THE EFFECT OF TEMPERATURE OF INCUBATION ON THE RESULTS OF TESTS FOR DIFFERENTIATING SPECIES OF COLIFORM BACTERIA

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The four tests most commonly employed for the differentiation of coliform bacteria are the indol, the methyl-red, the Voges-Proskauer, and the citrate tests. These tests, together with the routine tests for the fermentation of lactose, have been proved by long usage to be valuable not only in differentiating types of *Bacterium coli* from those of *Bact. aerogenes*, but also in differentiating those strains known as Intermediates, which have characteristics intermediate between those of *Bact. coli* and *Bact. aerogenes*. During recent years several workers have investigated the effect of the temperature of incubation in differentiating coliform bacteria. Thus Wilson, Twigg, Wright, Hendry, Cowell & Maier (1935), Clegg & Sherwood (1939), Sherwood & Clegg (1942), and Batty-Smith (1942) have found that the production of acid and gas in MacConkey broth at a temperature of 44° C. is a reasonably specific test for *Bact. coli* type I, only occasional cultures of other types giving a similar reaction. The subject has been reviewed extensively by Batty-Smith (1942).

During a study of the coliform flora of Windermere and its inflowing streams, and of certain other waters, some 3000 cultures of coliform bacteria were isolated by the author and classified by means of the four tests just mentioned. While investigating the characteristics of the types other than *Bact. coli* type I and *Bact. aerogenes* type I it was found that the reactions obtained were dependent in some cases on the temperature of incubation and on the chemical reagents used in the test. The methods used, the results obtained, and their bearing on the differentiation of types of coliform bacteria are discussed in the present paper.

METHODS EMPLOYED FOR THE CLASSIFICATION OF COLIFORM BACTERIA

Formation of indol was tested by the addition of Kovac's reagent to a culture of Bacto tryptone broth after incubation for 24 hr. at 37° C. The citrate medium used was that specified by the Ministry of Health (1939), and examinations were made after incubation for 2–3 days at 37° C. The Voges-Proskauer test was made on cultures in glucose-phosphate broth incubated for 24 hr. at 37° C.; 5 ml. of 10 % KOH were added to 5 ml. of the culture and examinations for the pink colour were made at subsequent intervals. For the methyl-red test cultures in glucose-phosphate broth were incubated at 37° C. for 3 days.

Types of coliform bacteria isolated

Table 1 shows the reactions of the cultures isolated to the four tests mentioned above. They are divided on this basis into nine groups, six of which correspond in their reactions to types named by Wilson *et al.* (1935), though it should be pointed out that these workers subdivided these six types further by applying the tests for production of acid and gas in MacConkey broth at 44° C. and for liquefaction of gelatin. A few irregular types were also isolated, but the numbers of these were small and they were not studied in detail.

Moreover, as further investigation showed that all cultures in group 7 when tested by a modified technique conformed in their reactions to those of *Bact. aerogenes* type I, they are not considered further here.

Table 1. *Types of coliform bacteria isolated from rivers, lakes, and streams*

	Test				
Group	Indol	M.R.	V.P.	Citrate	Type (following Wilson *et al.*)
1	+	+	−	−	*Bact. coli* type I
2	−	+	−	−	*Bact. coli* type II
3	−	+	−	+	Intermediate I
4	+	+	−	+	Intermediate II
5	−	−	+	+	*Bact. aerogenes* type I
6	+	−	+	+	*Bact. aerogenes* type II
7	+	+	+	+	Unnamed
8	−	+	+	+	Unnamed
9	−	−	−	+	Unnamed

Fermentation of lactose in MacConkey broth at different temperatures of incubation

The ability of cultures to produce acid and gas from lactose in MacConkey broth at different temperatures was tested by inoculating into tubes of

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MacConkey broth which were incubated in a thermostatically controlled water bath, the temperature of which was found to vary by not more than ± 0·07° C. when tested by an N.P.L. thermometer. The temperatures employed were 37, 40, 42, 43, 44, and in some cases 45 and 46° C. Temperatures were carefully checked by an accurate thermometer. The results are shown in Table 2.

