A COMPARISON OF TWO METHODS OF ASSESSING THE NUMBER OF DIFFERENT TYPES OF COLIFORM ORGANISMS IN WATER

By DORIS A. BARDSLEY, M.Sc.

From the Department of Bacteriology and Preventive Medicine, University of Manchester

In a report by Wilson and his colleagues (1935) serious objections were raised to the present method of ascertaining the proportion of different types of the *coli-aerogenes* group of organisms in water. The officially recommended technique (*Report*, 1934) consists in making serial dilutions of the sample and inoculating portions of each dilution, and of the undiluted sample, into MacConkey's bile-salt-lactose broth. The MacConkey cultures are incubated at 37° C. for 48 hr., when the highest dilution giving acid and gas is plated out on MacConkey agar, and a varying number of colonies are picked off and tested in pure culture by the usual biochemical methods. The strains are identified, the numbers of coliform organisms are calculated from suitable probability tables, and the proportion of the different types is obtained from the results of the colony identification.

It will facilitate subsequent discussion if the more important coliform types occurring in water in this country are immediately defined. Table I is abridged from that given in Wilson's report.

There is strong reason to believe that *Bact. coli* I is the predominant organism in the intestine and rarely occurs in water unless excretal pollution has taken place at some more or less recent period. Organisms of the intermediate-*aerogenes-cloacae* type, though also found quite frequently in the intestine, are generally believed to be numerically scanty in faeces compared with *coli* I; they appear to have their main habitat in soil and on grain. Since their presence in untreated water is not nearly such an important index of recent excretal pollution as that of *Bact. coli* I, and since they differ from this organism in growing in Koser's citrate medium and in failing to produce gas in MacConkey broth at 44° C., they may be conveniently classified together as a single group—intermediate-*aerogenes-cloacae*, abbreviated to I.A.C.

It is pointed out that the official method of examination suffers in its present form from two serious disadvantages. In the first place there is an error due to dilution. If one type of organism is present in much smaller numbers than another it will probably be diluted out during the process of making the original inoculations and will not appear among the colonies picked

for examination, since it is customary to plate only from the highest dilution giving acid and gas.

In the second place, there is an error due to the varying rates of growth of different coliform types. *Bact. coli* I has a strong tendency to overgrow I.A.C. Even if *Bact. coli* I and I.A.C. are present in approximately equal numbers in the original water it is not unusual for *Bact. coli* I to gain the ascendancy during growth in MacConkey broth, so that again the colonies on the plates will often consist of only one type of organism. The proportion of the two different types as estimated by the examination of colonies picked from plates made in this way represents not the distribution of *coli* and I.A.C. in the original water, but

Table I. *Classification of coliform strains*

Type	Methyl red	Voges-Proskauer	Citrate	Indole	Gas in MacConkey broth at 44° C.	Gelatin liquefaction
Bact. coli, type I	+	−	−	+	+	−
Bact. coli, type II	+	−	−	−	−	−
Intermediate, type I	+	−	+	−	−	−
Intermediate, type II	+	−	+	+	−	−
Bact. aerogenes, type I	−	+	+	−	−	−
Bact. aerogenes, type II	−	+	+	+	−	−
Bact. cloacae	−	+	+	−	−	+
Irregular I, *coli* I-like	+	−	−	+	−	−
Irregular II, *coli* I-like	+	−	−	−	+	−
Other irregular types						

merely the distribution of *coli* and I.A.C. in the fermented MacConkey broth from which the plate was prepared. If all the fermented tubes are plated out and six or more colonies are studied from each plate, the error due to dilution can be largely avoided, but even this extremely laborious procedure cannot prevent the results of overgrowth of one type of organism by another.

Wilson and his colleagues introduced a new method of examination to obtain a more accurate estimate of the real distribution of the different types of organisms in the original water. It is both simpler and quicker than the present method, and avoids the necessity of plating and of subsequent study of the developing colonies.

It consists essentially in subculturing *every* fermented tube of MacConkey broth into two highly selective liquid media, one selective for *Bact. coli* I, the other for the I.A.C. group. By this means both the dilution fallacy and the overgrowth fallacy are largely avoided, since every fermented tube is examined and since any organism of either type, even if present in minimum numbers, is enabled to grow in one or other of the selective media provided.

The two media used are MacConkey broth incubated in a specially controlled water-bath at 44° C., and Koser's citrate medium incubated at 37° C. Gas production within 2 days in MacConkey at 44° C. is indicative of the presence of *Bact. coli* I; growth within 2 days in citrate indicates the presence of organisms of the intermediate, *aerogenes*, *cloacae* or sometimes irregular types. This method is fully described on p. 204 of Wilson's report, and is referred to as

method IV, in contrast to the officially recommended method which is referred to as method I.

Comparing these two methods Wilson and his colleagues found that by method I it was usual to obtain evidence of the presence of only one type of organism, whereas by method IV both *Bact. coli* I and I.A.C. types could usually be demonstrated together in any one sample.

