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A medium for the rapid enumeration of *Escherichia coli* in the presence of other faecal coliforms in tropical waters

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SUMMARY

A selective membrane filtration medium is described for use in the rapid assessment of water quality in tropical countries where the incidence of faecal coliforms other than *E. coli* presents problems in the interpretation of results. The medium gives comparable results to MPN values obtained in the multiple tube dilution test using modified Gray's glutamate medium, and to membrane filtration counts obtained using M-FC broth and membrane-enriched Teepol broth, whilst differentiation of *E. coli* is enhanced.

INTRODUCTION

The use of a coliform count as an indicator of faecal pollution of water supplies is now generally supplemented by a faecal coliform (FC) count (D.H.S.S., 1969; W.H.O., 1971; A.P.H.A., 1981) in order to confirm the faecal origin of coliform contamination. Not all coliforms originate in the animal bowel (Moussa, 1965; Dutka, 1973). Also, the membrane filtration technique has often replaced the multiple tube dilution method for the enumeration of coliforms and FC.

Standard methods in the United States of America (A.P.H.A., 1981) and the United Kingdom (D.H.S.S., 1969) recommend the use of M-FC broth and membrane-enriched Teepol broth, incubated at 44·5° and 44·0 °C respectively, for the estimation of FC density by membrane filtration. Acid-producing colonies appearing on these media are accepted as presumptive *E. coli*. Whilst the weight of evidence suggests that this presumption is acceptable in countries with temperate climates, some authors have indicated that this may not be the case in tropical countries (Evison & James, 1973; Barrell & Rowland, 1979). Early experience of water quality testing for FC in Sierra Leone has confirmed the uncertainty of identity of colonies appearing on standard media; using M-FC broth, many pale blue colonies were apparent which were demonstrated to be indole negative, whilst using membrane-enriched Teepol broth, acid-producing (yellow) colonies of differing morphology were apparent, many of which (in some cases more than 90%) were again demonstrated to be indole negative; oxidase-positive aeromonads were not encountered frequently amongst the acid producers and both media gave high background counts.

A medium was therefore sought, for use primarily in Sierra Leone, but with potential for use in other countries, which would give rapid *E. coli* counts without the need for confirmatory tests of all colonies producing acid from lactose at the elevated incubation temperature. Sodium lauryl sulphate (LS) has recently been recommended (Joint Committee of the Public Health Laboratory Service and the Standing Committee of Analysts, 1980) as an acceptable selective ingredient in a membrane filtration medium for the estimation of coliforms and FC. The use of LS in such a medium was considered preferable to the use of Teepol 610 (the selective ingredient in membrane-enriched Teepol broth) or bile salts + rosolic acid (selective ingredients in M-FC broth) in terms of the chemically defined nature of LS, its ready availability in a pure form and the ability to include it in a dehydrated medium. Trial media in this study were therefore based on the use of LS as selective agent.

MATERIALS AND METHODS

Standard media

M-FC broth was prepared as described by A.P.H.A. (1981). Membrane-enriched Teepol broth and modified Gray's glutamate medium were prepared according to D.H.S.S. (1969).

Trial media

The sodium lauryl sulphate used in these media was a specially purified grade with a minimum assay of 99·0% (BDH No. 44215). Concentrations of all ingredients are given in g l^{-1}.

Medium 1: LS (1·0) was substituted for Teepol 610 in membrane-enriched Teepol broth.

Media 2–8: These consisted of a common basal nutrient medium of (all Oxoid) tryptose (20·0), yeast extract (6·0) and lactose (30·0), plus the following (all BDH).

Medium 2: LS (2·0) and phenol red (0·2).

Medium 3: LS (1·0) and phenol red (0·2).

Medium 4: LS (1·0) and bromocresol purple (0·2).

Medium 5: LS (4·0), phenol red (0·2) and aniline blue (0·4).

Medium 6: LS (2·0), phenol red (0·2) and aniline blue (0·2).

