THE SANITARY SIGNIFICANCE OF COLIFORM BACILLI IN SOIL

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Considerable conflict exists as to whether coliform bacilli may be found in the absence of faecal pollution. The opinion expressed in the Ministry of Health Report No. 71 in 1939 was that *Bacterium coli* type I and irregular I are of intestinal origin, but that the primary habitat of the other coliform types, the citrate users, probably lay in soil and on vegetation. Parr (1939), however, held the opinion that 'all of the coliform bacteria must be thought of as possibly faecal in origin'. This opinion was unsupported by Griffin & Stuart (1940), who found the distribution of 6577 strains of coliforms in milk, water, soil, grains and faeces to suggest that *Aerobacter (aerogenes)* group and intermediates constituted the normal coliform flora of non-faecal materials while *Escherichia (Bact. coli* type I) were normal to faeces. Taylor (1942), however, studied the results of previous workers on the ecology of the coliform bacteria and found insufficient evidence to conclude that *aerogenes* and intermediate types were normal inhabitants of the soil, grasses and grains. Bardsley (1948) reported that when soil plots were watered with suspensions of *Bact. coli* type I, intermediate type I and *Bact. aerogenes* type I, all three types tended to die out gradually and that there was no question of the *aerogenes* or intermediate types growing and multiplying. In 1949, Thresh, Beale & Suckling joined with such authorities as the Ministry of Health (1939) and Topley & Wilson (1946) in stating that the probable habitat of *Bact. coli* type II, the intermediate and the *aerogenes* types, lay outside the intestine in soil and vegetation. Taylor (1951), in an examination of soils collected from widely separated locations in the United Kingdom and in Denmark, found the most prevalent coliform organism isolated to be *Bact. coli* type I. Of 75 soils examined only 43 showed any coliform bacteria present in 15 g.; of these 63 % had less than 10 coliform organisms per gram and only 11 % had more than 100 coliforms per gram. Taylor considered the evidence supported the view that soil was not the natural habitat of either the intermediate types or *Bact. coli* type II. He thought it likely that the most important habitat of all major *coli-aerogenes* types was in faeces.

Using a primary isolation temperature of 30° C., Thomas, Jones & Franklin (1951) found *aerogenes-cloacae* type to predominate in soil-polluted waters. In 1952, Thomas & McQuillin, also using a primary isolation temperature of 30° C., found all types of coliforms in grass samples taken from both grazed and ungrazed herbage. *Aerogenes-cloacae* types were in the majority, and intermediate types were well represented.

In the work reported here samples of soil were examined in order to enumerate and identify the coliforms present. It was thought that bacteria living in their natural habitat would exist in large numbers, and quantitative analyses were thus

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considered essential in the survey. The usual method used by previous workers in surveys of this kind was to inoculate a fluid medium which, after incubation, was plated out on MacConkey's medium to isolate the coliforms. When the tests were done quantitatively several tubes of fluid media were inoculated with varying amounts of the sample. Preliminary trials with this technique showed that there could be great errors. It appeared a valid enough technique when a very highly selective solid culture medium could be used to separate the various species present in the broth, as, for example, in the separation of coliforms from salmonellae, but did not appear to be a good technique when trying to separate coliforms from one another when all were able to grow equally well on the solid medium on which the broth was plated. The problem is essentially the same as attempting to isolate salmonellae from faeces using only nutrient broth as a primary medium and sub-culturing on ordinary nutrient agar. The method of Wilson, Twigg, Wright, Hendry, Cowell & Maier (1935), in which a subculture is made from the 37° C. fermented MacConkey broth into (1) 44° C. MacConkey broth for the *Bact. coli* type I and (2) citrate at 37° C. for the citrate-using coliforms, may well be free from criticism on these grounds. Its use, however, was not entirely convenient, and accordingly it was decided to grow the samples of soil in a solid MacConkey's medium thus making an immediate total coliform count. This method had the added advantage of drawing attention easily to the samples most likely to offer interest.

METHODS
Primary cultures

Samples of soil or vegetation were collected in sterile 1 oz. screw-capped bottles using a sterile scoop to collect about 10 g. (bottle approximately one-third full). In the laboratory sterile quarter-strength Ringer's solution was added to the level of the neck of the bottle, i.e. approximately twice the weight of soil. The bottles were then well shaken by hand, and as the soil had necessarily been well broken up to get it into the bottle the shaking readily produced a good mixing. After shaking, the suspended particles were allowed to settle for 15 min.