It can be seen that 97 % of the cultures of *Bact. coli* type I formed acid and gas over the temperature range of 40–44° C., and that the percentage giving a positive result at 45° C. was only slightly less. At 46° C., however, little more than half the cultures were able to cause fermentation. The ability of this type to form acid and gas at 44° C. confirms the findings of Wilson *et al.* (1935), Clegg & Sherwood (1939), and Sherwood & Clegg (1942). The percentage of cultures which caused fermentation at 45° C. was much larger than that found by Clegg & Sherwood. Wilson *et al.* (1935) found that 42° C. was too low, and 46° C. too high, a temperature for separation of *Bact. coli* type I from other types. Other types found to cause fermentation at 44° C. were *Bact. coli* type II (28 %) and *Bact. aerogenes* type I (15 %). It can be seen that in the case of *Bact. coli* type II there was little difference between the percentage causing fermentation at 42, 43 and 44° C., but in the case of *Bact. aerogenes* type I each degree rise in temperature reduced the percentage appreciably. Cultures of *Bact. coli* type I differed from those of *Bact. coli* type II only in the ability to form indol. The percentage of cultures of *Bact. aerogenes* able to cause fermentation at 44° C. is higher than has been previously reported in this country, where the figure has usually been below 5 %. The inability of *Bact. aerogenes* type II to cause fermentation at 43° C. and of Intermediate type II to do so at 42° C. are points worthy of note. It can also be seen that the use of the temperature of 42° C. as a primary incubation temperature in the examination of water samples would fail to detect a large proportion of coliform bacteria other than *Bact. coli* type I.

Table 2. *Production of acid and gas in MacConkey broth by different types of coliform bacteria at different temperatures*

Type	No. of cultures tested	Percentage of cultures forming acid and gas at 37° C.	40° C.	42° C.	43° C.	44° C.	45° C.	46° C.
Bact. coli type I	96	100	97	97	97	97	93	52
Bact. coli type II	50	100	64	36	32	28	—	—
Intermediate type I	78	100	78	56	23	0	—	—
Intermediate type II	53	100	55	4	2	0	—	—
Bact. aerogenes type I	80	100	83	53	35	15	2	—
(or *Bact. cloacae*)	52	100	—	—	—	15	—	—
Bact. aerogenes type II	25	100	80	12	0	0	—	—

Factors affecting the indol test

Seventeen of the cultures of *Bact. coli* type II which had been found consistently to produce no indol at 37° C. were tested for their ability to produce indol in tryptone broth at 30° C. At this temperature three cultures were found to give positive results and all three were of the type found to be capable of fermenting lactose at 44° C. Similar tests made on cultures of Intermediate type I revealed no further cultures which produced indol. In order to see whether a difference in the source of nitrogen or of the temperature of incubation encouraged formation of indol, additional tests were made at temperatures of 20, 30 and 37° C. with cultures in tryptone broth and in tryptone broth fortified with tryptophane, but no further positive results were obtained.

Factors affecting the Voges-Proskauer and the methyl-red tests

For convenience in working, the number of cultures shown in Table 2 was reduced to between twelve and twenty in each group. All cultures were replated on eosin methylene-blue agar at frequent intervals, and the determination of the characters was repeated. It was found that after repeated laboratory culture some strains which had originally been positive gave a negative or weak reaction with the V.P. test when carried out by the method recommended by the American Public Health Association (1936). It was decided to investigate the methods of testing for acetylmethylcarbinol to see if more satisfactory results could be obtained.

O'Meara (1931) introduced a modification of the V.P. test in which creatine and caustic potash were added to the culture and a positive result was indicated by the appearance of a pink colour on standing. The modification was endorsed by Levine, Epstein & Vaughn (1934), and others, but was not included in the last edition of *Standard Methods* of the American Public Health Association (1936). Barritt (1936) intensified the V.P. reaction by the addition of α-naphthol to the culture in conjunction

with potassium hydroxide. Cultures were incubated at 37° C. When comparisons were made between his new method and the original method, it was found that with 290 cultures of *Bact. coli* types I and II and *Bact. aerogenes* types I and II the results of the two tests agreed, but that with a large proportion of the intermediate types the tests gave different results. Barritt found that of twenty-one strains of intermediate types which gave positive results in the methyl-red and the citrate tests, twenty-one strains were V.P. positive when tested by O'Meara's method, and fifty-four were positive when tested by his own method. The seventeen cultures were which found to be V.P. negative by the new technique were considered to be allied to *Bact. coli*, and the fifty-four positive cultures to *Bact. aerogenes*. In a later paper on the origin of acetylmethylcarbinol, Barritt (1937) concluded that 2.3-butylene glycol was the precursor of acetylmethylcarbinol. He also considered that