Medium 7: LS (1·0), phenol red (0·4) and aniline blue (0·4).

Medium 8: LS (1·0), phenol red (0·2) and aniline blue (0·1).

All trial media were adjusted to a pH of 7·4 with NaOH, brought to the boil and allowed to cool. No further sterilization was found to be necessary provided media were used on the day of preparation.

Sampling and analysis

All samples were obtained from water sources used as supplies of water for domestic use by 31 settlements in Moyamba District, Southern Province, Sierra Leone. All sources were unprotected and had already been shown to contain FC.

Initially, 10 water samples from different sources were examined by both the multiple tube method using modified Gray's glutamate medium and membrane

filtration using each of the eight trial media described above. Filters (Millipore, 0·45 μm, 47 mm diameter) were incubated for 18 h on absorbent pads soaked in the relevant medium. After evaluating results, the most suitable medium (medium 8) was subjected to further trials, as follows.

Thirty-one water samples from different sources were tested for FC by both multiple tube and membrane filtration methods. Counts were made of colonies appearing on filters at 10, 12, 15 and 18 h incubation. Three counts were made for each filter: green colonies, green plus yellow colonies and total colonies.

Thirty-one further samples, from the same sources used above, were tested for FC by membrane filtration, in triplicate: one filter was incubated on M-FC broth, one on membrane-enriched Teepol broth and the third on the new medium.

A third batch of 31 samples was tested for FC by both the multiple tube method and membrane filtration using a solidified version of medium 8 (Oxoid Agar No. 1, 1 % w/v, added before boiling).

All incubation was carried out in a water bath at 44·0 °C. The effect of direct incubation at 44·0 °C on results obtained in the multiple tube test was initially investigated by performing triplicate analyses on the 31 water sources under investigation, incubating one series of tubes at 37 °C throughout the period of incubation, one series at 37 °C for 4 h followed by 44·0 °C and the third series at 44·0 °C throughout. All positive (acid + gas production) tubes were tested for indole production, as described below. Results showed that, whilst the number of tubes showing acid + gas production was significantly ($P < 0·001$) higher in the series incubated at 37 °C throughout, the numbers of tubes confirmed as indole positive were not significantly ($P > 0·05$) different between series when results from subculture after 48 h incubation were compared. A comparison of results from subculture after 24 h incubation revealed a significantly ($P < 0·05$) lower number of indole-positive tubes from the two series incubated at the elevated temperature than from the series incubated at 37 °C throughout. Direct incubation at 44·0 °C was therefore considered useful in reducing the number of positive tubes produced in the multiple tube test which were not attributable to indole-positive organisms, provided incubation was maintained for 48 h.

All positive tubes occurring in the multiple tube test within 24 h were subcultured to tryptone water (D.H.S.S., 1969), incubated at 44·0 °C for 24 h and tested for indole production. Negative tubes in the multiple tube test were incubated for an additional 24 h and any further positives obtained were similarly subcultured to test for indole production. Most probable number (MPN) values were derived from:

(i) positive tubes occurring within 24 h in the multiple tube test;
(ii) above tubes which were shown to contain indole-positive organisms;
(iii) positive tubes occurring within 48 h in the multiple tube test;
(iv) above tubes which were shown to contain indole-positive organisms.

In order to confirm the identity of lactose-fermenting organisms, 20 pure cultures of indole-positive isolates causing a positive reaction in the multiple tube test within 24 h were obtained and these were tested for methyl red, Voges-Proskauer and citrate reactions as described by Cowan & Steel (1965). Similarly, 20 of the green colonies and 20 of the yellow colonies appearing on medium 8 (at

18 h) were subjected to these (IMViC) tests and their ability to produce a positive reaction in modified Gray's glutamate medium. The Gram-staining reaction of each isolate was also recorded. All isolates were selected randomly from the above experiment comparing the multiple tube method with membrane filtration using medium 8 (broth).

