Epidemiol. Infect. (1991), **106**, 345–354
Printed in Great Britain

Methods for enumerating *Escherichia coli* in subtropical waters

W. H. S. CHEUNG, D. K. K. HA, K. Y. YEUNG AND R. P. S. HUNG
Environmental Protection Department, 28/F., Southorn Centre, Wanchai, Hong Kong

(*Accepted 28 August 1990*)

SUMMARY

The standard membrane filtration method of the UK has been modified in order to improve its specificity for enumerating *Escherichia coli* in the subtropical waters of Hong Kong. This involves incorporating into the membrane lauryl sulphate (mLS) method either an *in situ* urease test (the mLS-UA method), or an *in situ* β-glucuronidase test (the mLS-GUD method). The false-positive errors of the mLS-UA and mLS-GUD methods are low, ranging from 3–5%. A comparison between the membrane filtration (mLS-UA) method and the multiple tube technique in testing *E. coli* in subtropical beach-waters has demonstrated that the former can give much more precise counts, and is the method of choice for such a purpose. The mLS-GUD method, for which automated counting of *E. coli* colonies is possible, is a good alternative to mLS-UA in routine enumeration of this bacterial indicator in environmental waters.

INTRODUCTION

The standard membrane filtration technique of the UK for testing *Escherichia coli* in environmental waters is the membrane lauryl sulphate (mLS) method, which was developed by the Joint Committee of the Public Health Laboratory Service and the Standing Committee of Analysts (PHLS and SCA) for the examination of potable water supplies [1, 2]. The method was later shown to be applicable to marine waters by Stanfield and Irving [3]. This membrane filtration method basically uses an elevated incubation temperature of 44 °C as a selective agent against other coliform bacteria and background organisms, and phenol red as the pH indicator for discriminating between colonies which ferment lactose (namely *E. coli*) and those which do not. There are bound to be false-positive and false-negative errors for such a method, as it has been known that many non-*E. coli* coliform bacteria are lactose fermenters (for instance 98% of *Klebsiella pneumoniae*), whilst some 10% of *E. coli* isolates do not produce acid from lactose [4]. This problem concerning the specificity of the mLS method becomes particularly apparent when the method is used for enumerating *E. coli* in tropical waters. The standard UK membrane filtration method has been modified by Wright [5, 6] to become the enriched lauryl sulphate-aniline blue (ELSAB) medium method, for enhancing the differentiation of *E. coli* from other non-*E. coli* faecal coliform colonies.

The mLS method has also been modified by Cheung [7, 8] to make it applicable to the subtropical waters of Hong Kong. This involves the incorporation into the standard UK membrane filtration method an *in situ* urease test (the mLS-UA method). Originally developed by Dufour and Cabelli [9], this *in situ* test is useful for differentiating *E. coli* from other thermotolerant coliforms on a membrane filter. *E. coli* isolates in general are urease-negative, whilst 95% of *K. pneumoniae* strains are urease-positive [4]. The mLS-UA method has been adopted in Hong Kong as the standard method for testing *E. coli* in environmental waters.

Another modification of the mLS method, by incorporating into it an *in situ* β-glucuronidase, test has recently been developed in Hong Kong for enumerating *E. coli* in subtropical waters (the mLS-GUD method). The use of fluorogenic assays for differentiating *E. coli* colonies from other coliforms on membrane filters was first reported by Feng and Hartman [10]. It has been observed that the production of β-glucuronidase is limited to *E. coli* and some *Salmonella*, *Shigella* and *Yersinia* strains in the family Enterobacteriaceae; and that over 97% of the *E. coli* isolates tested hydrolyse a non-fluorescent substrate, 4-methylumbelliferyl glucuronide (MUG), to produce 4-methylumbelliferone which fluorescences under long-wave u.v. light [11–13].

The purpose of this paper is to describe the modified UK standard membrane filtration methods (mLS-UA and mLS-GUD) for enumerating *E. coli* in subtropical waters, and compare the performance of the mLS-UA and multiple tube methods in testing this bacterial indicator in the beach-waters of Hong Kong.

MATERIALS AND METHODS

Water sampling

Water samples for bacteriological analysis were collected from bathing beaches and other coastal waters, and from streams and rivers. These waters are polluted to different extent either by human sewage, or by both sewage effluent and animal wastes. All the water samples were packed on ice, kept in the dark, and transported to the laboratory for analysis within 4–6 h.

