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ENUMERATION OF *E. COLI* IN ENVIRONMENTAL WATERS AND WASTEWATER USING A CHROMOGENIC MEDIUM

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ABSTRACT

CHROMagar Liquid ECC (CLECC) was compared with membrane lauryl sulphate broth plus urea (mLS-UA) used routinely in Hong Kong for *E. coli* enumeration. *E. coli* appear as distinctive greenish-blue colonies on CLECC while other faecal coliforms are red in colour. CLECC performance was comparable to mLS with mixtures of faecal coliforms, *E. coli* and non-faecal coliforms. Beach, river, ground and waste water samples were used for further comparison. Results given by the two media were significantly correlated (P = <0.001). CLECC was superior to mLS-UA in sensitivity (99.1% *vs* 87.2%) and specificity (96.9% vs 59.8%) for detecting *E. coli*. On CLECC 95% of *E. coli* colonies were correctly identified but only 66.9% on mLS-UA. On average, time required for performing one *E. coli* enumeration test with CLECC was 1.2min/sample less than with mLS-UA. CLECC may enable the development of complete automation for enumerating *E. coli*. © 1997 IAWQ. Published by Elsevier Science Ltd

KEYWORDS

Chromogenic; membrane lauryl sulphate; urea; *E. coli* enumeration; environmental waters.

INTRODUCTION

E. coli is used in Hong Kong for assessing faecal pollution in waters (Cheung *et al.*, 1985). Bacteria grown on membranes using membrane lauryl sulphate broth (mLS) (with phenol red) are subjected *in situ* to a urease test. The main disadvantage is that the cólony colour is stable for a short time so that *E. coli* counting has to be done shortly after the urease test rendering checking of counts at a later stage impossible. CHROMagar Liquid ECC (Chromagar™, France) includes the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronate (BCIG). As a result of β-D-glucuronidase activity in *E. coli*, BCIG is metabolised to form an insoluble indigo complex with the colonies having a distinctive greenish-blue colour while other faecal coliforms are red and non-faecal coliforms are colourless. CLECC was assessed for the enumeration of *E. coli* in subtropical waters of Hong Kong.

MATERIALS AND METHODS

mLS-UA method - samples were membrane filtered (0.45μm, Millipore) and the filters placed on a pad saturated with mLS broth (Oxoid). The filters were incubated for 4h, 30°C (resuscitation) followed by 44°C

for 14-16h. Faecal coliforms, if present, appeared as yellow colonies. Filters were laid on an absorbent pad soaked with a 2% urea solution (BDH, England) containing phenol red as pH indicator. Faecal coliforms other than *E. coli* possess urease which converts urea into an alkaline end-product giving red colonies. *E. coli* lacks urease and its colonies remain yellow after reaction with the urea substrate. Colonies remaining yellowish in colour 15min after urease reaction were enumerated as *E. coli*.

CHROMagar liquid ECC method - samples are filtered, the filter laid on a pad saturated with CHROMagar liquid ECC (CLECC) and the plates incubated at 44.5°C for 18-24h without resuscitation. *E. coli* appears as greenish-blue colonies while other faecal coliform colonies are red and non-faecal coliforms are colourless.

Controls using known bacterial cultures - the efficacy of CLECC and mLS-UA in enumerating *E. coli* was evaluated by using controls containing known concentrations of bacterial cultures. These included isolates of representative species of faecal coliforms and non-faecal coliforms often encountered in Hong Kong waters. *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Escherichia coli* (as representative faecal coliforms) and *Aeromonas* spp (non-faecal coliform) were separately cultured in brain heart infusion broths (BHI, Gibco) overnight. Two environmental isolates of *E. coli*, one lactose fermenter (Lac^+) and one non-fermenter (Lac^-) were cultured and added into the mixture. The concentration of each species in the BHI culture was checked before mixing by spread-plate count technique (in duplicate) using trypticase soy agar (Difco). An aliquot of 8ml from each of the cultures of *K pneumoniae*, *E cloacae* and *Aeromonas* spp ($9.3x10^8$, $1.37x10^8$ and $3.4x10^8$/ml respectively) was used to mix with 12ml of each of the *E. coli* cultures (Lac^+ $8.9x10^8$/ml; Lac^- $1.09x10^9$/ ml). The mixture was made up to 800ml with Ringer's solution (Oxoid). The final concentration of faecal coliforms was of the order of 10^5/100ml of which 55-60% were *E. coli*; non-faecal coliforms were of the order of 10^3/100ml. This aimed to simulate the proportion of faecal coliforms, *E. coli* and non-faecal coliforms routinely found in the subtropical environmental waters of Hong Kong. The control was divided into two equal portions, filtered and incubated on CLECC or mLS.

