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Comparison of Standard, Fluorogenic and Chromogenic Substrate Based Methods for Enumeration of *Escherichia coli* in Sasthamkotta Lake of Kerala, India

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Abstract

Discovery of chromogenic and fluorogenic substances has made the conventional method more hassle-free to detect specific bacteria. A comparative analysis was conducted to assess the effectiveness of fluorogenic and chromogenic substrates in aiding the enumeration of *E. coli* of Sasthamkotta Lake located in Kerala, India. The fluorogenic compound, 4-Methyl umbelliferyl-D-glucuronide (MUG) is employed for the detection of *E. coli* in food and water. Similarly, a chromogenic substance, viz., 5-Bromo-4-chloro-3-indolyl-ß-glucuronic acid (BCIG) is also employed for *E. coli* identification in food in ISO 16649-3. The present study compared the fluorogenic and chromogenic techniques with the standard method for enumerating *E. coli* by the most probable number (MPN) method. To enumerate the *E. coli,* a total of sixteen water samples were collected from Sasthamkotta Lake of Kerala, India and analyzed by 5-tube MPN method. Quantitatively, the results showed that, chromogenic approach revealed the highest *E. coli* count, followed by the fluorogenic and standard methods. Qualitatively, among the 16 samples, 11 samples were positive in standard method whereas for other methods, all 16 samples were positive. Hence it can be concluded that, in traditional enumeration technique, there is a substantial chance of under representation of *E. coli* by this method. On the other hand, there is chance of over-representation of *E. coli* in the chromogenic and fluorogenic aided methods. Based on the study, it is suggested that a combination of fluorogenic and chromogenic methods, i.e., EC-MUG followed by BCIG plating, would reduce the false positive or reduce the over representation of *E. coli* enumeration.

Keywords: *E. coli*, MPN method, Sasthamkotta Lake, EC-MUG Media, chromogenic media

Introduction

Escherichia coli is a commensal bacteria present in the warm-blooded animal intestines and is routinely monitored in the food and water samples for determination of quality (Visnuvinayagam et al., 2017). It is one of the main indicator bacteria recognized by most countries to check faecal contamination (Vaiyapuri et al., 2021). Generally, indicator bacteria are not harmful, but their presence suggests the existence of other pathogenic bacteria (Sivaraman et al., 2016; Visnuvinayagam et al., 2019). There is a high correlation between *E. coli* and the presence of *Salmonella* in the water and food contamination (Jones et al., 2014). As per the regulatory requirement, the level of *E. coli* in drinking water is 0 MPN / 100 mL (EC, 1998). It is well known that coliform and faecal coliform bacteria have been employed as markers of faecal pollution in water bodies (Koonse et al., 2005). The mere presence of faecal coliforms in water bodies does not always represent direct potential hazards; rather, it may be a sign of many more dangerous viruses and other pathogens that may be found in faeces (Visnuvinayagam et al., 2015). Before the millennium era, for the isolation of *E. coli,* a loopful of culture from the EC-broth positive test tubes were streaked on EMB agar for the appearance of metallic sheen (presumptive *E. coli*) and confirmed with IMViC tests as per American Public Health Association's method (Vanderzant & Splittstoesser,

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1992). However, screening of three colonies from each plate and carrying out the IMViC tests is laborious and time consuming. The United States Environmental Protection Agency (EPA) employed the fluorogenic method, i.e., 4-methyl umbelliferyl-D-glucuronide for confirmation of using EC-MUG broth as a quick and reliable technique for identifying *E. coli* in food samples (Fiello et al., 2014). Similarly, ISO employed a chromogenic medium, i.e., 5-Bromo-4-chloro-3-indolyl-ß-glucuronic acid (BCIG), for the confirmation of *E. coli* directly from the lactose broth/LST (ISO 16649-3). Even though both fluorogenic and chromogenic methods are rapid and hassle-free, accuracy vary between 95 % (Feng et al., 2020) and 97 % (Restaino et al., 1990) for detecting *E. coli,* for MUG and BCIG methods, respectively. In the present study, *E. coli* counts might have been over-represented in both fluorogenic and chromogenic medium compared to the standard method. Hence, a combined method might reduce the over-representation of the *E. coli* in routine water monitoring protocol.

