A STUDY OF COLIFORM ORGANISMS IN THE MELBOURNE WATER SUPPLY AND IN ANIMAL FAECES, WITH OBSERVATIONS ON THEIR LONGEVITY IN FAECES AND IN SOIL

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The results recorded in this paper were obtained from March 1938 to December 1940 during an investigation into the bacterial flora of the water supply of the City of Melbourne. The catchment areas in the Main Dividing Range are densely covered with temperate rain forest (the dominant species *Eucalyptus regnans*), so that soil erosion is rare except after severe bush fires as in January 1939. The three large impounding reservoirs, Yan Yean, Maroondah and O'Shannassy, provide storage facilities and the water receives no further treatment either by filtration or chlorination. These reservoirs form three independent systems of supply connected by open channel or by pipe line to service reservoirs near the city. Most of the catchment is closed to the public, but native animals and birds, which are abundant, undoubtedly introduce faecal organisms.

The main purpose of the investigation was to make a study of the coliform organisms occurring in the water, to trace these organisms to their source and, whenever possible, to assign each of the various types to its particular habitat. In order to assist in the correlation of type with source, samples of animal faeces collected on the water sheds were examined to discover whether the coliform organisms in Australian fauna are of the kind usually encountered in the intestines of warm-blooded animals and whether the ratio of the various types is the same. Tests were also made of the relative viability of *Bact. coli* I and the intermediate, *Bact. aerogenes* and *Bact. cloacae* (I.A.C.) group in animal faeces and in soil.

THE COLIFORM TEST

The technique adopted for the study of coliform organisms was Wilson's method IV (Wilson, Twigg, Wright, Hendry, Cowell & Maier, 1935). After incubation in MacConkey or in lactose broth at 37° C. for 48 hr. the positive presumptive tubes were subcultured into (i) MacConkey or lactose broth incubated in a water-bath at 44° C. for 48 hr., and (ii) into Koser's citrate medium incubated at 37° C. also for 48 hr. By this means it was possible to distinguish true faecal *coli* I, which produces acid and gas at 44° C., from the intermediate, *aerogenes*, *cloacae* types (known for convenience as the I.A.C. group), which do not produce gas in lactose at 44° C., but, in contrast to *coli* I, are able to grow in citrate. The I.A.C. group are more generally found in soil than in faeces.

The technique was extended by plating from at least one positive presumptive tube for the study of coliform bacilli in pure culture, so that the samples were, in fact, examined by method I as well as by method IV. Three colonies were isolated from each plate which, if proved to be of typical morphology and staining reaction, were submitted to the usual confirmatory tests and classified according to Wilson's method as in an earlier investigation dealing with the examination of water supplies in England (Bardsley, 1938). The numbers of coliform bacilli and the ratios of *coli* I and I.A.C. were calculated in both methods from McCrady probability tables (Min. of Health, 1939).

The chosen enrichment medium was MacConkey's bile salt lactose broth, but the supply of bile salt failed before the work was completed. Fresh ox bile was not satisfactory because it stimulated the growth of certain coliform types (Salter, 1919), and also introduced varying amounts of fermentable sugar. Dominick & Lauter's broth (1929) and eosin methylene blue liquid medium gave results similar to those obtained with standard lactose broth (American Public Health Association, 1933).

In order to compare MacConkey and lactose broth 165 inoculations from 11 polluted water samples were cultured in each medium. The number of fermentations was slightly higher in the latter case where 36·4 % showed acid and gas compared with 32·7 % in MacConkey, but 6 of the lactose broth cultures failed to confirm, which brought the number of confirmed positive tubes to 54 in each case. The number of tubes yielding *coli* I was 43 in MacConkey's medium compared with 35 in lactose broth, while the number of tubes yielding I.A.C. in MacConkey was correspondingly lower.

The lack of uniformity in the presumptive coli count due to the use of different media for enrichment purposes (Raghavachari & Iyer, 1936) is further emphasized by the results obtained from 1616 polluted water samples, of which 1136 were incubated in MacConkey and the remaining 480 in lactose broth, for while the MacConkey cultures included 18 (1·6 %) in which certain tubes failed to

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confirm, the lactose broth included 52 (10·8%). Many of these tubes were inoculated from high dilutions of polluted waters. The 18 samples which gave unconfirmed reactions in MacConkey all had very high presumptive coliform counts. Most of them yielded strains producing abundant gas in glucose, mannitol, maltose and salicin in 24 hr. but nothing in lactose even after several days. Among the 52 samples which gave unconfirmed reactions in lactose broth there were 44 in which certain tubes produced abundant gas but no acid. About 40% of these gave no growth on plating and 30% eventually yielded sluggish or attenuated bacilli of the I.A.C. group all requiring a week or 10 days for the production of a bubble of gas in lactose. Occasionally *Proteus morgani* was isolated, but never in association with *Streptococcus faecalis* or other lactose-fermenting coccus (Atkinson & Wood, 1938). Specific tests for *S. faecalis* (Min. of Health, 1939) carried out on 542 samples of water yielded this organism only in 4·06%. Since *S. faecalis* cannot influence the presumptive coliform results except when, by chance, it is associated with *Proteus morgani* or a similar organism, it is unlikely that its presence in such a small percentage of samples affected the coliform counts in this series. In England *Clostridium welchii* in soil sometimes gave positive presumptive results in MacConkey broth which could not afterwards be confirmed (Bardsley, 1934), but it was extremely rare in water cultures in MacConkey. Routine tests for *welchii* were therefore carried out on 2000 samples of potable water in Victoria, but only 5·3% were positive and the organisms seldom occurred in numbers exceeding 1 per 100 ml. of sample. Hence this organism could hardly have been responsible for the many unconfirmed positive presumptive results obtained in lactose broth.

