IMPROVED METHODS FOR DETERMINING THE MOST PROBABLE NUMBER OF *BACTERIUM COLI* AND OF *STREPTOCOCCUS FAECALIS*

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(With 3 Figures in the Text)

Methods have recently been published (Allen, Pasley & Pierce, 1952; Allen, Pierce & Smith, 1953) for obtaining colony counts of *Bacterium coli* and of *Streptococcus faecalis* in samples, such as polluted waters, which contain a heterogeneous flora. These methods give a more accurate value than the dilution count but they are applicable only to samples with a sufficiently high count to give a reasonable number of colonies in an inoculum of 5 ml. In less polluted liquids, as, for example, in waters approaching the quality of drinking water, a technique permitting the use of larger volumes of water is necessary. Determination of the Most Probable Number (M.P.N.) by inoculating measured quantities of the sample or of suitable dilutions of it into replicate tubes of a differential medium is probably the most convenient method. This is also the method of choice for samples of comparatively high count which are being examined in the field or in laboratories not equipped with apparatus for spinning bottles.

In the work described in this paper the existing methods of determining the M.P.N. of *Bact. coli* and of *Str. faecalis* are surveyed and modifications designed to improve the methods are introduced.

EXPERIMENTAL

In the examination of polluted waters, growth in bile salts-lactose broth at 44° C. has been found to be nearly specific for *Bact. coli*, type I, and growth in glucose-azide broth at 45° C. for *Str. faecalis* (cf. Allen, Brooks & Williams, 1949). In the 'direct' method of determining the M.P.N. inoculated tubes are incubated directly at these temperatures. In the 'subculture' method the inoculated tubes are first incubated at 37° C. for 24–48 hr., and positive tubes are then subcultured to fresh tubes of the same medium incubated at the higher temperature.

Allen *et al.* (1952, 1953), in a study of the factors affecting the colony counts of faecal bacteria, found that the inhibitory effect of selective media incubated at these higher temperatures was largely overcome if the bacteria were subjected to a short preliminary period of resuscitation in sugar broth at 37° C. This principle has been applied in the present work to the determination of the M.P.N. by the 'resuscitation' method. In this method the media are prepared in two parts, each of double strength for use with inocula of 1 ml., or of quadruple strength for use with inocula of 10 or 50 ml. One part consists of a nutrient sugar broth and the other part contains the selective agent, consisting of bile salts for *Bact. coli* and of

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sodium azide for *Str. faecalis*, and the indicator. Details of the media and of the technique finally used are given in the Appendix. In general, the inoculum was added to a measured quantity of the nutrient portion of the medium in a tube or bottle. This mixture was incubated in a water-bath at 37° C. for a period of resuscitation varying from 1 to 2 hr. in different experiments. The second portion of the medium was then added aseptically and the bottles or tubes were incubated in a water-bath accurately controlled at 44° C. for *Bact. coli* and at 45° C. for *Str. faecalis*. In the conventional method of detecting *Bact. coli* Durham's tubes are included in each test-tube or bottle of medium and they become filled with the liquid during sterilization. In the modified method this is not possible because the second portion of the medium is not added until after the inoculum. The difficulty was overcome by using screw-capped McCartney bottles. After adding the second portion of the medium the cap was screwed down and the Durham's tubes were filled by momentarily inverting the bottles.

Results with Bacterium coli

Preliminary tests with media containing neutral red showed that with many samples this indicator exerted an inhibitory effect on the growth of *Bact. coli*. In the remaining tests, therefore, 0·003 % of brom-cresol purple was employed as the indicator. The composition of the lactose-bile salts broth used as a medium is given in the Appendix.

Comparison of 'direct' method with 'subculture' method

Clegg (1941) concluded from the results obtained for the M.P.N. of *Bact. coli* in 413 samples of water, in which this organism was present, that the 'subculture' method gave significantly higher results than the 'direct' method; the medium he used for these tests was MacConkey broth containing Andrade's indicator. He obtained a higher count by the 'subculture' method with 248 samples and by the 'direct' method with 105 samples; sixty samples gave identical results by both methods. Using the binomial test of significance of consistency in sign, Buchanan-Wollaston (1941) showed that the probability (P) of the observed difference occurring by chance was less than 10^{-11}. Nearly two-thirds of the samples examined by Clegg were of stored or treated waters.

