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Quantitative determination of total coliforms and *Escherichia coli* in marine waters with chromogenic and fluorogenic media

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K. GEISSLER, M. MANAFI, I. AMORÓS AND J.L. ALONSO. 2000. This study compared the performance of LMX® broth (LMX), Chromocult Coliform® agar (CC) and Chromocult Coliform agar plus cefsulodin (10 μg ml^{-1}) (CC-CFS), with standard methods multiple tube fermentation (MTF), for the enumeration of total coliforms and *Escherichia coli* from marine recreational waters. LMX and CC are two media designed to concurrently detect total coliform (TC) bacteria and *E. coli* by the specific action of β-galactosidase (total coliforms) and β-glucuronidase (*E. coli*). Overall results for the TC test showed that LMX, CC and MTF recovered 2.63, 1.95 and 1.90 times as many TCs as CC-CFS, respectively. Data from the multiple range test showed significant differences ($P < 0.05$) between TC counts on CC-CFS and LMX. The traditional MTF was less sensitive for *E. coli* enumeration. However, there was no statistically significant differences between LMX, CC, CC-CFS and the MTF method for *E. coli* enumeration. Background interference was reduced on CC-CFS and the counts obtained reflected more accurately the number of TCs. Therefore, the contribution of β-galactosidase positive, non coliform bacteria (*Aeromonas* spp. and *Vibrio* spp.) to TC counts should not be neglected.

INTRODUCTION

Total coliforms (TCs), faecal coliforms (FCs) and *Escherichia coli* are used as indicators of marine recreational water quality. Among these, *E. coli* is generally considered the most reliable since its presence directly relates to faecal contamination with its implied threat of the presence of enteric disease (Rice *et al.* 1991). There are two standard methods for the enumeration of TCs and FCs from marine recreational waters. The multiple tube fermentation (MTF) technique provides a most probable number (MPN) analysis after growth of coliforms in liquid medium. The membrane filtration (MF) technique enumerates coliforms on the surface of agar by providing a cfu per 100 ml count (APHA 1995). A major limitation of the standard methods is the length of time required to complete the testing. The MTF with lauryl sulphate broth (LSB) and brilliant green lactose bile broth (BGLB) media takes up to 4 d to obtain results, is labour intensive, and unless *E. coli* medium with 4-methylumbelliferyl-β-D-glucuronide (ECMUG) is used, does not identify *E. coli* (Palmer *et al.* 1993). To overcome this problem, new enzymatic methods have been developed. The use of media containing chromogenic or fluorogenic substrates for the enzymes β-galactosidase (Lac) and β-glucuronidase (Gus) for simultaneous detection of coliforms and *E. coli* is increasing (Fricker & Fricker 1996). *E. coli* metabolizes both methylumbelliferyl-β-D-glucuronide (MUG) and indoxyl-β-D-glucuronide (IBG) (Haines *et al.* 1993). In coliforms, the enzyme β-D-galactosidase catalyses the breakdown of lactose into glucose and galactose. However β-D-galactosidase also has been found to be produced by some noncoliform bacteria, such as *Aeromonas* spp. and *Vibrio* spp. (Palmer *et al.* 1993; Davies *et al.* 1995; Alonso *et al.* 1996).

This study was undertaken to compare two commercial preparations of chromogenic (Chromocult Coliform® agar, CC, Merck, Darmstadt, Germany) and fluorogenic (Fluorocult LMX® broth, LMX, Merck) enzyme detection media with standard media (APHA 1995) for the simultaneous enumeration of TCs and *E. coli*. In addition, to determine

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whether we could eliminate aeromonads interference from TC counts, we compared recoveries of TCs and *E. coli* from marine recreational waters on membrane filters using CC agar without cefsulodin and CC agar with cefsulodin (CC-CFS).

MATERIALS AND METHODS

Sampling

A total of 26 water samples was collected from the beaches of Valencia (sites M1 and M2) and Alboraya (sites A1 and A2). All the samples were collected in sterile glass bottles, refrigerated and processed within 4 h of collection.