COMPARISON OF THE COLIFORM RESULTS BY METHODS I AND IV

At the beginning of the investigation 271 samples were examined only by the plating method, but the isolates were tested at 44° C. when the necessary water-bath became available. It is convenient to exclude these 271 samples for the moment and consider only the 1345 waters which were examined by both methods. The *coli* I and I.A.C. results are summarized in Table 1.

Table 1. *Showing the number of samples of water from which* Bact. coli *I and* I.A.C. *were isolated by methods I and IV*

Type	Method I	Method IV
Bact. coli I	1087	1202
I.A.C.	405	1098

Method IV gave a much larger proportion of I.A.C. than method I, showing that when the plating method was used I.A.C. was often missed. This is due in part to the differing rates of growth of *coli* I and the I.A.C. group in the enrichment medium, where *coli* I frequently becomes dominant and is the only organism to appear on spread plates. There is also an error due to dilution. The organisms present in smaller numbers tend to be diluted out in making the original inoculations for the presumptive test and will not therefore occur among the colonies subcultured, since it is customary to plate only from the highest dilution giving acid and gas.

The *coli* I results were in closer agreement, although even here there were 117 samples giving *coli* I by method IV, but not by method I, and conversely there were 2 samples in which *coli* I was found only by method I.

Among the 117 samples in which *coli* I was missed by the plating method there were 60 with coliform counts above 25/100 ml. and 77% of them had very high I.A.C. ratios, so that in these cases the dilution factor and the habit of plating from a single positive tube was responsible for an underestimate of the *coli* I content. There were also 22 samples with coliform counts below 5/100 ml. where the plating of more than one tube would probably have revealed the presence of *coli* I as well as I.A.C. by method I, since the two types of organism were shown to be in approximately equal numbers by method IV. The remaining 35 waters, with coliform counts between 2 and 25 per 100 ml., were a mixture of samples with low *coli* I, I.A.C. ratios and of samples where, by chance, a single tube had been plated giving only I.A.C. It happened occasionally, as will be shown later, that irregular strains were responsible for the production of gas at 44° C. by method IV. The 2 samples which yielded *coli* I by method I and not by method IV had coliform counts of 1 and 3 per 100 ml. respectively. There appeared to be no obvious explanation for the discrepancy in these results.

The I.A.C. group was always isolated by method IV when it was found by method I, and these organisms were rarely recovered by plating unless they formed a high percentage of the strains identified by method IV. Only 8 samples yielded I.A.C. by method I which had less than 25% I.A.C. by method IV.

A study of the frequency distribution of the ratios of *coli* I to I.A.C. by methods I and IV (Table 2) among the 1345 samples of polluted water examined, makes this point clearer.

By method I there were 1120 samples (83·27%) from which *coli* I or I.A.C. were isolated alone, and only 186 (13·83%) where the two types were found together. Method IV showed that in actual fact 963 (71·60%) of these samples contained both *coli* I and I.A.C. which were found alone only in 374 (27·80%) of the waters. Furthermore, *coli* I and I.A.C. were

rarely isolated from the same sample by the plating method unless they were present in relatively equal numbers, but by method IV the more even distribution of the ratios indicates that the organisms, particularly I.A.C., were detected even when present in extremely small numbers.

Table 2. *Frequency distribution of the ratios of* coli *I to* I.A.C. *in* 1345 *samples of polluted water*

Ratio *Coli* I : I.A.C.	Method I	Method IV
100 : 0	901	239
80–100 : 1	0	3
60–80 : 1	0	0
40–60 : 1	0	3
20–40 : 1	0	9
5–20 : 1	0	84
2–5 : 1	37	216
1 : 1	96	362
1 : 2–5	49	206
1 : 5–20	4	66
1 : 20–40	0	8
1 : 40–60	0	2
1 : 60–80	0	0
1 : 80–100	0	4
0 : 100	219	135
	1306*	1337†

* Thirty-nine samples showed only *coli* II, or irregulars, or both by method I.

† Eight samples showed no reaction in MacConkey at 44° C. and did not grow in citrate, indicating the probable presence of *coli* II or Irregular I.

THE 44° C. TEST FOR *BACT. COLI* I

Evidence of the specificity of the 44° C. test for *coli* I, under Australian conditions, is afforded by a study of the reactions given by 6986 strains of coliform bacilli isolated by the plating method from 1616 samples of polluted water (Table 3).