RESULTS

In order to avoid ambiguity, the term 'faecal coliform' or 'FC', as used herein, refers to those organisms capable of fermenting lactose in the multiple tube dilution test under the conditions of incubation described, and to those organisms producing acid from lactose in the membrane filtration test under the conditions described. The term is qualified by the indole reaction of cultures where this was determined. Use of the specific designation, *E. coli*, is restricted to those organisms demonstrated to give the IMViC reactions + + − − and to those organisms giving a characteristic colonial appearance on the membrane filtration media described.

Preliminary trials

Counts obtained on trial membrane filtration media were recorded as colony-forming units (c.f.u.) dl^{-1} and compared to MPN counts by means of the Wilcoxon signed rank test. Significant differences were looked for at the $P < 0{\cdot}05$ level.

Trial media 1, 3 and 4 gave counts of acid-producing (yellow) colonies equivalent to MPN values derived from positive tubes occurring in the multiple tube test (FC) within 48 h, but significantly higher than MPN values derived from positive tubes demonstrated to contain indole-positive organisms (indole-positive FC). Visual differentiation of acid-producing colonies from other colonies was poor, due to the high proportion of the latter.

Trial media 2, 5, 6 and 7 gave counts which were significantly lower than both the MPN FC and MPN indole-positive FC values (24 and 48 h). Visual differentiation on media 5, 6 and 7 was much improved, however; inclusion of aniline blue allowed a further differentiation of acid-producing colonies into green (those incorporating aniline blue) colonies and yellow colonies.

Trial medium 8 gave counts of acid-producing (green plus yellow) colonies equivalent to 24 h MPN FC values and counts of green colonies alone were seen to be equivalent to 48 h MPN indole-positive FC values.

From the above trials, it was concluded that sodium lauryl sulphate was a satisfactory selective ingredient for inclusion in a membrane filtration medium for the estimation of FC, that concentrations of sodium lauryl sulphate $> 1{\cdot}0$ g l^{-1} were inhibitory for some FC, that at least two groups of micro-organisms were capable of producing acid from lactose under the conditions described, that these two groups could be distinguished by their ability to incorporate aniline blue into the colony and that aniline blue at a concentration of $0{\cdot}4$ g l^{-1} was inhibitory for some FC.

Table 1. *Median FC counts of* 31 *samples obtained by membrane filtration using a new medium and by the multiple tube method using modified Gray's glutamate medium*

	Multiple tube			
	FC		Indole-positive FC	
Membrane filtration c.f.u. dl^{-1}	24 h MPN dl^{-1}	48 h MPN dl^{-1}	24 h MPN dl^{-1}	48 h MPN dl^{-1}
430–1100–2500*	930	2400	230	430

* The first count refers to green colonies, the second to green plus yellow colonies and the third to total colonies.

Table 2. *A comparison of results obtained with a new membrane filtration medium for the estimation of FC with membrane-enriched Teepol broth and M-FC broth*

Figures are the medians of 31 samples

Medium 8	Membrane-enriched Teepol broth	M-FC broth
110–210–440*	130–340–1100†	120–1300‡

* See footnote to Table 1.

† The first count refers to yellow colonies with a morphology typical of *E. coli*, the second count to all yellow colonies and the third count to total colonies.

‡ The first count refers to blue colonies and the second count to total colonies.

Further testing of suitable trial medium

Trial medium 8 was subjected to the further trials described. Significant differences were again looked for at the $P < 0{\cdot}05$ level.

A comparison of medians from results on medium 8 and MPN values for 31 samples (Table 1) showed that the new medium gave a count of acid-producing colonies at 18 h equivalent to the 24 h MPN FC value (but significantly less than the 48 h MPN FC value) and that the median count of green colonies was equivalent to both 24 and 48 h MPN indole-positive FC values. Colonies were visible on the new medium within 10 h incubation, although development of a green coloration did not occur until *ca.* 15 h. However, the count of yellow colonies with a typical *E. coli* morphology (low elevation, flat, with an entire edge and *ca.* 1 mm diameter) made at 12 h incubation was greater than 90% of the final (18 h) count of green colonies (Fig. 1). At 12 h, background counts of colonies not producing acid were zero and the number of acid producers not typical of *E. coli* was small.