In the present work similar tests, comparing the two methods, were made with thirty samples of river water, of sewage, and of sewage effluent. A higher count was obtained with 12 samples by the 'direct' method and with 14 samples by the 'subculture' method; identical results were recorded with 4 samples ($P = 0{\cdot}84$). If the value 0·2 is adopted for P as the level of significance, as suggested by Buchanan-Wollaston (1941) for bacteriological work, it may be concluded that in these tests the two methods did not give significantly different results.

The difference between these conclusions and those arrived at by Clegg (1941), as to the relative merits of the two tests, may be due to the comparatively low viability of organisms in stored or treated waters, which renders them more susceptible to the effects of direct incubation at 44° C. than are the organisms in

river water (cf. Mackenzie, Taylor & Gilbert, 1948). The results of a few tests with a medium containing neutral red suggested that the indicator may also influence the counts obtained by the two methods. Neutral red, for example, appeared to be more strongly inhibitory to the bacterial cells in a sample of water which was incubated with lactose-bile salts broth directly at 44° C. than it was to the progeny of the same cells after primary incubation at 37° C.

Comparison of 'resuscitation' method with 'subculture' method

With 50 samples, of which 31 came from rivers, 4 from canals, 2 from wells, 1 from a reservoir, and 12 from sewage works, the M.P.N. was determined by the 'resuscitation' method, in which the preliminary period of resuscitation at 37° C.

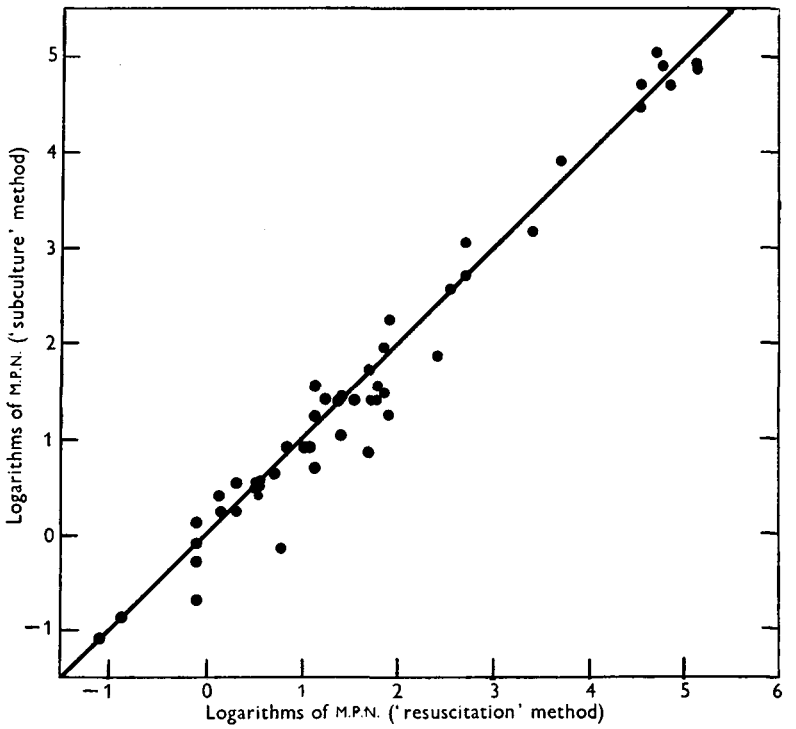

Fig. 1. Comparison of results of determination of the Most Probable Number (M.P.N.) of *Bact. coli* in lactose-bile salts broth by 'subculture' and 'resuscitation' methods.

was 1 hr., and by the 'subculture' method. Results are compared graphically in Fig. 1, in which the logarithms of the M.P.N. obtained by the two methods for each sample are plotted against each other. With 23 samples the 'resuscitation' method gave a higher count, with 15 samples the 'subculture' method gave a higher count, and with 12 samples the results with both methods were identical ($P = 0{\cdot}19$). The 'resuscitation' method therefore gave just significantly higher results than the 'subculture' method.