One ml. of the supernatant was added to a tube of MacConkey broth which was incubated at 37° C. for a time which depended upon the production of gas in the tube, but in any case did not exceed 3 days. One ml. of the supernatant fluid was added to each of two sterile tubes to which MacConkey agar medium (slightly modified, Clegg & Sherwood, 1947) was added at a temperature of 50° C. The tubes were 'rolled' under the cold water tap to set the mixture quickly; one tube was incubated at 37° C. to enumerate the total coliform population and the other was incubated in an accurate water-bath at 44° C.

Using this technique Clegg & Sherwood (1947) found in the examination of shell-fish that colonies appearing in the roll tube incubated at 44° C. were almost in-variably *Bact. coli* type I. From 30 samples of soil in this survey a total of 100 colonies grown in the roll tubes at 44° C. were examined and all were found to be *Bact. coli* type I. Accordingly, it was presumed throughout the remainder of the survey that well-defined red colonies appearing in the roll tubes incubated over-night at 44° C. were colonies of *Bact. coli* type I.

Isolation of strains

Colonies in the roll-tube cultures were fished by a sterile wire slightly bent at the end. The number of colonies picked in this way depended upon the counts at the different temperatures. For example, if there were approximately 10 colonies in a roll tube incubated at 37° C. all were sampled; when there were some 10 colonies in the tube incubated at 44° C. and approximately 100 in the tube incubated at 37° C. (a not uncommon type of result) 10–20 of the colonies of the latter tube were samples; samples yielding high counts at 37° C. and low or nil counts at 44° C. were specially sought. Consequently on some of the occasions, when only small numbers of colonies (1–10) were grown at 37° C. they were not picked for further study. On such occasions the coliforms were identified by an examination of the fermented MacConkey broth culture, and the sample was included amongst those shown in the tables as containing 2–10 coliforms per g. Also, when, as sometimes happened, coliforms were grown in the MacConkey broth but not in the roll tube, the count was entered in the tables as being one of 2–10 per g.

Coliforms grown in fermented MacConkey broth were always subcultured and identified except when *Bact. coli* type I appeared by roll tube culture at 44° C. to be the overwhelmingly dominant or only coliform present in the sample. In such a case when the presence of *Bact. coli* type I in the broth seemed likely a 44° C. MacConkey test was done for confirmation.

Colonies picked from the roll tubes and subcultures from the MacConkey broths were streaked on MacConkey agar plates and incubated at 37° C. overnight. It happened not infrequently that pink colonies growing in the roll tube incubated at 37° C. were found on further examination not to be true coliforms. Only those organisms which grew as red-coloured colonies on the MacConkey agar plates after subculture were considered as possible coliform bacilli. These cultures were tested for purity by plating out isolated colonies on to nutrient agar plates. After overnight incubation single colonies could be picked for differentiation.

Identification of strains

A well-isolated colony selected from those on the nutrient agar plates was carefully touched on its surface with a sterile platinum wire which was then dipped once into Koser's citrate medium (Oxoid product containing bromo-thymol-blue), taking care not to touch the side of the tube, and thereafter, without recharging, the wire was dipped into peptone water, glucose phosphate broth and MacConkey broth (all made according to Ministry of Health Report No. 71, 1939). Tests for indole production, methyl red reaction, Voges–Proskauer reaction, citrate utilization and fermentation of lactose were carried out in the following manner.

Indole test. The peptone water was incubated overnight at 37° C. About 1 ml. of ether was added to the culture which was vigorously shaken. It was allowed to stand for a few minutes when a few drops of Ehrlich's rosindole reagent were added.

Methyl-red reaction. From the glucose phosphate culture, incubated at 30° C.

for 3 days, about 5 ml. was withdrawn into a clean test-tube to which were added a few drops of methyl-red solution.

Voges–Proskauer test (Barritt's modification). Two ml. of the same glucose phosphate culture was withdrawn into a test-tube to which 0·6 ml. of a 5 % solution of α-naphthol and 0·2 ml. of a 40 % solution of potassium hydroxide was added. The tube was vigorously shaken and then allowed to stand.