Membrane filtration methods

Water samples were filtered using the 0·7 μm Millipore HC membranes. The procedures of the mLS-UA and mLS-GUD methods are summarized in Fig. 1.

The membrane lauryl sulphate (mLS) medium was reconstituted from dehydrated broth (Oxoid). The urea substrate for the *in situ* urease test consists of 2·0 g of urea and 0·01 g of phenol red in 100 ml of distilled water, with pH adjusted to 5·0–5·2.

For the *in situ* β-glucuronidase test, the membranes were placed on absorbent pads saturated with MUG-incorporated lauryl tryptose broth (Gibco) with the following formulation: 20 g peptone, 5 g lactose, 2·75 g monobasic and 2·75 g dibasic potassium phosphate, 5 g sodium chloride, 0·1 g sodium lauryl sulphate and 50 mg 4-methylumbelliferyl glucuronide in 1 l distilled water. A drop of 30% ammonia solution was placed adjacent to the membrane filter before the petri dish was capped for 10–15 min, to enhance the fluorescence of 4-methylumbelliferone released by β-glucuronidase activity [13]. The fluorescent colonies were counted as

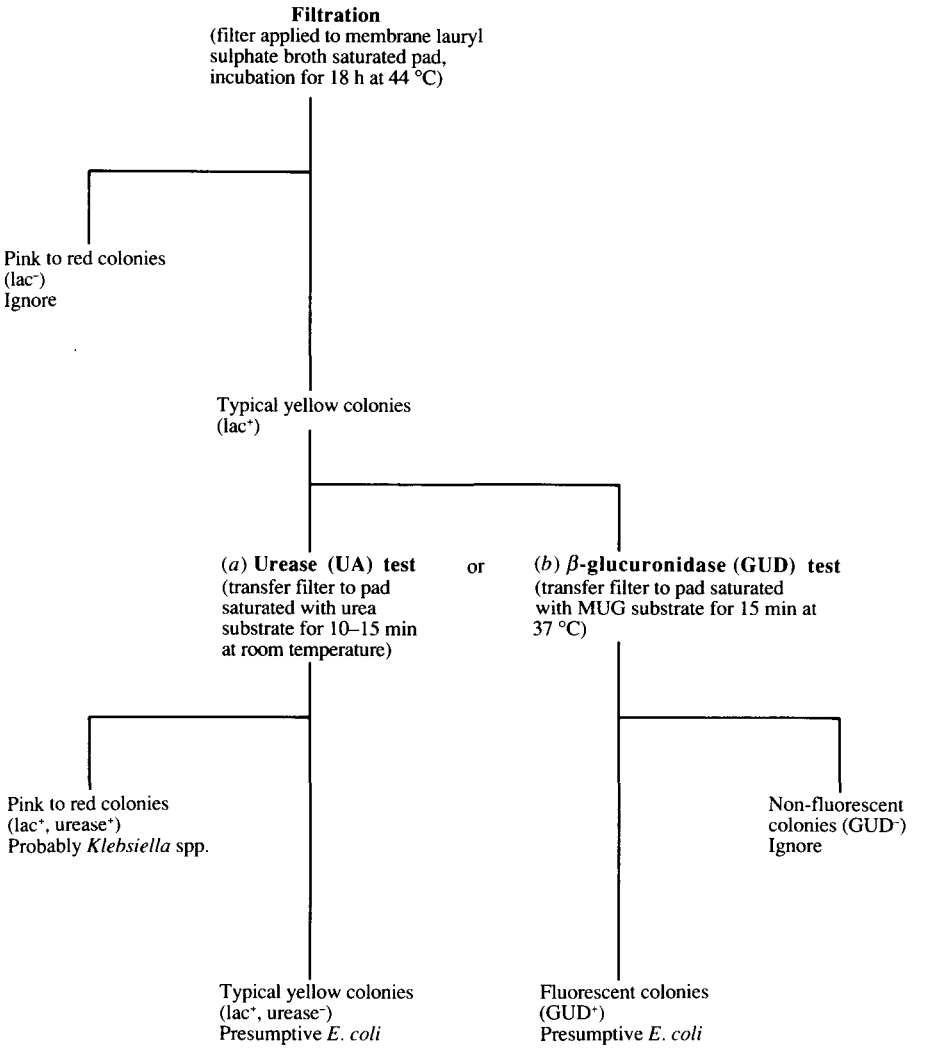

Fig. 1. mLS-UA and mLS-GUD methods for enumerating *E. coli* in subtropical waters.

presumptive *E. coli* by an image analyser (model 40–10, Analytical Measuring Systems) under long-wave u.v. light.