Relative times required for performance of the two tests

In the 'subculture' method the period of primary incubation at 37° C. must be prolonged for 48 hr. if the true count at this temperature is required. Although the majority of positive tubes is developed in 24 hr. a small but significant proportion of slowly growing organisms does not form gas until later. Only a fraction of these, however, consists of faecal coli forming gas at 44° C. Thus in 85 samples tested in the present work, in which there was a total of 854 positive tubes at 44° C., only four positive tubes arose from organisms which took more than 24 hr. to ferment lactose at 37° C. If the count of faecal coli is required, therefore, it seems reasonable to neglect the small fraction included in the late lactose-fermenters and to incubate at 37° C. for only 24 hr. A further period of incubation of 24 hr. is required at 44° C. Throughout the tests made in the course of this investigation no instance has been recorded where gas formation at 44° C. has taken place after 24 hr. The total time occupied by the 'subculture' test may therefore be taken as 48 hr. The 'resuscitation' test is completed in 18–24 hr. In the samples used in this work gas formation has never required more than 24 hr.

Specificity of 'resuscitation' method for Bacterium coli

During the course of the investigation isolations were made from 57 tubes, showing acid and gas at 44° C. in the 'resuscitation' method, by streaking the contents on MacConkey agar, incubating at 37° C. and subculturing representatives of the resulting colonies. Cultures so obtained were subjected to the indole, methyl red, Voges-Proskauer and Koser's citrate tests. Of 57 strains isolated from 19 samples of river water and 10 samples of sewage 56 strains conformed to the characters of *Bact. coli* type I; the remaining strain was Irregular, type II. The method therefore appeared to be nearly specific for 'faecal' coli.

Results with Streptococcus faecalis

The glucose azide broth used in comparative tests was based on the formula given by Hannay & Norton (1947). Details are given in the Appendix.

Comparison of 'direct' method with 'subculture' method

Apart from a passing reference by Ostrolenk, Kramer & Cleverdon (1947), unsupported by experimental data, the advantages of primary incubation at 37° C. when determining the M.P.N. of *Str. faecalis* in glucose azide broth do not appear to have been considered. Allen *et al.* (1953) showed that direct incubation at 45° C., as recommended by Hajna & Perry (1943) and by Hannay & Norton (1947), inhibited growth of an appreciable proportion of cells, particularly of those weakened by age or by long immersion in water.

Comparative tests, using the 'direct' and the 'subculture' methods, were made with 12 samples of sewage sludge, 10 of sea water polluted with sewage, 5 of lake deposits, 3 of inland river water, 6 of sewage, and 3 of sewage effluent. Of the 39 samples tested the M.P.N. was higher by the 'subculture' method with 28 samples

and by the 'direct' method with 8 samples; 3 samples gave the same result by both methods ($P = 0{\cdot}00086$). Significantly higher results are therefore given with *Str. faecalis* by the method involving primary incubation at 37° C.

The results obtained by the two methods are compared graphically in Fig. 2.