The observations recorded in Wilson's report were made on milk. In view of the much greater importance of the coliform test in water than in milk it seemed desirable to find out whether method IV was applicable to water, and whether the conclusions reached by Wilson and his colleagues could be confirmed by a study of the distribution of organisms of the *coli-aerogenes* group in water, as estimated by a comparison of the two methods.

TECHNIQUE

The present report concerns 1108 samples of water submitted to the laboratory for routine analysis. Of these, 550 samples contained coliform bacilli and were examined by methods I and IV. The polluted samples were collected from various sources including 374 from town supplies, 108 from wells, springs or pumps, fifty-eight from swimming baths and ten from docks, ponds, and other heavily polluted sources. 149 of the potable waters and thirty-nine of the bath waters were chlorinated. The other samples were not treated with chemical disinfectants, but some of them were filtered.

Preliminary enrichment for both methods is carried out in MacConkey's bile-salt-lactose broth. The sample is diluted with sterile tap water, and 1 ml. of each tenfold dilution and of the undiluted sample is inoculated into each of five tubes of ordinary strength MacConkey; 10 ml. of the sample are inoculated into each of five tubes of double-strength MacConkey, and there is one 50 ml. inoculation also carried out into double-strength medium. After 48 hr. incubation at 37° C. the results are recorded and the coliform count per 100 ml. of sample is estimated with probability tables from the number of MacConkey tubes giving acid and gas at 37° C. The examination is then continued by the two different methods.

Method I

A spread plate is made from the highest dilution giving acid and gas, and three colonies are isolated in pure culture and submitted to the usual confirmatory tests. All strains must be of typical morphology and staining reaction, and must produce acid and gas in lactose peptone water, and acid and usually clot in litmus milk. They are also tested for indole, the methyl red and Voges-Proskauer reactions, liquefaction of gelatin, growth in Koser's citrate, and production of acid and gas in MacConkey broth at 44° C. The *coli* I and I.A.C. counts are obtained from the ratio in which colonies of these types are present among those isolated from the MacConkey spread plate (Table II).

Though this is the method that was usually adopted, in a considerable

number of samples tubes were plated out from more than one dilution. As will be seen later, this rendered the results of method I more favourable than would otherwise have been the case.

Table II. *The Examination of water by method I: specimen result*

Amount of sample in ml....	50	10	1	0·1	Probable counts per 100 ml. of sample	
No. of tubes ...	1	5	5	5	Absolute numbers	Percentage of total
Tubes A.G. at 37° C.	1	5	5	3	Coliform 900	100
				1 tube plated, 3 strains isolated:		
				2 *coli* I	*coli* I 600	67
				1 *aerogenes*	*aerogenes* 300	33

Note. A.G. = acid and gas.

Method IV

All MacConkey tubes giving acid and gas at 37° C. are subcultured into MacConkey broth and into citrate. The MacConkey subcultures are incubated in a carefully regulated water-bath at 44° C. for 2 days and the production of acid and gas is noted; the citrate tubes are incubated at 37° C. for 2 days to see whether growth occurs. The results are expressed numerically. Absolute numbers and the percentage ratios are obtained for *coli* I from the number of tubes positive in MacConkey at 44° C., and for I.A.C. from the number of citrate-positive tubes at 37° C. The calculation may be expressed in general terms. Where x = probable coliform count per 100 ml. of sample, a = probability figure for *coli* I from the number of MacConkey tubes positive at 44° C., and b = probability figure for I.A.C. from the number of citrate tubes showing growth at 37° C.; the absolute number for *coli* I is $x \times a/(a+b)$, and for I.A.C. $x \times b/(a+b)$. The percentage proportions are $100 \times a/(a+b)$ and $100 \times b/(a+b)$ (Table III).

Table III. *The examination of water by method IV: specimen result*

Amount of sample in ml. ...	50	10	1	0·1	Probable counts per 100 ml. of sample		
No. of tubes ...	1	5	5	5	Probable numbers	Absolute numbers	Percentage of total
MacConkey tubes A.G. at 37° C.	1	5	5	3	Coliform 900	900	100
MacConkey tubes A.G. at 44° C.	1	5	5	2	*Coli* I 600	568	63
Citrate tubes positive at 37° C.	1	5	5	1	I.A.C. 353	332	37

Note. A.G. = acid and gas.

Results of the water examinations

The positive presumptive test for the coliform group carried out in MacConkey broth at 37° C. was confirmed in each of the 550 samples of polluted water. In no instance was acid and gas formation in the medium found to be due to any other organism than a coliform bacillus. The results obtained from

examination by methods I and IV are summarized in Table IV. *Bact. coli* I and I.A.C. only are given, *Bact. coli* II and irregular types being excluded for the moment.

