COLI-AEROGENES BACTERIA IN SOILS

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In a review of the ecology of coliform bacteria (C. B. Taylor, 1942) it was concluded that there was no valid published evidence that *coli-aerogenes* organisms multiply in grasses, grain or soil, and that *Bacterium aerogenes* and the so-called intermediate types of organisms are not normal inhabitants of these substrates. More recent experimental work (Bardsley, 1948), in which soil plots were watered with suspensions of *Bact. coli*, intermediate type I and *Bact. aerogenes*, has shown that all these organisms tend to die out gradually in soil. Nevertheless, authoritative text-books and manuals on water examination (E. W. Taylor, 1949; Ministry of Health, 1939) still state that soil is a probable normal habitat of *Bact. coli* type II and of the intermediate types, and that vegetation is the normal habitat of *Bact. aerogenes* types I and II.

The experimental work now presented was designed to assess the prevalence of different types of *coli-aerogenes* organisms in a range of soils from several distinct habitats, varying in their exposure to faecal contamination.

EXPERIMENTAL METHODS

Samples of soil were collected aseptically from Cornwall, Surrey, Hampshire, Hertfordshire, Bedfordshire, Norfolk, Yorkshire, Northumberland, Cumberland, the Welsh Border, Snowdonia, Scotland and Denmark, and also from some of the classical plots at Rothamsted Experimental Station. The surface soil ($\frac{1}{4}$–$\frac{1}{2}$ in.) was scraped away and samples taken from the underlying $2\frac{1}{2}$–3 in. The samples were examined as soon as they were received at the laboratory, transit time ranging from 1 hr. to several days.

Types and numbers of *coli-aerogenes* organisms were determined by a modification of the method of Wilson, Twigg, Wright, Hendry, Cowell & Maier (1935).

Presumptive test. Sets of five tubes of MacConkey broth were each inoculated with 0·1, 0·01 and 0·001 g. of soil, added as 1 ml. of an aqueous dilution series, and with weighed portions of 1 g. soil; a boiling tube containing 20 ml. of MacConkey broth was inoculated with 10 g. of soil. Thus in all 15·555 g. of soil were used. The tubes were placed in an incubator at 37° C. and examined for the production of acid and gas after intervals of 24 and 48 hr.

Confirmed tests. Streak cultures on eosin-methylene blue (E.M.B.) agar plates were made from all positive tubes. These plates were incubated at 37° C. Tubes of 2% brilliant green bile broth were also inoculated from the positive tubes and incubated in a water-bath at 44 ± 0·1° C. These tubes were examined after intervals of 24 and 48 hr. for the production of gas. The E.M.B. agar plates were inspected after 24 and 48 hr., and all colonies showing marked differences in appearance were subcultured on slopes of nutrient agar.

Cultures which grew on the slopes were further confirmed by the Gram stain, the indole, methyl red, Voges-Proskauer, growth in citrate tests and the ability to produce gas in MacConkey medium at 37° C. Attention was also paid to organisms which produced acid alone or acid with a trace of gas in this confirmatory test. For such organisms incubation of the broth was continued for 7 days, and repeat tests were frequently carried out at 30° C.

It is important to stress that the quantitative methods have been carried out in accordance with official recommendations for the examination of water (Ministry of Health, 1939; American Public Health Association, 1946), and that in both the presumptive and confirmed tests the amount of gas necessary to constitute a positive reading was the complete filling of the concavity of the Durham tube.

The experiment has been so designed that it is possible to express the presumptive and confirmed numbers of *coli-aerogenes* organisms in the soil samples either as 'most probable numbers' (Ministry of Health, 1939) or as the 'number of fertile tubes' (N.F.T.) (Buchanan-Wollaston, 1941), and to determine the types of bacteria present.