Table 3. *Effect of temperature and test reagent on V.P. reactions of* 221 *coliform strains (after Levine,* 1941)

	Test reagent	
	KOH	KOH + α-naphthol
Period of incubation 24 hr.:		
No. of positives at 37° C.	21	44
No. of positives at 30° C.	45	51
Period of incubation 48 hr.:		
No. of positives at 37° C.	26	44
No. of positives at 30° C.	48	51

all coliform organisms formed some acetylmethylcarbinol, but that the amount formed by *Bact. coli* was normally too small to detect; Kluyver & Molt (1939) confirmed this. Tests made by the Metropolitan Water Board (1936) with the Barritt technique confirmed that a very much higher proportion of V.P. positive results could be obtained by this method than by the original tests or by the O'Meara modification. Of 150 cultures with positive methyl-red and citrate reactions, V.P. positive reactions were obtained with thirty-seven cultures by the original method, with fifty-seven by the O'Meara method, and with sixty-two by the Barritt method. Cultures giving positive methyl-red and V.P. (Barritt) reactions were replated and were found to be pure.

Wilson *et al.* (1935) compared the original method with that of O'Meara in the examination of sixty-five *aerogenes-cloacae* strains and found that ten of these which were negative by the old method were positive by O'Meara's method, though no strain was positive by the old method that was negative by the O'Meara method.

Levine (1941) has recently compared the original method with Barritt's technique when the cultures were incubated for 24 and 48 hr. at 30 and 37° C. The results of this work are shown in Table 3.

It can be seen that a temperature of 30° C. gave a much greater number of positives than a temperature of 37° C., that the Barritt modification gave a greater number of positives than the original method, and that when the modified method was used no additional positives were obtained by extending the period of incubation to 48 hr.

Batty-Smith (1941) compared the O'Meara and Barritt methods, but did not state the temperature used. It was found that of 125 cultures which produced acetylmethylcarbinol by one or other method the O'Meara technique detected 82·4% after incubation for 2 days, and 91·2% after incubation for 3 days, whereas the Barritt technique detected 96% after incubation for 2 days. Some strains which were tested after repeated purification were found to be both methyl-red and V.P. positive.

Table 4. *Reactions to O'Meara's test at* 30° C. *of strains of coliform bacteria, classified as Intermediate types I and II and giving a negative reaction at* 37° C. *both by the original method and by the O'Meara modification*

Type	No. of cultures	No. positive	No. negative
Intermediate type I	62	21	41
Intermediate type II	35	24	11
Total	97	45	52

In view of Levine's results it was decided to test by the original method and by O'Meara's method, both at 30 and at 37° C., some of the strains shown in Table 1 which had been found to be V.P. negative by the original method, particularly strains of Intermediate type I and strains in group 9. Preliminary tests having shown that several strains gave positive reactions at 30° C. but not at 37° C., and also that O'Meara's method gave a higher number of positives than the old method, it was decided to investigate more closely all the available cultures in the laboratory which had been classified as *Bact. coli* type II, Intermediate type I, and Intermediate type II. Duplicate tubes of glucose phosphate broth were therefore inoculated with each strain and incubated at 30° C. for 2 days. The V.P. reaction was then tested by the O'Meara and by the Barritt modifications. It was soon found that the Barritt technique was unsatisfactory, almost every culture tested giving a more or less intense positive reaction. Tests on cultures of *Bact. coli* type I, for example, showed that some of them produced enough colour to be regarded as positive. Tests by this method were therefore discontinued. The results of the O'Meara test are shown in Table 4.

