proskauer reaction, but 9 of these may be excluded because they liquefied gelatine. The remaining 151 strains gave the typical reactions of *B. coli* in lactose, gelatine and milk, and a large proportion of them, 49 per cent., were able to produce indol in a medium containing peptone.

Table VI.

Total number of colonies which gave acid and gas in lactose, and which were	Gelatine	Milk	Indol	Number
1. M.R. + V.P. 0 = 1256	−	+	+	1116
	−	+	−	128
	−	−	+	0
	−	−	−	0
	+	+	+	11
	+	+	−	1
	+	−	+	0
	+	−	−	0
2. M.R. 0 V.P. + = 160	−	+	+	77
	−	+	−	74
	−	−	+	0
	−	−	−	0
	+	+	+	1
	+	+	−	6
	+	−	+	2
	+	−	−	0

Thus of the 1416 strains which were isolated from water during the present investigation, there were 1395 which could be classified in the *B. coli* group as a result of the lactose, gelatine and milk tests, but when the methyl red, and the Voges and Proskauer reactions were considered, 10·8 per cent. of these strains were identified with the *B. lactis aerogenes* type.

RESULTS CONSIDERED ACCORDING TO SAMPLES.

Of the 265 samples which gave acid and gas in MacConkey's bile salt medium there were two which only yielded organisms which gave anomalous methyl red and Voges Proskauer results. The further results obtained with the remaining 263 waters are considered in Table VII.

Among the 263 samples which were studied only one was found which failed to yield a bacillus which was non-gelatine liquefying.

The remaining 262 waters all contained an organism of the *B. coli* type as determined by the fermentation of lactose, the non-liquefaction of gelatine and the clotting of milk, and 244 of these samples contained an organism which produced indol in peptone water.

When the methyl red test is applied, however, it is found that 15 of these

Table VII.

Total number of samples which yielded a bacillus which was	Gelatine	Milk	Indol	Number
1. M.R. + V.P. 0 = 247*	−	+	+	236
	−	+	−	11
	−	−	+	0
	−	−	−	0
	+	+	+	0
	+	+	−	0
	+	−	+	0
	+	−	−	0
2. M.R. 0 V.P. + = 16†	−	+	+	8
	−	+	−	7
	−	−	+	0
	−	−	−	0
	+	+	+	0
	+	+	−	0
	+	−	+	1
	+	−	−	0

* Among these 247 samples there were 21 which included M.R. 0 V.P. + organisms as well as the M.R. + V.P. 0. In 3 of these samples the M.R. + V.P. 0 type was the more numerous. In 12 the two types were found in the same quantity of water. In 6 the M.R. + V.P. 0 organisms were less frequent than the M.R. 0 V.P. +. 11 samples contained no indol producing organisms, but among the 236 which did yield an indol positive type, there were 63 samples which also included an indol negative organism.

† None of these 16 samples gave a M.R. + V.P. 0 organism. Among the 8 samples which gave an indol producing bacillus there were 7 samples which also yielded an indol negative organism. The remaining 7 samples (containing non-gelatine liquefying bacilli) included no organism which produced indol.

samples, 5·7 per cent., yielded only bacilli which produced a negative reaction, and it may therefore be assumed that these waters contained no lactose fermenting organisms except those of the *B. lactis aerogenes* group.

Sugar fermenting results.

961 of the strains which were isolated during the early part of the work were classified according to Kligler's method on the basis of the fermentation of saccharose and salicin, but the results obtained appeared to have little significance.

SUMMARY AND CONCLUSIONS.

The present investigation deals with the routine bacteriological examination of 525 samples of water, 265 of which contained lactose fermenting bacilli; 1441 strains were isolated and various differential tests were used in the classification of these organisms.

All the strains were tested in dextrose phosphate culture for the methyl red and the Voges and Proskauer reactions, and although most of them gave normal results (M.R. + V.P. 0 or M.R. 0 V.P. +), there were 25 strains, 1·73 per cent., which gave either double positive or double negative reactions. Similar cases have already been discussed in an earlier portion of this paper, and they leave no doubt that there are types which may vary from the normal as far as the methyl red and the Voges and Proskauer reactions are concerned. At the same time these abnormal types are not sufficiently numerous to make any real practical difficulties in the use of these tests for routine purposes.

The Koser test, considered by many bacteriologists to be of great differential value, was found to give imperfect correlation with the other reactions.

1395 strains were isolated which could be classified in the *B. coli* group as a result of the production of acid and gas in lactose, the clotting of milk, and the non-liquefaction of gelatine, but when the methyl red and the Voges and Proskauer results were considered it was found that nearly 11 per cent. of these strains were of the *B. lactis aerogenes* type, and were therefore of no value as indicators of faecal pollution.

The indol reactions were somewhat variable. Among 1244 strains of the *B. coli* type (M.R. + V.P. 0), more than 10 per cent. failed to produce indol; while among 151 strains of the *B. lactis aerogenes* type (M.R. 0 V.P. +), nearly 51 per cent. were indol producers. Preliminary extraction with ether was carried out with 448 of these strains before Böhme's reagent was added. With this technique it was found that among 400 methyl red positive types there were 87 strains (22 per cent.) which produced no indol, and among 48 methyl red negative strains there were 12 (25 per cent.) which gave the reaction. Chen and Rettger (1920) were unable to establish any definite correlation between the indol test and the other reactions.

When the sample results were studied, it was found that although 262 waters contained organisms which were included in the *B. coli* group on the basis of the lactose, milk and gelatine tests, nearly 6 per cent. of these samples contained only organisms which gave a methyl red negative, Voges and Proskauer positive reaction, and were probably associated with pollution from soil washings rather than contamination from faecal sources. This means that the application of the methyl red and Voges and Proskauer reaction does make a considerable practical difference in the interpretation of results in the bacteriological examination of water.

More detailed examination as regards the fermentation of the various carbohydrates is useful for purposes of classification, but is of no significance, in the light of our present knowledge, in assessing the probability of excretal pollution.

It is suggested that, in considering the results of a bacteriological examination of water, organisms should be regarded as significant which are of typical morphology and staining reaction, produce acid and gas in lactose, fail to liquefy gelatine, clot milk, and give a positive methyl red and a negative Voges and Proskauer reaction. The production of indol in a medium containing peptone may be added as an extra test if desired, but the available evidence does not suggest that failure to produce indol is a sufficient basis for the exclusion of an otherwise typical bacillus from the *B. coli* group.

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