The majority of acid-producers on medium 8 with a morphology atypical of *E. coli* were high convex, glistening colonies, with an entire edge and equivalent in size to typical (green) colonies at 18 h incubation (*ca.* 2 mm diameter).

A comparison of median results on medium 8 with median results obtained using standard membrane filtration media (Table 2) showed that the new medium gave

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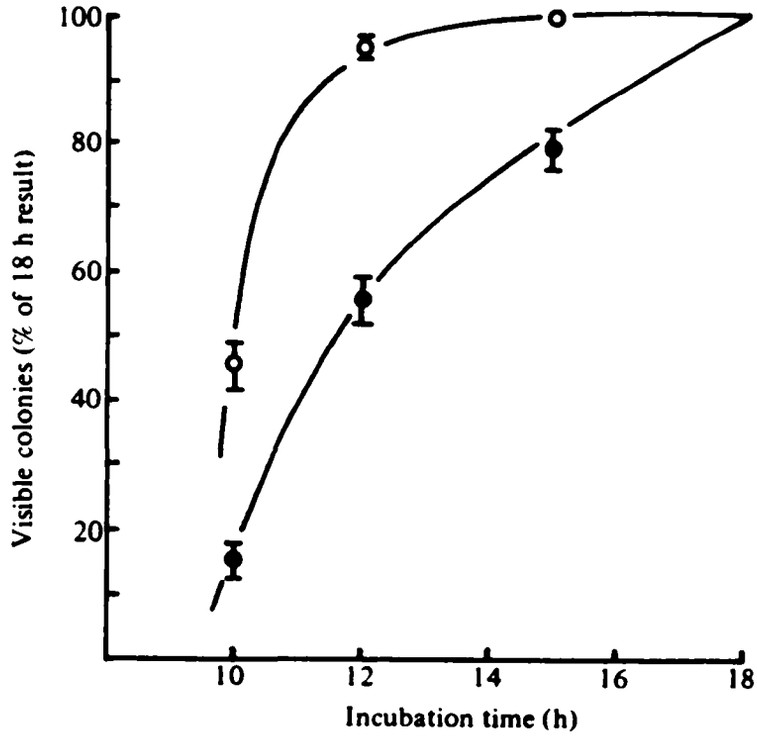

Fig. 1. Proportion of colonies apparent on medium 8 after 18 h incubation which were visible at earlier incubation times. Counts were made at 10, 12 and 15 h incubation of acid-producing colonies with a typical *E. coli* morphology and acid producers with an atypical morphology; counts were converted to percentages of the total numbers of typical (○) and atypical (●) colonies apparent at 18 h incubation respectively and expressed as arithmetic means (of 31 samples) and 95% confidence limits.

counts of colonies which were characteristic of *E. coli* equivalent to the standard media, counts of non-characteristic acid producers significantly less than on membrane-enriched Teepol broth and background colony counts significantly less than on both standard media. It was noted that differentiation between colonies on medium 8 improved on standing at room temperature after incubation, whereas differentiation on membrane-enriched Teepol broth and M-FC broth became poorer.

Medium 8 was therefore considered to be more inhibitory to FC other than *E. coli* and 'background' organisms than the standard media, whilst allowing equivalent counts of *E. coli* to be made.

The solidified version of medium 8 gave lower results relative to MPN FC and MPN indole-positive FC than the broth, although the difference was not significant.

Variation in the colour of media containing aniline blue was experienced using batches of aniline blue from different suppliers and of different ages. Generally, fresh aniline blue was almost colourless at the original pH of the medium and the colour was thus determined by the phenol red. Older aniline blue gave a blue colour independent of pH and the medium took on a much darker, mauve coloration. Differentiation of colonies was possible with either background; however, the darker medium produced a much better contrast against the yellow and green colonies and was preferred.