It can be seen that with a temperature of incubation of 30° C. and the use of O'Meara's test almost half the cultures shown consistently to be V.P. negative by the old method gave a positive reaction. Fifty-two negative strains were tested during a period of 2 days at a temperature of incubation of 20° C., but no further positives were obtained by this means. Tests made on cultures incubated at 30° C. and examined after different periods of incubation showed that as strong a reaction was obtained after incubation for 20 hr. as after 2–4 days. In no case was acetylmethylcarbinol destroyed during an incubation period of 4 days.

Levine (1941) points out that Clark and Lubs originally stipulated that for accurate results in the methyl-red test cultures should be inoculated at 30° C. for 5 days, but it is now more usual to incubate at 37° C. for 3 or 4 days. It may be seen that, since forty-five of the cultures (Table 4) now gave a positive test for acetylmethylcarbinol when incubated at 30° C. and tested by O'Meara's method, they failed to show an inverse correlation with the methyl-red test carried out when the cultures had been incubated at 37° C. It was therefore decided to repeat the methyl-red test on all the cultures shown in Table 4, using a temperature of incubation of 30° C. and applying the test for 5 days. Table 5 shows the results obtained and indicates the correlations between the methyl-red and the V.P. tests when the temperature of incubation of 30° C. was employed in both tests.

Table 5. *Methyl-red reactions of cultures incubated at* 30° C. *for* 5 *days and previously found to be positive at* 37° C. *Correlations between methyl-red and V.P. tests at* 30° C.

No. of strains	Originally classified as	M.R. test at 30° C. +	M.R. test at 30° C. −	Correlations between M.R. and V.P. tests at 30° C. + − or − +	Correlations between M.R. and V.P. tests at 30° C. + +	Correlations between M.R. and V.P. tests at 30° C. − −
62	Intermediate type I	41	21	62	0	0
35	Intermediate type II	16	19	28	6	1
6	Group 7 (unnamed)	0	6	6	0	0
13	Group 8 (unnamed)	0	13	13	0	0

It may be seen that just as a considerable proportion of Intermediate strains were negative to the V.P. test at 37° C. and positive at 30° C., a similar proportion was positive to the methyl-red test at 37° C. and negative at 30° C. The inverse correlation between the two tests was therefore preserved in most cases. It is interesting to note that when a temperature of incubation of 30° C. was adopted the characteristics of the cultures in group 7 conformed with those of *Bact. aerogenes* type II and the characteristics of the cultures in group 8 with those of *Bact. aerogenes* type I. Incubation at a temperature of 30° C. and the use of the O'Meara modification did not alter in any way the reaction which had been obtained at 37° C. for strains of *Bact. coli* types I and II or of *Bact. aerogenes* types I and II.

A similar investigation was made of twelve cultures obtained from another laboratory engaged in the bacteriological examination of water. These twelve cultures were originally classified as Intermediate type I, the methyl-red and V.P. tests having been performed at 37° C. and the latter by the O'Meara modification. When a temperature of incubation of 30° C. was used and the O'Meara test was applied after 24 hr. and the methyl-red test after 7 days, it was found that five cultures were methyl-red negative and V.P. positive by these tests, the remaining seven cultures being methyl-red positive and V.P. negative.

Effect of temperature of incubation on the citrate test

Ability to utilize citrate as a source of carbon has for a long time been used as one of the standard tests for differentiation of Intermediate-*aerogenes-cloacae* types from *Bact. coli* types I and II. Levine (1941) found no difference in the ability of Intermediate strains to utilize citrate at 30 or 37° C., but Stuart, Mickle & Borman (1940) reported that a number of their strains utilized citrate when the temperature of incubation was 20° C., though they were unable to do so at 37° C. In retesting the ability of cultures of coliform bacteria to use citrate at 37° C., the temperature normally employed, it has been found occasionally that after many subcultures some strains have shown growth in citrate medium, though they had previously been reported as negative. It has not been possible to show whether these cultures have gained this characteristic or whether, in previous tests, either the inoculum or the time allowed for growth had been insufficient. It was found that in general the cultures which showed this characteristic were those which had been grouped as *Bact. coli* type II. Ability to utilize citrate would classify them as Intermediate type I.