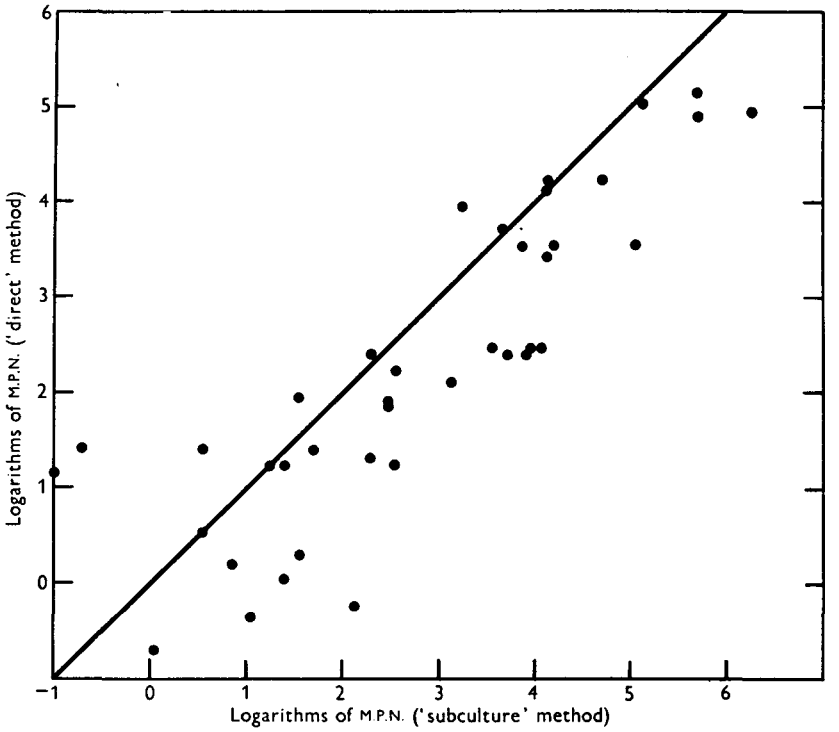

Fig. 2. Comparison of results of determination of the Most Probable Number (M.P.N.) of *Str. faecalis* in glucose azide broth by 'direct' and 'subculture' methods.

Comparison of 'resuscitation' method with 'subculture' method

Two series of tests were performed, with samples of water from rivers, reservoirs, and wells and with samples of sewage and sewage effluent, in order to compare the M.P.N. obtained by the 'subculture' method with those obtained by the 'resuscitation' method. In the first series the period of resuscitation was 1 hr. and in the second series it was 2 hr. Results are expressed graphically in Fig. 3.

Of a total of 28 samples in the first series of tests, 15 gave higher counts by the 'resuscitation' method, 11 gave a higher count by the 'subculture' method, and 2 gave identical results by both methods ($P = 0{\cdot}56$). In the second series 68 samples were tested; the 'resuscitation' method gave a higher count on 38 occasions and the 'subculture' method on 24 occasions; 6 samples gave identical results by both methods ($P = 0{\cdot}076$).

The 'resuscitation' method therefore tends to give higher counts than the 'subculture' method, significantly so if the period of resuscitation lasts 2 hr. When determining the M.P.N., there is no danger, as there is with the colony count, that too long a period of resuscitation will give too high a count through proliferation

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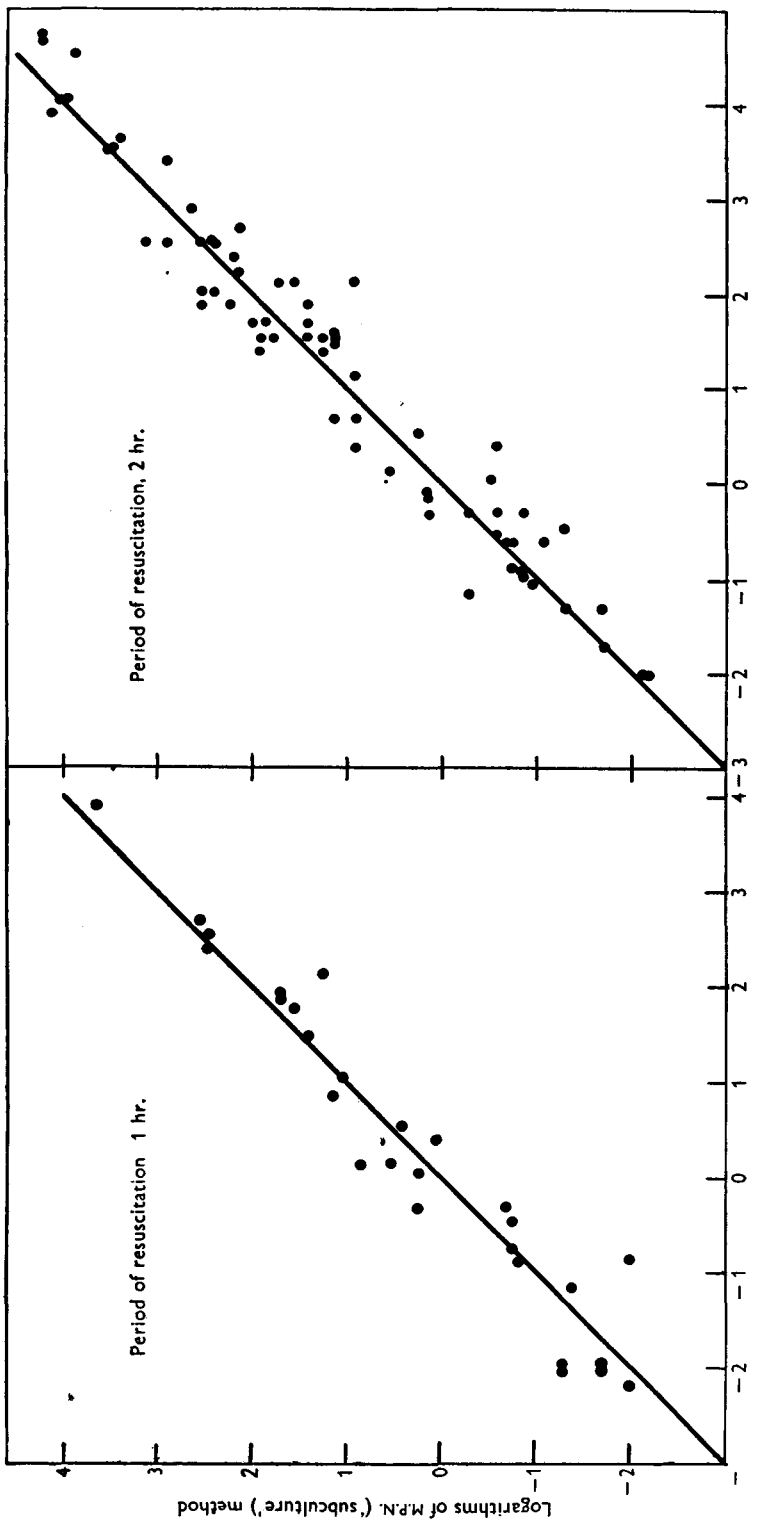

