International Council for the Exploration of the Sea.

CM 1955
Shellfish Committee
No. 31.

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A COMPARISON OF METHODS FOR THE BACTERIOLOGICAL EXAMINATION OF MOLLUSCAN SHELLFISH

by

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Introduction

The object of the work described here was to compare some of the principal methods used for the bacteriological examination of molluscan shellfish. For this purpose the roll tube method of Clegg and Sherwood (1947), somewhat modified as the result of several years experience (Reynolds and Wood, 1955), was used as the standard and each of four other methods was compared with it in turn. The four methods were (1) the Fishmongers' Company method described by Knott (1951), (2) Bigger's method (1934), (3) the Canadian M.P.N. as described for the I.C.E.S. Summer Meeting 1954, (4) the phenol broth method described by Ladouce (1954). In each case a number of samples of shellfish was examined simultaneously by the roll tube method and the one being compared with it. When possible the same shellfish were examined by the two techniques, otherwise sub-samples from the same sample were used.

Comparisons of the Methods

(a) Fishmongers' Company Method

In this method 0.2 ml. of each shellfish is tested for the presence or absence of *B. coli* by inoculation in lactose bile broth at 44°C. and the result expressed as % clean.

The procedure followed was that laid down by Knott (1951). For the roll tube method the procedure was modified slightly so that the same ten shellfish could be used for both methods, thus reducing the sampling error and making it possible to obtain a result by each method for the same shellfish. After taking from each shellfish the 0.2 ml. inoculum required for the Fishmongers' Co., the remaining liquors and tissues were pooled. The amount of shell fluid was estimated from a few extra shellfish and the volume of tissue in the pool calculated. This was then diluted with sterile distilled water in accordance with Clegg and Sherwood, making allowance for the volume of shell fluid already present. In some cases six roll tubes were inoculated in place of the usual four. Otherwise the procedure was identical with that used for the routine examination of shellfish. The results are given in Table 1.

Sub-sampling from the same sample showed that wide variations occurred within samples when examined by the Fishmongers' Company Method, e.g. samples 6 and 7 which gave 90% and 40% clean, and samples 10 - 14 which varied from 90% to 60% clean. These differences were sufficient to place the samples in different sanitary grades (Sherwood and Scott-Thomson, 1953), whereas the results of the roll tube method varied but little in comparison, and in no case did the sanitary grading of the sub-samples differ.

(b) Bigger's Method

This was a liquid culture method depending on the formation of acid and gas within 24 hours, in lactose bile broth at 37°C., for the identification of *Bact. coli*. A sample consisted of ten mussels, the number of *Bact. coli* in each mussel was assessed as less than 50, 50-250, 250-1,250, or over 1,250. The sample was classed as "pass" or "fail" according to the number of mussels

having more than a certain number of Bact. coli. (Bigger, 1934). The method had the advantages associated with the individual examination of mussels. If more than seven contained more than 50 Bact. coli the sample failed, likewise if more than three contained over 250, or more than one contained over 1,250 B. coli. On this system a sample containing nine clean mussels and one heavily polluted would pass. This situation is most unlikely to arise with a sample taken direct from the beds but does occasionally occur with purified mussels when one mussel fails to function during an otherwise satisfactory treatment.

Nine samples were examined by this method (Table 2). Three of them fell into Grade I of the classification of Sherwood and Scott-Thomson (1953) and were passed by Bigger's test. Of two in Grade II one passed and one failed by Bigger's test, and of four in Grade III three failed and one passed. This last sample (ref. 3323) was near the border-line by both methods (18 B. coli per ml. of flesh) and had some of the fermentation tubes which were read as "ag" been read as "AG" the sample would have failed.

In general the two methods agreed well in their assessment of the samples.

In eight of the trials Astell bottles were inoculated from the Bigger's method liquor and incubated at 44°C. Counts of faecal Bact. coli per ml. of flesh obtained from these were compared with those from the roll tube method done on ten similar mussels (Table 2a). Of the cases in which there were more than 10 B. coli per ml. of flesh the count from the roll tube method was higher than that from Bigger's method four times out of five. This suggested that the shaking in a closed vessel advocated by Clegg and Sherwood was a more efficient way of releasing and distributing the bacteria than the cutting up and mixing with a pipette recommended by Bigger.

