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Hydroxyapatite powder cake filtration reduces false positives associated with halophilic bacteria when evaluating *Escherichia coli* in seawater using Colilert-18

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ABSTRACT

Escherichia coli is an important fecal indicator bacterium that is used to evaluate the microbiological quality of water. The Colilert-18 (Quanti-Tray/2000) is a widely used, rapid, and simple quantitative method for detecting *E. coli* in drinking water, bathing water, and wastewater. However, Colilert-18 method is less reliable for seawater; false positives are often caused by halophilic bacteria such as *Vibrio*. While false positives can be avoided by diluting the sample by 10 times or more, the resulting decrease in detection limit makes it difficult to quantify *E. coli* in seawater. In this study, we combined cake filtration, using hydroxyapatite powder, with the Colilert-18 method to remove salinity without diluting the water sample. When quantifying *E. coli* in river water, the *E. coli* concentration obtained from the cake filtration/Colilert-18 method showed a high quantitative value of 90% or more, on average, compared to the concentration obtained with the original Colilert-18 method. The *E. coli* concentrations in seawater determined using the developed method were similar to those determined using the modified m-TEC method, with no false positives. Highly reliable quantitative values can be obtained using the proposed method because it is possible to measure 100 times as much sample compared to the dilution method. Thus, the developed method is expected to be a powerful tool that can eliminate the problem of false positives.

1. Introduction

To protect public health, regular monitoring of fecal contamination in recreational bathing water is important (WHO (World Health Organization), 2003). Fecal indicator bacteria (FIB), including total coliforms, fecal coliforms, *Escherichia coli*, and enterococci, have long been used to evaluate fecal contamination in bathing water (NRC (National Research Council), 2004). Detecting and quantifying FIB are typically achieved using culture-based methods (APHA–AWWA–WEF, 2012; EU (European Union), 2006). *E. coli* specifically indicates fecal contamination more than total coliforms and fecal coliforms, which also contain species derived from the natural environment (Gavini et al., 1985; Leclerc et al., 2001; Mclellan et al., 2001). Consequently, *E. coli,* along with enterococci, are used as FIB for recreational waters (EU (European Union), 2006; USEPA (U.S. Environmental Protection Agency), 2012). However, recent findings have demonstrated that some *E. coli* strains can survive for long periods of time and regrow in extraintestinal environments such as soil, sand, and sediment (Beversdorf et al., 2007; Ishii et al., 2007; Ishii et al., 2006; Walk et al., 2007). Recent epidemiological studies found no relationship between gastrointestinal illness in bathers and *E. coli* levels (Fewtrell and Kay, 2015). Despite these findings, *E. coli* is currently considered a useful FIB because it is affordable, simple, and rapidly quantified using defined substrate techniques. Furthermore, no appropriate alternative indicators have been identified to replace *E. coli*.

Culture-based methods for detecting and quantifying *E. coli* are mainly based on defined substrate techniques that use *E. coli's* specific enzyme activity as an indicator. Various types of defined substrate methods have been developed and compared to standard methods to analyze natural waters (Geissler et al., 2000; Horman and Hanninen, 2006; Tiwari et al., 2016; Valente et al., 2010). For example, the Colilert-18 (Quanti-Tray/2000) method has a short incubation time (18 h), making it superior to other culture methods in terms of rapidity and convenience. The Colilert-18 method is widely used to analyze

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recreational water, drinking water, and wastewater (IDEXX Laboratories, 2013). Its medium contains *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) and 4-methylumbelliferyl-β-D-glucuronide (MUG) as specific enzyme substrates and is hydrolyzed by the β-D-galactosidase and β-D-glucuronidase expressed by *E. coli*. By measuring the color change (yellow) and noting the presence or absence of fluorescence for metabolites, *E. coli* can be easily quantified using this method without conducting additional tests.

However, the Colilert-18 method has several specificity problems. For example, the non-target bacteria *Aeromonas* spp., *Vibrio* spp., and *Flavobacterium* spp. exhibit β-D-galactosidase activity (Covert et al., 1989; Davies et al., 1995; Landre et al., 1998), while *Shigella* spp., *Salmonella* spp., *Flavobacterium* spp., *Vibrio* spp., and *Yersinia* strains are non-target bacteria with β-D-glucuronidase activity (Baudart et al., 2009; Feng and Hartman, 1982; Petzel and Hartman, 1986; Ralovich et al., 1991; Trepeta and Edberg, 1984). Thus, when these non-target bacteria are present in the sample and not selectively suppressed during culturing, the substrates' metabolism in the medium can result in false positives for *E. coli*. In fact, serious false positives have been reported in the analyses of seawater samples using the Colilert-18 method. For example, in measurements of seawater around Tampa Bay, Florida, Pisciotta et al. (2002) detected *E. coli* at levels that were 10–100 times higher than the level of fecal coliforms. These results were caused by halophilic bacteria such as *Vibrio* spp. Therefore, the effect of halophilic bacteria should be considered when measuring *E. coli* in seawater using the Colilert-18 method.