Environmental waters and wastewater - 100 samples (9 river, 15 waste, 66 beach and 10 ground waters), polluted to different extents by human sewage or both sewage effluent and animal wastes, were tested. All samples were kept on ice during transportation and were analysed within 4-6h of collection. Each well-mixed sample was divided into two equal portions, membrane-filtered and laid onto pads saturated with either CLECC or mLS. Yellowish colonies on mLS after incubation were tested for urease activity. Identities of presumptive faecal coliforms, *E. coli* and non-faecal coliform colonies were confirmed using Vitek AMS-GNI cards (bioMerieux). A total of 228 and 310 representative colonies isolated from mLS and CLECC, respectively, were selected for such identity confirmation. *E. coli* concentrations of all 100 environmental samples enumerated by CLECC and mLS-UA were also plotted for correlation analysis.

Table 1. Comparison of CLECC and mLS-UA methods to enumerate bacteria in controls

Original concentration of bacteria added to the controls (estimated by plate count)	Bacterial number/100mL	Concentration estimated by	
		mLs-UA	CLECC
Faecal coliforms	$5.3x10^5$	$3.0x10^5$	$5.7x10^5$
E. coli (Lac^+ and Lac^-)	$3.0x10^5$	$1.8x10^5$	$3.3x10^5$
Non-FC	$3.4x10^3$	Not enumerated	Not enumerated

RESULTS

Comparison between CLECC and mLS-UA using controls - the actual concentrations of microorganisms added to the controls are shown in Table 1. The concentrations of lactose-fermenting and non-lactose-fermenting *E. coli* in the bacterial mix were $1.4x10^5$ and $1.6x10^5$/100ml respectively. The *E. coli* concentrations estimated by both CLECC and mLS-UA were of the same order of magnitude (i.e.

10^5/100ml) as the original concentrations added. The non-lactose-fermenting *E. coli* were misidentified as non-faecal coliforms by the mLS-UA method.

Comparison of CLECC and mLS-UA methods using environmental samples - *E. coli* concentrations for the 100 samples were plotted for correlation analysis. The correlation coefficient (r) was found to be 0.986 and the two sets of results were statistically correlated with P = <0.001 (Figure 1). The scatter plot gave an impression that CLECC was less sensitive than the mLS-UA method (with resuscitation) in detecting low concentrations of *E. coli* when concentration of *E. coli* was below 2/100ml. Among 100 environmental samples analysed, colony counts in four samples on mLS treated with urea varied after 30min. Colony counts in all the samples on CLECC remained the same even after 24h.

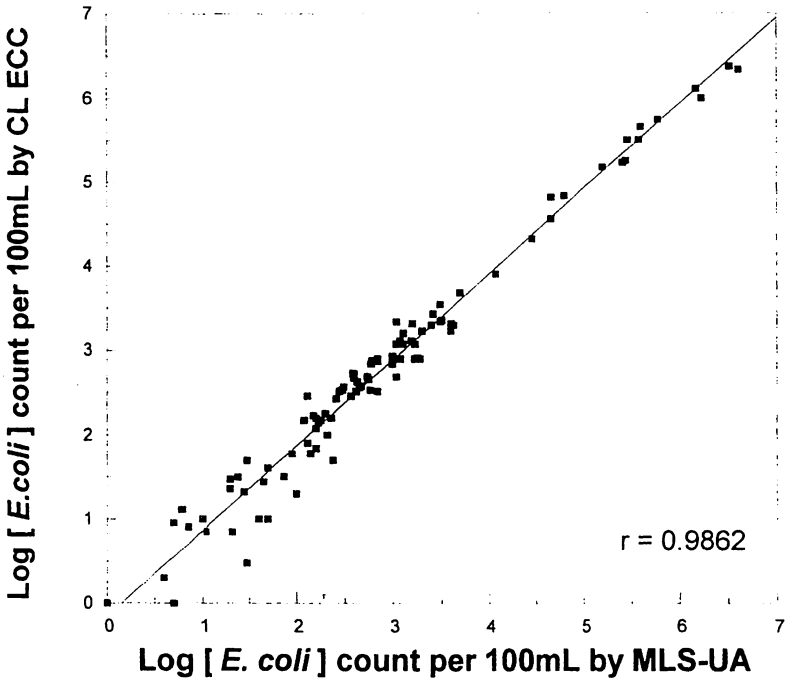

Figure 1. Scatterplot of *E. coli* enumeration using mLS-UA and CLECC methods.