(c) Canadian M.P.N. Method

The procedure adopted was that laid down in report No. 10 to the International Council, Summer 1954. Polluted oysters were opened under aseptic conditions and the liquor and meats pooled in a measuring jug. Approximately 200 ml. were obtained, and the volume then doubled using sterile "dilution water". This was homogenised in the first two experiments with a home-made mixer, but for the rest of the samples a commercially manufactured machine was used. Dilutions of this extract were then made in sterile dilution water and five tubes of lactose broth were inoculated with each of three dilutions, fifteen tubes in all. After incubation at 37°C. for 48 hours the presumptive positive tubes were confirmed by sub-culture to 2% Brilliant Green Bile broth at 37°C. for a further 48 hours. The Most Probable Number of Bact. coli per 100 ml. of oyster meat was determined from Hoskins probability tables (1934).

As a final check the positive B.G.B. tubes were sub-cultured into the same medium at 44°C. and the figure for faecal coliforms was obtained.

In order to compare the efficacy of the roll tube method using the same sample and extract as the M.P.N. method, this extract was used to inoculate roll tubes in the later experiments (Table 3).

For the comparable roll tube examination four oysters of the same sample as that used for the M.P.N. method were examined by the normal method.

The figures in Table 3 may not give a fair comparison of the two methods since, towards the end of the series of experiments, it was realised that by leaving the lactose broths for 48 hours before sub-culturing, many of the lower dilution gas-producing tubes were not confirmed. This was considered to be the result of suppression of coliforms by more vigorous non-coliform organisms. Thus in the samples giving the higher counts these figures were an underestimate of the real number of coliforms present.

TABLE 1

Comparison of the Fishmongers' Co.'s and Roll Tube Methods for examination of Oysters and Mussels

	Oysters															Mussels		
Sample No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	1	2	3
Fishmongers' Co. % clean	20	10	10	70	60	90	40	90	100	90	70	80	80	60	60	50	10	70	70	0
Roll tube B. coli per ml. of Flesh	7.0	6.3	6.3	1.3	2.3	0.0	2.5	1.0	0.0	1.5	0.5	1.5	0.0.	1.5	15.3	11.0	29.2	2.0	0.0	95.0

Samples bracketed together are sub-samples from the same sample.

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TABLE 2

Comparison of Bigger's Method and Roll Tube Method

Ref. No.	Bigger: No. of mussels having *B. coli* content of over 1,250	1,250-250	250-50	Less than 50	Result	Roll Tube: Sanitary Grade	*B. coli* per ml. of flesh
3319	0	3	5	2	Fail	III	24
3321	0	2	7	1	Fail	II	11
3323	0	2	4	4	Pass	III	18
3326	2	6	2	0	Fail	III	43
3330	5	5	0	0	Fail	III	108
3334	1	0	5	4	Pass	II	14
3336	0	0	1	9	Pass	I	1
3346	1	0	3	6	Pass	I	2
3350	0	0	0	10	Pass	I	1

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TABLE 2a

Comparative Roll tube counts done (a) on the mussel extract of Bigger's Method, and (b) on the extract of the Roll Tube Method

Count per ml. of	(Bigger Extract	10	9	32	168	11	3	4	0.5
	(Roll Tube Extract	24	18	43	108	14	1	2	1.5

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TABLE 3

Comparison of the M.P.N. method with the Roll Tube method.
For convenience of comparison all results have been reduced to counts per 1 ml.

Sample No.	M.P.N.		Roll tube counts	
	Coliform Presumptive B.G.B. 37°C. per 1 ml.	Confirmed B.G.B. 44°C. per 1 ml.	per 1 ml. flesh	per 1 ml. M.P.N. extract
1	2.10	1.40	2.00	
2	2.10	0.68	0.0	
3	0.20	0.20	0.0	
4	3.40	2.10	1.50	
5	2.70	2.20	2.00	
6	2.20	1.70	0.0	
7	0.82	0.82	0.0	
8	2.00	2.00	1.00	
9	2.60	2.10	0.50	3.50
10	4.90	Not done	0.0	0.0
11	Dilution insufficient for result	Not done	316.00	Not done
12	4.90	4.90	1.00	3.50
13	1.20	Not done	1.50	5.50
14	Dilution insufficient	-	-	-
15	8.30	Not done	4.50	17.00
16	20.00	20.00	42.00	39.00
17	10.00	8.30	129.00	103.00
18	150.00	120.00	214.00	304.00

(d) Phenol Broth Method

This method was described by R. Ladouce in his contribution to the I.C.E.S. Summer Meeting 1954. It differed from those so far considered in that it did not depend on the fermentation of lactose for the identification of Bact. coli. The formation of indol in phenol broth at 42°C. was taken as the criterion for the presence of this bacterium. It was essentially a M.P.N. method. For shellfish, as opposed to seawater samples, a preliminary 48 hours incubation in peptone broth was given; this was then sub-cultured into phenol broth for a further 48 hours. The purpose of this was to avoid errors caused by bacteria growing on shellfish tissue introduced with the initial inoculum.