Citrate utilization test. The citrate culture was incubated at 30° C. for 5 days and examined daily for turbidity and blue coloration.

Fermentation of lactose. The MacConkey broth culture was incubated at 37° C. for up to 5 days and examined daily for gas production. An organism which produced sufficient gas to fill a third or more of the inverted Durham tube within 48 hr. at 37° C. was considered to be 'typical' in respect of the fermentation of lactose. 'Atypical' strains, even those which produced a mere bubble of gas after 5 days' incubation, were placed with whichever species they had most characters in common.

44° C. *MacConkey test*. In the event of an indole-positive, methyl red-positive, Voges–Proskauer-negative and citrate-negative result being obtained in the previous tests, a subculture was made from the fermented MacConkey broth into a fresh MacConkey broth preheated in the water-bath to 44° C. This was incubated for 2 days when the production of gas at 44° C. could be observed. This restricted application of the 44° C. MacConkey test did not allow for the differentiation of irregular II and irregular VI.

The gelatine liquefaction test was not used in this work. This test differentiates *Bact. cloacae* from *Bact. aerogenes* type I and also helps to differentiate irregular III and irregular IV from *Bact. coli* type II and intermediate type I respectively.

RESULTS

The samples of soil and vegetation collected from many different sources were roughly grouped as follows:

Group I. Decaying garden vegetation mixed with soil (56 samples).

Group II. Garden soil apparently free from manure (102 samples).

Group III. Woodland soils (40 samples).

Group IV. Soil from ungrazed grassy sites (110 samples).

Group V. Soil from special grassy sites (98 samples). These 98 samples were taken from the protected grassy slopes beside enclosed reservoirs.

The chances of pollution were thought from inspection to vary from group to group being greatest in group I and descending in order to group V.

Group I. 56 samples—decaying garden vegetation

Nine samples yielded no coliforms either in the roll-tube culture or in the MacConkey broth culture. It was, therefore, concluded that no coliforms were present in 0·5 g. of each of these samples. The three coliform organisms most frequently isolated in the remaining 47 samples were *Bact. coli* type I, intermediate type I and *Bact. aerogenes* type I. The numbers in which they were found are shown in Table 1.

Table 1

	No. of samples containing			
Type of coliform	2–10/g.	10–100/g.	100–2000/g.	>2000/g.
Bact. coli type I	10	23	7	4
Intermediate type I	2	9	0	0
Bact. aerogenes type I	0	4	0	0

Two strains of *Bact. coli* type II and one of *Bact. aerogenes* type II were also isolated. They appeared to be present in only small numbers. No intermediate type II or irregular strains were isolated from these 56 samples.

Numbers of coliforms exceeding 2000 per g. were found in only four samples and the coliform isolated on each of these four occasions was *Bact. coli* type I, the numbers present in both the roll tubes incubated at 37° C. and at 44° C. being too numerous to count.

From no sample was a count exceeding 100 per g. of either *aerogenes* or intermediate strains obtained.

Bact. coli type I was by far the predominating coliform and was isolated from 44 (78·5 %) of the 56 samples. In fact from only 3 samples was a coliform isolated in the absence of *Bact. coli* type I. Two of these were samples which contained intermediate type I in numbers of less than 10 per g. The third sample was found to contain 100 coliforms per g. Apparently equally represented in this number were *Bact. aerogenes* type I, *Bact. aerogenes* type II and intermediate type I.

Intermediate type I was isolated from only 11 (19·6 %) and *Bact. aerogenes* type I from only 4 (7·1 %) of the 56 samples.

Group II. 102 samples—garden soils

Seventy-three of the 102 samples were found to contain coliform bacilli. No coliforms were isolated from 0·5 g. amounts in the remaining 29 samples. The coliform types that were found and the numbers in which they occurred are shown in Table 2.