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

	Method I	Method IV
Bact. coli I	417	446
I.A.C.	213	449

Method IV yielded a very much higher proportion of I.A.C. than method I, showing that by the plating method I.A.C. is often missed. The *coli* I results showed more agreement. This was apparently due to the fact that *coli* I usually overgrows I.A.C. in MacConkey broth and has therefore the better chance of isolation. Nevertheless, in the present series even *coli* I was found more often by method IV.

The results of analysing the 550 samples containing coliform bacilli may be examined in more detail according to the number of tubes plated out in method I (Table V).

Table V. *Distribution of* Bact. coli *I and* I.A.C. *according to the number of positive tubes plated out in method I*

	A		B		C		D	
Group	1 tube or a selection of tubes plated out		Only one MacConkey tube positive		One dilution and more than one tube positive. All positive tubes plated		More than one dilution positive. All positive tubes plated	
Method ...	I	IV	I	IV	I	IV	I	IV
Coliform	279	279	131	131	19	19	121	121
Bact. coli I	253	271	76	78	6	7	82	90
I.A.C.	52	241	55	82	14	14	92	112

Organisms of the I.A.C. group were very often missed by method I when only one, or a few tubes were plated for examination (Table V, column A).

When only one dilution was positive and all tubes were plated out the two methods yielded substantially the same results (column C). When more than one dilution was positive, even though all positive tubes were plated, the number of I.A.C. was less by method I than by method IV (column D). The discrepancy between the results recorded in columns C and D is most easily explained on the assumption that, when organisms are present in unequal numbers in the original sample, one or other is likely to be diluted out and missed by method I.

The fact that in column B, when only one tube of one dilution was positive, the number of I.A.C. was less by method I than by method IV, indicates that I.A.C. is not infrequently outgrown by *Bact. coli* I in MacConkey broth, and is therefore likely to be absent from plates inoculated from this tube.

In order to test the truth of this explanation mixed cultures of coliform

bacilli were added to MacConkey broth, incubated at 37° C. for 48 hr., and recovered by the plating method, twenty colonies being picked off each plate. Fifteen experiments were carried out. In six tests *coli* I and I.A.C. were added in approximately equal numbers; four of them yielded no I.A.C. at all on plating, while one showed twice as many *coli* I as I.A.C. and the other four times as many. In three experiments *coli* I and I.A.C. were added in the ratio of 1 : 2 and recovered in the ratio of 20 : 1, 4 : 1 and 20 : 0. In five experiments *coli* I and I.A.C. were added in the ratio of 1 : 5; the ratios on recovery from plates were 2 : 1, 4 :1, 4 : 1, 20 : 1, 20 : 0. In the last experiment *coli* I and I.A.C. were added in the proportion of 1 : 50, and only *coli* I was recovered.

These experiments were carried out with different strains of *coli* I, intermediate and *aerogenes*, and in consequence showed some irregularity of result. The main conclusion, however, is clear, namely, that *coli* I in liquid MacConkey broth tends to overgrow the other coliform types. Plates made from such cultures are likely to reveal only the dominant type, i.e. *Bact. coli* I.

In the present series of water examinations I.A.C. were never found by method I when they were not also found by method IV, and were in fact seldom isolated by plating unless they constituted at least 25 % of the strains revealed by method IV. There were only six samples yielding I.A.C. by method I which had less than 25 % I.A.C. by method IV.

In 550 samples *Bact. coli* I was missed thirty times by method I but only once by method IV. In most of the thirty samples *Bact. coli* I was present in very small numbers. In twenty-three out of the thirty samples less than five *Bact. coli* I per 100 ml. were present. In five out of seven of the samples in which there were more than five *Bact. coli* I per 100 ml., I.A.C. were present in fairly large numbers, while in the other two samples Irregular II was present.

It may be concluded that if *Bact. coli* I is present in small numbers in relation to I.A.C. it is liable to be missed by method I. In the single sample in which *Bact. coli* I was detected by method I and not by method IV only one coliform organism was present per 100 ml., and the strain of *Bact. coli* I isolated by method I produced gas very slowly at 44° C.

Frequency distribution of ratios of Bact. coli *I to* I.A.C. *in water*

A further comparison of methods I and IV is afforded by a study of the frequency distribution of the ratios of *Bact. coli* I to I.A.C. in the 550 samples of water examined (Table VI).

It will be seen that by method I the ratios tend to be grouped about equality and the two extremes, whereas by method IV they are more evenly distributed. By method I *Bact. coli* I or I.A.C. was isolated alone in 450 of the samples; in only ninety samples were both organisms found together. By method IV, on the other hand, the two organisms were demonstrated together in no fewer than 349 of the samples. It is clear therefore that I.A.C. is often missed by method I, particularly when it is present in smaller numbers than *coli* I.