Isolate identity verification is shown in Table 2. Only 138/228 (60.5%) isolates identified on mLS presumptive identifications were correct. 7/34 presumptive non-faecal coliform colonies on mLS were confirmed to be non-faecal coliforms most being *Klebsiella, Enterobacter* or *Citrobacter*. 36/52 (69.2%) presumptive non-*E. coli* faecal coliforms and 95/142 (66.9%) presumptive *E. coli* from mLS were correctly identified. In comparison, of the 310 colonies selected for identification on CLECC, the presumptive identities of 81 (98.8%) non-faecal coliforms, 103 (95.4%) non-*E. coli* faecal coliforms and 114 (95%) *E. coli* colonies were confirmed. For *E. coli*, CLECC was overall superior to mLS-UA in terms of sensitivity and specificity. The initial testing of CLECC with a known bacterial mix indicated that it was better than mLS-UA for enumeration of *E. coli* and faecal coliforms, particularly when samples contained non-lactose-fermenting *E. coli*. Further testing of CLECC with environmental samples confirmed that the *E. coli* counts, even without resuscitation, correlated significantly (P = <0.001) with those obtained with mLS-UA. At first sight, CLECC appears to be less sensitive in detecting *E. coli* when concentrations are low. However, mLS-UA only identified 66.9% correctly while 95% were correctly identified with CLECC. This probably explains why the mLS-UA method often resulted in higher counts of *E. coli* than those obtained on CLECC, giving a false impression that CLECC was less sensitive in detecting *E. coli* when present at low concentrations. Among the 142 presumptive *E. coli* isolates obtained from mLS-UA and subsequently

subjected to identity confirmation, 34 were selected from 10 beach water samples with low *E. coli* counts, only 16 (47%) proving to be *E. coli*. This further eliminated the doubt of lower sensitivity of CLECC.

Table 2. Verification of environmental isolates using mLS-UA and CLECC methods

Presumptive identification of isolates using mLS-UA	Verification			Presumptive identification of isolates using CLECC	Verification		
	+ve	-ve	% id correct		+ve	-ve	% id correct
34 non-faecal coliforms	7	27	20.6	82 non-faecal coliforms	81	1	98.8
52 non-EC coliforms	36	14*	69.2	108 non-EC coliforms	103	5**	95.4
142 *E coli*	95	47	66.9	120 *E coli*	114	6	95.0
For detection of *E coli* using mLS-UA				For detection of *E coli* using CLECC			
Sensitivity[1] = 95/109 = 87.2%				Sensitivity[1] = 114/115 = 99.1%			
Specificity[2] = 70/117 = 59.8%				Specificity[2] = 189/195 = 96.9%			

[1] Sensitivity = no. of *E coli* correctly identified ÷ actual number of *E coli* tested x 100%

[2] Specificity = no. of non-*E coli* correctly identified ÷ actual number of non-*E coli* tested x 100%

CLECC has also been shown to have a higher specificity for identification of other faecal coliforms. Although *E. coli* is the only bacteriological parameter stipulated in the Hong Kong Water Quality Objectives, faecal coliforms are used as standards in other countries. The high misidentification rate of faecal coliforms as non-faecal coliforms by the mLS-UA method (79.4%) would result in a lower reported concentration of faecal indicators in analysed samples. This deficiency of mLS was not observed with CLECC which could identify correctly 98.8% of non-faecal coliforms. Hence, CLECC would be suitable for use where faecal coliforms are used for quality control purposes. For the presumptive non-*E. coli* faecal coliform colonies on CLECC, it is expected that some colonies would turn out to be *E. coli* as the production of ß-D-glucuronidase is the characteristic of only 96-97% of *E. coli* (Damare *et al.*, 1985). These β-D-glucuronidase-negative colonies, biochemically confirmed to be *E. coli*, occurred at a rate of 0.5% on CLECC. Higher occurrence rates around 6% have been reported on other BCIG-containing media (Ciebin *et al.*, 1995).

CLECC for enumerating *E. coli* has advantages over mLS-UA. The detection of urease formation on mLS depends on the production of alkaline end-product which changes phenol red to red but the colours of both red and yellow colonies fade away quickly. These changes in colour production were particularly prominent with the wastewater samples so that enumeration on mLS has to be carried out soon after the urease test. Furthermore, non-lactose-fermenting *E. coli* (approximately 10% of the species) would be misidentified as non-faecal coliforms by the mLS-UA method unlike CLECC where there were no problems. The colour stability with CLECC (at least 24h) allows counting and subsequent rechecking at anytime during the day. It appears that CLECC would be more expensive than mLS-UA but the time required for one *E. coli* test with CLECC was found to be, on average, 1.2min/test less than with mLS-UA and with more reliable results. Preliminary results also revealed that only those greenish-blue colonies of *E. coli* formed on CLECC became visible when viewing through a red filter (unpublished data); this also paves the way to developing the automation of the routine *E. coli* enumeration process.

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