Characterization of faecal coliforms

Sixteen of the 20 isolates from positive tubes occurring in the multiple tube test were IMViC + + − −, i.e. *E. coli* type I (classification of Wilson *et al.*, 1935), 2 were IMViC + + − + (*Citrobacter freundii* type II) and one each were + − − + and + − + + (irregular or intermediate types). The IMViC type + + − + was included in the Escherichia group by the International Subcommittee on the Taxonomy of the Enterobacteriaceae (1963).

All 20 isolates of green colonies from the new membrane filtration medium were IMViC + + − − (*E. coli*). Eighteen of the 20 isolates of yellow colonies from the same medium were IMViC − − − + and two were − − + + (both members of the Klebsiella–Enterobacter group as defined by the International Subcommittee). All 40 isolates from membrane filters produced a positive reaction in modified Gray's glutamate medium within 24 h.

All 60 isolates tested were Gram-negative rods.

DISCUSSION

The new medium (broth version) described is considered more satisfactory for the analysis of tropical, untreated waters for the presence of *E. coli* than either M-FC broth or membrane-enriched Teepol broth. It is also considered more satisfactory for such waters than the membrane-enriched lauryl sulphate broth (equivalent to trial medium 1 of this study) recommended as an alternative to membrane-enriched Teepol broth (Joint Committee of the Public Health Laboratory Service and the Standing Committee of Analysts, 1980). The rapidity with which results can be obtained (12 h for a presumptive *E. coli* count based on all acid-producing colonies visible at this time; 18 h for a final *E. coli* count based on the number of green colonies) constitutes a significant advantage over the use of the multiple tube method in developing countries such as Sierra Leone, where laboratory facilities are scarce and the provision of a constant source of power for incubation presents problems. The production of isolated colonies allows subcultures to be made for confirmatory tests without the need for purification. A rapid micro-method (Arnold & Weaver, 1948) is currently being assessed for the detection of indole production by colonies appearing on the new medium; preliminary results indicate the formation of readily detectable levels of indole within 6 h by subculture of green colonies to 1 ml tryptone broth (tryptone, 10 g l^{-1}, Lab-Lemco, 3 g l^{-1} – both Oxoid – adjusted to a pH of 7·4 and sterilized at 115 °C for 15 min) and incubation at 44·0 °C.

Although the oxidase test was not performed on acid-producing colonies during trials, aeromonads had not proved to be a major cause of false-positive colonies appearing on M-FC broth and membrane-enriched Teepol broth during preliminary testing, and the fact that the colonies tested for the characterization of FC during the trials were demonstrated to produce gas from lactose in modified Gray's glutamate medium tends to confirm that growth of aeromonads is not a major problem with the new medium.

The double indicator system (phenol red + aniline blue) allows simultaneous counts to be made of all acid-producing colonies and *E. coli*. The proportion of the latter within the former group may prove to be a useful tool in the diagnosis of the source(s) of pollution or the time of pollution. *E. coli* is the commonest coliform organism in human faeces. Indole-negative FC may be present in human faeces in lower numbers but have a longer survival time in water (D.H.S.S., 1969); they may also originate from other sources (Moussa, 1965). It should be noted that the ratio of indole-negative to indole-positive FC in water samples was sometimes so high that filtration of an appropriate volume of sample to obtain a reasonable indole-positive FC count on the new medium would not be possible. An inhibitory agent still needs to be found, therefore, for indole-negative FC, for inclusion in a medium to be satisfactory for use under such conditions, assuming that an *E. coli* count is desired rather than an FC count.

Results of FC analysis of tropical waters using standard media which have not been confirmed, at least by testing for indole production, must be considered to represent mixtures of the Escherichia group with other FC, the relative proportions of which are unknown. Until the origin(s) of the indole-negative FC have been determined and their relative survival characteristics elucidated in tropical waters, the sanitary significance of such unconfirmed counts is in doubt.

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