A study of Table VII, in which the ratios of the two organisms are recorded in relation to the numbers of coliform bacilli present, shows that by method I *Bact. coli* I is usually present in much larger numbers than I.A.C. It is not surprising therefore that I.A.C. is frequently diluted out and altogether missed by method I. In method IV *coli* I and I.A.C. are much more frequently found together in the same fermented tube than in method I, so that the ratio of

Table VI. *Frequency distribution of the ratios of* Bact. coli *I to* I.A.C. *in* 550 *samples of polluted water*

Ratio *Coli* I : I.A.C.	Method I	Method IV
100 : 0	327	97
80–100 : 1	0	8
60–80 : 1	0	0
40–60 : 1	0	20
20–40 : 1	0	11
5–20 : 1	4	43
2–5 : 1	32	72
1 : 1	33	92
1 : 2–5	21	72
1 : 5–20	0	21
1 : 20–40	0	2
1 : 40–60	0	4
1 : 60–80	0	0
1 : 80–100	0	4
0 : 100	123	100
Totals	540*	546†

* Ten samples showed in method I only *Bact. coli* II or irregulars or both.

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

Table VII. *Frequency distribution of the ratios of* Bact. coli *I to* I.A.C. *in water in relation to the number of coliform bacilli present*

Probable coliform count per 100 ml.	Corresponding to coliform bacilli present in	No. of samples	Percentage ratios	
			Method I *coli* I : I.A.C.	Method IV *coli* I : I.A.C.
1	100 ml.	131	100 : 71	94 : 100
2–5	50 ml.	153	100 : 42	95 : 100
6–25	10 ml.	111	100 : 34	100 : 87
26–250	1 ml.	102	100 : 18	100 : 58
>250	<1 ml.	53	100 : 25	100 : 73

coli I to I.A.C. is uniformly lower by method IV than by method I. Both methods, however, agree in showing that the ratio of *coli* I to I.A.C. increases with the degree of pollution. This is largely in accordance with expectation, since *coli* I in excretal material greatly outnumbers organisms of the I.A.C. group. In very heavily polluted waters the ratio appears to diminish again—presumably owing to actual growth of organisms of the I.A.C. group in the water.

It will be seen from the results just presented that the conclusions drawn by Wilson and his colleagues from the study of milk are amply confirmed by this examination of water.

It may, however, be objected that the success of method IV depends too much on the assumption that a positive 44° C. MacConkey test is indicative of the presence of *Bact. coli* I and a positive citrate test of the presence of I.A.C. To find out how far these assumptions are justified a series of special tests were carried out which may now be described.

Observations on the specificity of the 44° C. MacConkey test for *Bact. coli* I

Examination of water

In sixty-two samples of water the MacConkey tubes producing gas at 44° C. were plated out and colonies were picked for subculture. *Bact. coli* I was isolated from all samples. The actual number of tubes tested was 180. 178 of them yielded *coli* I, and the other two contained Irregular II, which agrees with *coli* I in its ability to produce gas in MacConkey medium at 44° C.

Further confirmation of the specificity of this test was obtained from a study of the reactions given by 2840 strains of coliform bacilli isolated by method I from 550 samples of polluted water (Table VIII).

Table VIII. *Classification of* 2840 *strains of coliform bacilli isolated by method I from* 550 *samples of polluted water*

Type	No. of strains isolated	No. of strains producing gas in MacConkey broth at 44° C.
Bact. coli I	1609	1609
Bact. coli II	64	0
Intermediate I	536	0
Intermediate II	36	0
Bact. aerogenes I	434	0
Bact. aerogenes II	49	0
Bact. cloacae	25	0
Irregular I	21	0
Irregular II	25	25
Irregular IV	3	0
Irregular V	5	0
Irregular VI	2	2
Unclassified, irregular	31	13
Totals	2840	1649

(See Wilson's report for the reactions of Irregular IV, V and VI).

Apart from the 1609 strains of *Bact. coli* I there were only forty irregular strains which produced gas in MacConkey broth. Of these, twenty-five were of the Irregular II type, which closely resembles *Bact. coli* I; two were of the Irregular VI type, which resembles *Bact. aerogenes* I; three resembled Intermediate I or II; one resembled *Bact. aerogenes* II; while nine were unclassifiable. In other words, only six out of a total of 1086 strains of I.A.C. were able to produce gas in MacConkey broth at 44° C. The specificity of this test for *Bact. coli* I or Irregular II, which closely resembles it, appears therefore to be very high.

Examination of faeces

One hundred samples of faeces were examined in the same way as water by methods I and IV, using a suspension standardized to a turbidity equivalent to 100×10^6 *coli* per ml. The results are given in Table IX.

Table IX. *Results of the examination of* 100 *samples of human faeces by methods I and IV*

Type	Producing gas in MacConkey broth at 44° C.	No. of samples containing the type as indicated by Method I	Method IV
Coliform	...	100	100
Bact. coli I	+	96	100
I.A.C.	–	10	61
Bact. coli II	–	5	–
Irregulars	–	0	–

It will be seen not only that *Bact. coli* I is undoubtedly the dominant coliform type in human faeces, but that not a single strain of any other type was isolated by Method I which gave a positive 44° C. MacConkey test. Further confirmation is afforded by the fact that from every one of 116 positive 44° C. MacConkey tubes inoculated from six samples of faeces *Bact. coli* I was isolated.

