FURTHER STUDIES OF INCUBATION AT 44° C. AS A TEST FOR 'FAECAL *COLI*'

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(With 2 Figures in the Text)

Introduction

In an earlier paper (Clegg & Sherwood, 1939) experiments were described on the incubation of cultures in MacConkey's bile-salt broth of organisms isolated from polluted shellfish. These were carried out in accurately controlled water baths, of which details were given, at temperatures between 37 and 46° C.[1] The object of these experiments was to ascertain what temperature would permit lactose-fermentation by *Bacterium coli* while inhibiting other lactose-fermenting coliforms. The use of such a temperature, by providing a basis for a simple test for faecal pollution in shellfish, depending on the detection of *Bact. coli* alone, would eliminate misleading results due to multiplication of certain coliform types in purified shellfish, as investigated by Dodgson (1936, 1937, 1938), who concluded that incubation at 44°, as already suggested by Prof. G. S. Wilson, would probably meet the requirements.

The data then obtained demonstrated the efficacy of incubation at 44°. At temperatures above 44° it was found that the numbers of *Bact. coli* were significantly reduced, but lower temperatures permitted gas production by other types of coliforms.

Attention has been drawn by the Ministry of Health (1939) to the need for further data about the specificity of 44° as a test for *Bact. coli* in connexion with the bacteriological examination of water. Among those whose investigations have been concerned with other aspects of this problem and who have reported favourably on incubation at that temperature are: Wilson *et al.* (1935), Bardsley (1938), Ferramola & Monteverde (1938, 1939), Ferramola (1940), Banerjea & Sen (1940), Raven, Peden & Wright (1940), and Clegg (1941).

The present paper greatly extends the earlier data not only to polluted shellfish from areas not represented in the previous samples, but also to sewage and to human and other faeces.

Nomenclature

A word on nomenclature in this paper is desirable. The classification used is that proposed by Wilson *et al.* (1935). The title refers to 'faecal *coli*'. The use of this term does not infer that there is a non-faecal *coli*. '*Bact. coli*' will

[1] Temperatures are in degrees Centigrade throughout.

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not serve for 'faecal *coli*' in this respect because it has come to mean *Bact. coli* type I, which, already by definition positive at 44° (Wilson *et al.* 1935; Ministry of Health, 1939), is the most characteristic and constantly occurring coliform in faeces. *Bact. coli* type II though found in faeces is comparatively uncommon. Thus 'faecal *coli*' refers to cultures which have the reactions indole +, methyl red +, Voges-Proskauer −, citrate −, regardless of their temperature reactions.

The term 'intermediate-*aerogenes-cloacae* types' (I.A.C.) is not used. In its place is substituted 'other coliforms than *Bact. coli*'. There is a twofold reason for this. First, as stated below, no differentiation is made between types on the basis of gelatin liquefaction. Thus this eliminates the '*cloacae*' members of the I.A.C. group. Secondly, it is desired that 'other coliforms' should include the irregulars; the term I.A.C. does not embrace these.

Experiments

The bacterial cultures for this work were isolated from freshly obtained material by the general method previously employed. Dilutions of the material to be examined were plated out in lactose bile-salt peptone neutral-red agar (MacConkey's agar) and incubated at 37° for 24 hr. From the red colonies on these plates 100 cultures capable of fermenting lactose at 37° were isolated from each sample. This differed slightly from the procedure adopted in the previous work where 100 colonies were pricked out from 48 hr. plates regardless of appearance. These colonies were cultured on agar slopes, tested in MacConkey's broth at 41, 44, 45 and 46°, and in appropriate media for the indole, methyl red, Voges-Proskauer and citrate ('IMViC') tests, and were classified according to the types recognized by Wilson *et al.* (1935), except that no attempt was made to distinguish between types which differ only in the gelatin test. Cultures suspected of being mixtures were plated repeatedly on eosin methylene blue or nutrient agar until the reactions were constant. All cultures on examination were found to be Gram-negative, non-spore-forming short rods.