Table 3. *Classification of* 6986 *strains of coliform bacilli isolated by method I from* 1616 *samples of polluted water*

Type	No.	Percentage
Bact. coli I	4470	64
Bact. coli II	243	3·5
Intermediate I	971	13·9
Intermediate II	475	6·8
Bact. aerogenes I	385	5·5
Bact. aerogenes II	98	1·4
Bact. cloacae	32	0·5
Irregular I	206	2·9
Irregular II	19	0·27
Irregular III	3	0·04
Irregular IV	21	0·3
Irregular V	16	0·22
Irregular VI	3	0·04
Irregular VII	3	0·04
Irregular VIII	0	—
Unclassified	41	0·6
	6986	100·01

It will be seen at once that, apart from the 4470 strains of *Bact. coli* I, there were only 19 strains of irregular II and 3 strains of irregular VI which were able to produce gas in MacConkey broth at 44° C. Of these only irregular VI has affinities with the I.A.C. group rather than *coli* I and therefore the production of gas at 44° C. is very rare in organisms of the I.A.C. type.

THE CITRATE TEST FOR ORGANISMS OF THE I.A.C. GROUP

Among 16 samples of water enriched in MacConkey there were 38 % which yielded only I.A.C. from citrate-positive tubes, 25 % yielded *coli* I as well as I.A.C. and 37 % had no I.A.C., but *coli* I and non-lactose fermenters were present. The actual number of citrate-positive tubes plated was 44 and 89 strains were studied of which 65 % were I.A.C.

Among 28 samples of water enriched in lactose broth there were 46 % which yielded only I.A.C. from citrate-positive tubes, 15 % yielded *coli* I and I.A.C. and 39 % showed only non-lactose fermenters. The actual number of tubes plated out was 40, and 80 strains were studied of which 40 % were I.A.C. In both series the I.A.C. count was augmented in method IV by the growth of *coli* I and non-lactose fermenters, particularly *Pseudomonas pyocyanea*. The figures given here suggest that the error is much the same with MacConkey as with lactose broth but, in the latter case, some positive presumptive tubes were discarded because they could not be confirmed by plating (method I); had they been included, the percentage of samples yielding only non-lactose fermenters in citrate-positive culture would have been higher.

ASSOCIATION OF *BACT. COLI* II AND IRREGULARS I AND II WITH OTHER ORGANISMS IN WATER

There is some uncertainty with regard to the natural habitat of *coli* II and irregulars I and II and, therefore, when these bacteria are found in water it is important to note the source of the supply and the organisms associated with them in order to get some idea of their origin. The association of these types with other coliform organisms is given in Table 4.

Bact. coli II occurred in 74 samples. It was found 41 times with *coli* I, 31 times with I.A.C., 10 times with irregulars and 17 times alone.

The samples which yielded *coli* II were not all heavily polluted, 7 of them had a coliform count of 1 per 100 ml. and 17 others had less than 10. There were, however, 35 samples with coliform counts varying between 11 and 100, and the remaining 15 samples gave counts of more than 100. *Bact. coli* II seems to occur both in heavily polluted water and in

water which is lightly contaminated, and it is found rather more frequently with *coli* I than with I.A.C. The evidence accumulated from water in Victoria does not solve the problem of the origin of this bacillus.

Irregular I occurred in 72 samples. It was found 45 times with *coli* I, 38 times with I.A.C., 9 times with *coli* II, 3 times with other irregular types and 11 times alone. It occurred 8 times in water which gave only 1 coliform bacillus per 100 ml., 19 times where the coliform count was between 2 and 10; 40 times where it was between 11 and 100, and 5 times where there were more than 100 coliform bacilli per 100 ml.

IRREGULAR STRAINS WHICH WERE NOT CLASSIFIED

The high colloidal organic content of many samples of water caused the bacteria to clump together, and it was sometimes difficult to obtain pure cultures except after repeated plating especially when lactose broth was the enrichment medium. There were 41 irregular strains, isolated from 21 samples, which could not be classified by any known method. Their reactions, which were confirmed, are given in Table 5.

The origin of these organisms is unknown.

Table 4. *The association of* Bact. coli *I and irregulars I and II with other coliform bacilli in water*

Organisms found	No. of samples containing *Bact. coli* II	Irregular I	Irregular II
Alone	17	11	2
With *Bact. coli* I	21	16	4
With I.A.C.	10	12	0
With *Bact. coli* I and I.A.C.	16	21	0
With *Bact. coli* I, I.A.C. and irregulars	4	1	0
With *Bact. coli* I and irregulars	0	2	1
With I.A.C. and irregulars	1	0	0
With irregulars	5	0	0
With *Bact. coli* II	0	3	1
With *Bact. coli* I and II	0	2	0
With *Bact. coli* I, II and I.A.C.	0	3	0
With *Bact. coli* II and I.A.C.	0	1	0
	74	72	8

Table 5. *Showing the reactions of the unclassified irregular strains isolated from water*

Reactions of strains						No. of samples yielding type	No. of strains isolated
MR	VP	Citrate	Indol	44° C. MacConkey	Gelatin		
+	−	+	+	−	+	6	10
+	−	+	+	+	−	3	5
+	−	+	−	+	−	1	3
+	−	−	+	+	+	1	1
−	+	+	+	−	+	4	8
−	+	−	+	−	−	2	6
+	+	+	−	−	−	3	5
+	+	−	−	−	−	1	3
						21	41

of sample. Like *coli* II this bacillus seems to be found both in polluted and in relatively pure water and occurs in association with I.A.C. almost as frequently as with *coli* I.