Incidentally, it may be noted that while organisms of the I.A.C. type were demonstrated in only 10% of the samples by method I, they were demonstrated by method IV in no fewer than 61%. Since our present knowledge of the frequency and numbers of organisms of the I.A.C. group in human faeces is very incomplete, it may be well to record the frequency distribution of the ratios of *Bact. coli* I to I.A.C. observed by methods I and IV (Table X).

Table X. *Frequency distribution of ratios of* Bact. coli *I to* I.A.C. *in* 100 *samples of human faeces*

Ratio *coli* I : I.A.C.	Method I	Method IV
100 : 0	90	39
>10,000 : 1	0	13
1000 : 1–10,000 : 1	0	8
100 : 1–1000 : 1	0	11
10 : 1–100 : 1	0	13
2 : 1–10 : 1	2	8
1 : 1	3	2
1 : 2–1 : 10	1	3
1 : 10–1 : 100	0	2
1 : 100–1 : 1000	0	0
1 : 1000–1 : 10,000	0	0
<1 : 10,000	0	1
0 : 100	4	0
	100	100

By method I I.A.C. was detected only when it was present in about equal numbers with, or in greater numbers than, *Bact. coli* I. Method IV yielded a very different distribution. It will be seen, however, that even by method IV

Bact. coli I was the preponderating organism in 92% of samples. In only 1% did I.A.C. greatly outnumber *Bact. coli* I.

The 100 samples of faeces were also examined by direct plating on MacConkey agar, and 327 strains of coliform bacilli were isolated (Table XI).

Table XI. *Classification of* 327 *strains of coliform bacilli isolated from* 100 *samples of human faeces by direct plating*

Type	No. of strains isolated	No. of strains producing gas in MacConkey broth at 44° C.
Bact. coli I	273	273
Bact. coli II	13	0
Intermediate type I	14	0
Intermediate type II	5	0
Bact. aerogenes I	11	0
Bact. aerogenes II	1	0
Irregular I, *coli* I-like	10	0
Totals	327	273

Again it will be seen that not a single strain of coliform organism other than *Bact. coli* I gave a positive 44° C. MacConkey result.

Taking all this evidence into consideration there seems to be no reasonable doubt that the production of gas in MacConkey broth at 44° C. is practically diagnostic of the presence of *Bact. coli* I. The only other type that gives this reaction is Irregular II. Since this organism is relatively uncommon and since, when it is found, it is often associated with *Bact. coli* I (see p. 320), it is unlikely to give rise to any serious difficulty in the interpretation of water examinations.

Observations on the specificity of the Koser citrate test for organisms of the I.A.C. group

Two hundred and eighty-two tubes of Koser's citrate medium inoculated from a corresponding number of fermented 37° C. MacConkey broths, which had been seeded from eighty-one samples of water, were plated out on MacConkey agar. Colonies were picked off and studied in the usual way. The following distribution of organisms was noted (Table XII).

Table XII. *The results of plating citrate-positive strains (method IV) in eighty-one samples of water*

Type	No. of samples containing this type
I.A.C.	73
Bact. coli I	16
Irregular strains	1
Non-lactose-fermenting strains	13

The non-lactose-fermenting strains were mostly MR+ VP−, citrate−, 44° C. MacConkey−, gelatin+, producing acid but no gas in glucose, maltose and mannitol.

It seems clear that a positive citrate test is not entirely specific for organisms of the I.A.C. group. In eight out of eighty-one samples of water other organisms were responsible for a positive reaction. In five of these samples non-lactose-fermenting organisms were responsible, in two samples *Bact. coli* I, and in one sample a mixture of non-lactose-fermenting and irregular strains. With one exception all the citrate tubes giving false positive reactions in these samples had been inoculated from fermented MacConkey tubes which had been seeded with comparatively large quantities of polluted water. Since neither *Bact. coli* I nor the non-lactose-fermenters isolated were able to grow in citrate in pure culture, it is possible that organic matter had been carried over from the fermented MacConkey tubes in sufficient quantity to enable a limited degree of growth to occur. Whether this could be avoided in future by the use of a smaller inoculum it is impossible to say.

ORIGIN OF *BACT. COLI* II AND OF IRREGULARS I AND II

Since these strains may affect the interpretation of method IV it is important to ascertain, if possible, their source of origin.

(*a*) *Frequency of these organisms in human faeces.* Table IX (p. 317) shows that *Bact. coli* II was isolated from five samples of faeces by method I. Table XI (p. 318) shows that among 327 organisms isolated from the same series of samples by direct plating there were thirteen strains of *coli* II and ten strains of Irregular I. It is evident that *coli* II can be detected in small numbers by plating, but it does not appear to be present any more frequently than strains of intermediate or *aerogenes* type.

