

THE SIGNIFICANCE OF *BACT. AEROGENES* IN WATER

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THE publication of a new method for the differentiation of coliform organisms (Brown, 1921) has recently directed attention to the alleged correlation between the type of prevalent organisms in, and the nature of the source of, water supplies (see Koser, 1923). Since Escherich (1885) separated *Bact. aerogenes* from the coliform group, and Houston divided the group in "typical" and "atypical" *B. coli*, it has been recognised that, in general, fermentative characteristics of these organisms are too variable to be suitable as criteria for the identification of types (see Gurney Dixon, 1919). Most of the recent observations have been based upon the differentiation of *B. coli* from *Bact. aerogenes*. The criteria used to identify *Bact. aerogenes* are the high gas ratio (carbon dioxide : hydrogen) (Theobald Smith, 1895); the negative methyl red test (Levine, 1916); the positive Voges-Proskauer reaction (1898) and the ability to utilise carbon, the sole source of which is contained in the citrate radical (Brown, 1921). As defined by these criteria, each of the *B. coli* and *Bact. aerogenes* groups, of course, contains organisms exhibiting widely different fermentative characteristics.

The *B. coli* type has been described as the form predominant in faeces and the *Bact. aerogenes* type as that characteristically found on grains and grasses (Rogers *et al.* 1914, 1915, 1916 and Winslow and Cohen, 1918, 1). From this, the deduction has been made that the presence of *Bact. aerogenes* in water supplies is indicative of contact with merely grains and grasses and not with faecal material (see Hulton, 1916 and Levine, 1916). Were this deduction correct, the differentiation of two types would assume considerable importance in the assessing of the sanitary status of a drinking water. Although recommended by the American Public Health Association (1923), the methyl red and Voges-Proskauer tests have been said to give results so lacking in correlation with the known sanitary qualities of the waters concerned, that little reliance could justifiably be placed on them as indices of sanitary purity (Winslow and Cohen, 1918, 1; Koser, 1924, 1; and Pawan, 1925). The citrate test, however, did show some degree of correlation with the known standard of the water in the hands of Koser (1924, 1 and 2).

The work described in this paper was carried out in an attempt to assess the significance of the finding of a predominance of *Bact. aerogenes* in water supplies. It has been shown clearly that the organism is practically universally present, although in small numbers, in the normal faeces of man, and certain

domestic and wild animals; and that *B. coli* is present in soil, the contamination of which by faecal matter is decidedly improbable. The distribution in nature of the two types is, therefore, sufficiently wide and intermingled to warrant considerable care in making deductions as to the source of the organisms when isolated from water. This need for care is supported by the isolation of both types from waters of high sanitary quality and from waters exhibiting varying degrees of both recent and remote contamination. It is evident, therefore, that both types are almost certain to be present at one time in the history of any upland water, *i.e.* whether the water has merely drained virgin soil or has been grossly contaminated with faecal material. Their relative distribution will, of course, depend on the source of the water, the amount and nature of the contamination and the relative viability of the two types.

Throughout this work, the proportion of the two types has been carefully studied in relation to the degree and date of the contamination of the water containing them. The criteria used were:

(1) *Methyl red test* as described by Mackie and McCartney (1931) except that the organism was allowed to grow for *five* days in the standard glucose medium prior to the addition of the methyl red solution. Wilson (1929) has stressed the necessity of allowing 4 or 5 days to elapse, for both *B. coli* and *Bact. aerogenes* produce acid and gas with the glucose; therefore, both would give a methyl red positive result in the early stages of the fermentation. Ayres and Rupp (1918) have suggested that in the case of *Bact. aerogenes* the reversal of the reaction is due to the production of basic carbonates from secondary decomposition of organic acids. The writer believes that the discrepancy between the results with the methyl red and citrate tests described by Koser (1924, 1) may be in part due to a falsely large number of methyl red positive results owing to an insufficient period of incubation having been allowed before the performance of the methyl red test. The irregularity and variability of the methyl red tests encountered in a number of cultures by Koser (1924, 1) may be due to the same cause.