Enumeration of indicators organisms

MTF/MPN method. For TC counts a series of five fermentation tubes of lauryl sulphate broth (LSB) (Merck) were inoculated with appropriate volumes of 10-fold dilutions of water samples, and incubated at 37 °C for 48 h. All gas-positive LSB tubes were subcultured to tubes of brilliant green lactose bile broth (BGLB) (Merck) and incubated at 37 °C for 48 h. Gas-positive BGLB tubes were considered positive for the presence of TCs (AHPA 1995). Gas-positive LSB tubes were subjected to further analysis with EC broth (Merck). The EC tubes were incubated at 44.5 °C for 24 h. EC positive tubes were confirmed for the presence of *E. coli* by indole production into tryptone water (Merck). Gas and indole production were considered to be positive for the presence of *E. coli* (AHPA 1995).

The LMX/MPN method. The five-tube MPN test was performed by adding appropriate decimal quantities (multiplies and submultiplies of 1 ml of sample) to LMX (Merck) tubes. The tubes were then incubated at 37 °C for 24 h, and all negative tubes were incubated for an additional 24-h period. Development of a blue–green colour indicated the presence of TCs in the tubes. Each positive TC tube was exposed to a hand-held long-wavelength (366 nm) ultraviolet light lamp (Merck). Fluorescence in the tube denoted the presence of *E. coli*. The following control strains were used in the interpretation of LMX reactions: *E. coli* CECT 678 (Lac^+ Gus^+), *Aeromonas media* CECT 4232 (Lac^+ Gus^-) and *Pseudomonas aeruginosa* (Lac^- Gus^-). A total of 303 TC blue-green positive tubes and 180 *E. coli* fluorescence positive tubes were streaked on CC agar (Merck) to confirm β-D-galactosidase and β-D-glucuronidase activities. Salmon to red (Lac^+) and dark-blue to violet (Lac^+ Gus^+) colonies were selected from CC agar and identified on the basis of cytochrome oxidase activity and growth on TSI agar. Oxidase positive and negative colonies were further identified using the API 20E system (bioMerieux, Marcy l'Etoile, France).

The CC/MF and CC-CFS/MF methods. Ten-fold dilutions of the water samples were made, and duplicates samples of 100 ml of each dilution were filtered through sterile 0.45 μm pore size membranes (Whatman, Maidstone, UK) by a standard membrane filtration technique (AHPA 1995). One membrane of each set of duplicates was placed on a prepared layer of CC agar in a 47-mm-diameter Petri dish. The second membrane of each duplicate pair was placed on CC-CFS (final concentration of cefsulodin 10 mg ml^{-1}) (Alonso *et al.* 1996). The membranes placed on CC and CC-CFS media were incubated at 37 °C for 24 h. All dark-blue to violet colonies were counted as *E. coli*, and all salmon to red colonies were counted as TCs. Salmon to red and blue colonies were randomly selected from CC and CC-CFS media and identified as described above.

Statistical analysis

TC and *E. coli* counts from MTF, LMX, CC and CC-CFS media were logarithmically transformed to make the variables more normal for statistical analysis. Results were analysed by linear regression to verify the linearity of the relationship between the TC and *E. coli* obtained with different media. A variance analysis (ANOVA) was performed to determine whether TC and *E. coli* counts from these media differed significantly. A multiple comparison procedure (multiple range test) was then used to find which means of methods were significantly different. The method used to discriminate among the means was Fisher's least significant difference (LSD) procedure. All statistics were obtained using Statgraphics Plus 3.1 software (Manugistics Inc., Rockville, USA).

RESULTS

Comparison of MTF, LMX, CC and CC-CFS media for enumeration of TCs

The counts of TCs at the four sampling sites are showed in Table 1. The 26 marine water samples tested had TC counts ranging from 1.4×10^2 cfu $100\,mL^{-1}$ to 8.0×10^4 cfu $100\,ml^{-1}$. The regression lines indicated good agreement between the media MTF, LMX, CC and CC-CFS. Positive correlations ($P < 0.01$) were found with the highest correlation coefficient between CC and CC-CFS ($r = 0.964$) and the lowest correlation coefficient between LMX and MTF ($r = 0.780$). Of the TC assays, the highest levels were obtained with LMX broth. Overall results for the TC test showed that LMX, CC and MTF recovered 2.63, 1.95 and 1.90 times as many TCs as CC-CFS, respectively. Data from the ANOVA performed on TC counts showed no difference between media (Table 2). However, data from the multiple range test (Table 3) demonstrated significant differences ($P < 0.05$) between TC counts on CC-CFS and LMX.