Materials and Methods

Sixteen sampling points were identified in the Sasthamkotta Lake; at every 2 km, one sampling point was identified, which was around 600 m to 1 km distance from the lakeshore area. A total of 16 water samples were taken by the Niskin water sampler in freshwater water at a depth of around 1.5 meter in the Sasthamkotta Lake in Kerala. The collected water samples were maintained under chilled conditions during transportation.

All 16 water samples were subjected to 5-tube-MPN (Multiple Tube Fermentation) method as described by American Public Health Association (Vanderzant & Splittstoesser, 1992) for the enumeration of *E. coli*. Initially, presumptive coliform count (PCC) was carried out in lactose broth, then the positive tubes (gas production) were confirmed as faecal coliform by inoculating the culture into EC-MUG broth. After the 48 h incubation at 44.5 ^{o}C, the positive tubes (gas production) were streaked on the EMB agar. Three to five greenish metallic sheen colonies were purified on a non-selective agar and subjected to the IMViC tests i.e., Indole, Methyl red, Voges–Proskauer and Citrate Utilization. Confirmation of the *E. coli* as per the conventional method based on the IMViC result, + + - - for the *E. coli* biovar-1 and - + - - for the *E. coli* biovar-2. However, as per the standard method, after the IMViC test, the colonies are subjected to polymerase chain reaction (PCR) for further confirmation (Fig. 1).

The confirmation of *E. coli* was based on the amplification of *uidA* gene. PCR reaction was carried out in thermal cycler (Veriti, Applied Biosystem) and the amplified products were analyzed in 2 % agarose gel in 1 x TAE buffer at 5 V/cm for 1 h and visualized in a gel documentation system (Vaiyapuri et al., 2016; 2021) (Fig. 2).

The enumeration of the *E. coli* using fluorogenic method is the continuation of the conventional method, where takes positive by lactose broth is inoculated to EC-MUG broth. EC-MUG (50 mg/L) positive broth is checked under the UV-long wavelength (UV-366 nm) and the appearance of blue fluorescence was considered as a direct confirmation of the *E. coli* (Fig. 3). The results are directly checked in 5-tube MPN chart for the count.

Enumeration of the *E. coli* using chromogenic method was modification of the conventional method; i.e., a loopful of culture from the positive lactose broth tubes (24-48 h) were further streaked

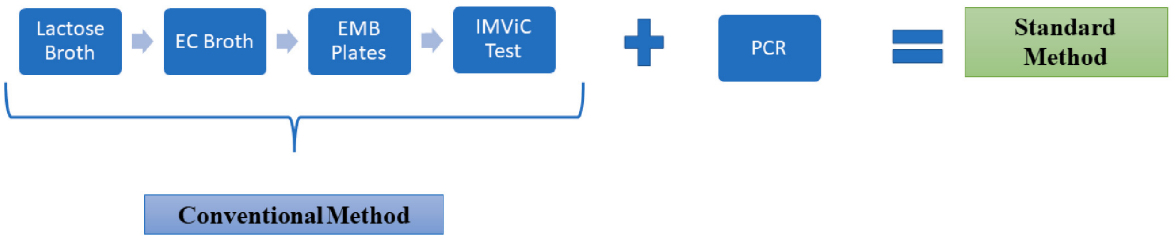

Fig. 1. Standard protocol for the detection of *E. coli* employing MPN method

on the TBX agar plates [Tryptone (20 g/L), Bile Salts No. 3 (1.5 g/L), Agar (15 g/L) and BCIG (0.075 g/L)]. Initial incubation of the TBX plates were at 37 ºC for 4-5 h followed by the incubation at 44 ºC up to 18 h. The appearance of the bluish green colonies indicates confirmation of the *E. coli*. Finally, the results were directly checked in 5-tube MPN chart for enumeration.