(2) *Voges-Proskauer test* as described by Mackie and McCartney (1931) except that the test was carried out after 1, 3 and 5 days' incubation (see Chen and Rettger, 1920) till a positive result was obtained. This is necessary, as, according to Paine (1927), the acetyl-methyl-carbinol on which the test depends, may be destroyed by the continued growth of the organism. This too may explain the irregularity and variability of the Voges-Proskauer test (Koser, 1924, 1).

(3) *The citrate test*. The medium elaborated by Koser (1923) (see below) was inoculated with the organism and the presence or absence of growth noted after incubation at 37° C. for 48 hours. It was essential to make the inoculation of the medium extremely light in order to obtain sharp demarcation between *B. coli* and *Bact. aerogenes*. Brown and his workers (1924) state that the enhancement of growth exhibited by *Bact. aerogenes* and the inhibition of growth shown by *B. coli* are trustworthy enough when restricted to citrate, as tests for the identification of the bacterial types found in domestic water supplies. Yet sometimes only slight growth resulted. The method of lead precipitation (Brown, 1921) was then found useful in distinguishing between the two types. During the experiments, which lasted for over a year, there was never noted any alteration in the power of the organisms isolated to utilise citrate during prolonged subculture. This is in keeping with the findings of Koser (1923 and 1924, 3).

1. THE ISOLATION OF *BACT. AEROGENES* FROM FAECES.

A synopsis of the work previously carried out by others with human faeces is given by Winslow and Cohen (1918, 2). Unfortunately, in the works mentioned, different tests have been applied for the identification of the organisms. The results of the different workers are, therefore, not completely comparable either with each other or with those of the writer.

J. Cruickshank (1930), in an unpublished communication to the Pathological Society of Great Britain and Ireland upon "the occurrence of *Bact. aerogenes* in the faeces," described his results, using the medium elaborated by Koser (1923), and depending on Brown's observation regarding the ability of the organism to use the carbon of the citrate radical (Brown, 1921). Of 135 samples of human faeces, Cruickshank failed to isolate *Bact. aerogenes* from only thirty-seven. In twenty-four of the latter he ascribed his failure to an overgrowth of *B. pyocyaneus*, which can grow luxuriantly in the medium, and he suggested that *Bact. aerogenes* was probably almost universally present in normal faeces.

It has been shown elsewhere (Gray, 1931) that *B. pyocyaneus* is inhibited by 0.8 per cent. of lithium chloride which allows of the free growth of most other organisms, including *Bact. aerogenes*. With a view to inhibiting by means of lithium the growth of *B. pyocyaneus* in cultures from faeces, various modifications (B, C, D and E) of Koser's medium (A) were made (see Table I).

Table I.

	Media employed				
	A (Koser)	B	C	D	E
Distilled water (c.c.)	1000	1000	1000	1000	1000
Sodium chloride (gram.)	5.0	—	—	—	—
Lithium chloride (gram.)	—	5.0	5.0	8.0	11.0
Magnesium sulphate (crystalline) (gram.)	0.2	0.2	0.2	0.2	0.2
Ammonium phosphate (gram.)	1.0	1.0	1.0	1.0	1.0
Di-potassium hydrogen phosphate (gram.)	1.0	1.0	1.0	1.0	1.0
Sodium citrate (anhydrous) (gram.)	2.0	2.0	—	—	—
Lithium citrate (anhydrous) (gram.)	—	—	2.0	2.0	2.0

Each medium was sterilised in the autoclave and its pH adjusted to between 6.7 and 6.9. About 6 c.c. was placed in each tube.

In addition, it was recently found by the writer that barium chloride in concentrations ranging from 2.5 to 6 per cent. also inhibited the growth of *B. pyocyaneus*. For the 5 gram. of sodium chloride in Koser's medium there was therefore substituted 5 gram. of barium chloride. This was labelled modification F.