To avoid false positives when determining *E. coli* in seawater using the Colilert-18 method, original samples are diluted by a factor of 10 or more to lower the salt concentration (ISO 9308-2, 2012). However, when using the Quanti-Tray/2000 system, the maximum sample volume is 10 ml, making it difficult to reliably quantify *E. coli* at low concentrations in diluted seawater samples. Furthermore, *E. coli* generally does not survive in marine conditions because of salinity, sunlight inactivation, suppressed photoreactivation, and predatory activity (Anderson et al., 1979; Chan and Killick, 1995; Chandran and Hatha, 2005; Oguma et al., 2013; Sinton et al., 2002). Therefore, except in waters strongly affected by fecal contamination sources (e.g., sewage), *E. coli* are thought to be present at low concentrations in seawater samples. For this reason, it is preferable not to lower the salt concentration via dilution as a means of avoiding false positives.

In this study, to avoid false positives caused by halophilic bacteria when analyzing seawater samples with the Colilert-18 method, we introduce a pre-treatment method based on cake filtration to remove salt without dilution. Hydroxyapatite (HAP) powder was used as the cake material in the cake filtration step. Initial co-culturing tests of *E. coli* and HAP confirmed that HAP did not affect *E. coli* growth during culturing or the visibility when observing color change. Cake filtration served as a pre-treatment step for the Colilert-18 method. The combined cake filtration/Colilert-18 method was applied to quantify *E. coli* in both river water and seawater. For river water samples, *E. coli* concentrations, using the new combined method and the Colilert-18 method alone, were compared. For seawater, the results of the combined method were compared to those of both the Colilert-18 method and a reference method. Finally, our new method's ability to reduce false positives was evaluated by verifying the presence or absence of *E. coli* in tested samples using polymerase chain reaction (PCR).

2. Materials and methods

2.1. Escherichia coli strain and culture conditions

E. coli strain ATCC11775 was used for co-culture tests. Frozen stock of *E. coli* stored at −80 °C in Luria-Bertani (LB) broth containing 1.0% tryptone, 0.5% yeast extract, and 1.0% NaCl with 15% glycerol was inoculated into LB broth and incubated at 36 °C for 20–24 h. Subsequently, 10 μl of the cultured broth was transferred into fresh LB broth and incubated at 36 °C for 20–24 h. Prior to use, the number of *E. coli* grown in the early stationary phase was adjusted to 10 and 10^2 cells/ml in phosphate buffered saline (PBS) through serial 10-fold dilutions.

2.2. Collection of water samples

Water samples from eight different rivers running through Hiroshima prefecture, Japan, were collected in sterile 500-ml glass bottles between November 2015 and July 2016. The samples were kept in a cool box at < 10 °C and examined for *E. coli* within 12 h of collection. Similarly, a total of nine 2000-ml seawater samples were collected from three different water areas in Hiroshima Bay, Japan, in November 2015. The collected samples were kept in sterile plastic bottles in a cool box at < 10 °C and examined for *E. coli* within 12 h of collection.

2.3. Hydroxyapatite powder

The HAP powder used in this series of experiments had a median particle diameter of 20 μm (AP-20C; Sekisui Plastics, Japan). The HAP powder was dry-heat sterilized in an electric drying oven (DRN620DA; Advantec–Toyo, Japan) for 1 h at 180 °C before use.

2.4. Filter cake filtration of hydroxyapatite powder

A 47-mm membrane filter holder base with a 250-ml glass funnel was connected to a vacuum filtration manifold (Advantec-Toyo) with a tube leading to a circulating aspirator (WJ-20; Shibata Scientific Tec., Japan). A sterile mixed cellulose ester (MCE) membrane filter (Advantec-Toyo) with a pore size of 0.45 μm served as a supporting filter on the membrane filter holder base. PBS (200 ml) containing 0.5 g HAP was poured into the funnel and filtered by negative pressure filtration. An additional 300 ml of PBS was then poured into the funnel to form a HAP cake layer on the upper surface of the membrane filter (precoat filtration). Following the filtration of 100 ml of river water or 1000 ml of seawater, the HAP filter cake, along with the supporting filter, was transferred to a Colilert vessel (IDDEX Laboratories, Westbrook, ME, USA) containing 100 ml of sterile deionized water. The HAP filter cake was suspended by inverting gently several times, and the suspension was then used for *E. coli* quantification with the Colilert-18 method.