In a personal note Prof. Wilson stated that among 125 strains which he isolated from fifty samples of cow dung two were *coli* II, three were Irregular I, and four were Irregular II. Among 118 strains isolated from thirty-six samples of human faeces there were no *Bact. coli* II or Irregular II, but there were twelve strains of Irregular I.

(*b*) *Association of these organisms with* Bact. coli *I and* I.A.C. *in water.* Table XIII gives the association of *Bact. coli* II and Irregular I and II with other coliform organisms in water.

Table XIII. *The association of* Bact. coli *II and Irregulars I and II with other coliform organisms in water*

Organisms found	No. of samples containing		
	Bact. coli II	Irregular I	Irregular II
Alone	6	0	1
With *Bact. coli* I	5	9	3
With I.A.C.	7	2	2
With *Bact. coli* I and I.A.C.	2	1	2
With *Bact. coli* I, I.A.C. and irregulars	1	0	0
With irregular *or* with I.A.C. and irregular	2	0	0
	23	12	8

Bact. coli II occurred in twenty-three samples; it was found eight times with *coli* I, twelve times with I.A.C., and six times alone. This distribution does not suggest a particularly close association with *Bact. coli* I. Since *Bact. coli* II appears to be relatively uncommon in human and bovine faeces, but to be found quite frequently in hay and straw (Wilson *et al.* 1935), it seems probable that it is not primarily of faecal origin. Its occasional presence in the intestine suggests rather that it is an organism of passage, i.e. an organism ingested with the food and surviving for a shorter or longer time in the intestine, but not one that lives permanently in this situation.

Irregular I was found in twelve samples of polluted water; in ten of these it was present in company with *Bact. coli* I. This close association with an organism of known faecal origin, taken in conjunction with the fact that Irregular I seems to be commoner in faeces than in any other situation, suggests very strongly that its main habitat is the mammalian intestine.

Irregular II was isolated from eight samples of water; in five of these it was associated with *coli* I, in four with I.A.C., and in one it was present alone. Its association with *Bact. coli* I was therefore less striking than that of Irregular I. Though Irregular II may be found in cow dung, its presence in human faeces seems to be uncommon. From the available data it is very difficult to express an opinion on the probable natural habitat of this organism, but the fact that in our series of waters it was associated slightly more frequently with *Bact. coli* I than with I.A.C., and the fact that except for indole formation it is indistinguishable from *Bact. coli* I, suggest that for the moment its presence in water should be regarded with grave suspicion.

False reactions by method IV

The numerical estimation of *Bact. coli* I may be inaccurately recorded if Irregular I or Irregular II is present.

Irregular I, it will be remembered, though almost certainly of excretal origin, does not produce gas in MacConkey medium at 44° C. By method IV, therefore, it is missed. In this series of water examinations it was never found alone, but it occurred in two samples in the absence of *Bact. coli* I. The faecal *coli* count was therefore underestimated in about 0·4 % of samples.

Irregular II produces gas in MacConkey broth at 44° C. and is therefore included in the *coli* I count. In this series it occurred alone in only one sample of the 550 tested, but in two others it was associated with I.A.C. in the absence of *coli* I. The *coli* I count was therefore overestimated in approximately 0·5 % of the samples. If Irregular II is of excretal origin this error is of no importance, but, as was pointed out in the last section, its real habitat is still doubtful.

The total error in what we may call the faecal *coli* count due to the presence of Irregulars I and II was 0·9 %. Since the two errors just described are equal and work in opposite directions, Irregular I giving too low and Irregular II too high a count, it follows that in any large series of waters the faecal *coli*

count as given by method IV is approximately correct. In individual samples a small error, insignificant in relation to the experimental error of the technique as a whole, may be occasionally expected from the presence of one or other of these two organisms.

Bact. coli II produces no gas in MacConkey broth at 44° C. and does not grow in citrate. Like Irregular I, therefore, it is missed by method IV. Since, however, there is reason to believe that *coli* II is not mainly of faecal origin, this failure is probably of little importance. In the 550 samples examined, it was present alone only six times, each time in numbers less than five per 100 c.c.

It may be remarked that the presence of either Irregular I or of *Bact. coli* II may be suspected if, in method IV, subcultures made from fermented 37° C. MacConkey broths fail to grow both in MacConkey broth at 44° C. and in Koser's citrate at 37° C. In such an event the 37° C. MacConkey broths may be plated, and the presence of Irregular I or *Bact. coli* II confirmed by examination of the resulting colonies. In the present series *Bact. coli* II was detected in this way in four out of the six samples in which it was present alone.