Results and Discussion

Sasthamkotta Lake is the largest freshwater lake of Kerala and is the main source of water for the entire Kottayam district of Kerala. To assess the water quality of the lake, 16 sampling points were identified covering the entire Sasthamkotta Lake, Kerala, and 16 water samples were collected for the enumeration of *E. coli.* and, to compare the efficiency of the different media for the enumeration of *E. coli* by most probable number (MPN) method. All the samples were subjected to the 5-tube-MPN method to assess the presumptive coliform count, confirmatory faecal coliform count and confirmatory *E. coli* count in each water sample.

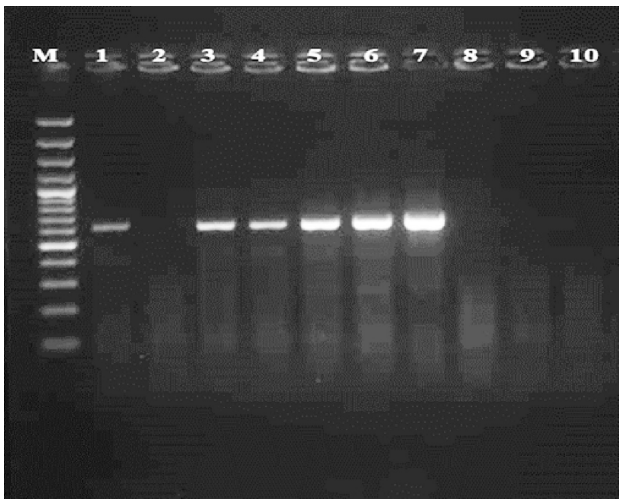

Fig. 2. Amplification of *uidA* gene (603 bp amplicon size indicates the confirmation of *E. coli*) M: 100 bp molecular weight marker; Lane 1: Positive control; Lane 2: Negative control; Lane: 3-7 test samples

It has been reported that the enzyme β-glucuronidase is present in most strains (95 %) of *E. coli* (Feng et al., 2020). The enzyme cleaves 4-Methyl umbelliferyl—D-glucuronide (MUG) chemical compound and produce fluorogenic compound; which emits blue fluorescence in the UV at 366 nm. The chemical property of the metabolic end products of MUG was exploited for the rapid method for detection and verification of *E. coli* in food, water and environmental samples. Organisms other than *E. coli* such as *Salmonella, Shigella, Staphylococcus, Streptococcus,* etc., also possess the enzyme β-glucuronidase. β-glucuronidase positive *E. coli* produces blue fluorescence. The fluorogenic compound MUG is employed in the United States Environmental Protection Agency as a quick technique for the detection of *E. coli* in water (EPA, 2002) and the accuracy ranged from 94 % to 96 % (Manafi et al., 1991).

Another chromogenic substrate can also be used to detect *E. coli*, i.e., 5-Bromo-4-chloro-3-indolyl- ß-glucuronic acid (BCIG). The appearance of blue/bluish green colonies in the media is considered as *E. coli*. The BCIG also known as X-GLUC, which generates a blue hue when cleaved by B-glucuronidase (Restaino et al., 1990). In ISO 16649-3 method, BCIG is employed for *E. coli* identification (ISO, 2001). A variation in the accuracy for the detection was observed by various researchers. Kilian & Bülo (1976) found that the accuracy of the BCIG was 97 % and Turner et al. (2000) reported that the accuracy was 96 %.