Forty samples of faeces from ten healthy adult males were examined. Each sample was plated directly on MacConkey's medium, and in each of the six citrate media, A to F. After 24 hours' incubation, five organisms were isolated from the MacConkey plate, and after a further 24 hours' incubation a loopful of each of the six inoculated citrate media was subcultured on

MacConkey's medium. After incubation, five further organisms were isolated from each of the plates so inoculated. It was soon found that no advantages accrued from the use of the media B, C and D, and their use was therefore discontinued, A, E and F being the only citrate media used. The twenty organisms isolated from each sample of faeces were then subjected to the methyl red, Voges-Proskauer and citrate tests.

The use of a modification of MacConkey's medium containing saccharose, in place of the lactose usually present, was tried for the differentiation of the saccharose-fermenting organisms, including *Bact. aerogenes* from the non-saccharose-fermenting organisms, including the typical variety of *B. coli*. The fermentation of the saccharose by numerous members of the *B. coli* group rendered the modified medium of little value, and its use was discontinued. It was found much easier to distinguish by the naked eye the colonies of *Bact. aerogenes* from those of *B. coli* when the plates had been left at room temperature for a period of 24 hours subsequent to incubation. After such an interval, the colonies of *Bact. aerogenes* become viscid and "pearly," in complete contrast to those of *B. coli*.

Of the 200 organisms isolated from the MacConkey's medium which had been directly inoculated with the faeces, all conformed to the *B. coli* type; yet by means of the citrate media thirty-seven of the forty samples of faeces were proved to contain *Bact. aerogenes*. It was found that the isolation of the organism was facilitated by lightness of the inoculation of the citrate media. The three negative samples were all from the same individual. Only thirty-four of the thirty-seven positive samples showed the presence of *Bact. aerogenes* by the sodium citrate medium, but all of the thirty-seven showed it by both the lithium and barium modifications. The three samples which gave positive results with the modifications but negative results with the original sodium citrate, were all found to contain numerous *B. pyocyaneus*.

The results of these experiments showed definitely that the isolation of *Bact. aerogenes* from faeces in which *B. pyocyaneus* were either absent or at least not numerous, was not appreciably facilitated by use of the lithium or barium modifications of the original sodium citrate medium. When, however, *B. pyocyaneus* were numerous, they tended in the original medium to overgrow the other organisms present, so that with such faeces, the isolation of *Bact. aerogenes* was much more readily accomplished by use of either of the modifications, the constituents of which inhibited the growth of the *B. pyocyaneus*.

The methods used for the isolation of *Bact. aerogenes* from human faeces were also successfully applied to the faeces of the horse, cow, sheep, pig, dog, cat, wild rabbit, wild rat and wild mouse. Similar findings were obtained in the case of the horse (MacConkey, 1909), the cow (Clemesha, 1912 and Rogers *et al.* 1914), and the pig (Heinick, 1920).

## 2. BACTERIOLOGICAL EXAMINATION OF UNPOLLUTED SOIL.

Six samples of soil, the contamination of which by faecal matter of either humans or domestic animals was improbable, were obtained from central Perthshire. Emulsions of them were inoculated directly on MacConkey's

medium. In this way, without having recourse to the use of citrate, *Bact. aerogenes* was isolated from every sample, showing that the organism was much more readily isolated than from normal faeces. Nevertheless, it was not present in large numbers. In addition, *B. coli* identified as methyl red positive, Voges-Proskauer negative and citrate negative, was isolated from five of the six samples of soil. Koser (1926) states that the *B. coli* isolated from soil, in contrast to that isolated from faeces, is usually citrate positive. The correlation described by Koser (1926) between the results of the citrate test and the origin of the strains examined was, therefore, not confirmed. In addition, occasionally faecal strains of *B. coli* were definitely citrate positive.

Re-examination of the soils demonstrated a fairly rapid decrease in the number of both types of organisms, until eventually, in about a fortnight's time, all the samples had become sterile on aerobic culture. There was no marked difference in the rates of disappearance of the two organisms. These results are in close keeping with those of Skinner and Murray (1926).

### 3. THE RELATIVE PROPORTION OF *B. COLI* AND *BACT. AEROGENES* IN LIVERPOOL WATER (a) AS DELIVERED FROM THE MAIN, AND (b) AFTER STORAGE FOR VARYING PERIODS AT ROOM TEMPERATURE IN WINCHESTER BOTTLES.