Considerable importance was attached to the amount of indol produced as indicating whether the pollution was remote or recent. Tubes yielding a putrid fermentation, even if no indol could be detected, indicated massive pollution. From such tubes it was possible to obtain Bact. coli and confirm it by the Eijkmann and IMV.C reactions. It seemed possible that in such cases the rapid development of a heavy bacterial growth resulted in the early formation of indol, and its breakdown later, but this was not confirmed by experiment.

Two water samples were examined by this method; 50 ml. of water were mixed with 50 ml. of phenol broth, distributed in 10 ml. lots in 10 tubes and incubated at 42°C. for 48 hours. Five Astell roll tubes, each inoculated with 2 ml. of water, were incubated at 44°C. overnight. In the first sample there were 7 Bact. coli per 10 ml. (700 per litre) and each of the ten phenol broth tubes gave a strong reaction, indicating the presence of over 200 per litre. In the second sample there was 1 per 10 ml. (100 per litre) and no reaction was obtained in any of the phenol broth tubes even after amyl alcohol had been added.

One sample of mussels was examined individually by the two methods. The roll tube method showed slight pollution to be present, varying from 0-4 Bact. coli per ml. of flesh. Of the phenol broth cultures made from the same ten mussels two gave a faint indol reaction and eight were negative, but all had a putrid fermentation.

Six further samples of mussels were examined by the two methods. In each case the pooled liquor of ten mussels was examined by the phenol broth method as recommended for a seawater sample, with preliminary incubation in peptone broth, and the pooled liquor of ten similar mussels was examined by the roll tube method (Table 4).

TABLE 4

Comparative *B. coli* counts on mussel samples done by Roll Tube and Phenol Broth Methods

Ref. No.		3388	3396	3398	3400	3403	3406
Bact. coli per ml.		45	40	33	13	34	45
No. of tubes positive	(5 ml.	0	0	1	0	1	-
out of 10 inoculated	(1 "	0	1	3	6	7	0
with following volumes	(0.1 "	5	10	10	9	8	10
of mussel extract	(0.01 "					4*	10

* Only these four tubes showed growth in this set. Heavy growth in all tubes in all other sets.

Discussion

It is useful at this stage to consider what is wanted of a method for the bacteriological examination of molluscan shellfish. Obviously the method must tell us whether a particular sample is polluted or not, but to be a good method it must do much more than this. Not only do we want to be able to detect the presence or absence of pollution but we want to know how much is there, is it a slight amount which may be ignored, or is it present in dangerous quantities? We need a method that is easy to do so that we are not deterred from the taking of an adequate number of samples by the difficulty of doing them. Similarly the method should be one which does not need unduly large quantities of glassware or special apparatus. Not only is this important for the laboratory that handles such samples regularly, but perhaps even more so for the laboratory that may only be called upon to examine shellfish once or twice a year. It is most important that the result of the examination should be available quickly.

In survey work the results obtained from samples taken one day will often help in the planning of the next day's work. In commercial practice consignments are sometimes held up in the market pending the result of examination, clearly here rapidity of getting an accurate result is of prime importance. Last, but not least, the method should be as specific as possible for recent human faecal pollution. Unfortunately it is not yet possible to distinguish between human and other mammalian (or avian) sources, but at least we can make the test reasonably specific for Bact. coli type I, which is widely recognised as the most useful indicator for this purpose.

We can now consider each of the methods in turn (Table 5), leaving the question of liquid v solid media for special consideration at the end.

TABLE 5

	Specific for B. coli	Quick to do	Individual Results	Count of B. coli	Answer in 24 hours
Roll Tube	✓	✓	✓	✓	✓
Fishmongers' Co.	✓		✓		✓
Bigger	✓		✓	½	✓
Canadian M.P.N.				✓	
Phenol Broth	✓	✓	✓		

(a) The method used by the Fishmongers' Company is intended to show the presence or absence of pollution in 0.2 ml. of each shellfish in the sample (normally 10). The result is expressed as the % not showing pollution in this amount, but as a measure of the degree of pollution it is very crude and, as has been mentioned (p. 2), may give widely varying results from a given sample. The method is rather more difficult than the roll tube method and in particular trouble was experienced in cutting up the flesh of mussels in one half shell without losing any of the shell water. The result is obtained quickly (overnight) and it is specific for Bact. coli, since it depends on the production of acid and gas in lactose bile broth at 44°C., which is probably the most specific single test available.