RESULTS

Of seventy-five soil samples examined, fifty-five (73 %) gave a positive presumptive test, but the presence of *coli-aerogenes* bacteria was confirmed in only forty-three (57 %) (Table 1). The numbers of presumptive and confirmed *coli-aerogenes* in the

Table 1. *Distribution of types of* coli-aerogenes *bacteria in all soils examined*

No. of soils			Percentage occurrence of types of *coli-aerogenes* bacteria in confirmed positive soils						
			Bact. coli		Inter-mediate		*Bact. aerogenes*		
Examined	Positive in presumptive test	Confirmed positive	Type I	Type II	Type I	Type II	Type I	Type II	Anomalous
75	55	43	67	9	30	5	23	2	14

Table 2. *The range of 'most probable numbers' of* coli-aerogenes *organisms/g. soil*

	Presumptive test					Confirmed test			
Most probable numbers ...	<1	1–9	10–99	100–180	>180	<1	1–9	10–99	>100
Percentage of soils examined	11	12	30	30	17	33	30	26	11

different soil samples are expressed in Table 2 in the form of a frequency distribution. In the presumptive test 60 % of the soils had a probable number of 10–180 organisms/g.; in the confirmed test 63 % of the soils had less than 10 organisms/g. Only 17 % of the soils had a probable number of presumptive positives greater than 180 organisms/g. and 11 % greater than 100 organisms/g. in the confirmed test. Thus, in most of these soils, *coli-aerogenes* organisms form a numerically insignificant part of the bacterial microflora.

The figures in Table 3 show the number of positive tubes (Buchanan-Wollaston, 1941) in the presumptive test, and the percentage of these confirmed as *coli-aerogenes* organisms, for soils arranged according to vegetation type. It will be seen that the production of gas in MacConkey medium in the presumptive test is an unreliable indication of the presence of *coli-aerogenes* organisms; only 6 % of the positive tubes from the hill, moorland, swamp and heath soils, and only 28 % of the tubes from arable soils, could be confirmed as containing *coli-aerogenes* organisms.

The frequency of occurrence of different types of *coli-aerogenes* organisms is shown in Tables 1 and 4. *Bact. coli* type I was by far the most common organism

Table 3. *Confirmation of presumptive positive MacConkey tubes arranged according to vegetation type of soil*

Vegetation type	No. of presumptive positive tubes	Percentage confirmed
Arable	172	28
Pasture	174	54
Woods	134	52
Hills, moorland, heath and swamp	118	6

Table 4. *Number of isolations of types of* coli-aerogenes *organisms isolated, arranged according to vegetation type of soil*

	No. of soils		Analysis of positive cultures (number of soils in which each *coli-aerogenes* type occurred)						
			Bact. coli		Inter-mediates		*Bact. aerogenes*		
Vegetation type	Examined	Positive	Type I	Type II	Type I	Type II	Type I	Type II	Anomalous
Arable	12	11	7	1	6	0	3	0	0
Pasture	14	10	9	2	4	1	1	1	3
Woods	18	11	7	0	1	1	3	0	2
Hills, moorland, heath and swamp	22	4	2	0	1	0	1	0	1

(Table 1); it was found in 67 % of all confirmed positive soils. Intermediate type I was found in 30 % of the soils. *Bact. aerogenes* type I was found in 23 % of the soils. These data are analysed according to vegetation type in Table 4. *Coli-aerogenes* organisms were found in all but one of the arable soils, in ten of the fourteen pasture soils, in eleven of the eighteen soils from woods, but in only four of the twenty-two moorland, heath and swamp soils. *Bact. coli* type I was the most common type in all localities. Intermediate type I was relatively common in arable and pasture soils. The significance of the data relating to the distribution of other types is doubtful, but none approached the two just mentioned in frequency of occurrence.

Effect of manurial treatment

Table 5 shows the results obtained from examination of samples from those arable plots receiving no manurial treatment, annual application of farmyard manure or annual application of a complete inorganic fertilizer in Broadbalk Field, Rothamsted Experimental Station. All positive MacConkey tubes were sub-cultured, and all the *coli-aerogenes* organisms isolated were examined in detail. These figures indicate that the application of farmyard manure has increased the numbers of positive presumptive tests, but that this increase is not nearly so marked in the confirmed test. Preliminary experiments in 1949, not reported in detail here, showed much smaller differences in the presumptive positive tests between manured and unmanured plots. It seems probable that any increases in *coli-aerogenes* organisms produced by the application of farmyard manure are quite transient.