Table 1 Summary of total coliform counts by site and method

Method*	No. of samples	TC counts 100 ml^{-1} Mean†	SD	Minimum	Maximum
Site M1					
MTF	8	3.4×10^3	5.2×10^3	2.6×10^2	1.6×10^4
LMX	8	1.3×10^4	2.7×10^4	1.4×10^2	8.0×10^4
CC	8	7.7×10^3	1.2×10^4	8.7×10^2	3.8×10^4
CC-CFS	8	3.1×10^3	3.4×10^3	4.4×10^2	1.1×10^4
Site M2					
MTF	7	3.7×10^3	5.6×10^3	2.3×10^2	1.6×10^4
LMX	7	7.1×10^3	1.0×10^4	1.1×10^3	3.0×10^4
CC	7	3.8×10^3	4.5×10^3	5.7×10^2	1.4×10^4
CC-CFS	7	2.1×10^3	2.4×10^3	5.0×10^2	7.3×10^3
Site A1					
MTF	6	4.9×10^3	5.6×10^3	5.0×10^2	1.6×10^4
LMX	6	5.7×10^3	6.2×10^3	2.4×10^2	1.7×10^4
CC	6	8.7×10^3	1.5×10^4	4.0×10^2	4.0×10^4
CC-CFS	6	2.9×10^3	3.5×10^3	2.2×10^2	9.8×10^3
Site A2					
MTF	5	2.1×10^4	3.9×10^4	5.0×10^2	9.0×10^4
LMX	5	1.2×10^4	2.1×10^5	1.1×10^3	5.0×10^4
CC	5	6.4×10^3	8.7×10^3	3.5×10^2	2.2×10^4
CC-CFS	5	3.6×10^3	4.6×10^3	3.4×10^2	1.2×10^4

* For a complete description of methods, see the text. † Arithmetic mean.

Table 2 Statistical comparison (ANOVA) of TC and *Escherichia coli* counts from different sampling sites and methods

Parameter	Source	SC	DF	MS	*F*-ratio	*P*-value
TC						
	Method	1.813	3	0.604	2.090	0.107
	Site	0.651	3	0.217	0.750	0.525
	Total	30.563	103			
E. coli						
	Method	0.060	3	0.020	0.050	0.986
	Site	4.424	3	1.475	3.620	0.016*
	Total	43.989	103			

* Denotes a statistically significant difference at the 95% confidence level.

In general, it was easy to see the blue–green colour in coliform-positive LMX tubes. Only in the lowest dilution step (10 ml to water added into 10 ml LMX broth double concentrated) was the colour difficult to detect. In these tubes, the coliform presence could be detected by the confirmation reaction using CC agar. Of the 303 LMX TC-positive tubes, 294 (98%) were confirmed for β-galactosidase reaction using CC agar. The above nine tubes which fluoresced were negative with CC agar suggesting that the breakdown of lactose by β-galactosidase was not the cause of the colour change during the original test. In the MF assay, occasional β-galactosidase positive microalgae were observed on CC agar. However, most of the false positive β-D-galactosidase TC in LMX and CC were due to *Aeromonas* spp. Of the 56 Lac$^+$ colonies isolated on CC agar from LMX broth, 20 colonies (35.7%) were oxidase positive, and of 62 Lac$^+$ colonies isolated from CC agar, 25 colonies (40.3%) were oxidase positive. A total of 24 pink colonies isolated from LMX broth

Table 3 Multiple range test by method

Contrast	Total coliforms	*Escherichia coli*
CC – CCCFS	0.229	−0.040
CC – LMX	−0.132	−0.035
CC – MTF	0.095	0.017
CCCFS – LMX	−0.361*	0.005
CCCFS – MTF	−0.135	0.058
LMX – MTF	0.227	0.052

* Denotes a statistically significant difference at the 95% confidence level.

were confirmed with the API20E system, and 19 out of 24 were members of the total coliform group, and four out of 24 to be of *Aeromonas* spp. One profile could not be identified by the API20E data base. The 15 colonies isolated on CC agar were identified as *Aeromonas* spp. (eight strains) and coliforms (six strains). One strain could not be identified in the API20E data base. A lower percentage of oxidase positive colonies (Lac^+) was recovered from CC-CFS agar than from LMX broth and CC agar. Of 51 Lac^+ colonies isolated from CC-CFS agar, four colonies (7.8%) were oxidase positive.