Table 2

	No. of samples containing			
Type of coliform	2–10/g.	10–100/g.	100–2000/g.	>2000/g.
Bact. coli type I	28	18	2	2
Intermediate type I	11	15	1	1
Bact. aerogenes type I	4	3	1	0

Only three samples were found to contain more than 2000 coliforms per g. In two of these cases the coliform isolated was *Bact. coli* type I, the number of colonies in both roll tubes being too numerous to count. From the third sample the red-coloured colonies grown in the roll tube incubated at 37° C. were too numerous to count whilst none developed in the roll tube incubated at 44° C. Thirty of these colonies were picked and plated; five failed to grow on the Mac-Conkey agar plate; three grew as colourless non-coliform colonies and the

remaining 22 were identified as intermediate type I. Of these, however, only three showed 'typical' gas production in MacConkey broth at 37° C.; the others produced little more than a bubble of gas in 5 days. It thus appeared that this sample contained some thousands of intermediate type I coliforms per gram, but that the majority of them were micro-aerogenic forms. Surprisingly, the only coliform obtained from the sample by MacConkey broth culture was *Bact. coli* type I. It was supposed that this organism was present in the sample in only very small numbers, but that nevertheless it had overgrown the intermediate strains.

One further specimen of this group deserves special mention. This gave a count of 2000 *Bact. aerogenes* type I strains per gram of soil (20 colonies were picked for identification and this was the highest count of an *aerogenes* strain found in soil). Fifteen of the twenty strains isolated were found to produce only slight amounts of gas in MacConkey broth at 37° C. even after prolonged incubation; the remaining five showed 'typical' acid and gas production at 37° C., as did the *aerogenes* type I isolated by broth culture.

As shown in the table *Bact. coli* type I was the predominating coliform isolated, occurring altogether in 50 (49 %) of the 102 samples. Intermediate type I was found in 28 (27·4 %) and *Bact. aerogenes* type I in 8 (7·8 %) of the samples.

Group III. 40 samples—woodland soils

Twenty-nine of the 40 samples yielded coliforms from 0·5 g. amounts. As shown in Table 3 the numbers found were not large.

Table 3

	No. of samples containing			
Type of coliform	2–10/g.	10–100/g.	100–2000/g.	> 2000/g.
Bact. coli type I	16	6	0	0
Intermediate type I	2	6	1	0
Bact. aerogenes type I	4	7	0	0

A strain of intermediate type II was also isolated from one of these samples.

Only one count exceeded 100 per g. The coliform isolated on this occasion was identified as an intermediate type I which fermented lactose feebly.

As can be seen in Table 3 the predominating coliform was again *Bact. coli* type I, found in 22 (55·0 %) of the 40 samples. Intermediate type I was found in 9 (22·5 %) and *Bact. aerogenes* type I in 11 (27·5 %) of the samples. This was the highest percentage occurrence of *aerogenes* strains in the five groups.

Group IV. 110 samples—grassland soils

Coliforms were isolated from 0·5 g. amounts of 97 of these samples. Table 4 shows the types found and the numbers in which they occurred in the samples.

Bact. coli type II also was isolated by MacConkey broth culture from two of the samples.

Only three samples were found to contain more than 2000 coliforms per g., and one of these contained only *Bact. coli* type I. The other two samples yielded inter-

mediate type I in numbers too high to count. Not only did the vast majority of these appear to be micro-aerogenic forms but they were also somewhat atypical in that growth in Koser's citrate at 30° C. proceeded slowly; after 3 days only a faint flocculent turbidity was shown, and not until after 5 days' incubation was full turbidity and colour change apparent.

Table 4

	No. of samples containing			
Type of coliform	2–10/g.	10–100/g.	100–2000/g.	>2000/g.
Bact. coli type I	12	5	2	1
Intermediate type I	40	17	15	2
Intermediate type II	7	2	0	0
Bact. aerogenes type I	5	6	3	0

The MacConkey broth culture of one of these two samples yielded in addition to a 'typical lactose-fermenting' intermediate type I a strain of *Bact. coli* type II. It was possible that this strain was represented in the dense colony growth in the roll tube incubated at 37° C. but sampling (20 pickings were made) failed to detect it. The MacConkey broth culture of the other sample yielded only an intermediate type I strain.

Intermediate type I was by far the predominating coliform and was isolated from 74 (67·2 %) of the 110 samples. *Bact. coli* type I was isolated from only 20 (18·1 %) of the samples. As shown in Table 4 intermediate type II was also found in small numbers in 9 (8·1 %) whilst *Bact. aerogenes* type I was isolated from 14 (12·7 %).

Group V. 98 samples—protected grassland soils

As in group IV these samples were chosen from grassy banks and the results obtained were very similar. Coliforms were isolated from 0·5 g. amounts in 75 of these 98 samples. The numbers in which they were found are shown in Table 5.