Cultures for examination were obtained from the following samples:

Shellfish. 500 cultures from five samples of mussels taken from polluted beds at Bangor, Boston, and Morecambe, and a sewage-bearing channel at Conway (two samples).

Sewage. 500 cultures from five samples of sewage from three collecting tanks near Conway, which deal with 100–500 houses each. The tanks are emptied by automatically controlled pumps as required. Two tanks were sampled twice to give as far as could be judged minimum and maximum faecal contents, i.e. during 'washing day' and later in the week.

Faeces. (*a*) 500 cultures from five samples of fresh faeces (i.e. plated within two hours of deposit), 200 of human origin and 100 each from sea birds, a sheep and a cow. (*b*) 100 cultures from an additional sample of fresh human faeces of special interest (see Table 1, sample 16).

RESULTS

The result of examination of these samples is given in Tables 1–3.

(*a*) *Distribution of types*

Table 1 shows the distribution of types. The figures for samples 11–15 which were almost identical are not shown separately, there being only one culture in the 500 which was not positive at 44°.

Sample 16 is additional to the work as first planned, and is included because of its bearing on the sanitary significance of the various members of the coliform group. The incidence of such samples is referred to on p. 52.

Bact. coli I was well represented in all the samples; it ranged from *ca.* 25% in samples 1 and 6, up to 100% in the faeces (excluding sample 16). Other 44°-positive types together did not exceed 7% in any of the samples and were entirely absent from the faeces. Almost the same applies to irregular I. Among the mussel and sewage samples 44°-positive types formed respectively half and slightly more than half of the total. The mussel contents closely resembled the sewage samples in their composition, differing markedly from the faeces, from which cultures of the 44°-positive irregular types were entirely absent, and only one of irregular I was present.

The result chiefly to be noted is the closeness of the combined totals for 44°-positives and for + + − − 'IMViC' types; in fact incubation at 44° proved to be an almost exact procedure for ascertaining the total number of + + − − lactose fermenters, the comparatively few + + − − cultures which gave a negative result at 44° being numerically balanced by the presence of the 44°-positive irregular types II and VI. Bardsley (1938) has already drawn attention to the fact that by incubation at 44° the *Bact. coli* count was underestimated by the presence of irregular I but overestimated by irregular II. The errors she experienced by these two types were 0·4 and 0·5% respectively, which being almost equal and working in opposite directions, counterbalanced one another; the incidence of irregular VI was so small (two cultures out of a total of 2840) that she took no cognisance of it.

This balance is perhaps more easily understood when the figures are displayed in a slightly different form as in Table 2, and summarized graphically in Fig. 1.

In every case the total positives at 44° (of all types) closely approximates to the total + + − − (both 44°-positive and negative) types. A glance at Table 1 will reveal that this situation occurs in all sixteen samples. Thus it is not merely a chance similarity of the totals.

In their report on the bacteriological examination of water supplies, the Ministry of Health (1939) state: 'Practically every strain forming gas at 44° C. appears to belong to the faecal coli type, but whether every strain of faecal coli will form gas at 44° C. is still in doubt.'

Table 1. *Distribution of types among* 1600 *lactose fermenters isolated from mussels, sewage and faeces*

Sources of samples numbered 1–16 below:

1 and 2: Conway River near Llandudno Junction sewer outfall.
3: Menai Straits near Bangor main sewer outfall.
4: Boston 'Lays' near mouth of Witham.
5: Morecambe 'Skear' near main sewer outfall.
6, 7: Conway Morfa sewage pumping station.
8, 9: Llandudno Junction sewage pumping station.
10: Deganwy sewage pumping station.
11–15: Two human, one sea bird's, one sheep's, one cow's faeces.
16: An extra sample of human faeces from an individual whose faeces when sampled elsewhere on four previous occasions were found to contain no *Bact. coli* in the dilutions employed.