The specificity of both mLS-UA and mLS-GUD methods was assessed. For each method, all the colonies from 18–20 membrane filters (each containing at least 20 presumptive target colonies) were isolated by growing on MacConkey or nutrient agar at 37 °C. They were identified using the API-20E or Vitek identification system. The results of specificity testing were expressed as the false-positive error and undetected target error. The former was calculated by dividing the number of false-positive target colonies by the total presumptive target colony count. The latter was determined by dividing the number of undetected target (false-negative) colonies by the sum of the verified target colonies and undetected target colonies.

https://doi.org/10.1017/S0950268800048494 Published online by Cambridge University Press

Multiple tube (MPN) method

Water samples were tested by 5-tube most probable number (MPN) procedures using the lauryl tryptose lactose broth as recommended by PHLS and SCA [14] and the Department of the Environment and the Department of Health and Social Security (DoE and DHSS) [2]. Lauryl tryptose lactose broth as recommended by PHLS and SCA [15] and tryptone water were used in the confirmatory tests for gas and indole production, respectively.

Comparison between membrane filtration and MPN methods

Two separate comparative studies were carried out. Firstly, water samples collected from nine beaches as part of the routine beach-water quality monitoring programme were analysed using both the membrane filtration and MPN methods, at fortnightly intervals over a 16-month period. These nine beaches are those where an epidemiological study of beach-water pollution was undertaken in 1987 [16]. The enumeration of *E. coli* in these samples by the mLS-UA method was undertaken by the laboratory of the Environmental Protection Department; and for the MPN testing, by two Public Health Laboratories of the Medical and Health Department. The two sets of data were compared by paired *t* test.

Secondly, a single 20 l sample of seawater was collected from a beach. After mixing, this was divided into 40 aliquots of 500 ml each. Twenty of these aliquots (without special labels) were sent together with other water samples to the laboratory of the Environmental Protection Department for testing by the mLS-UA method; the rest were submitted in the same way to a Public Health Laboratory for multiple tube analysis. This experiment was carried out at three beaches with different levels of pollution, namely Repulse Bay, Lido, and Old Cafeteria. The precision of the two methods was compared in terms of the standard deviation of the geometric mean *E coli* counts for each beach.

RESULTS

Specificity of mLS-UA and mLS-GUD methods

Table 1 presents the confirmation of target and non-target colonies as *E. coli* after the mLS-UA and mLS-GUD procedures, and the specificity of the two methods for enumerating *E. coli* in the environmental waters of Hong Kong. Of the 416 urease-negative, yellow colonies of the mLS-UA method, 96% were confirmed as *E. coli*; and for the 379 fluorescent β-glucuronidase-positive colonies of the mLS-GUD method, 97% were verified as such. The false-positive errors for the mLS-UA and mLS-GUD methods were 4 and 3%, respectively. The undetected target error for the mLS-UA method was 7%; and for the mLS-GUD method, 6%.

The identities of the urease-positive and negative thermotolerant coliform colonies after the mLS-UA test, or of the fluorescent and non-fluorescent colonies after the mLS-GUD procedures, are shown in Tables 2 and 3. It is notable that *Klebsiella pneumoniae* represented 81% of the urease-positive thermotolerant coliform colonies, and 65% of the non-fluorescent colonies on membrane filters.

https://doi.org/10.1017/S0950268800048494 Published online by Cambridge University Press

Table 1. *Confirmation of target and non-target colonies on membrane filters as* E. coli

Method	Sample (and no. tested)	Target colonies: No. of presumptive colonies	Target colonies: No. verified as *E. coli* (and %)	Non-target colonies: No. of presumptive colonies	Non-target colonies: No. verified as *E. coli* (and %)	Specificity indicates (%)*: False positive error	Specificity indicates (%)*: Undetected target error
mLS-UA	Freshwater (8)	258	248 (96)	124	18 (15)	4	7
	Marine water (10)	158	150 (95)	199	12 (6)	5	7
	Overall (18)	416	398 (96)	323	30 (9)	4	7
mLS-GUD	Freshwater (10)	224	218 (97)	73	12 (16)	3	5
	Marine water (10)	155	151 (97)	125	11 (9)	3	7
	Overall (20)	379	369 (97)	198	23 (12)	3	6

* False positive error is expressed as: (no. of false-positive target colonies)/(no. of presumptive target colonies) × 100. Undetected target error: (no. of undetected target colonies)/(total no. of verified target colonies and undetected target colonies) × 100.