Table 5

	No. of samples containing			
Type of coliform	2–10/g.	10–100/g.	100–2000/g.	>2000/g.
Bact. coli type I	8	2	0	0
Intermediate type I	36	13	11	0
Intermediate type II	6	2	0	0
Bact. aerogenes type I	3	4	1	0

No counts of more than 2000 coliforms per g. were recorded. Eleven of the samples yielded more than 100 per g. of intermediate type I, and one sample yielded more than 100 *Bact. aerogenes* type I. In this latter case all the *aerogenes* present appeared to ferment lactose feebly.

As in group IV, intermediate type I was by far the predominant coliform being isolated from 60 (61·2 %) of the 98 samples. *Bact. coli* type I was found in only

10 (10·2 %) of the samples. Intermediate type II and *Bact. aerogenes* type II were isolated on 8 occasions (8·1 %).

Whilst these examinations failed to demonstrate the regular isolation of *aerogenes* or intermediate coliforms in large numbers from soil and vegetation, an unexpectedly high number of samples was found to contain one or more coliforms. It is possible that the successful isolation of some of these coliforms merely illustrates their ubiquity and the widespread nature of faecal pollution. However, a comparison of the results in the different groups indicates that the intermediate types at least were apparently unassociated with faecal pollution.

In the table that follows (Table 6) the proportion of samples within each group yielding coliforms of different varieties is expressed as a percentage. The number of times no coliforms were obtained is similarly expressed. *Bact. aerogenes* type I and *Bact. aerogenes* type II are considered together as are intermediate type I and intermediate type II.

Table 6

	No. of samples	No. of samples containing						
		No. coliforms		*Bact. coli* type I		Inter- mediates		*Bact. aerogenes*
		No.	%	No.	%	No.	%	No.	%
Group I	56	9	16·0	44	78·5	11	19·6	4	7·1
Group II	102	29	28·4	50	49·0	28	27·4	8	7·8
Group III	40	11	27·5	22	55·0	10	25·0	11	27·5
Group IV	110	13	11·8	20	18·1	79	71·8	14	12·7
Group V	98	23	23·4	10	10·2	63	64·2	8	8·1

From a topographical consideration of the sites from which the samples were chosen it was expected that the number of polluted samples would be greatest in group I, then group II, then III, then IV, and then V. The order of incidence of samples containing *Bact. coli* type I within the groups was found, as is shown in the table, to be as follows: most in group I, then III, then II, then IV, and then V. This agrees closely with the expected order of pollution above and is thus in favour of *Bact. coli* type I being the chosen index of pollution.

On the other hand, if all the various types of coliforms were derived from the intestine and if all coliforms were of equal sanitary significance, the occurrence of negative samples should be most numerous in those groups taken from sites least exposed to pollution, i.e. in descending order groups V, IV, III, II and I. The actual descending order of incidence of negative samples was groups II, III, V, I and IV. There is no agreement here, and it would seem that the various types of coliforms do not equally indicate faecal pollution. It is possible that intermediates survive longer in soil and thus some of the results might be explained; mere survival, however, offers no explanation of the fact that a greater proportion of the less polluted soils yielded intermediates. It is possible, on the other hand, that these intermediates were established in soil, but if so, one might have thought that on occasion they would have been found in exceedingly large numbers.

FURTHER STUDIES OF SAMPLES OF SOIL FROM SELECTED GRASSY SITES

Samples of soil from the grassy slopes surrounding a protected reservoir in the Cardiff City boundary had shown very little evidence of faecal pollution as judged by the presence of *Bact. coli* type I. On the other hand, a very high proportion of the samples from these sites had yielded intermediate type I strains. It was thought desirable to make further tests for evidence of faecal pollution. It was thought that if other tests confirmed the absence of such pollution the probability that the intermediate type I did not indicate faecal pollution, would be increased. Accordingly, 33 more samples were collected, and in addition to the tests already described, special tests were made for *Clostridium welchii* and *Streptococcus faecalis*.

Methods

Media

In addition to the media used and described previously in the examination of 406 samples of soil and vegetation the following were used. Double-strength MacConkey broth in 10 ml. amounts was used to examine larger amounts of soil for coliforms. Wilson–Blair medium was used for the isolation of *Cl. welchii*. Both of these were made according to the Ministry of Health Report No. 71, 1939. Azide medium used in the isolation of *Strep. faecalis* was made according to Childs & Allen (1953).