Type	IMViC reactions	Mussels 1	2	3	4	5	Total	Sewage 6	7	8	9	10	Total	Faeces 11–15	16	Combined total
1. Producing acid and gas* at 44° C.																
Bact. coli type I	+ + − −	26	65	41	43	59	234	22	53	38	66	85	264	499	39	1036
Irregular II, *coli*-like 2	− + − −	3	2	1	6	1	13	1	0	0	5	1	7	0	0	20
Irregular VI, *aerogenes*-like 2	− − + +	0	0	0	1	2	3	3	4	2	1	0	10	0	0	13
Total (compare 4 below)		29	67	42	50	62	250	26	57	40	72	86	281	499	39	1069
2. Not producing acid and gas at 44° C.																
Bact. coli type II	− + − −	0	0	6	7	0	13	0	1	1	4	1	7	0	16	36
Intermediate type I	− + − +	11	11	30	12	10	74	3	15	5	14	9	46	0	45	165
Intermediate type II	+ + − +	2	12	4	8	14	40	5	4	6	2	0	17	0	0	57
Bact. aerogenes type I	− − + +	38	8	12	10	6	74	43	26	25	2	1	97	0	0	171
Bact. aerogenes type II	+ − + +	13	1	2	10	3	29	8	2	3	0	0	13	0	0	42
Irregular I, *coli*-like 1	+ + − −	0	0	4	1	2	7	0	6	6	6	3	21	1	0	29
Other irregular types		7	1	0	2	3	13	4	0	14	0	0	18	0	0	31
Total		71	33	58	50	38	250	63	54	60	28	14	219	1	61	531
3. Total all types		100	100	100	100	100	500	89	111	100	100	100	500	500	100	1600
4. Total + + − − (IMViC) types (i.e. *Bact. coli* I plus irregular I)		26	65	45	44	61	241	22	59	44	72	88	285	500	39	1065

* 'Gas' here denotes sufficient to fill the hemispherical portion of the Durham's tube, i.e. *ca.* 10% of its capacity. Any smaller quantity of gas is taken to indicate that the incubation temperature is too near the thermal death-point to count as a satisfactory positive result for the purpose of this investigation, as concluded from previous observations (Clegg & Sherwood, 1939).

Table 2. *Percentages of* Bact. coli *in relation to* 44°*-positive and* + + − − *lactose fermenters among* 1600 *cultures examined*

	Mussels	Sewage	Faeces	Total
Bact. coli type I	46·8	52·8	99·8	64·7
Irregular II, *coli*-like 2	2·6	1·4	0·0	1·3
Irregular VI, *aerogenes*-like 2	0·6	2·0	0·0	0·8
44° positives (all types)	50·0	56·2	99·8	66·8
Bact. coli type I	46·8	52·8	99·8	64·7
Irregular I, *coli*-like I	1·4	4·2	0·2	1·8
Total + + − − (both 44° positive and negative)	48·2	57·0	100·0	66·5
% of *Bact. coli* among 44° positives	93·6	94·0	100·0	96·9