The numerical estimation of the I.A.C. group may be inaccurately recorded by method IV if other organisms grow in the citrate medium. In this series there were eight out of eighty-one samples (9·9 %) in which the I.A.C. count was overestimated by method IV owing to this cause. As has already been pointed out, this type of fallacy occurred when subcultures were made into citrate tubes from fermented 37° C. MacConkey broths inoculated with comparatively large quantities of polluted water. It is possible that sufficient organic matter was carried over in this way to enable citrate-negative organisms, such as *Bact. coli* I and certain non-lactose-fermenters, to undergo a limited multiplication. Since such polluted waters would be condemned in any case on their high *coli* I count, the occasional overestimation of the numbers of I.A.C. present is probably of little practical importance.

DISCUSSION

The importance of detecting organisms of the intermediate-aerogenes-cloacae (I.A.C.) *group in water, with particular reference to chlorination*

The results of this investigation leave no doubt that method IV gives a very much more reliable estimate than method I of the real frequency of different coliform types in the original water. Unless I.A.C. is present alone or in large numbers in relation to *coli* I, it is generally missed by method I. Even with *Bact. coli* I method I appears to be rather less sensitive than method IV, since in the present series it failed to detect this organism in thirty out of 446 samples in which it was demonstrated by method IV, while method IV was only once negative when method I was positive.

The advantage of a highly sensitive method of detecting the presence of *Bact. coli* I need not be stressed; but the advantage of such a method for

detecting the presence of organisms of the I.A.C. group is less obvious and requires some discussion.

In the past it has been believed, as the result of examinations by method I, that *Bact. coli* I is far and away the predominant coliform type in British waters (Bardsley, 1934). The present investigation, in which method IV was used as well as method I, throws some doubt on the truth of this conclusion. Though by method I *Bact. coli* I was easily the commonest coliform organism present, method IV showed that in the whole series of 550 waters the average ratio of *coli* I to I.A.C. was 100 : 88. In the very slightly polluted waters containing not more than 5 coliform organisms per 100 c.c., I.A.C. proved by method IV to be actually more frequent than *coli* I, the *coli* I : I.A.C. ratio being about 95 : 100 (Table VII).

Analysis of the figures further showed that I.A.C. was relatively commoner in chlorinated than in non-chlorinated waters. Among 225 samples of untreated town water the ratio of *coli* I : I.A.C. was 100 : 28 by method I and 100 : 64 by method IV; among 149 samples of chlorinated town water the ratio of *coli* I : I.A.C. was 100 : 50 by method I and 100 : 98 by method IV. Similarly, among nineteen samples from open-air non-chlorinated baths the ratio of *coli* I : I.A.C. was 100 : 68 by method I and 100 : 79 by method IV, while among thirty-nine samples of chlorinated bath water the ratios of *coli* I : I.A.C. were 88 : 100 by method I and 47 : 100 by method IV. Thus in chlorinated drinking water the two organisms were present by method IV in approximately equal numbers, while in chlorinated bath water, in which of course the concentration of chlorine was higher than in drinking water, I.A.C. was about twice as common as *coli* I.

Examination of 100 samples of human faeces by method IV revealed the presence of *Bact. coli* I in every sample, and of I.A.C. in no fewer than 61 % of the samples, even though by method I it could be detected in only 10 %. It is true that in 92 % of the total samples examined by method IV *Bact. coli* I was numerically much the more important organism, but it is none the less true that in 8 % of the samples I.A.C. was present in equal or greater numbers than *coli* I, being in one sample greatly superior to *coli* I.

Taking into consideration, therefore, the fact that (1) I.A.C. is nearly as frequently present in polluted water as *Bact. coli* I, and may exceed it where the pollution is only slight; (2) I.A.C. tends to be the dominant organism in polluted water that has been subjected to chlorination; and (3) I.A.C. is often present in human faeces, and may sometimes constitute the dominant coliform type—it is difficult to escape the conclusion that organisms of the I.A.C. group can no longer be disregarded in the interpretation of water analysis in this country. Their presence is likely to be of particular significance in waters of a fairly high degree of purity such as are usually distributed to our larger urban populations. Evidence is available to suggest that members of the I.A.C. group, or at least some of them, are more resistant than *Bact. coli* I to chlorination, and even in non-chlorinated water tend to survive longer than *Bact. coli* I.

The occurrence of small numbers of I.A.C. in water in the absence of *coli* I

may well indicate the existence of a mild degree of excretal pollution, and even though they may have come from some less dangerous source, their presence can hardly be neglected with safety. In view of the growing practice of chlorination the importance of these organisms will probably increase rather than diminish. For these reasons we believe that method IV, with its much greater sensitivity than method I in the detection of organisms of the I.A.C. group, is likely to prove of considerable value in future, especially in the examination of high-grade waters.