Fig. 3. Comparison of results of determination of the Most Probable Number (M.P.N.) of *Str. faecalis* by 'subculture' and 'resuscitation' methods.

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taking place at 37° C. Whether a tube recorded as positive after incubation at 45° C. initially contained a single cell or a group of several cells makes no difference to the M.P.N.

Relative times required for performance of the two tests

The 'subculture' method requires a period of incubation of 2 days at each temperature. Although the majority of positive tubes can be identified as such after 24 hr. a significant proportion of the total would be missed if no observations were recorded after that period. Thus, of a total of 793 tubes which were positive at 37° C. in the series of tests described here, 489 were positive after 24 hr.; at 45° C., 455 of the 577 positive tubes were recorded after 24 hr. The 'resuscitation' method requires a period of incubation of 48 hr. only, with the majority of positive tubes apparent after 24 hr. Of 1357 tubes and bottles recorded as positive throughout the present investigation 1084 were positive after 24 hr.

Effect of phosphate on the count

It had been found previously (Allen *et al.* 1953) that, if both phosphate and peptone were included in the medium before autoclaving, the number of cells of *Str. faecalis* able to form colonies on glucose-azide agar at 45° C. was lower than was recorded if a solution of the phosphate was sterilized and added separately. Tests were made to see whether phosphate exerted a similarly depressing effect on the M.P.N. by the 'resuscitation' method. The M.P.N. of 20 samples of polluted waters were determined in two batches of media. The first batch contained half the total phosphate in each of the two portions of the medium before autoclaving; in the second batch all the phosphate was included in the azide portion of the medium and none was autoclaved in the presence of peptone. Higher counts were recorded on eight occasions in the first medium and on nine occasions in the second medium; identical results were obtained with both media on three occasions. It appeared, therefore, that the effect of phosphate on the M.P.N. was insignificant.

Specificity of 'resuscitation' method for Streptococcus faecalis

From 67 tubes, positive at 45° C. in the 'resuscitation' method, pure cultures were isolated by streaking on glucose-yeast extract agar, incubating at 37° C. and subculturing from the resulting colonies. These cultures were tested as described by Allen *et al.* (1949). Of 67 strains isolated from 34 samples of polluted waters all were Gram-positive cocci, in pairs and short chains, which grew at pH 9·6 and in broth containing 6·5 % sodium chloride, which survived heating at 60° C. for 30 min., and which acidified litmus milk. The method, therefore, appeared to be specific for *Str. faecalis*.