In the present study, the level of *E. coli* was determined in all three methods, i.e., Standard, fluorogenic and chromogenic. Based on the result, it has been observed that only 2 sample showed similar MPN values out of 16 samples in all three methods in MPN values. For better understanding, the correlation coefficient between the different methods were calculated (Table 1) and it was found that the chromogenic method gave a higher *E. coli* value than the fluorescent and standard method indicating that the chromogenic media was better in detecting *E. coli* (Table 2).

The levels of *E. coli* in the water samples of Sasthamkotta Lake as determined by the chromogenic method ranged from 4-80 MPN/100mL. The

Table 1. Correlation between the Standard, fluorogenic and chromogenic methods for *E. coli* detection

	Standard	MUG	BCIG
Standard	1		
MUG	0.685476	1	
BCIG	0.521929	0.742296	1

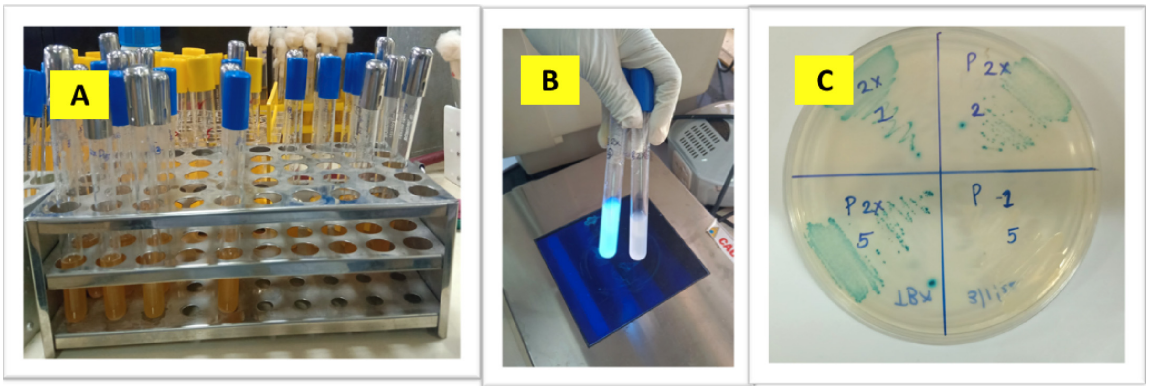

Fig. 3. Standard, chromogenic and fluorogenic methods for the enumeration of *E. coli.* A. Standard MPN method (EC-Broth) B. Appearance of blue fluorescence under UV at 366 nm (EC-MUG Broth) C. *E. coli* colonies appear bluish green on Chromogenic media (TBX agar)

E. coli levels were still lower by other methods which indicated that the quality of water was very good. The European Union Standard (Directive 2006/7/EC) for the limit of inland water is 500 CFU/100mL (EC Directive, 2006). Since the *E. coli* level in the Sasthamkotta is lesser than the limit prescribed by EU, the quality of water can be defined as "Excellent". In India, Central Pollution Control Board (CPCB) has designated different waters and their pollution limit. The total coliforms count in drinking water, after disinfection of water should be ≤ 50 MPN/100mL. In the present study, based on the microbiological results of standard MPN method, it has been observed that the quality of water of the Sasthamkotta Lake was good even before any treatment criteria.

Variation in the accuracy was reported in fluorogenic method from 94 % to 96 % (Manafi et al., 1991) and chromogenic medium from 96 to 97 % (Turner et al., 2020; Kilian & Bülo, 1976). It is hypothesized that the combination of fluorogenic and chromogenic methods would give better accuracy, and simplifies the protocol for the detection of *E. coli*. Hence, instead of IMViC tests followed by PCR, employing fluorogenic medium and chromogenic medium can be used for better precision (Fig. 4).