Comparison of MTF, LMX, CC and CC-CFS media for enumeration of *E. coli*

The counts of *E. coli* at the four sampling sites are presented in Table 4. The 26 marine water samples tested had *E. coli* counts ranging from 10 cfu $100\,ml^{-1}$ to 8.4 cfu $100\,ml^{-1}$. The regression lines demonstrated good agreement between the media MTF, LMX, CC and CC-CFS. Positive correlations ($P < 0.01$) were found with the highest correlation coefficient between CC and CC-CFS ($r = 0.961$) and the lowest correlation coefficient between LMX and MTF ($r = 0.713$). The traditional MTF method was the least sensitive for *E. coli* enumeration. The overall results for the *E. coli* test showed that LMX, CC-CFS and CC recovered 1.60, 1.64 and 1.39 times as many *E. coli* as MTF, respectively. However, there were no statistical significant differences between LMX, CC, CC-CFS and the MTF method for *E. coli* enumeration (Table 2). The CC-CFS agar exhibited higher *E. coli* counts than CC agar due to the elimination of nearly all the background flora.

The combined use of two reference strains, *E. coli* MUG positive and *Aer. media* MUG negative made detection of fluorescence easy. No *Pseudomonas* spp. interference in the fluorogenic assay was observed in this study. Of the 180 LMX fluorescence positive tubes, 104 (96%) were confirmed for β-glucuronidase production using CC agar. From LMX broth, 11 blue colonies isolated on CC agar were confirmed as *E. coli* with the API20E system. Two strains of *Citrobacter freundii* and two strains of *Enterobacter agglomerans* exhibited β-glucuronidase production.

DISCUSSION

In the MF assay, occasional β-galactosidase positive microalgae were observed on CC agar. Davies *et al.* (1994) observed that the presence of some microalgae at high concentrations may interfere with the detection and enumeration of coliform bacteria by methods which are based on the production of β-galactosidase, such as Colilert-Marine Water (Colilert-MW). It is also possible that in our study, some interfering microalgae would have showed β-D-galactosidase activity in LMX tubes (2%). However, most of the false positive β-D-galactosidase TC in LMX and CC were due to *Aeromonas* spp. Similar problems were experienced by other workers. Höller *et al.* (1995) found a high percentage of *Aeromonas* spp. in BGLB-MUG. Ley *et al.* (1993) observed that *Aeromonas* spp. represented 76% of β-D-galactosidase colonies isolated on mX-Gal. Covert *et al.* (1989) found *Serratia* spp., *Hafnia alvei*, *Vibrio fluvialis*, and *Aeromonas* spp. constituted 25.0% of isolates after 24 h and 40.8% of isolates after 28 h of incubation with Colilert. Fricker & Fricker (1996) reported that most of the false positive results for coliforms in LMX broth were due to *Aeromonas* spp. Palmer *et al.* (1993) suggested that, depending on the relative abundance of false positive organisms (*Aeromonas hydrophila*, *Vibrio cholerae* non-O1 and *Kluyvera* spp.) in a given coastal area, the Colilert-MW test could yield falsely elevated numbers of TCs. The potential for marine vibrios to cause false positive reactions in coliform assays based on β-galactosidase activity has been demonstrated (Davies *et al.* 1995). Marine vibrios have a competitive advantage over coliforms in seawater, since it is their natural habitat (Davies *et al.* 1995). The frequency of false positive reactions by vibrios in LMX broth will be dependent on the abundance of these organisms in coastal waters. Several studies also provides estimates of TC false positives when standard media were used in a MPN format. Ramteke *et al.* (1992) reported 32.6% of isolates from MacConkey broth were *Aeromonas*. Most *Aeromonas* spp. are β-galactosidase positive (Sakazaki & Balows 1981). Disagreement between the CC-CFS and, LMX, CC and MTF media for TC enumeration was primarily due to false positive results. Alonso *et al.* (1996) found that CC agar recovered 2.46 times as many TCs as CC-CFS agar. Background interference was reduced on CC-CFS, and the TC counts that were obtained with this medium reflected more accurately the number of TCs in the marine water samples analysed.