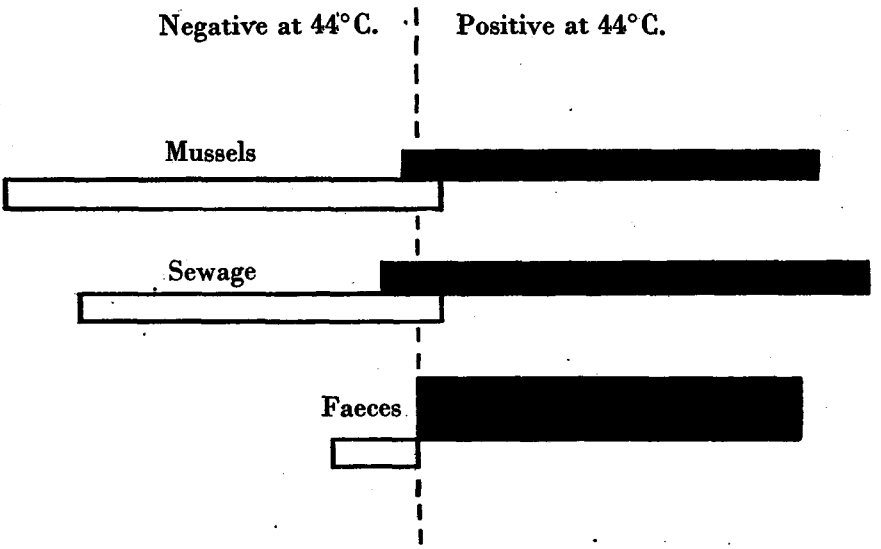

Fig. 1. Areas representing percentage of + + − − cultures (black) and other types (outline) in relation to incubation at 44° C. in 1600 cultures from mussels, sewage and faeces. Black projections to the left of the dividing line represent irregular type I, outline projections to the right irregular types II and VI.

Table 2 supplies information on this point. It can be seen that the percentage of the + + − − type which does not form gas at 44° (irregular I) is virtually the same as those irregulars which do form gas at 44° (types II and VI). These 44°-positive and negative categories constitute respectively 1·8 and 2·1% of all the cultures. The existence of irregular types II and VI does not appear to be regarded by the Ministry of Health as a significant source of error.

(*b*) *Comparison of incubation temperatures*

Table 3 and Fig. 2 give the results of inoculating all the cultures into MacConkey's broth at different temperatures.

Table 3 and Fig. 2 show that the rise in temperature from 37 to 44° had

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Table 3. *The result of different incubation temperatures on* 1600 *cultures inoculated into MacConkey's broth: percentages of cultures showing acid and gas**

Left-hand columns of each pair refer to + + − − types, right-hand columns to other coliforms.

Incubation temp. °C.	Individual samples															
	Mussels												Total no. of cultures		Percentages of total no.	
	1		2		3		4		5							
37	26	74	65	35	45	55	44	56	61	39			241	259	48·2	51·8
41	26	10	65	4	41	13	43	28	61	14			236	69	47·2	13·8
44	26	3	65	2	41	1	43	7	59	3			234	16	46·8	3·2
45	21	2	47	1	31	0	35	5	50	0			184	8	36·8	1·6
46	4	0	20	0	7	0	10	2	20	0			61	2	12·2	0·4
	Sewage															
	6†		7†		8		9		10							
37	25	75	53	47	44	56	72	28	88	12			285	215	57·0	43·0
41	25	34	50	17	42	13	72	11	85	11			277	84	55·4	16·8
44	25	4	48	4	38	2	66	6	85	1			264	17	52·8	3·4
45	14	0	34	0	31	0	59	4	77	1			218	5	43·6	1·0
46	0	0	17	0	2	0	33	0	43	0			97	0	19·4	0·0
	Faeces															
	11		12		13		14		15							
37	100	0	100	0	100	0	100	0	100	0			500	0	100	0
41	100	0	100	0	100	0	100	0	100	0			500	0	100	0
44	100	0	100	0	100	0	100	0	99	0			499	0	98·8	0
45	65	0	100	0	78	0	98	0	95	0			436	0	87·2	0
46	15	0	0	0	35	0	4	0	39	0			92	0	18·4	0

Sample 16. + + − −: 39 at 37–44°, 33 at 45°, 30 at 46°. Other types: 61 at 37°, 10 at 41°, none at 44°.

* See footnote to Table 1.

† Percentages to nearest whole numbers from totals of 89 and 111. All other samples already comprised 100 cultures each.

little effect on the + + − − cultures, only twenty-nine of them (2·7%) being cut out. Above 44° the effect was so great that 786 out of 1036 cultures positive at 44° (75%) were cut out. In the samples of faeces (excluding sample 16, the significance of which is discussed on p. 52) there were no types other than + + − −. The curves for mussels and sewage are much alike both for + + − − and for other types. These other types were much reduced between 37 and 41°, above which the reduction continued more gradually until at 46° almost all were eliminated. At 44°, sixteen (3·2%) and seventeen (3·4%) respectively of these other types remained from mussels and sewage.