To compare the results obtained at temperatures of incubation of 30 and 37° C. twenty-two cultures of *Bact. coli* type II which had always proved to be citrate negative were inoculated into citrate

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medium and incubated at the two temperatures. It was found that two cultures showed visible growth after incubation for 2 days at 30° C. but no growth after 4 days at 37° C.

Differentiation of Intermediate types

Although Bergey (1939) provides two genera—*Escherichia* for the *Bact. coli* type and *Aerobacter* for the *Bact. aerogenes* type—the position of those species which have some characteristics common to both those types is uncertain. The basis for the differentiation of coliform types at the present time is largely that of their reactions to the indol, methyl-red, V.P., and citrate tests, but many other tests have been tried with varying degrees of success. Werkman & Gillen (1932) found that certain coliform bacteria produced trimethylene glycol from glycerol. They described seven such species and proposed that the generic name *Citrobacter* be adopted for them. These organisms give a positive or indefinite reaction in the methyl-red test at 37° C. and a negative or very faint reaction in the V.P. test at 30° C. Tittsler & Sandholzer (1935) criticized the formation of such a genus for, during a study of twenty-nine methyl-red positive, V.P. negative Intermediate strains they were unable to find any one test which would differentiate them from *Bact. coli* or *Bact. aerogenes*. Levine *et al.* (1934) found that a characteristic common to all strains of *Citrobacter* was the formation of hydrogen sulphide in a peptone-ferric-citrate agar medium which contained 0·3 % boric acid. Carpenter & Fulton (1937) studied 117 cultures of Intermediates (indol negative, methyl-red positive, V.P. negative, citrate positive) which had been isolated from faeces. It was found that all cultures reduced nitrates, but did not liquefy gelatin; with one exception acid or acid and gas was formed from cellobiose. The ability of the cultures to utilize the nitrogen of uric acid or to produce H_2S was not determined.

In order to see whether the application of further tests might assist in classifying coliform bacteria of the Intermediate type, eighty-four cultures whose indol, methyl-red, V.P., and citrate reactions corresponded with those of Intermediate type I, of Intermediate type II, or of *Bact. aerogenes* type II were tested for their ability to form H_2S, to utilize uric acid as a source of nitrogen, and to ferment cellobiose. The results are given in Table 6.

These results show that the uric acid test distinguished cultures of Intermediate type I from those of *Bact. aerogenes* type II since none of the former were able to grow in this medium, while all cultures of *Bact. aerogenes* type II were able to do so. The diagnostic value of the test for hydrogen sulphide was not quite so good as that of the uric acid test, but it was found that whereas 77 % of Intermediate type I cultures formed H_2S, none of the cultures of *Bact. aerogenes* type II was able to do so. The results of the cellobiose test were disappointing for, although all cultures of *Bact. aerogenes* type II formed acid and gas from this carbohydrate, about two-thirds of the cultures of Intermediate type I formed acid only, a small proportion formed both acid and gas, and the remainder gave no reaction.

Table 6. *Results of uric acid,* H_2S, *and cellobiose tests applied to Intermediate types I and II and* Bact. aerogenes *type II*

Type	No. of cultures studied	No. showing growth in uric acid medium	No. producing H_2S	Fermentation of cellobiose	
				No. producing acid and gas	No. producing acid only
Intermediate type I (− + − +)	40	0	31	6	27
Intermediate type II (+ + − +)	10	4	2	6	1
Bact. aerogenes type II (+ − + +)	34	34	0	34	0

DISCUSSION

The importance of incubating cultures of coliform bacteria at the optimum temperature when applying differential tests for the purpose of classification has been demonstrated in the present work. Although the sanitary significance of the various groups of coliform bacteria remains a matter of controversy, there is a general tendency to regard *Bact. coli* type I as a far more important indicator of pollution, particularly of recent origin, than any of the other groups. This, therefore, entails a separate estimate of that type, either by the selective fermentation of lactose at 44° C., a method often adopted in this country, or by typing the cultures on the basis of several differential tests. Of the five tests more commonly used—44° C., the methyl-red, the V.P., the indol, and the citrate tests—the first three depend on fermentation of carbohydrates.