Irregular II was found only in 8 samples. It was never associated with I.A.C. in this series of results, but it was present 5 times with *coli* I, once with *coli* II, once with irregular I, and twice alone. The coliform counts on these 8 samples were 1, 1, 8, 11, 13, 13, 30 and 50 respectively, so the organism apparently occurs both in polluted and in relatively unpolluted waters. The close association with *coli* I suggests that irregular II may also be of faecal origin.

FALSE POSITIVE REACTIONS BY METHOD IV

The numerical estimation of faecal coliform organisms may be distorted if irregular I or irregular II are present.

Irregular I, which is generally believed to be of faecal origin, produces no gas in lactose at 44° C. and is, therefore, missed by Wilson's method. Among the 1345 samples tested it was found alone 11 times and in 16 other samples it occurred in the absence of *coli* I. Assuming that irregular I is an index of faecal pollution, the use of method IV resulted in an

underestimate of the faecal coli count in about 2% of the samples.

Irregular II, which is not usually regarded as a faecal type, produces gas in lactose at 44° C. and its presence augments the *coli* I count. The results in this series of examinations suggest that it may in fact be faecal, in which case there is no error involved by including the type with *coli* I. If it is not of intestinal origin, however, the results were wrongly recorded by method IV in about 0·2% of samples, which includes two where it occurred alone and one in which it was associated with *coli* II.

Coli II, which is of doubtful origin, gives no gas in lactose at 44° C. and is unable to grow in citrate so that, like irregular I, it is missed by method IV. In this investigation *coli* II occurred in 5·5% of the samples. If it is an intestinal organism, the faecal count was underestimated in 33 samples (2·4%) where *coli* II occurred in the absence of *coli* I. If it is non-intestinal, there is no sanitary significance attached to its presence and it may safely be discounted in the final assessment of results.

Eight samples which yielded only irregular I or *coli* II by the plating method gave no reaction at all by method IV.

It has already been pointed out that the I.A.C. count is frequently overestimated by method IV because the citrate test is not entirely specific for the intermediate, *aerogenes, cloacae* group. Irregulars IV, VI and VII, which grow well in citrate, have affinities with I.A.C. and are very rarely isolated so that their influence on the findings by method IV can be ignored.

RATIO OF *COLI* I TO I.A.C. IN WATER FROM DIFFERENT SOURCES

The ratio of *coli* I to I.A.C. varies at different points on the system of supply (Table 6).

The results obtained both by method I and method IV show that in the catchments where animals and birds are plentiful *coli* I is dominant; after storage in Maroondah and in O'Shannassy reservoir I.A.C. are dominant, but by the time the water reaches the city distributing services, after passing through the open channels, the ratio of *coli* I again increases.

In a personal note Manzanete (Madrid) stated that, in his experience, I.A.C. are always most numerous in samples from stored or from stagnant water where aeration is poor. This might account for the high ratio in Maroondah reservoir and in the storm channels, but in the Yarra River and in seepage water the predominance of this group is the probable result of soil contamination.

The *coli* II ratios are everywhere low except at the source of the Yarra River and as there was only one series of collections at this point, the high ratio might have been an isolated instance and in the nature of an accident.

Table 6. *Comparison of the* coli *I:* I.A.C. *ratios in* 1345 *samples of polluted water from different sources*

Source of samples	No. of samples	Ratios: Method I: *Coli* I	Method I: *Coli* II	Method I: I.A.C.	Method I: Irregular	Method IV: *Coli* I	Method IV: I.A.C.
(1) Yan Yean supply:							
City Main	179	63	5	28	4	46	53
(2) Maroondah supply:							
Catchment	132	78	6	13	3	70	30
After storage	12	24	5	62	9	17	83
Open channel	480	74	2	20	4	54	46
City-entering main	253	85	0·5	11	3	64	36
(3) O'Shannassy supply:							
Catchment	52	72	6	17	5	59	39
After storage	24	15	8	73	4	10	90
Open channel	25	72	4	20	4	68	28
City Service reservoir	145	63	2	32	3	41	58
(4) Other sources:							
Service reservoirs	11	68	0	24	8	39	61
Source of Yarra	7	23	44	19	14	44	42
Yarra River near city	3	31	8	49	12	45	55
Storm and seepage water	19	37	4	50	9	27	73
Screen and channel deposits	3	100	0	0	0	88	12

EXAMINATION OF SCREEN AND CHANNEL DEPOSITS

The sloping walls of the open channels are covered with a growth of algae of many different species which multiply very rapidly during the hot summer

months. Later, when the growth cycle ceases, they slough away and accumulate in the sumps and settlement basins along the aqueducts. Some of this material was examined bacteriologically a few hours after collection and also after 4 days' and after 7 days' incubation at 22° C. Samples of fine charcoal and silt were also examined from a screen and a sump following the extensive bush fires of 1939 when the water carried unusual amounts of suspended matter, and in contrast to these polluted substances, 2 samples of algae were taken from one of the aqueducts at a point remote from Melbourne where coliform organisms were rarely present. One of these samples was also stored at 22° C. and retested from time to time. Table 7 gives the results of these various examinations.