It was noticed that some of the LMX tubes needed more than 24 h of incubation for fluorescence development, although isolations were not made from positive tubes to determine whether the increased incubation time yielded false

Table 4 Summary of enumeration results of *Escherichia coli* by site and method

Method*	No. of samples	*Escherichia coli* counts 100 ml^{-1}			
		Mean†	SD	Minimum	Maximum
Site M1					
MTF	8	8.1×10^2	7.5×10^2	1.0×10^2	2.2×10^3
LMX	8	8.2×10^2	1.0×10^3	0.8×10^2	3.0×10^3
CC	8	1.5×10^3	1.7×10^3	0.6×10^2	5.1×10^3
CC-CFS	8	1.5×10^3	1.7×10^3	0.4×10^2	5.1×10^3
Site M2					
MTF	7	4.1×10^2	5.8×10^2	0.4×10^2	1.7×10^3
LMX	7	8.5×10^2	1.0×10^3	0.8×10^2	3.0×10^3
CC	7	6.6×10^2	1.3×10^3	0.1×10^2	3.8×10^3
CC-CFS	7	6.9×10^2	1.3×10^3	0.3×10^2	3.9×10^3
Site A1					
MTF	6	1.4×10^3	1.4×10^3	3.0×10^2	3.0×10^3
LMX	6	1.4×10^3	2.0×10^3	2.3×10^2	5.0×10^3
CC	6	1.9×10^3	3.0×10^3	0.6×10^2	7.3×10^3
CC-CFS	6	2.2×10^3	3.1×10^3	0.6×10^2	7.7×10^3
Site A2					
MTF	5	9.2×10^2	9.9×10^2	1.3×10^2	2.8×10^3
LMX	5	1.1×10^3	1.9×10^3	0.9×10^2	5.0×10^3
CC	5	1.9×10^3	3.0×10^3	0.2×10^2	7.8×10^3
CC-CFS	5	1.9×10^3	3.2×10^3	0.4×10^2	8.4×10^3

* For a complete description of methods, see the text. † Arithmetic mean.

positive results after 48 h. Hahn & Wittrock (1991) showed that LMX broth detected *E. coli* with greater sensitivity at 48 h incubation than at 24 h. In Colisure™, continuing incubation between 28 and 48 h increased the MUG response of *E. coli* (McFeters *et al.* 1995). Edberg & Edberg (1988) reported that injured *E. coli* required longer incubation times to produce fluorescence.

Turbidity due to heavy bacterial growth in the lactose-based MUG media often made reading the fluorescence of the MUG reaction difficult (Shadix & Rice 1991). The MUG positive (*E. coli* MUG$^+$) and MUG negative (*Aer. media* MUG) controls were needed for comparison. The use of MUG in *E. coli* detection offers the benefit of reducing the workload by simply counting the number of fluorescent tubes at the end of the desired incubation time without additional confirmatory testing (Neidhardt *et al.* 1995), although the indole reaction can be performed in LMX tubes because the medium contain tryptophane.

Petzel & Hartman (1985) encountered problems in distinguishing the natural fluorescence of pseudomonads from the fluorescence produced during MUG breakdown by *E. coli*. Some *Pseudomonas* strains produce a green fluorescent pigment, but the fluorescence signal is usually too small for visual detection (Manafi *et al.* 1991) and the green fluorescence is clearly different from the blue fluorescence produced by *E. coli*. In the present study, no *Pseudomonas* spp. interference in the fluorogenic assay was observed.

Two strains of *Cit. freundii* and two strains of *Ent. agglomerans* exhibited β-glucuronidase production. Although β-glucuronidase activity has been reported in some coliform strains (Manafi & Kneifel 1989), its occurrence appears to be very infrequent (Sartory & Howard 1992). The reason for the production of β-glucuronidase by these organisms is not known, but other investigators (Brenner *et al.* 1993) have suggested that the reaction may be plasmid mediated.

In summary, the results of this study indicate that the combination of CC agar with cefsulodin (CC-CFS) is highly efficient for the inhibition of growth of *Aeromonas* spp. and other oxidase-positive organisms (e.g. *Vibrio* spp.) from marine recreational water samples. The LMX and CC media are a viable alternative to the traditional MTF procedure for the enumeration of *E. coli*. Furthermore, the results may be obtained more easily and more rapidly than by traditional MTF.

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