Table 2. *Species distribution of thermotolerant coliform colonies after the* in situ *urease test**

	Organism	% of isolates		
		Freshwater	Marine-water	Overall
Lac$^+$, urease$^-$ (yellow)	*Escherichia coli*	96	95	96
	Klebsiella pneumoniae	2	2	2
	Citrobacter freundii	1	2	1
	Hafnia alvei	0	1	0·5
	Escherichia hermavii	0·4	0	0·2
	Unidentified	0·4	0	0·2
Lac$^+$, urease$^+$ (red or pink)	*Klebsiella pneumoniae*	82	80	81
	Escherichia coli	9	3	6
	Enterobacter cloacae	1	10	5
	Citrobacter freundii	3	1·4	2
	Acinetobacter calcoaceticus var. *antitratus*	2	1·4	2
	Serratia oderifera	1	0	0·6
	Enterobacter sakazakii	1	0	0·6
	Serratia liquefaciens	0	1·4	0·6
	Yersinia intermedia	0	1·4	0·6
	Yersinia pseudotuberculosis	0	1·4	0·6

* A total of 416 urease-negative and 161 urease-positive thermotolerant coliform colonies on 18 membrane filters were identified.

Table 3. *Species distribution of fluorescent and non-fluorescent colonies after the* in situ *β-glucuronidase (GUD) test**

	Organism	% of isolates		
		Freshwater	Marine-water	Overall
GUD$^+$ (fluorescent)	*Escherichia coli*	97	97	97
	Klebsiella pneumoniae	2	0	1
	Escherichia hermavii	0·4	0	0·3
	Pseudomonas aeruginosa	0	0·6	0·3
	Klebsiella ozaenae	0	0·6	0·3
	Escherichia vulneris	0	0·6	0·3
	Unidentified	0·4	0·6	0·5
GUD$^-$ (non-fluorescent)	*Klebsiella pneumoniae*	67	63	65
	Escherichia coli	16	9	12
	Enterobacter sakazakii	5	4	5
	Acinetobacter calcoaceticus var. *antitratus*	5	5	5
	Enterobacter cloacae	3	10	7
	Citrobacter freundii	1·4	3	2·5
	Klebsiella ozaenae	0	1·6	1
	Hafnia alvei	1·4	0·8	1
	Arizona spp.	0	1·6	1
	Yersinia pseudotuberculosis	0	0·8	0·5
	Serratia liquefaciens	0	0·8	0·5
	Yersinia intermedia	0	0·8	0·5

* A total of 424 fluorescent and 200 non-fluorescent colonies on 20 membranes were identified.

https://doi.org/10.1017/S0950268800048494 Published online by Cambridge University Press

Table 4. *Comparison of* E. coli *densities of nine bathing beaches measured by the membrane filtration (MF) and MPN methods*

Beach	No. of samples†	Geometric mean density per 100 ml: MF‡ count (S.D.)	Geometric mean density per 100 ml: MPN count (S.D.)	Mean MF/MPN ratio§
Shek O	63	*107·0 (0·81)	195·0 (0·72)	0·55
Repulse Bay	73	*196·0 (0·65)	349·0 (0·81)	0·56
Stanley Main	65	*148·0 (0·71)	284·0 (1·03)	0·60
Lido	75	*103·0 (0·52)	142·0 (0·49)	0·73
Butterfly	101	177·0 (0·72)	222·0 (0·63)	0·80
Deep Water Bay	42	40·0 (0·48)	39·0 (0·83)	1·03
New Cafeteria	68	230·0 (0·68)	216·0 (0·57)	1·06
Old Cafeteria	61	779·0 (0·70)	657·0 (0·72)	1·19
Clear Water Bay	35	85·0 (0·60)	70·0 (0·98)	1·21
All 9 beaches	583	*161·0 (0·80)	206·0 (0·91)	0·78

* Significantly different from mean MPN count ($P \leqslant 0{\cdot}05$, paired t test).