The water supply of Liverpool is examined bacteriologically twice daily (see Beattie, 1930). The samples are obtained by means of a special fitting direct from the main which supplies the laboratory and adjacent property. The examination consists of the enumeration of the total viable bacteria and the quantitative estimation of *B. coli*.

The viable bacteria present are enumerated by mixing 1 c.c. of the water with 15 c.c. nutrient agar (pH 7.8) which has been melted and cooled to 50° C. The mixture is then poured into a Petri dish and allowed to solidify. After incubation for 3 days at 37° C. the colonies are counted and each taken to represent one viable organism in the original water. The number of colonies viable at 22° C. is also estimated, gelatin being employed in place of the agar, and the plates incubated at 22° C. instead of 37° C.

Throughout this work the organisms viable at 22° C. have been almost invariably more numerous than those developing at 37° C.—presumably due to the preponderance of saprophytes. The reverse occurred only when a sample had been heavily and recently contaminated (*vide infra*).

The quantitative estimation of *B. coli* is made by a method which excludes, as far as possible, fallacies due to the irregular distribution of the organism in the water. To five large tubes, each containing 20 c.c. and one tube containing 10 c.c., double strength MacConkey's fluid medium, equal amounts of the water are added. After incubation for 48 hours, the presence of acid and gas is noted in the tubes. The results are read as *B. coli* absent in 20(5-x) c.c. and present in 20(6-x) c.c., where x is the number of 20 c.c. tubes showing acid and gas. In polluted waters, the 10 c.c. tube may show acid and gas, in which case *B. coli* is described as being present in that amount of water, and when necessary, as in the case of raw river water, etc., appropriate dilutions with sterile water are made prior to inoculation. Confirmation of the presence of *B. coli* in the tubes showing acid and gas is, of course, made by subsequent isolation of the organism on solid MacConkey medium, and the testing of its biochemical characters.

For the purpose of this special investigation, viz. to determine the proportion of *B. coli* and *Bact. aerogenes*, the contents of each of the six tubes were subcultured on plates of solid MacConkey's medium, *i.e.* whether they showed no fermentative change, acid only, or acid and gas. Five lactose-fermenting colonies from each plate were then isolated and tested as regards the morphology, staining reaction to gram's stain, methyl red, Voges-Proskauer and citrate tests. Thirty organisms were, therefore, obtained at each examination of each sample of water. Admittedly, a method whereby the organisms were isolated by direct plating of the water on MacConkey's medium, as was done with the heavily contaminated samples, would have been more accurate, theoretically, for assessing the proportion in the water of the two types. The scantiness of the organisms in the water, however (*e.g.* *B. coli* present in 80 c.c. and absent in 60 c.c.), would have necessitated the use of an unwieldy amount of both water and media. For experimental purposes the two methods were carried out in parallel experiments with a heavily contaminated sample, and the differences in the proportion of *B. coli* to *Bact. aerogenes* as determined by the two methods was small.

The thirty organisms obtained at each examination of each sample were classified by the usual tests into: (1) *B. coli* group, (2) *Bact. aerogenes* group, and (3) those organisms which exhibited characteristics not in keeping with either of the other two groups. Koser (1924, 1) states that such organisms occur chiefly among soil cultures. Many were consistently both methyl red positive and citrate positive. Yet the writer does not agree that such are necessarily non-faecal in origin.