SUMMARY

Three methods have been investigated for determining Most Probable Numbers, using lactose-bile salts broth for *Bacterium coli* and glucose-yeast extract-azide broth for *Streptococcus faecalis*. In the 'direct' method inoculated tubes of medium are incubated directly in a water-bath, at 44° C. for *Bact. coli* or at 45° C. for

Str. faecalis. In the 'subculture' method primary incubation at 37° C. is followed by subculture of positive tubes to tubes of fresh media which are incubated at the higher temperature. In the 'resuscitation' method each medium is divided into 2 parts. The inoculum is mixed, in McCartney bottles or tubes, with the first part of the medium, containing the sugar and the peptone, and the mixture is held at 37° C. for 1–2 hr. to resuscitate organisms weakened by age or by long immersion in water. The second part of the medium, containing the bile salts or the azide, is then added and the bottles or tubes are incubated at 44 or 45° C. With samples of polluted waters the 'subculture' method gives significantly higher counts than the 'direct' method for *Str. faecalis* but not for *Bact. coli*. The 'resuscitation' method gives significantly higher results than the 'subculture' method for both organisms, it requires less time for completion, and is specific for the organism concerned.

These results were obtained with media containing 0·003 % brom-cresol purple as indicator; neutral red was found to be inhibitory.

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APPENDIX

Media and methods for determining M.P.N.

Bacterium coli

'Subculture' method

Medium. Peptone, 20 g.; NaCl, 5 g.; bile salts,* 1·5 g.; lactose, 5 g.; brom-cresol purple, 2 ml. of 1·6 % alcoholic solution; distilled water, 1000 ml.

(1) Dissolve peptone, sodium chloride, and bile salts in 900 ml. of the water. Heat in steamer for 1 hr., adjust pH value to 7·0, and replace in steamer for a further 30 min. Filter through pulp filter.

(2) Dissolve lactose in 100 ml. water and add brom-cresol purple. Mix solutions (1) and (2), distribute in 5 ml. quantities in test-tubes containing Durham's tubes, and autoclave at 15 lb. for 20 min.

Procedure. Tubes inoculated with serial tenfold dilutions of the sample are incubated in a 37° C. air incubator. After 18–24 hr. positive tubes are subcultured to fresh tubes of medium which are incubated in a water-bath at $44 \pm 0{\cdot}2$° C. Tubes remaining in the 37° C. incubator are examined and similarly treated after a further period of 24 hr. Positive tubes at 44° C. are recorded after incubation for 24 hr.

For use with inocula of 10 ml. or 50 ml. a medium of double the above strength is prepared and distributed respectively in 10 and 50 ml. quantities.

'Resuscitation' method

Double-strength broth. Peptone, 40 g.; NaCl, 10 g.; lactose, 10 g.; distilled water, 1000 ml.

Dissolve peptone and sodium chloride in 900 ml. water, heat in steamer for 1 hr.,

* The concentration of bile salts required depends on the purity and strength of the commercial article. Guidance can usually be obtained from the manufacturer.

adjust pH value to 7·0, and replace in steamer for further 30 min. Dissolve lactose in remaining 100 ml. water. Mix the two solutions and fill into McCartney bottles. Autoclave at 15 lb. for 20 min.

Double-strength bile salts solution. Bile salts,* 3 g.; brom-cresol purple, 4 ml. of 1·6 % alcoholic solution; distilled water, 1000 ml.

Procedure. For 1 *ml. inocula.* Use $\frac{1}{2}$ oz. McCartney bottles, containing $1\frac{3}{8} \times \frac{5}{16}$ in. Durham's tubes and 2·5 ml.† double-strength broth. Care should be taken that the broth is not drawn up the Durham's tube. Place the tubes in the 37° C. water-bath for about 1 hr. before use. Add the 1 ml. inocula of the sample or of suitable dilutions of it and replace in the water-bath for 1 hr.

Add 2·5 ml.† bile salts solution to each bottle, mix and invert bottles momentarily to fill Durham's tubes. Place in water-bath at $44 \pm 0{\cdot}2°$ C. Record positive tubes after 18–24 hr.