Table 5. *'Most probable numbers' of* coli-aerogenes *organisms/g. in soils from differently manured plots of Broadbalk Field, Rothamsted*

Treatment during 1950	'Most probable numbers'		Types isolated
	Presumptive	Confirmed	
Farmyard manure	> 1800 (> 1800)*	5	*Bact. coli* I (1)†
			Intermediate I (4)†
			Bact. aerogenes I (2)†
Complete inorganic fertilizer	18 (18)*	1·3	*Bact. coli* I (2)†
			Intermediate I (1)†
Control, no manure	70 (350)*	1·2	Intermediate I (6)†

* Tests carried out at 30° C. † Number of isolations.

The non-confirmation of presumptive positive tubes of MacConkey broth

A marked feature of the present investigation has been the high proportion of MacConkey broth tubes which were found to be positive after incubation for 48 hr. at 37° C. in the presumptive test but which could not be confirmed. The proportions actually confirmed were: hills, moorland, etc., 6%; arable, 28%; woods, 52%; pasture, 54% (Table 3). In some cases where no growth took place on the eosin-methylene-blue agar plates, it is probable that the false positive reactions were caused by anaerobic bacteria, not necessarily *Clostridium welchii*, since normally no gas was produced in brilliant green bile broth; gas production in this medium is a characteristic reaction of *Cl. welchii* according to Mackenzie, Taylor & Gilbert (1948). From the majority of the unconfirmed tubes of MacConkey broth, cultures were isolated which appeared to possess all the characteristics of *coli-aerogenes* bacteria but which were unable to produce significant amounts of gas in MacConkey broth. The predominant organisms resembled intermediate type I or *Bact. aerogenes* type I in their indole, methyl-red, Voges-Proskauer and citrate reactions, but lactose was fermented slowly with the production of little or no gas. The types of bacteria isolated from Broadbalk soils are shown in Table 6. A detailed examination of many of these cultures which failed to ferment in MacConkey broth at 37° C. showed that the time and temperature of incubation

and the composition of the culture medium were important factors in gas production. At an incubation temperature of 30° C., many of these cultures, particularly those forming acid or acid and a bubble of gas after prolonged incubation at 37° C., produced significant amounts of gas after 2 or more days' incubation; some could be trained by successive subculture at increasingly higher temperatures to ferment at 37° C. With some soils all the cultures isolated from eosin-methylene-blue agar plates were of this type. Gas production by some cultures, particularly at 35–37° C., was much more vigorous in lactose broth than in MacConkey broth; the employment of lactose broth in the confirmed test would have given a greater number of positive tubes and thus slightly higher counts.

DISCUSSION

This survey of the density and types of *coli-aerogenes* bacteria in soils from a number of widely spaced localities in the United Kingdom and elsewhere has shown that such organisms are only present in very small numbers. Of the

Table 6. *Types of bacteria isolated on eosin/methylene-blue agar from presumptive positive MacConkey tubes*

Figures in parentheses refer to number of isolations.

Broadbalk soils	No. of presumptive positive tubes	Reactions in MacConkey broth of organisms typed by the indole, methyl-red, Voges-Proskauer and citrate tests: Gas, 2 days	Gas, 7 days	Acid, or acid and gas bubble, 7 days	No reaction
Farmyard manure	20	*Bact. coli* I (1) Intermediate I (4) *Bact. aerogenes* I (2)	Intermediate I (5) *Bact. aerogenes* I (6)	Intermediate I (12) *Bact. aerogenes* I (6)	*Bact. coli* II (2) *Bact. aerogenes* I (1)
Complete inorganic fertilizer	10	*Bact. coli* I (2) Intermediate I (1)	Intermediate I (3)	Intermediate I (6) *Bact. aerogenes* I (4)	*Bact. coli* II (2) Intermediate I (3) *Bact. aerogenes* I (2)
No manurial treatment	13	Intermediate I (6)	Intermediate I (1) *Bact. aerogenes* I (2)	Intermediate I (10) *Bact. aerogenes* I (2)	Intermediate I (1) *Bact. aerogenes* I (1)

samples examined, 27 % contained no *coli-aerogenes* bacteria in 15·5 g. soil; of the soils which contained *coli-aerogenes* types only 17 % had presumptive counts greater than 180 per g. and only 11 % had confirmed counts greater than 100 per g. The number of organisms was closely related to the probable animal population. Numbers were greatest in pasture lands and least in soils from remote hills, moorlands and heaths. These results confirm the work of Bardsley (1934) on acid moorland soils and of Koser (1926) and Minkewitsch (1930) on a variety of soils.