† The samples were collected fortnightly from September 1987 to December 1988, as part of the routine beach-water quality sampling programme.

‡ The mLS-UA method was used.

§ Arithmetic mean of the ratios for individual samples.

S.D., Standard deviation for geometric mean *E. coli* count.

Comparison between membrane filtration and MPN methods

Table 4 shows the *E. coli* densities in water samples collected from nine coastal bathing beaches in Hong Kong over a 16-month period, measured by the mLS-UA and multiple tube methods. There was significant difference in the *E. coli* counts obtained by the two methods at four out of the nine beaches. The average ratios of mLS-UA to MPN counts for individual samples from these beaches ranged from 0·55 to 1·21; and the overall ratio was 0·78 for all the nine bathing beaches.

The distributions of *E. coli* counts for 20 aliquots of a single sample collected from three Hong Kong beaches, as measured by the mLS-UA or multiple tube method, are shown in Fig. 2. The geometric mean *E. coli* densities and their standard deviation for the beaches are also given. It was found that the geometric mean *E. coli* densities as obtained by the two methods were similar for Lido and Repulse Bay Beaches. They were significantly different for Old Cafeteria Beach ($P \leqslant 0{\cdot}05$), with the count obtained by the mLS-UA method being higher. In general, the log standard deviation of the *E. coli* densities obtained by the MPN technique was significantly different from that for the mLS-UA method ($P \leqslant 0{\cdot}05$, Bartlett's test). It ranged from 0·06 to 0·08 for mLS-UA counts; and for the MPN counts, from 0·33 to 0·63. The precision of the mLS-UA method was thus higher than the MPN technique.

DISCUSSION

The *in situ* urease test originally developed by Dufour and Cabelli [9] is compatible with mLS, the standard UK membrane filtration medium, as both the urea substrate and the medium use phenol red as the pH indicator. This simple test can considerably improve the specificity of the mLS method in enumerating

https://doi.org/10.1017/S0950268800048494 Published online by Cambridge University Press