Some samples of cow dung were resampled at intervals to determine whether any change occurred in the ratio of the coliform types after leaving the animal body.

TECHNIQUE OF EXAMINATION

Emulsions of the faeces were prepared using 0·5 g. in 100 ml. of sterile water. These were plated out on MacConkey or eosin-methylene-blue agar and colonies were picked for subculture. The suspensions were then diluted and examined in the same way as water by methods I and IV. Twenty-five samples were enriched in MacConkey and 48 in lactose broth. There were no unconfirmed positive presumptive tubes.

Table 7. *Examination of nine samples of leaves, algae and other debris*

Material	Days of incubation at 22° C.	Coliform count per 100 g.	*Cl. welchii* per 100 g.	Total counts per g. 22° C.	Total counts per g. 37° C.
Screen deposit of decaying algae	0	2,500	180,000	4,600,000	206,000
	4	1,800,000			
	7	170,000,000			
Colloidal matter from sump	0	600,000	60,000	5,300,000	1,520,000
	4	1,400,000			
	7	35,000,000			
Leaves under screen	0	25,000,000	180,000	7,250,000	4,260,000
	4	1,700,000,000			
Algal felt from channel wall in section where pollution was heavy	0	300	1,800	1,065,000	1,665,000
	7	11,000			
Fungus growth on screen	0	18,000,000	18,000	1,805,000	1,160,000
	7	90,000,000,000			
Screen deposit after fires	—	160,000	350	1,755,000	68,500
Sump deposit after fires	—	3,500,000	3,500	4,700,000	128,500
Algal felt from channel wall where pollution was light	—	0*	—	—	250
Algal felt from channel wall in section where pollution was light	0	25†	—	—	180
	8	5,500			
	16	8,500			

* After handshaking in water.

† After shaking in a machine.

The counts are enormously high in all the screen and sump deposits and the coliform figures are further increased after storage at 22° C. Even allowing for the sampling error, which must be very great with material of this kind, there can be no doubt that vegetable matter not only harbours bacteria but also provides food material for growth and multiplication.

EXAMINATION OF ANIMAL FAECES

Seventy-three samples of freshly voided animal faeces were examined for coliform bacilli. They include 33 samples from cow, 15 from kangaroo, 10 from wombat, 7 from rabbit, 6 from sheep, 1 from wallaby and 1 from fox.

RESULTS

The results of examination of the faeces by these three methods are given in Table 8.

Coli I is undoubtedly the dominant organism in the animal faeces tested, since it was found in every sample by methods I and IV and was present on 68 of the 69 plates which gave a growth of coliform organisms by direct culture.

The other types were comparatively rare by each of the plating methods, for I.A.C. occurred only in 5 (7%) of the samples examined by method I and in 8 (12%) of the direct plates, showing that I.A.C. is missed sometimes by method I, because it is overgrown by *coli* I in MacConkey broth. The use of Wilson's method IV, however, raised the number of

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samples containing I.A.C. to 63%. It was noted that the 6 samples of sheep dung were free from I.A.C., but that this group was isolated by method IV in at least some samples from all other animals. Kangaroo droppings always contained I.A.C., but this would have been missed in 13 samples by method I and in 10 samples by direct plating. Wilson's method also shows that 71% of the rabbit samples, 60% of the wombat and nearly 55% of the cow contained I.A.C.

Table 8. *Types of coliform organisms isolated by three methods of examination from seventy-three samples of animal faeces*

Type	Direct plating method	Wilson's method IV	Plating after enrichment (method I)
Coliform	69	73	73
Bact. coli I	68	73	73
I.A.C.	8	46	5
Bact. coli II	0	—	1
Irregular I	0	—	0
Irregular II	5	—	1
Other irregulars	0	—	0

Coli II was isolated from the single sample of wallaby faeces tested. It was intended to collect more wallaby dung but these animals became rare after the bush fires and further samplings were not possible.

The occurrence of irregular II is interesting because the origin of this organism is somewhat doubtful and therefore its presence in dung is significant. It was found 5 times by direct plating. It occurred once in sheep droppings in the absence of *coli* I, but was found with *coli* I in another sample from sheep, twice from cow and once from kangaroo. Like I.A.C. it seems to be overgrown in MacConkey broth by *coli* I, for it was isolated only once by method I, namely, in the sample of kangaroo dung which showed this same bacillus on the plate spread directly from the faecal emulsion. Irregular II produces gas in lactose at 44° C. and, therefore, causes an increase in the *coli* I count by method IV. This increase is of no importance if, as seems possible, it can be proved that this organism is of faecal origin.

CONFIRMATION OF THE CITRATE POSITIVE RESULT IN METHOD IV

The water examinations showed that growth in citrate was sometimes due to *coli* I or to non-lactose fermenters. In the faeces experiments the citrate-positive tubes from the highest dilutions were plated from each sample and all yielded I.A.C. on subculture from at least some of the positive tubes. Not all tubes were, however, confirmed in every sample, which means that the I.A.C. count was sometimes lower in reality than it appeared to be by Wilson's method alone.