SUMMARY AND CONCLUSIONS

1. A bacteriological examination was made of 1108 samples of water submitted to the laboratory for routine analysis. Of these, 550 contained coliform bacilli and were examined by two different methods. The first method, referred to as method I and officially recommended by the Ministry of Health, involves enrichment in MacConkey broth at 37° C., followed by plating and subsequent identification of selected colonies. The second method, described by G. S. Wilson and his colleagues in their report on milk and referred to as method IV, consists in subculturing every positive 37° C. MacConkey tube into MacConkey broth at 44° C. for *Bact. coli* I and into Koser's citrate at 37° C. for organisms of the intermediate-*aerogenes-cloacae* group (I.A.C.). In both methods the absolute numbers of coliform bacilli and of the different types are calculated from probability tables.

2. From every fermented tube of MacConkey broth at 37° C. that was plated coliform organisms were isolated. In not a single instance was a false positive, due to non-coliform bacilli, encountered. This merely confirms the general experience in this country of the high degree of specificity of MacConkey broth for members of the coliform group. Altogether 2840 strains were isolated by method I and classified according to the scheme given in Table I (p. 310).

3. In the 550 samples *Bact. coli* I was demonstrated 417 times by method I and 446 times by method IV. The corresponding numbers for I.A.C. were 213 and 449. Method IV therefore proved rather more delicate for the detection of *Bact. coli* I, and very much more delicate for the detection of I.A.C.

4. Evidence is brought to show that the frequent failure of method I to detect I.A.C. is due partly to a dilution effect and partly to the overgrowth of I.A.C. by *Bact. coli* I. The chances of demonstrating I.A.C. by method I are remarkably small unless these organisms are present alone, or in numbers greater than or approximately equal to those of *coli* I.

5. The specificity of the 44° C. MacConkey test for *Bact. coli* I, and for the infrequent Irregular II which closely resembles it, is very high. For instance, among 2840 strains tested, only fifteen produced gas in MacConkey at 44° C. which could not be classified as *Bact. coli* I or Irregular II. Only six out of a total of 1086 I.A.C. strains reacted positively to this test. Finally not a single strain, other than *Bact. coli* I, isolated from 100 samples of human faeces was found capable of producing gas in MacConkey broth at 44° C.

6. The specificity of the citrate test for I.A.C. is not so high as that of the 44° C. MacConkey test for *Bact. coli* I. In eight out of eighty-one samples of water a positive citrate test was given by organisms other than I.A.C. Examination showed that these consisted of *Bact. coli* I, non-lactose-fermenters, or of irregular types. Since neither *Bact. coli* I nor the non-lactose-fermenters grew in citrate in pure culture, and since they were found in citrate tubes that had been subcultured from fermented MacConkey broths inoculated with relatively large quantities of polluted water, it seems possible that their growth was due to the carrying over of small amounts of organic matter into the citrate medium.

7. Observations on the association of *Bact. coli* II, Irregular I and Irregular II with other coliform types render it probable that *Bact. coli* II is not mainly of faecal origin, that Irregular I is almost certainly of faecal origin, and that Irregular II may be of faecal origin, but that further evidence will be required to establish this definitively.

8. Owing to the fact that Irregular I does not produce gas in MacConkey broth at 44° C., the faecal *coli* count by method IV is underestimated, and owing to the fact that Irregular II does produce gas at 44° C., the faecal *coli* count is overestimated. In this series of examinations the faecal *coli* count was falsely estimated owing to the presence of Irregular I in 0·4 % and owing to the presence of Irregular II in 0·5 % of samples. The combined error of 0·9 % is insignificant in relation to the experimental error of the technique as a whole.

9. By the use of method IV it is possible to demonstrate that (*a*) I.A.C. is nearly as frequently present in polluted water as *Bact. coli* I, and when the pollution is only slight may exceed it; (*b*) I.A.C. tends to be the dominant organism in polluted water that has been subjected to chlorination; and (*c*) I.A.C. is often present in human faeces (61 % of 100 samples), and may sometimes constitute the dominant coliform type. In view of these facts it would seem unwise to neglect organisms of the I.A.C. type in the interpretation of water analyses in this country. Their presence is likely to be of most significance in water of a fairly high degree of purity in which, owing to chlorination or other causes, *Bact. coli* I can no longer be detected.

10. It is therefore concluded that it would be advisable in future to adopt a method of analysis, such as method IV, which affords a more delicate index of the presence of *Bact. coli* I, and particularly of I.A.C., in water than the method officially recommended at the present time. The fact that method IV is both simpler, quicker, and cheaper than Method I provides an additional recommendation for this change.

REFERENCES

BARDSLEY, D. A. (1934). *J. Hyg., Camb.*, **34**, 38.

Report (1934). *Rep. Publ. Hlth Med. Subj., Lond.*, No. 71.

WILSON, G. S., TWIGG, R. S., WRIGHT, R. C., HENDRY, C. B., COWELL, M. P. & MAIER, I. (1935). *Spec. Rep. Ser. Med. Res. Coun., Lond.*, No. 206.

(*MS. received for publication* 8. II. 38.—Ed.)