Primary cultures

Samples of soil in approximately 50 g. amounts were collected in large sterile bottles which had been weighed. By weighing the bottle with its sample the weight of soil was known. Twice the weight of sterile saline was added and the bottle vigorously shaken. After shaking, the suspended particles were allowed to settle for 15 min.

Isolation of coliforms

As before, 1 ml. of the supernatant was added to each of two roll tubes and to 5 ml. of MacConkey broth (single strength). In addition 10 ml. were added to 10 ml. MacConkey broth (double strength).

One roll tube was incubated at 37° C. the other at 44° C., whilst both MacConkey broth cultures were incubated at 37° C. Isolation and identification of strains was made as before.

Enumerations

Once again it was assumed that all the bacteria were released from the soil into the liquor. Therefore, the number of coliforms contained in 1 g. of soil was obtained by multiplying the count at 37° C. by two. In all, 12 ml. of supernatant were cultured at 37° C. for coliforms and this was equivalent to 6 g. of soil. The numerical description 2–10 per g. again included isolation by MacConkey broth culture only.

Isolation of Clostridium welchii

Five ml. of the supernatant, equivalent to $2\frac{1}{2}$ g. of soil, was heated to 80° C. for 10 min. and added to 15 ml. of Wilson–Blair agar medium which had been melted and allowed to cool to 50° C. A plate was poured and incubated anaerobically at 37° C. When *Cl. welchii* was present the large black colonies were easily recognized and a colony was picked and tested by the Nagler reaction (Mackie & McCartney, 1953).

Isolation of Streptococcus faecalis (*resuscitation method of Childs & Allen,* 1953)

Ten ml. of the supernatant fluid, equivalent to 5 g. of soil, were added to a tube containing 5 ml. of quadruple strength nutrient broth and incubated at 37° C. for 2 hr. After this period of resuscitation 5 ml. of quadruple strength azide solution was added and the tube incubated in a water-bath at 45° C. Tubes showing growth were subcultured on MacConkey agar and the presence of *Strep. faecalis* confirmed by morphological appearances.

Results

The amount of soil sampled was greater in this series than in the series recorded under group V, and it was expected that a smaller proportion would be free from coliforms and *Bact. coli* type I in particular.

Occurrence of coliforms

Only one sample failed to yield coliforms. The numbers of coliforms obtained from the remaining thirty-two samples are shown in Table 7.

Table 7

	No. of samples containing			
Type of coliform	2–10/g.	10–100/g.	100–2000/g.	> 2000/g.
Bact. coli type I	6	1	3	0
Intermediate type I	13	4	4	1
Intermediate type II	9	0	1	0
Bact. aerogenes type I	4	1	2	0

It can be seen that only one count above 2000 per g. was recorded. This was from a sample which yielded 3000 intermediate type I strains, the majority of which appeared to be atypical in respect of lactose fermentation. Of 20 colonies picked 18 produced only a bubble of gas in MacConkey broth after 5 days' incubation at 37° C. The remaining two showed typical gas production within 2 days, as did the intermediate type I strain isolated by MacConkey broth culture.

Two samples which yielded more than one hundred *Bact. aerogenes* type I per g. are worthy of special mention. From one of these a count of 780 coliforms per g. was obtained. Twenty of the colonies were picked and 3 were identified as intermediate type I and 17 as *Bact. aerogenes* type I, i.e. by proportion about 700 of the total number per gram were *aerogenes*. Every one of the 17 *aerogenes* colonies picked was atypical in respect of lactose fermentation and only inter-

mediate type I was isolated by MacConkey broth culture. In the other sample a count of 600 *Bact. aerogenes* type I, all apparently slow fermenters, was obtained. Surprisingly, however, the MacConkey broth culture of this sample yielded an intermediate type I and an intermediate type II strain but no *aerogenes* strains. It is possible that these intermediate strains, though numerically inferior, outgrew the *aerogenes* strains in the broth culture.

Frequency of isolation of strains

Bact. coli type I was isolated from 10 specimens, i.e. from 30 % as compared with 10 % when the smaller sample was cultured in the first series recorded under group V.

Intermediate type I strains were isolated from 22, i.e. 66 % as compared with 61 % when the smaller sample was cultured.