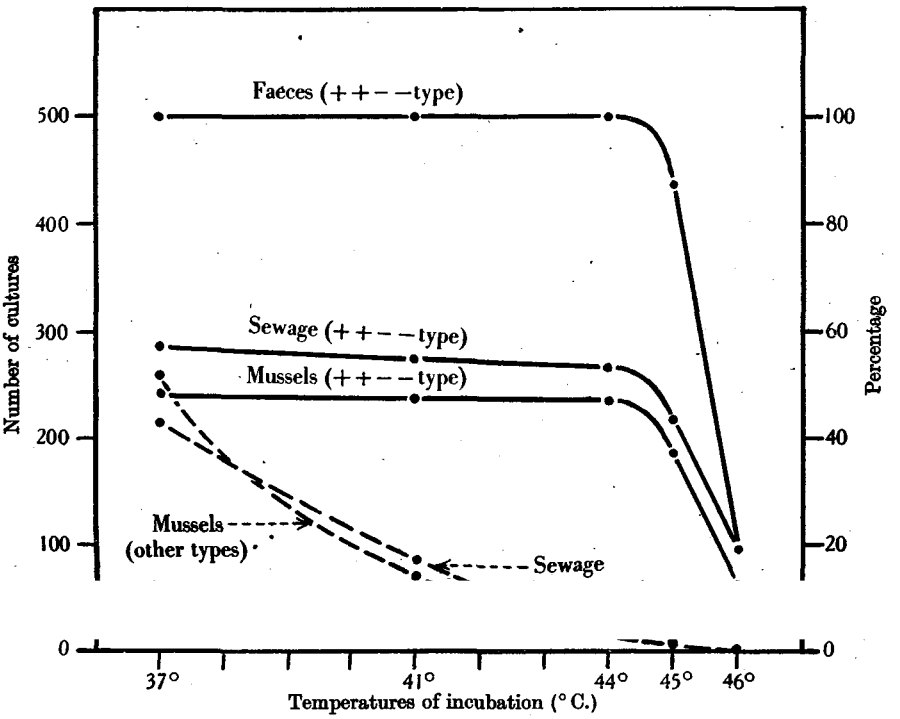

Fig. 2. Showing effect of different temperatures of incubation on 500 lactose fermenters each from mussels, sewage and faeces.

A notable feature in Table 3 is the similarity of trend among all the samples. The specificity of incubation at 44° for 'faecal *coli*' is thus strongly confirmed.

This marked fall in the number of the *Bact. coli* cultures between 44 and 46° differs from the results of Hajna & Perry (1939), who showed that almost all of their *Bact. coli* were able to produce gas at 46°. However, it must be pointed out that Hajna & Perry found their modified Eijkman medium more suitable than MacConkey's broth for the production of gas by *Bact. coli* at 46°. But they also admit that their modified Eijkman has its defects, because at 44° this medium permits a much higher proportion of other coliforms to produce gas than does MacConkey's broth. Thus merely because the cultures

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of *Bact. coli* examined by these workers did not fail to produce gas at 46°, this does not necessarily imply that theirs is a better method. The root of the problem remains in striking a balance between the number of *Bact. coli* which can produce gas at a certain temperature and the number of other coliforms which are inhibited at that temperature. At present the situation seems to be more adequately met by using MacConkey's broth at a temperature of 44° than Hajna & Perry's modified Eijkman broth at 46°.

General considerations

Whereas the chief feature of the samples of faeces was the presence mainly of *Bact. coli* I, the sanitary significance of which has never been disputed, the bacterial flora of the polluted mussels examined contained, in addition to *Bact. coli*, an assortment of coliform types which much resembled that found in the sewage. This is only to be expected, as mussels are polluted with sewage and not directly by faeces.