Table II. *Sample A.*

Day of storage	Total count		<i>B. coli</i>		Proportion of types		
	37° C.	22° C.	Absent in	Present in	<i>B. coli</i>	<i>Bact. aerogenes</i>	"Group 3"
0	25	31	60	80	17	8	5
1	23	30	60	80	13	7	10
3	21	27	80	100	15	6	9
5	28	35	60	80	18	10	2
7	29	33	80	100	12	12	6
10	21	26	80	100	13	8	9
14	16	27	80	100	14	15	1
18	18	22	100	—	11	13	6
21	10	13	100	—	9	15	6
28	8	16	100	—	12	17	1
35	6	11	100	—	10	19	1
42	1	9	100	—	7	21	2
49	4	2	100	—	6	20	4
56	3	5	100	—	7	19	4

It will be noted that the total viable bacterial counts made immediately on withdrawal of the water are remarkably low. After a week's storage there was a small increase in the total count coincident with a marked increase in the proportion of *Bact. aerogenes* to *B. coli*. No cause, such as a sudden alteration in temperature, could be adduced as an explanation. While admitting that the increase is so small as not to exclude the possibility of being due to some

undetected fault in technique, its occurrence coincident with the increase in proportion of *Bact. aerogenes* to *B. coli* suggests that it was due to an actual multiplication of the *Bact. aerogenes*. Clemesha (1912), however, states that while at a certain stage in natural purification of a water, the organism multiplies, it does not do so in laboratory experiment when the water is contaminated with faeces and kept in a bottle. Reference to Table II shows the gradual increase in the ratio of *Bact. aerogenes* to *B. coli*. These results are closely in keeping with those of Winslow and Cohen (1918, 1 and 2).

The statistics in Table III were obtained with Sample B. Similar results were obtained with Sample C. Originally, all three samples, A, B and C, were obtained at the same time, from the same source, and with the same precautions. Immediately after withdrawal, all were examined. Four litres of Sample B were then contaminated with a spoonful of fresh, normal, human faeces, and to a similar quantity of Sample C there was added a like amount of fresh human faeces from which *Bact. aerogenes* could not be isolated. As was to be expected, B, and especially C, then exhibited a great preponderance of *B. coli* over *Bact. aerogenes*, but on storage, as with Sample A, the ratios became reversed.

Table III. *Sample B.*

Day of storage	Total count		<i>B. coli</i>		Proportion of types		
	37° C.	22° C.	Absent in	Present in	<i>B. coli</i>	<i>Bact. aerogenes</i>	"Group 3"
0*	23	30	60	80	16	8	6
0	6150	6230	—	10	22	3	5
1	5930	5920	—	10	20	3	7
3	3120	3010	—	10	17	6	7
5	3010	3110	—	10	11	10	9
7	860	920	—	10	10	9	11
10	69	81	—	10	12	11	8
14	72	79	—	10	9	12	9
18	93	113	—	10	11	9	10
21	65	79	—	10	11	14	5
28	59	76	—	10	12	13	5
35	48	53	—	10	10	15	5
42	48	56	—	10	9	12	9
49	40	43	—	10	7	13	10
56	35	51	10	20	6	20	4

\* Before contamination.

These experiments indicated that a predominance of *Bact. aerogenes* in a water supply was characteristic of high sanitary quality, as in Sample A, or of remote contamination as in Samples B and C. The question then arose—might such a predominance of *Bact. aerogenes* be taken as an index of safety from infection of the water with enteric or dysenteric organisms? Samples of water were therefore contaminated with *B. typhosus*, *B. paratyphosus* B and *B. dysenteriae* Y. In order to simulate as closely as possible the conditions likely to occur in nature, faeces from actual cases of infection with these organisms were used in preference to the emulsions of cultures used by the earlier investigators (see Robertson, 1898; Konradi, 1904; Jordan *et al.* 1904).

*Experiments with B. typhosus.*

Of two identical samples of water D and E, E only was contaminated, as in the above experiments, with fresh faeces from an actual case of typhoid fever. In addition to the total count, and estimation of the proportion of *Bact. aerogenes* to *B. coli* as with Samples A, B and C, attempts were made daily to isolate *B. typhosus* from Sample E by means of the brilliant green enrichment method of Browning, Gilmour and Mackie (1913). The organism was isolated on the first two days only, negative results being obtained thereafter. No differences were noted in the subsequent results with these samples from the results previously obtained with Samples A and B.