Thus, increasing the amount of soil examined increased the proportion yielding *Bact. coli* type I three times, but the proportion yielding intermediate type I remained constant. This in itself indicates that the intermediate type I strains were more numerous in the soil. A study of Table 7 shows this to be true but not markedly so.

Ten samples were found to contain intermediate type II, i.e. from 30 % of the samples as compared with 8 % of the samples when a smaller amount of soil was cultured.

Bact. aerogenes type I was isolated from 7 samples, i.e. from approximately 21 % as compared with 8 % when a smaller sample was cultured. Only one of these contained *Bact. coli* type I.

Of the 22 samples yielding intermediate type I only 4 yielded *Bact. coli* type I; of the 11 samples not yielding intermediate type I, 6 yielded *Bact. coli* type I. There appeared, therefore, to be no association between the occurrence of these two coliforms in soil. The absence of association might be thought to be more apparent than real as there would appear to be many problems in the identification of several species in mixed culture when no reliable selective culture medium is available to help the separation. This problem is being further studied.

Occurrence of Clostridium welchii

Cl. welchii has been found in soil, water, milk, dust, sewage and the intestinal canal of man and animals (Topley & Wilson, 1946). Thresh *et al.* (E. Windle Taylor, 1949) comment that, though a few strains of the spore-bearing anaerobes may have their natural habitat in soil and decaying animal and vegetable matter, they are for the most part essentially associated with the intestine of man and animals. In the Ministry of Health pamphlet on the Bacteriological Examination of Water Supplies (Report No. 71, 1939) the presence of *Cl. welchii* in water is taken as an indication of the occurrence of faecal pollution.

Twenty-nine of the thirty-three samples of soil contained *Cl. welchii*. None of the four negatives yielded *Bact. coli* type I, but three of them yielded other coliforms in small numbers.

Assuming that *Cl. welchii* is in fact indicative of faecal pollution this result would seem to be further evidence of the virtual impossibility of obtaining a soil entirely free from animal pollution.

Occurrence of Streptococcus faecalis

Strep. faecalis occurs principally in the faeces of man and animals, which may contain up to many millions per gram. According to Thresh *et al.* (E. Windle Taylor, 1949) they are absent from virgin soil and unpolluted water. Houston (1910) found that streptococci died out rapidly in water, and Savage & Wood (1918) indicated that when human faeces, animal excreta or domestic sewage was added to water the streptococci died out rather more quickly than *Bact. coli*. On the other hand, Horrocks (1901) found streptococci to persist in sewage after the disappearance of *Bact. coli*. It would seem likely, therefore, that any source yielding *Strep. faecalis* has at some time or other been associated with manurial pollution.

Strep. faecalis was isolated from 8 of the 33 samples, and from 6 of these *Bact. coli* type I was also isolated. *Strep. faecalis* was not isolated from a sample which did not also contain *Cl. welchii*.

These further tests for *Cl. welchii* and *Strep. faecalis* failed to confirm the absence of faecal pollution. Of the 33 samples there were only 4 from which neither of these indicators of pollution was obtained. One of these 4 samples contained no coliforms in the amounts tested, 2 yielded intermediate type I by MacConkey broth culture only (i.e. in very small numbers) and 1 yielded 40 coliforms per g. (28 *Bact. aerogenes* and 12 intermediate type I).

It was not found possible to make any striking comparisons between the association of *Bact. coli* type I with the other indicators of pollution, *Cl. welchii* and *Strep. faecalis*. The occurrence of *Cl. welchii* and *Strep. faecalis* in these samples would appear to indicate contamination at some time more or less remote.

SUMMARY

Coliforms were readily obtained from many soils. In samples taken from sites exposed to animal contamination *Bact. coli* type I occurred predominantly. A large number of samples taken from grassy banks, only remotely exposed to contamination, were found to contain intermediates. It was found that the greater the probable degree of contamination of the soil the higher was the proportion of *Bact. coli* type I, and that the less the probable degree of contamination the higher was the proportion of intermediates. It is difficult to explain this merely on the basis of the prolonged survival of intermediates in soil, and it would appear obvious that the source of these intermediates lay elsewhere than in animal faeces. The numbers in which they were found in soil, however, were not of the order expected of bacteria living in their natural environment, and consequently it was equally difficult to conclude that these intermediates were actively established in soil.

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