Available evidence shows that the great majority of *Bact. coli* type I are able to produce acid and gas from lactose at 44° C., but different workers have

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found rather different proportions of *Bact. aerogenes* type I which are also able to do so. The proportion of *Bact. aerogenes* found to be positive at 44° C. in the work reported in the present paper (15 %) is by far the greatest reported by workers in this country, but confirms earlier work (Taylor, 1941) where a high proportion (9·2 %) was found. Batty-Smith (1942) considers that the distribution of such types is localized. In addition, a fair proportion of cultures of *Bact. coli* type II was found in the present work to be positive at 44° C.; these are strains which Wilson *et al.* (1935) designated Irregular type II. As three of the cultures of *Bact. coli* type II which were positive at 44° C. were found to give a positive indol reaction when the test was repeated at 30° C., and as almost all the cultures of this type which were unable to cause fermentation at 44° C. were able to do so between 40 and 42° C., it seems reasonable to suggest that strains of *Bact. coli* type II which are positive at 44° C. may be strains of *Bact. coli* type I which have lost their ability to form indol at 37° C. Strains of *Bact. coli* type II which are negative at 44° C. might equally well be considered as citrate negative strains of Intermediate type I.

The adoption of a temperature of incubation of 30° C. for the V.P. test, as recommended by Levine (1941), together with O'Meara's modification of adding creatine, resulted in a positive reaction with many Intermediate strains of coliform bacteria which were negative to the V.P. test when carried out by the method recommended by the American Public Health Association (1936). Further, when a temperature of 30° C. was also adopted for the methyl-red test and a sufficiently long period of incubation was allowed, there was an inverse correlation between this and the V.P. reaction for most of the cultures tested. It seems clear that 37° C. is not the optimum temperature for the fermentation of glucose by strains of the Intermediate type. It seems likely that some strains of coliform bacteria reported by different workers as both methyl-red and V.P. positive would have yielded negative methyl-red reactions if the period and temperature of incubation had been modified, and that some cultures which have been reported as Intermediate type I would have been classified as *Bact. aerogenes* type I if a lower temperature of incubation had been employed.

It is believed that the great sensitiveness of the Barritt test which has been recorded here, and by other workers, results in an abnormally high number of positive V.P. reactions. This finding and the results of Utermohlen & Georgi (1940) who found that by more sensitive methods formation of H_2S by *Bact. coli* could be detected, suggests that these delicate modifications in technique do not aid present methods of classification of bacteria.

SUMMARY

1. A study has been made of different types of coliform bacteria with particular reference to (*a*) their ability to ferment lactose in MacConkey broth at different temperatures, and (*b*) the effect of using different temperatures of incubation for the indol, methyl-red, V.P., and citrate tests.

2. It was found that 97 % of the cultures of *Bact. coli* (indol positive, methyl-red positive, V.P. negative, citrate negative) examined could ferment lactose with production of acid and gas between 40 and 44° C. The number was not appreciably reduced at 45° C. but was markedly reduced at 46° C. 28 % of the cultures of *Bact. coli* (indol negative, methyl-red positive, V.P. negative, citrate negative) and 15 % of *Bact. aerogenes* (indol negative, methyl-red negative, V.P. positive, citrate positive) were found to be positive at 44° C.

3. The adoption of a temperature of incubation of 30° C. for the V.P. test as advocated by Levine (1941) and the use of O'Meara's test showed that many cultures previously regarded as unable to produce acetylmethylcarbinol were in fact able to do so. Employing a temperature of 30° C. for 5, or in some cases 7, days for the methyl-red test, it was found that with nearly all the cultures tested there was an inverse correlation between the results of the methyl-red test and those of the V.P. test. With these modifications in technique some cultures originally designated as Intermediate type I were found to have reactions corresponding with those of *Bact. aerogenes* type I. Similarly, many cultures originally classified as Intermediate type II should have been typed as *Bact. aerogenes* type II.

4. It was found that all cultures of Intermediate type I classified as such by the new technique were incapable of using the nitrogen of uric acid for growth, but that the majority produced hydrogen sulphide. Cultures of *Bact. aerogenes* type II, on the other hand, grew well in uric acid medium, but produced no hydrogen sulphide.

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