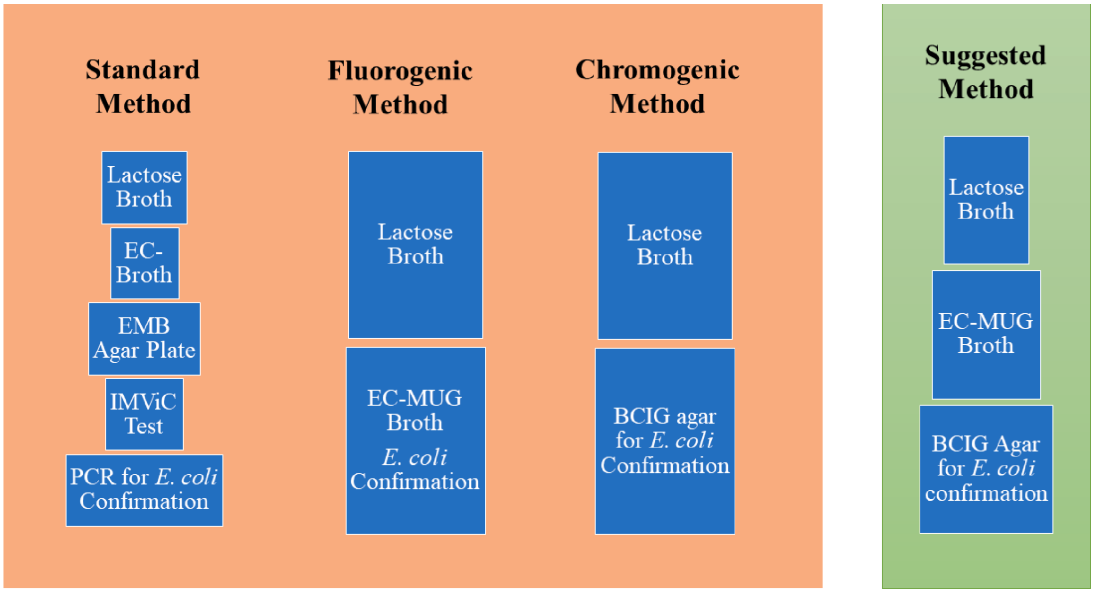

Fig. 4. Recommended methods of detection of *E. coli* from the MPN tubes

Table 2. Comparison of standard fluorogenic and chromogenic method for the detection of *E. coli*

Sample code	*E. coli* (MPN/100mL) Standard Method	*E. coli* MPN/100mL MUG Method	*E. coli* MPN/100mL BCIG Method	Difference in *E. coli count* between MUG Method and Standard Method	Difference in *E. coli* count between BCIG Method and Standard Method
	I	II	III		
A	11	11	17	0	6
B	11	11	50	0	39
C	17	17	30	0	13
D	22	22	22	0	0
E	13	13	17	0	4
F	11	11	11	0	0
G	0	22	50	22	50
H	22	22	80	0	58
I	13	13	17	0	4
J	17	17	50	0	33
K	0	4	4	4	4
L	4	4	4	0	0
M	13	13	17	0	4
N	0	8	8	8	8
O	0	8	8	8	8
P	0	4	8	4	8
	Samples positive for *E. coli*- 11/16	**Samples positive for *E. coli*- 16/16**	**Samples positive for *E. coli*- 16/16**	**Samples with *E. coli* counts different from standard method- 5/16**	**Samples with *E. coli* counts different from standard method- 13/16**

Standard method = Conventional method + PCR confirmation

Even though the standard method is more accurate, there is a substantial chance of under reporting of *E. coli* numbers since only loopful of bacteria from the EC borth is transferred to the EMB agar plate in the traditional enumeration technique. On the other hand, there is a chance of over-representation of *E. coli* in the chromogenic and fluorogenic methods (Restaino et al., 1990; Feng et al., 2020). Based on our study, it is suggested that a combination of fluorogenic and chromogenic method i.e., EC-MUG followed by BCIG plating, would reduce the over-representation of the *E. coli*. The suggested method needs to be validated with large number of samples to establish it as a user-friendly method with better accuracy for the enumeration of the *E. coli* in water samples.

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