Among the 84 citrate-positive tubes from 25 samples enriched in MacConkey there were only 2 that failed to confirm; 1 contained *coli* I and the other a non-lactose fermenter which grew well in citrate. Among the 188 citrate-positive tubes cultured from 48 samples enriched in lactose broth there were 141 with I.A.C., 21 which yielded only *coli* I, 23 with irregular VI, and 3 with non-lactose fermenters. Irregular VI grows in citrate and produces gas in MacConkey at 44° C., hence the presence of this type augments both the *coli* I and the I.A.C. count by method IV. This bacillus occurred only in cow dung and the reactions were confirmed by replating and retesting in every case. It was not isolated by either of the other methods of examination.

The strains isolated from citrate tubes cultured from lactose broth showed the same tendency towards the 'clumping' of *coli* I and I.A.C. that was noticed in water. The growth on plates spread from citrate was very mixed; it was often necessary to replate twice or even 3 times before pure cultures could be obtained.

FREQUENCY DISTRIBUTION OF *COLI* I TO I.A.C.

Although I.A.C. was found in 46 samples of faeces by Wilson's method, it was present in much smaller numbers than *coli* I. The frequency distribution of the ratios of *coli* I to I.A.C. by methods I and IV are given in Table 9.

Table 9. *Frequency distribution of* coli *I to* I.A.C. *in animal faeces by methods I and IV*

Ratio *Coli* I : I.A.C.	No. of samples giving ratio indicated by Method I	Method IV
100 : 0	68	27
> 10,000 : 1	0	12
1000 : 1–10,000 : 1	0	10
100 : 1–1,000 : 1	0	10
10 : 1–100 : 1	0	8
2 : 1–10 : 1	1	2
1 : 1	4	4
< 1 : 1	0	0
	73	73

It will be noted that I.A.C. was never found in greater numbers than *coli* I by either method I or IV, and it was only present by method I when the numbers of the two types were approximately equal. These results confirm the findings in England when 100 samples of human faeces showed I.A.C. more often by method IV than by method I, and where I.A.C. outnumbered *coli* I only in 1% of the samples even by method IV.

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The small number of I.A.C. compared with *coli* I suggests that these organisms, when found in faeces, are organisms of passage rather than true faecal bacteria which have their normal habitat in the intestines of animals.

CLASSIFICATION OF STRAINS ISOLATED FROM SAMPLES OF ANIMAL FAECES

There were 207 strains isolated from the 73 samples of animal faeces by direct plating, 341 by subculture of the citrate positive tubes in method IV and 269 from plating out the positive presumptive tubes by method I. The classification of these 817 strains by Wilson's method is given in Table 10.

Table 10. *Classification of* 817 *strains of coliform bacilli isolated from* 73 *samples of animal faeces*

	No. of strains isolated by		
Type of organism	Direct plating	Plating cit. +tubes method IV	Plating pre-sumptive +tubes method I
Bact. coli I	189	78	251
Bact. coli II	0	0	3
Intermediate I	1	19	2
Intermediate II	0	28	0
Aerogenes I	12	176	10
Aerogenes II	0	15	0
Cloacae	0	0	0
Irregular I	0	1	0
Irregular II	5	0	3
Irregular VI	0	23	0
Irregular (not classified)	0	1	0
	207	341	269

The number of *coli* I and I.A.C. strains isolated from faeces depended entirely on the method used; method I favoured the growth of *coli* I and method IV favoured the isolation of I.A.C. *Bact. aerogenes* apparently occurred more often in animal faeces than intermediate type although it is rarer in water, for it was by far the commonest type among the I.A.C. group isolated from the citrate-positive tubes (method IV). These strains were nearly all weak lactose fermenters and were slow in clotting milk, and this is extraordinary because *aerogenes* is usually regarded as a very vigorous fermenter. Among the 191 *aerogenes* isolated from citrate 118 (62 %) gave only a bubble of gas in lactose after 5–10 days' incubation at 37° C. The *aerogenes* isolated by direct plating gave gas in lactose in 24 hr. but 50 % of *aerogenes* found by method I also showed late gas production. Occasionally, too, the indicator was slowly bleached by cultures of *aerogenes* so that in the end the tubes contained gas but seemed to contain no acid, and they resembled the unconfirmed positive presumptive reactions already noted in water.

Slow lactose-fermenting *coli* I and intermediate type were rare. Six intermediate type out of the 47 isolated showed delayed fermentation, but only two *coli* I cultures in the whole series were slow in producing gas. In England intermediate type is sometimes a slow gas producer in lactose (Bardsley, 1934), but *aerogenes* and *coli* I very rarely need more than 24–48 hr. incubation at 37° C. to give a positive reaction.

The single irregular unclassified strain was from cow dung. It was MR −, VP +, citrate +, indol +, 44° C. +, gelatin −, and might have been a mixed culture, for it was never replated and retested.