(b) Bigger's method is more elaborate and is intended to give a more accurate count of the numbers of faecal Bact. coli in each mussel. The results are available overnight and agree well with those obtained by the roll tube method. The closeness of the agreement is interesting since this method uses incubation at 37°C. and not 44°C. The main disadvantage is the very laborious nature of the method. For each sample sixty fermentation tubes have to be inoculated. Many samples could not be examined by this method in a single day without a large staff. The large quantities of glassware needed is also a serious drawback.

Although this method gives more information about the degree of pollution than the preceding one, the mussels are still only classified as containing 0-50, 50-250, 250-1,250 or over 1,250 Bact. coli. This is a very crude assessment, especially having regard to the labour involved.

(c) Canadian Most Probable Number Method

This is a method which is important because it is very widely used. It is the standard one for both Canada and the United States of America. It is also essentially similar to one used in some English laboratories where shellfish are cut up and shaken with sterile water, and the resultant liquor is examined by the method recommended by the Ministry of Health (1939), for water supplies. The results obtained are usually expressed as number of Bact. coli per 100 ml. of flesh; to facilitate comparison with the roll tube method they have been reduced to counts per 1 ml. in Table 3. The general agreement between the two methods was reasonably good, but it will be noticed that in samples 1-9, where pollution was light, the M.P.N. method tended to give the higher count, but that the reverse was true in samples 16-18, where pollution was heavy. Sample 11 (where all the dilution tubes gave a positive result) exposes one fundamental weakness of this method. It is essential to have some idea of the degree of pollution before the test is made in order that the appropriate range of dilution tubes may be set up. The test is a very slow one in that four days elapse before the result is known. In our opinion this is a very serious drawback, the more so because it is not accompanied by any compensating advantage of accuracy or detailed information. Confirmation is normally done at 37°C. and not at 44°C. which is widely accepted for this purpose. Only the average bacterial content of the "pooled" sample is given, there is no information as to the pollution of the individuals in the sample as is given in both of the preceding methods. This information is particularly to be desired in the case of purified shellfish in order to distinguish between a sample having a moderate pollution uniformly distributed, due to a failure of the purification technique, and a sample containing one moribund mussel which has failed to function during an otherwise satisfactory purification. The use of a macerator results in a very homogeneous extract and to that extent is a good thing. It is liable to carry in its train difficulties of organisation if many samples are to be examined in one day, unless a good supply of the rather expensive macerator jars is available. This method also necessitates the use of a large number of fermentation tubes and two different culture media.

(d) The phenol broth method is so different from the others that one should not be surprised to find that there was some difficulty in correlating the results obtained with those given by the roll tube method. We found no difficulty in making up the various solutions employed and got clear colour reactions in the dilute indol solutions used as standards, but we could get no satisfactory results from the method for examining shellfish. The fundamental difficulty is that there seems to be no clear distinction between a positive and negative culture tube. One which shows the presence of indol is undoubtedly positive, but one which does not may be positive or negative according to whether or not it shows a putrid fermentation. This made the assessment of results from a set of dilution tubes impossible since often only a few of the lower dilution tubes (1:1 or 1:9) would be indol positive, while most if not all of the higher dilutions (1:99) would be positive, just the reverse from what one would expect. In our hands it was not possible to make a count of the numbers of Bact. coli in a pool of mussels. The method can be used for the individual examination of mussels in which case the presence or absence in each individual is recorded. Here again the difficulty of the tubes which contain Bact. coli, but which are indol negative arises. The information yielded by this method would be similar to that given by the Fishmongers' Co. method, but would take four days instead of one to obtain.

We found the method fairly quick to do and the amount of glassware, etc. needed was reasonable, but this was more than counter-balanced by the four day wait for the result.

Liquid v. solid media

All the four methods discussed so far involve cultivation in a liquid medium with some form of estimation of the M.P.N. of Bact. coli present, whereas the Clegg and Sherwood method uses a solid medium giving a count of actual colonies present. Since an agar plate count is very widely used in bacteriological research as a method of determining the number of bacteria in suspensions, it is clear that its value is not seriously doubted. The very careful work of Wilson (1922) showed that, given a suitable culture medium, a plate count gave an accurate count of the number of viable bacteria present. It will be recalled that he used this method, in conjunction with a microscope count of total cells, to estimate the proportion of living cells in young cultures. It was obvious from his work that the colony count properly done yielded very consistent and accurate results.