For 10 *ml. inocula.* Use 1 oz. McCartney bottles containing $1\frac{3}{8} \times \frac{5}{16}$ in. Durham's tubes. Add 5 ml. quantities of broth and of bile salts solution, each of quadruple strength.

For 50 *ml. inocula.* Use 4 oz. McCartney bottles containing $3 \times \frac{7}{16}$ in. Durham's tubes. Add 25 ml. quantities of broth and of bile salts solution, each of quadruple strength.

Streptococcus faecalis

'Subculture' method

Medium. Peptone, 10 g.; Yeastrel, 3 g.; K_2HPO_4, 5 g.; KH_2PO_4, 2 g.; glucose, 5 g.; NaCl, 5 g.; sodium azide, 0·25 g.; brom-cresol purple, 2 ml. of 1·6 % alcoholic solution; distilled water, 1000 ml.

Dissolve the peptone, phosphates and sodium chloride in water. Autoclave the Yeastrel in 50 ml. of water at 15 lb. for 20 min., mix the two solutions and make up the volume to 1 l. Steam for 1 hr. Adjust pH to 6·9 if necessary.

Add the remaining constituents to the hot solution and shake thoroughly to dissolve. If necessary filter through pulp filter.

Distribute in tubes in 5 ml. quantities and sterilize at 15 lb. for 20 min.

For use with inocula of 10 or 50 ml. a medium of double the above strength is prepared and distributed, respectively, in 10 and 50 ml. quantities.

Procedure. Tubes inoculated with successive tenfold dilutions of the sample are incubated in a 37° C. air incubator. As soon after formation of acid as possible, which varies from 18 to 48 hr., positive tubes are subcultured to fresh tubes of medium which are incubated in a water-bath at $45 \pm 0{\cdot}2°$ C. Positive tubes at 45° C. are recorded after incubation for 48 hr.

'Resuscitation' method

Double-strength broth. Peptone, 20 g.; Yeastrel, 6 g.; K_2HPO_4, 5 g.; KH_2PO_4, 2 g.; glucose, 10 g.; NaCl, 5 g.; distilled water, 1000 ml.

* The concentration of bile salts required depends on the purity and strength of the commercial article. Guidance can usually be obtained from the manufacturer.

† For convenience in pipetting this quantity was reduced to 2 ml. during part of the investigation. The tests under these conditions were still found to be specific for *Bact. coli*, type I.

Dissolve the peptone, phosphates and sodium chloride in water. Autoclave the Yeastrel in 50 ml. of water at 15 lb. for 20 min., mix the two solutions and make up the volume to 1 l. Steam for 1 hr. Adjust pH to 6·9 if necessary. Add the glucose to the hot solution and shake thoroughly to dissolve. If necessary filter through pulp filter.

Distribute in test tubes or McCartney bottles. Autoclave at 15 lb. for 20 min.

Double-strength azide solution. Sodium azide, 0·5 g.; brom-cresol purple, 4 ml. of 1·6 % alcoholic solution; NaCl, 5 g.; K_2HPO_4, 5 g.; KH_2PO_4, 2 g.; distilled water, 1000 ml.

Dissolve all the constituents in the water. Sterilize in the autoclave at 15 lb. for 20 min.

Procedure. For 1 *ml. inocula.* Use $6 \times \frac{5}{8}$ in. test-tubes containing 2·5 ml.* double-strength broth. Place the tubes in a 37° C. water-bath for about an hour before use. Add the 1 ml. inocula of the sample, or of suitable dilutions of it, and replace in the water-bath for 2 hr.

Add 2·5 ml.* azide solution to each tube. Place in water-bath at $45 \pm 0{\cdot}2°$ C. Record positive tubes after 24–48 hr.

For 10 *ml. inocula.* Use 5 ml. quantities of broth and of azide solution, each of quadruple strength, in $6 \times \frac{3}{4}$ in. test-tubes.

For 50 *ml. inocula.* Use 25 ml. quantities of broth and of azide solution, each of quadruple strength, in 4 oz. McCartney bottles.

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* For convenience in pipetting this quantity was reduced to 2 ml. during part of the investigation. The tests under these conditions were still found to be specific for *Str. faecalis*.

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