VIABILITY OF COLIFORM ORGANISMS IN BOVINE FAECES

Five samples of cow dung were allowed to remain in the meadow and subsamples were collected at intervals, at and below the surface, for periods up to 49 days. It was found that *coli* I was present in all samples, the reactions were unchanged and the numbers were only gradually reduced. There was a greater reduction in dry-surface scrapings than in moist samples collected well below the surface. The I.A.C. group were never selected by method I or by direct plating, but they were occasionally isolated by method IV in collections from 2 samples, while in the other three the group were constantly present in very small numbers, the highest ratio being 2·76 %. There was no significant difference in the I.A.C. ratio in any of the samples, even after 49 days' exposure.

A few laboratory experiments were designed to detect possible changes in the relative numbers of *coli* I and I.A.C. by storing cow dung in a series of moist chambers at temperatures of 37°, 22° and 8° C., and also under dry conditions at room temperature. The damp chambers were of a simple type, each being a large Petri dish containing wet cotton-wool and enclosing a smaller dish of faeces. Dry storage was carried out in a glass bottle through which was drawn a current of air sterilized and dehydrated by passage through concentrated sulphuric acid. Coliform counts were made on composite samples of the faeces (Table 11).

Under moist conditions the number of coliform organisms declined more slowly than under dry conditions. At the first sampling I.A.C. formed 0·06 % of the total coliform population, but were never isolated again, indicating that they did not increase at any of the incubation temperatures. The experiment at 37° C. was discontinued after 20 days on account of heavy growths of *Mucor*.

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Table 11. *Coliform counts on bovine faeces stored at different temperatures under moist and under dry conditions*

Days	Moist 37° C.	Moist 22° C.	Moist 8° C.	Dry Room temperature
0	160,000	160,000	160,000	90,000
2	30,000	250,000	60,000	—
5	—	—	—	25,000
7	9,000	180,000	35,000	—
10	—	—	—	6,000
20	1,100	70,000	3,500	—
84	—	11,000	1,800	—

the sampling was continued. The presumptive coliform counts were carried out on a composite of 20 subsurface samples and were incubated in lactose broth because there was no bile salt available at the time of the experiment. The results are given in Table 12.

The occurrence of positive presumptive results which could not be confirmed made the reading of the tests very difficult, particularly in the control samples where there were coliform counts as high as 2500 per 100 g. in which not a single fermented tube yielded coliform organisms on plating. The same difficulty was encountered in the inoculated plots

Table 12. *Results obtained from sampling soil plots after watering with suspensions of* coli *I, intermediate I or* aerogenes *I*

Coliform type added		Organisms recovered per 100 g. of soil: Winter experiment (days) 0	15	57	120	Summer experiment (days) 0	8	12	28
Coli I	Coliform count per 100 g.	25×10^6	9×10^6	350	0	16,000	2500	6000	0
	Percentage *coli* I (method IV)	> 99·99	100	100	—	100	99·92	100	—
Intermediate I	Coliform count per 100 g.	60×10^6	6×10^6	90	0	250	90	900	0
	Percentage I.A.C. (method IV)	> 99·99	> 99·99	100	—	100	100	100	—
Aerogenes I	Coliform count per 100 g.	40×10^4	35×10^5	2500	35	25×10^4	6000	900	0
	Percentage I.A.C. (method IV)	99·73	> 99·99	100	100	99·86	100	100	—
Control	Coliform count per 100 g.	3500	6000	0	0	0	35	0	0
	Percentage I.A.C. (method IV)	81·39	100	—	—	—	100	—	—

THE LONGEVITY OF COLIFORM ORGANISMS IN SOIL

Experiments were devised to discover how long coliform organisms of different types survive in soil. The soil available for these tests was a podzol, poor in organic matter and overgrown with a mixed stringy bark (*Eucalyptus cinerea*) and tea-tree (*Leptospermum flavescens*) scrub. Four soil plots measuring 2 × 1 m. and each surrounded by a border 1 m. wide were fenced off to keep animals away and left fallow for 6 weeks. On 1 July 1940 (winter experiment) three of the plots were sprinkled with 350 ml. of an aqueous suspension of *coli* I, intermediate I, and *aerogenes* I respectively, and each suspension contained approximately 1000×10^6 bacilli per ml. The last plot received 350 ml. of sterile water and served as a control. On 11 November (summer experiment) the plots were each rewatered with 350 ml. of suspension containing 500×10^6 organisms per ml. and towards the end of each experiment when the added organisms began to die out. Wilson's method was no help because non-lactose fermenting citrate-positive strains were very common in soil and they caused confusion in the I.A.C. count. Unconfirmed fermented MacConkey broth cultures of soil were troublesome in England (Bardsley, 1934), where *Clostridium welchii* was responsible for the false reaction. *Welchii* was present here in all the false positive tubes tested, but it cannot be assumed that the production of gas in the unconfirmed tubes was due to *welchii* alone because water cultures, apparently free from *welchii*, frequently gave the same reaction. The results had to be determined by plating every lactose broth tube giving a positive presumptive result for coliform organisms at 37° C. and for *coli* I at 44° C., as well as every citrate culture showing growth at 37° C.