Although in water examinations organisms of the coliform group other than *Bact. coli* are regarded as having sanitary significance, this does not apply to shellfish samples, because the multiplication of these organisms in purified shellfish, as reported by Dodgson (1936), renders misleading interpretation of their presence possible.

The precise sanitary significance of the various members of the coliform group other than *Bact. coli* is in any case still doubtful. The widely held view that *Bact. aerogenes* is derived from non-faecal sources, e.g. grain and soil, has been criticized in a review of the literature by Taylor (1941). Sample 16 contained intermediate I and *Bact. coli* II as of faecal origin, though a relatively small but nevertheless substantial proportion of *Bact. coli* I was also present. Samples of faeces such as this, or with a much lower content of *Bact. coli* I down to none, are uncommon, according to Bardsley (1938) and Parr (1938). Bardsley examined 100 specimens of faeces and found *Bact. coli* I in every specimen; in 92% *Bact. coli* I was the dominant organism. Parr, in an examination of 235 stools from 100 persons, found eleven samples which yielded no coliform organisms, but most of these were from infants or invalids. Seven other samples yielded no *Bact. coli*, but contained only intermediate and *Bact. aerogenes* types. The remainder of the stools contained *Bact. coli*. This places these three types of stools respectively as 4·7, 3·0 and 92·3% of the total. He found, however, that the examination of additional samples from those persons whose faeces at one time yielded *Bact. coli*-free samples, subsequently showed the presence of this organism.

Ample evidence as to the faecal origin of sample 16 is afforded by the percentage of *Bact. coli* found, viz. 39. This bears out the view of the Ministry of Health (1939) that organisms of the I.A.C. group are of no practical interest in the presence of more than minimal numbers of *Bact. coli*. The method of detection of *Bact. coli* by incubation at 44° as advocated by Dodgson (1938)

still appears to be adequate, and for shellfish purposes the detection of other coliforms appears to have little, if any, useful purpose.

There remains the question of the importance of irregular I. We have previously experienced a small percentage of this type from polluted shellfish, which, in a bath closely-regulated at 44°, showed variability in gas production both on different occasions and also between replicate tubes on the same occasion. A similar difficulty was reported by Clegg (1941) in connexion with water examinations. Thus there exists a border-line type which cannot be classified definitely as either *Bact. coli* I or irregular I. Although the faecal origin of irregular I is clear, it does not appear to be of great importance; it did not form more than 14% of the + + − − group in any one of the sixteen samples, and only 2·7% of all the + + − − cultures together. As already pointed out this loss from the 44°-positive category was numerically balanced by the inclusion of other 44°-positive types. Hence the present investigation is thought to give substantial confirmation of the value of incubation at 44° for the detection of faecal pollution.

SUMMARY AND CONCLUSIONS

1. The authors concluded in a previous paper that the most reliable single test for the detection of *Bact. coli* was incubation in MacConkey's broth at 44° C., such a test for *Bact. coli* alone being necessary in shellfish because of occasional multiplication of other coliforms in purified mussels. The present investigation is an attempt to verify this finding on a broader basis.

2. A description is given of the isolation of 1600 cultures of coliform organisms from polluted mussels, sewage, and faeces (human, cow, sheep and sea birds). Their classification according to 'IMViC' reactions and the production of gas in MacConkey's broth at temperatures of 37, 41, 44, 45 and 46° C. is given.

3. Of the + + − − type, 1036 out of 1065 cultures (97·3%) were found to produce gas in MacConkey's broth at 44° C. A balance is noted between the + + − − type which does not produce gas at 44° C. (irregular I) and the two irregular types which do so (irregular II and irregular VI). Such a balance was reported by Bardsley (1938).

4. From the cultures examined it is confirmed that incubation at 44° C. is the most suitable temperature for use with MacConkey's broth for permitting the maximum number of *Bact. coli* to produce gas while inhibiting the maximum number of other coliforms.

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