*Experiments with B. paratyphosus B.*

These experiments were carried out on Samples F and G, in a manner similar to those on Samples D and E. In addition to employing the brilliant green method for the isolation of the *B. paratyphosus* B, the lithium method was also used (Gray, 1931). By both methods the presence of the organism was demonstrated in the first two days, but the lithium method alone was successful on the third day. The results were comparable to those already obtained.

*Experiments with B. dysenteriae Y.*

These experiments, carried out on the lines above indicated, did not give such satisfactory results in that the *B. dysenteriae* was never isolated from the sample of water J, which had been contaminated with the dysenteric faeces. On storage, however, the usual reversal of the *B. coli* : *Bact. aerogenes* ratio was observed.

Water was also contaminated with the samples of soil from Perthshire, and, as was to be expected, *Bact. aerogenes* predominated over *B. coli* from the outset.

## 4. EXAMINATION OF STORED FAECES.

In addition to observing the ratio of the two types in the various samples of water, the samples of faeces which had been used to contaminate the waters were frequently examined. These experiments with both the normal and infected faeces fully confirmed the results published by Jordan (1926). In both, the *B. coli* first increased enormously in numbers (even when the faeces were left at room temperature). An irregular decline in the numbers of the *B. coli* followed coincident with an increase in the proportion of *Bact. aerogenes* present. As in the water experiments, it was difficult to determine whether this increase in the *Bact. aerogenes* was absolute or only relative to the decreasing *B. coli*. In enteric and dysenteric stools there was no evidence of any multiplication of the pathogenic organism during the storage at room temperature.



All these experiments, therefore, support the belief that a predominance of *Bact. aerogenes* is an index of safety in a water supply. Even if pathogenic bacteria do survive for long periods, as Winslow (1928) emphasises, their presence from the epidemiological standpoint is of little or no practical significance, as shown by Houston (1908). The possibility of more than one source of contamination of a water has been considered, and it can be definitely stated that a water exhibiting a predominance of *Bact. aerogenes* if contaminated by fresh faecal material immediately and invariably shows a huge increase in the numbers of the *B. coli* type present.

Little danger appears to accrue from the ingestion of *Bact. aerogenes* even in large numbers. Although Archibald (1930) has reported a case of bacillaemia due to the organism, there is no suggestion that the infection was acquired by ingestion.

#### SUMMARY.

1. *Bact. aerogenes* is practically universally present, although in small numbers in the stools of normal adult humans. The stools of one individual, however, repeatedly gave negative results. The organism has also been isolated from the faeces of horse, cow, sheep, pig, dog, cat, wild rabbit, wild rat and wild mouse.

2. The use of modifications of the citrate medium, containing lithium or barium, is useful in the isolation of *Bact. aerogenes* when *B. pyocyaneus* is abundant.

3. *Bact. aerogenes* predominates over *B. coli* in soil, the contamination of which by faecal material was unlikely. The *B. coli* isolated from such soil do not show differences by the tests used from typical faecal strains of *B. coli*.

4. In the municipal water supply of Liverpool the proportion of *Bact. aerogenes* to *B. coli* is relatively high and increases on storage.

5. In water contaminated with faeces, the proportion of *Bact. aerogenes* to *B. coli* is relatively low but is rapidly reversed on storage. This is largely due to the death of the *B. coli*, but may in part be also due to multiplication of the *Bact. aerogenes* present.

6. Preponderance of *Bact. aerogenes* over *B. coli* in a water supply is indicative of either (a) contact with soil which is not contaminated with fresh faeces, or (b) long past faecal contamination.

7. Preponderance of *Bact. aerogenes* over *B. coli* in a water supply may, for practical purposes, be regarded as an indication of freedom on the part of the water from pathogenic organisms, including *B. typhosus* and *B. paratyphosus* B.

8. The repeated examination of faeces during storage showed a marked increase followed by a gradual decrease in the total number of organisms present. The decrease coincided with an increase in the numbers of *Bact.*

*aerogenes* relative to the organisms present. Whether an absolute increase in the numbers of *Bact. aerogenes* occurred, was not determined.

9. A plea is made for uniformity among the tests adopted by future workers for the identification of coliform organisms, and an exact statement of the methods employed.

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