Application of farmyard manure to the Rothamsted plots produced no permanently large population of *coli-aerogenes* organisms. The presence of relatively high numbers of these organisms in soils from woods remote from domestic animals and man suggest appreciable contamination by small wild animals. Although soils from acid heaths populated by rabbits or from acid hill pastures grazed by sheep were frequently sterile, low pH alone is not necessarily the cause of such sterility, for cultures of *Bact. coli* type I were isolated from a pine plantation soil of pH 3·6 and from other soils of pH < 4·5.

The predominant type of *coli-aerogenes* bacteria isolated was *Bact. coli* type I, whatever the location or vegetation type of the soil. Intermediate type I, *Bact. aerogenes* type I and *Bact. coli* type II were the only other types of any numerical significance. Intermediate type I was more prevalent in arable and pasture soils than in other locations. The evidence supports the view that soil is not the natural habitat of either the intermediate types or *Bact. coli* type II. It is strange that this theory should still exist in view of the fact that crude sewage, improperly purified sewage effluents, liquors from septic tanks or other materials of faecal origin may contain very high populations of intermediate-type *aerogenes* organisms. It seems likely that the most important habitat of all major types of *coli-aerogenes* bacteria is in faeces.

In such soils as are subject to intermittent excretal contamination, there appear to be fluctuating populations of Gram-negative rods with characteristic coliform reactions to the indole, methyl-red, Voges-Proskauer and citrate tests, but whose reactions on lactose at 37° C. vary between the production of slight concentrations of acid to vigorous and rapid formation of gas. The evidence obtained suggests that most of those types producing little or no gas are either lactose-degraded *coli-aerogenes* organisms, or paracolon bacteria, and that they are largely of the intermediate I and *aerogenes* I types of organism. Such coliform organisms with weak lactose-fermenting powers have been termed *Paracolobactrum* by Bergey (1948). Further, though it may well be that *Bact. coli* I does not persist as long in sewage or soil as other *coli-aerogenes* organisms, the results presented above suggest that in soil this may be masked by the loss of lactose-fermenting abilities by the intermediate-*aerogenes* types, which are consequently not usually detected at 37° C.

It seems likely that false positive reactions, particularly from arable and pasture soils, are due to bacterial types which produce very small amounts of gas in the MacConkey tubes of the presumptive test but negligible amounts when isolated and tested in pure culture.

SUMMARY

The prevalence of the different types of *coli-aerogenes* bacteria has been determined in various soils collected from widely separated locations in the United Kingdom and in Denmark. Of the soils examined, 27 % contained no *coli-aerogenes* organisms in 15 g. of soil tested. The number of these organisms in the positive soils was very low; only 11 % had confirmed counts greater than 100 organisms/g. and 33 % less than 1 organism/g.

The most prevalent organism isolated was *Bacterium coli* type I, whatever the location or vegetation type of the soil; intermediate type I, *Bact. aerogenes* type I and *Bact. coli* type II were the only other organisms of numerical importance. The annual application of large amounts of either farmyard or inorganic manures produces no permanently large population of *coli-aerogenes* organisms in soils.

Presumptive tests in MacConkey broth were unreliable, since only 6 % of the

positive tubes from hill and moorland soils and 28% from arable soils were confirmed as containing *coli-aerogenes* bacteria. Such discrepancies are considered to be partly due to coliform bacteria with weak lactose-fermenting properties.

It is considered that there is now sufficient experimental evidence to discredit statements that soil is the natural habitat of *Bact. aerogenes* or intermediate types of *coli-aerogenes* organism.

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