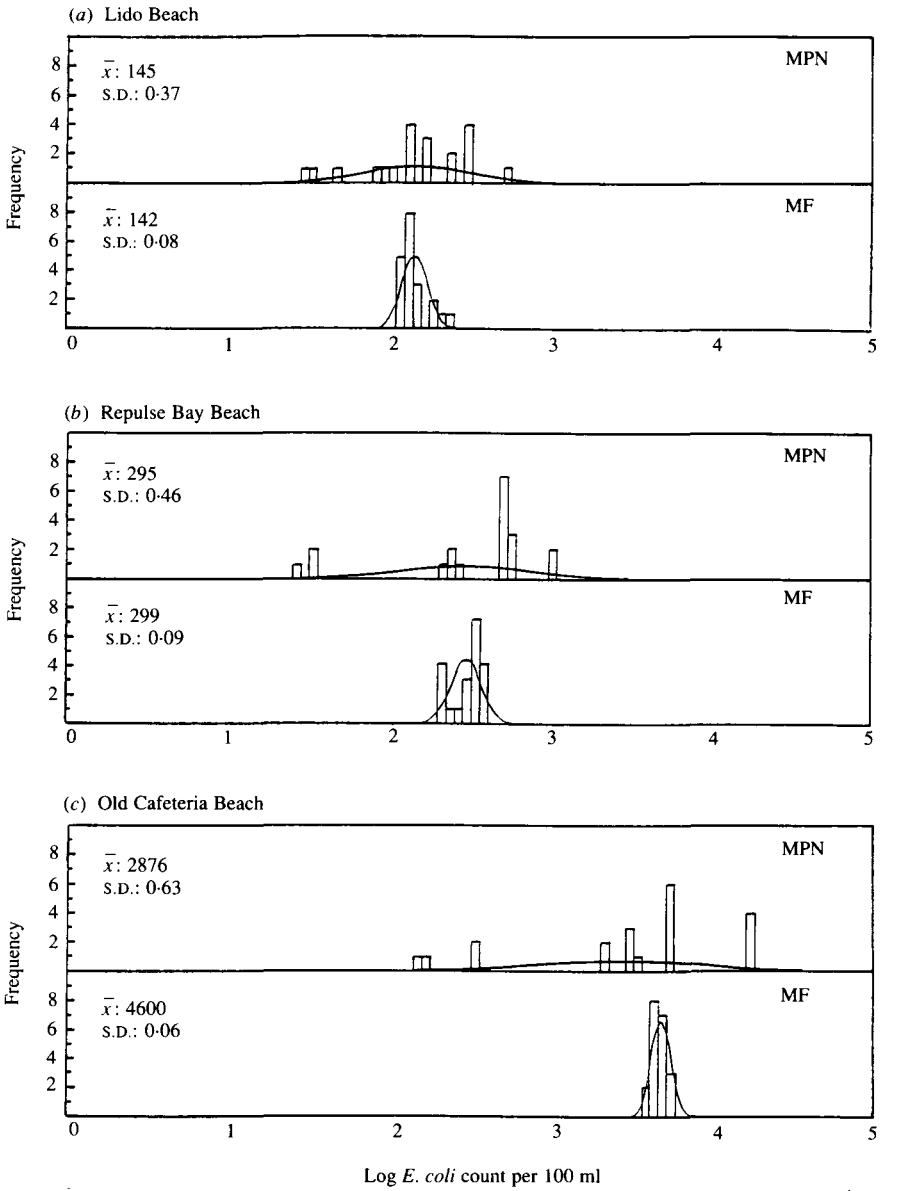

Fig. 2. Distribution of *E. coli* counts by analysing 20 aliquots of a water sample collected from three beaches of Hong Kong, using the membrane filtration and MPN methods. $\bar{x}$, geometric mean count per 100 ml; S.D., log standard deviation.

E. coli in environmental waters by helping to differentiate *E. coli* from other non-*E. coli* thermotolerant coliform colonies, mainly *K. pneumoniae*.

Klebsiella spp. represent most of the urease-positive non-*E. coli* thermotolerant coliform colonies. Their densities in waters could be estimated by first counting all the thermotolerant coliform colonies and subtracting from such a count the number of presumptive *E. coli* colonies obtained after the *in situ* test.

In a recent study on the health effects of beach-water pollution, it was found that *E. coli* on average only represent 57% of the faecal coliform group in the

https://doi.org/10.1017/S0950268800048494 Published online by Cambridge University Press

subtropical coastal beaches of Hong Kong [16]. This suggests that non-*E. coli* thermotolerant coliforms, which may not originate from faecal pollution sources, are prevalent in subtropical waters. A similar phenomenon in relation to tropical waters has previously been reported by Grabow, Hilner and Coubrough [17]; Wright [5, 6]; and Jesus and Hazen [18]. It may not be appropriate to adopt methods which have been developed in temperate countries for enumerating *E. coli* in tropical or subtropical waters, without having assessed the applicability of these methods to testing such waters in the first instance.

There is a limitation of the mLS and mLS-UA methods which is associated with their use of phenol red as the pH indicator – namely that they are not amenable to automated counting of *E. coli* colonies. The yellow target colonies could not be satisfactorily differentiated from the pink or red non-*E. coli* colonies on the membranes by an image analyser. On the contrary, mLS-GUD procedures have the advantage that automated counting of *E. coli* colonies is possible, as the fluorescent *E. coli* colonies and other non-fluorescent colonies on a membrane can be easily differentiated by an image analyser under long-wave u.v. light.

The *in situ* β-glucuronidase test, like the *in situ* urease test, can considerably improve the specificity of the mLS method. The former has the potential for use as an alternative to the mLS-UA method in testing *E. coli* in water samples. It is particularly useful in routine beach-water quality monitoring or intensive surveys where a large number of water samples are taken in one day, and automated counting of *E. coli* colonies by an image analyser is desirable.

The present study has also shown that the multiple tube method is an imprecise method for enumerating *E. coli* in environmental waters. It is not suitable for use for measuring the density of the indicator bacteria in beach-waters and predicting the health risk levels associated with swimming. The precision of the membrane filtration method is much higher. It is the method of choice for health-related monitoring of the microbial water quality of bathing beaches and other environmental waters.

It has been found for some beaches, the geometric mean *E. coli* densities over a 16-month period as measured by the multiple tube and membrane filtration methods are significantly different. Because the two methods may give results which are not comparable, it is not appropriate to state in beach-water quality standards that either method can be used for enumerating this indicator.