Coli I, intermediate I, and *aerogenes* I all tended to die out in the soil. There was no question of *aerogenes*

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or intermediate growing and multiplying under the conditions provided by the experiment, and there was no suspicion that any of the three organisms suffered profound changes in their reactions as a result of prolonged sojourn in the soil, although strains were found in all three inoculated plots which gave only a bubble of gas in lactose and showed weak clotting ability in milk after 7–10 days incubation at 37° C. There is, however, a possibility that *coli* I may die out rather more rapidly than I.A.C. In the first experiment *aerogenes* I was still viable after 4 months although the numbers were greatly depleted, whereas the plot sown with intermediate I gave a relatively low count after 2 months and the fermented tubes obtained at the next sampling (120 days) could not be confirmed. In the control plot I.A.C. was dominant at the beginning of the experiment, it was also found in the absence of *coli* I more than 4 months later after several samples had yielded only unconfirmed positive tubes. *Aerogenes* I was the organism present in each of the positive samples from the control.

It often happened that coliform bacilli other than those added were present in the samples. Thus I.A.C. was occasionally found in the *coli* I plot, and *coli* I in the I.A.C. plots. When this happened the number of invading bacteria was usually small and might have been carried by birds, blown dust or rain water. It is obvious that I.A.C. in the *coli* I plot was not due to growth of organisms originally present because the plots sown with intermediate I and *aerogenes* I did not show any sign of increase as the weeks went by, but agreed with *coli* I in a gradual decline in numbers. *Coli* II occurred once in soil with *coli* I.

The bacteria died out much more rapidly in the summer experiment, partly because there were fewer added, but also because the hot weather had a desiccating effect.

A 'soil' sample was collected from the site of an old cowshed on the catchment where the authorities had demolished a farm 3 years earlier. The following results were obtained on examination: total count per g. at 22° C., 238,500,000; and at 37° C., 71,500,000. Coliform organisms per 100 g. 2,500,000. The strains isolated by method I were *coli* I and the ratio of *coli* I: I.A.C. was 89·29: 10·71 by method IV. It is evident that in the warm moist conditions on the catchment coliform organisms die out extremely slowly, and washings from soil holding accumulated animal faeces may be the source of heavy *coli* counts in the water supply long after the land has ceased to be used as pasture.

SUMMARY

The bacteriological examinations of 1616 samples of polluted water from the Melbourne supply revealed that the production of acid and gas in MacConkey or in lactose broth was not always evidence of the presence of a coliform bacillus. There was, however, a marked difference in the number of confirmed samples in the two media. Among 1136 samples incubated in MacConkey broth coliform organisms were isolated by plating in 98·4%, but among 480 samples enriched in lactose broth 10·8% failed to confirm. MacConkey's broth is therefore recommended as the better medium for the presumptive coliform test in Melbourne water.

The technique used in the examination of 1345 samples of water and 73 samples of animal faeces was Wilson's method IV extended by plating from at least one tube giving a positive presumptive reaction at 37° C. to include method I also. In both series of tests *coli* I appeared to be by far the more numerous type by method I, but when method IV was used the intermediate, *aerogenes*, *cloacae* group (abbreviated to I.A.C.) was detected in almost as many samples in water and in 63% of the faeces where the numbers were often very low compared with *coli* I. Unless I.A.C. was present alone or in large numbers in relation to *coli* I it was frequently missed by the plating method. Even *coli* I was more often found by method IV which proved, therefore, to be a more delicate test than method I for both types of organism.

The 44° C. test for *coli* I proved to be highly specific in Victoria, since few organisms were able to produce acid and gas in MacConkey broth at 44° C. except *coli* I and organisms which had affinity with *coli* I rather than I.A.C. This contrasts with results reported from Madras (Raghavachari, 1939) and Singapore (Boizot, 1941) where the test appears to be less specific, but is in agreement with British (Bardsley, 1938; Batty-Smith, 1942) and Argentine (Ferramola, 1940) experience where the 44° C. test has also proved to be of high differential value.

Growth in citrate in method IV was sometimes due to *coli* I, non-lactose fermenters or *Pseudomonas pyocyanea*, particularly when lactose broth was the enrichment medium.

Bact. coli II and irregular I were found in heavily and in lightly polluted waters, and were associated rather more often with *coli* I than with I.A.C. They were seldom found in animal faeces. Irregular II rarely occurred among the organisms cultured, but the evidence suggests that it may be of faecal origin.

The coliform counts on decaying leaves, algae and other vegetable debris found in the sumps and on the screens were very high, and the figures showed a heavy increase after incubation.

Periodic sampling of cow dung left to dry in the open meadow showed that reduction in coliform count was very gradual and largely dependent on the moisture content, and that *coli* I was still overwhelmingly dominant 3–7 weeks after the samples

were voided. Laboratory experiments, in which cow faeces were stored in moist and in dry chambers, gave similar results.

Soil plots low in organic matter were watered with cultures of *coli* I, intermediate I or *aerogenes* I and sampled at intervals. All three types tended to die out gradually and there was no question of intermediate I or *aerogenes* I growing and multiplying under the conditions of the experiment, neither were any profound changes apparent in the biochemical reactions of the three types as a result of prolonged sojourn in soil. Soil which had been heavily contaminated with cow manure 3 years previously still contained large numbers of viable *coli* I.

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