The M.P.N. method on the other hand is known to be lacking in such accuracy. Prescott, Winslow and McCrady (1945) discuss this in some detail, quoting Halvorson and Ziegler's (1933) finding that with a bacterial density of about 1.5 per ml., of counts made with 5 tubes each of 3 dilutions 97% will fall in the range of 30% to 360% of the correct value. If 40 tubes were used for each of the three dilutions the range would be reduced to 62% to 147% of the correct value. Following on from this Prescott et al. conclude that "some 50 to 100 tubes with each of three dilutions would be needed to furnish an estimate equal in accuracy to a single satisfactory plate count."

Because of the rather high temperature of incubation, and the presence of inhibiting substances such as bile salts, the MacConkey agar roll tube does not give a full count of the numbers of Bact. coli present, but it does give a constantly high proportion of the true count, and is much less variable than the M.P.N. count. This was clearly shown by Clegg and Sherwood (1947).

The usual criticism made of the use of a solid medium is that it does not permit of the detection of gas formation from lactose, and so colonies which would produce only acid are counted as well as those which would produce acid and gas. This point was specially investigated by Clegg and Sherwood who found that of 1,000 red colonies from tubes incubated at 44°C., 969 were IMViC ++--, i.e. Bact. coli I and Irregular I, both of which are considered to be of intestinal origin. Hence any error introduced in this way is very small, of the order of 3%. Further experience in plating out red colonies from these roll tubes has shown that it is very rare to find a colony that does not produce full acid and gas in MacConkey broth at 44°C. Even these may be genuine Bact. coli since anaerogenic strains have been reported (Glantz and Dunne 1955). There is in fact no reason to doubt that for all practical purposes a bacterium which produces a red colony in MacConkey agar at 44°C. is Bact. coli.

The roll tube method of Clegg and Sherwood fulfils all the requirements, mentioned earlier, for the examination of shellfish and marine waters. It gives an accurate measure of the degree of pollution present expressed in simple units, it is easy to do, so that five or six samples may be examined individually, or perhaps up to a dozen examined as "pools", in one day by a single operator. Although some special apparatus is desirable the method can be used with the glassware normally found in any bacteriological laboratory. The result is available overnight, sixteen hours incubation is sufficient to make all the colonies visible.

In our opinion this method yields more information than any of the others we have tried, and the information given is more accurate. For these reasons alone it is the method to be recommended; the ease with which it may be used and the speed with which the results become available are additional advantages which, in our opinion, place it in a class of its own.

Bibliography

BIGGER, J. W., 1934. The Bacteriological Examination of Mussels. J. Hyg. 34, 172-194.

CLEGG, L. F. L. and SHERWOOD, H. P., 1947. Bacteriological Examination of Molluscan Shellfish. J. Hyg. 45, 504-521.

GLANTZ, P. J. and DUNNE, H. W., 1955. Isolation of Anaerogenic E. coli 026:B6 serotype from a case of calf scours. Science, 121. No. 3156, p.902.

HOSKINS, J. K. 1934. Publ. Hlth. Rep. Wash. 49, 393.

KNOTT, 1951. "Memorandum on the principals and standards employed by the Worshipful Company of Fishmongers in the Bacteriological Control of Shellfish in the London Markets". Fishmongers Co., London.

LADOUCE, R., 1954. "Evaluation de la Salubrité des Eaux et des Coquillages en France". I.C.E.S. Shellfish Committee. Paris, 1954.

MINISTRY OF HEALTH, 1939. The bacteriological examination of water supplies. Rep. Publ. Hlth. Med. Subj., Lond., No. 71.

PRESCOTT, S. C., WINSLOW, C. A. and McCRADY, M.H., 1945. Water Bacteriology. Chapman and Hall, London.

REYNOLDS, N. and WOOD, P.C., 1955. Improved techniques for the Bacteriological Examination of Molluscan Shellfish and Water (in the press).

SHERWOOD, H.P. and SCOTT-THOMSON, 1953. Bacteriological examination of shellfish as a basis for sanitary control. Monthly Bull. of the Min. of Health and P.H.L.S. May, 1953. 103.

HALVORSON, H. O. and ZIEGLER, N. R., 1933. Quantitative Bacteriology. Burgess Publishing Co. Minneapolis.

WILSON, G. S., 1922. The proportion of viable Bacteria in young cultures with especial reference to the technique employed in counting. J. Bact. 7, part 4, 405.