ACKNOWLDEGEMENTS

We thank Messrs K. K. Leung, S. M. Leung, W. S. Ng, K. W. Tse, M. C. To, and Misses K. L. Lo and S. H. Tam of the Environmental Protection Department for technical assistance. We are grateful to the Director of Environmental Protection, Hong Kong Government for permission to publish this paper.

REFERENCES

1. Joint Committee of the Public Health Laboratory Service and the Standing Committee of Analysts. Membrane filtration media for the enumeration of coliform organisms and *Escherichia coli* in water: comparison of Tergitol 7 and lauryl sulphate with Teepol 610. J Hyg 1980; **85**: 181–91.

2. Department of the Environment, Department of Health and Social Security, Public Health Laboratory Service. The bacteriological examination of drinking water supplies 1982, reports on public health and medical subjects No. 71, methods for the examination of waters and associated materials. London: Her Majesty's Stationery Office, 1983.
3. Stanfield G, Irving TE. A suitable replacement for Teepol 610 in the selective isolation of coliforms from marine waters and sewage. Water Res 1981; **15**: 469–74.
4. Kelly MT, Brenner DJ, Farmer JJ. Enterobacteriaceae. In: Lennette EH, Balows A, Hausler WJ, Shadomy HJ, eds. Manual of clinical microbiology. 4th ed. Washington DC: American Society For Microbiology, 1985; 263–77.
5. Wright RC. A medium for the rapid enumeration of *Escherichia coli* in the presence of other faecal coliforms in tropical waters. J Hyg 1982; **88**: 265–73.
6. Wright RC. A new selective and differential agar medium for *Escherichia coli* and coliform organisms. J Bacteriol 1984; **56**: 381–8.
7. Cheung WHS. Standardization of methods for enumerating coliform bacteria in water: a review with recommendations for Hong Kong. Environmental Protection Agency, Hong Kong Government, 1983. EPA/TM26/83.
8. Cheung WHS. *Escherichia coli*: A bacterial indicator of faecal pollution. In: Proceeding of the Joint Meeting of the Hong Kong Society of Microbiology and the Guangdong Society of Microbiology, 2–5 June 1985; 1985: 30–43.
9. Dufour AP, Cabelli VJ. A membrane filter procedure for enumerating the component genera of the coliform group in seawater. Appl Microbiol 1975; **29**: 826–33.
10. Feng PCS, Hartman PA. Fluorogenic assays for immediate confirmation of *Escherichia coli*. Appl Environ Microbiol 1982; **43**: 1320–9.
11. Kilian M, Bulow P. Rapid diagnosis of Enterobacteriaceae. I. Detection of bacterial glycosidases. Acta Pathol Microbiol Scand Sect B 1976; **84**: 245–51.
12. Trepeta RW, Edberg SC. Methylumbelliferyl-β-D-glucuronide-based medium for rapid isolation and identification of *Escherichia coli*. J Clin Microbiol 1984; **19**: 172–4.
13. Freier TA, Hartman PA. Improved membrane filtration media for enumeration of total coliforms and *Escherichia coli* from sewage and surface water. Appl Environ Microbiol 1987; **53**: 1246–50.
14. Joint Committee of the Public Health Laboratory Service and the Standing Committee of Analysts. A comparison between minerals-modified glutamate medium and lauryl tryptose broth for the enumeration of *Escherichia coli* and coliform organisms in water by the multiple tube method. J Hyg 1980; **85**: 35–49.
15. Joint Committee of the Public Health Laboratory Service and the Standing Committee of Analysts. A comparison of confirmatory media for coliform organisms and *Escherichia coli* in water. J Hyg 1981; **87**: 369–75.
16. Cheung WHS, Chang KCK, Hung RPS, Kleevens JWL. Health effects of beach-water pollution in Hong Kong. Epidemiol Infect 1990; **105**: 139–62.
17. Grabow WOK, Hilner CA, Coubrough P. Evaluation of standard and modified M-FC, MacConkey, and Teepol media for membrane filtration counting of faecal coliforms in water. Appl Environ Microbiol 1981; **42**: 192–9.
18. Jesus SM, Hazen TC. Comparison of four membrane filter methods for faecal coliform enumeration in tropical waters. Appl Environ Microbiol 1987; **53**: 2922–8.