2.5. Escherichia coli enumeration

(i) *Colilert-18 method.*

Colilert-18 regent (IDDEX Laboratories) was dissolved in 100 ml of undiluted water sample, the sample's 10-fold dilution in deionized sterile water, or the sample processed using HAP filter cake filtration. The sample was then transferred to the Quanti-Tray/2000 (IDDEX Laboratories) and cultured at 36 °C for 18–20 h. Wells with yellow color (Y) and blue-white fluorescence (F) under excitation with a 6-W, 365-nm ultraviolet light in a dark environment were considered positive for *E. coli*. The number of *E. coli*-positive wells (hereafter termed YF wells) was translated into a most probable number (MPN) estimate for *E. coli* according to the manufacturer-provided MPN table.

(ii) *Reference method: Modified m-TEC method.*

Concentrations of *E. coli* were also measured using the modified m-TEC method, which is a standard method of the U.S. Environmental Protection Agency (2009) (Method 1603) applied for seawater tests, as a reference method. Seawater samples (100 ml) were filtrated through the MCE membrane (Advantec-Toyo; pore size, 0.45 μm; diameter, 47 mm). Filtered membrane was placed on the HiCrome m-TEC agar

(Sigma-Aldrich, USA) and incubated at 35 °C for 2 h and then at 44.5 °C for 22 h. Red or magenta colonies, which hydrolyzed of 5-bromo-6-chloro-3-indolyl-β-D-glucuronide in m-TEC medium by β-D-glucuronidase, were counted as *E. coli*.

2.6. Isolation of Vibrio species

To confirm the growth of *Vibrio* species, 1 µl of culture medium from the Quanti-Tray/2000 YF-positive wells, corresponding to undiluted seawater samples and seawater samples pretreated by HAP filter cake filtration, were plated on thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Oxoid, Basingtoke, UK). After cultivation at 36 °C for 18 h, yellow or green colonies formed on the medium were identified as *Vibrio* spp.

2.7. DNA extraction and amplification of Escherichia coli 16S rRNA gene by PCR

To confirm the presence or absence of *E. coli* in YF-positive wells, the *E. coli* 16S rRNA gene was screened from the culture by PCR. To extract *E. coli* genomic DNA, 1 ml of culture fluid from each YF-positive well, randomly selected 4 or 5 wells from each tray, was aseptically collected and centrifuged for 10 min at 20,000 ×*g*. The pellet was then re-suspended in 50 µl of sterile ultra-pure water and heated at 100 °C for 10 min in a heat block. After cooling and centrifuging at 20,000 ×*g* for 5 min, the supernatant was used as a template for PCR. Primer pairs with ECA75F and ECR619R (Sabat et al., 2000) were used for the amplification of *E. coli* 16S rRNA gene. Amplification reactions were performed in a total volume of 25 µl containing 2.5 µl of the supernatant fluid, 12.5 µl of 2× Quick Taq HS DyeMix (TOYOBO, Japan), and 10 pmol of each primer. The expected PCR product size was 544 bps. PCR reaction was basically carried out according to the method described by Sabat et al. (2000). Briefly, 1 cycle of reaction was conducted at 94 °C for 2 min followed by 40 cycles at 94 °C for 45 s for denaturing, 72 °C for 45 s for annealing and extraction, and 72 °C for 10 min for terminal extension. The reaction mixture was then kept at 4 °C until the reaction stopped.

3. Results

3.1. Influence of hydroxyapatite powder on Escherichia coli growth in Colilert-18 medium

Co-culture tests of *E. coli* and HAP were performed to confirm whether HAP powder affected *E. coli* growth or the quantification results. An *E. coli* suspension was inoculated with 0.5 g HAP in 100 ml of Colilert-18 broth and cultured in a Quanti-Tray/2000 at 36 °C for 18–20 h. As a control, the same experiment was performed without adding HAP to the culture medium.

Yellow (Y) color and blue-white fluorescence (F) were clearly observed in the Quanti-Tray/2000 wells with HAP as a result of *E. coli* growth, and the positive and negative wells were easily distinguished (Figs. 1A and B). The MPN estimates for *E. coli* determined from the numbers of YF-positive wells with and without HAP in the inoculated samples ($n = 3$) were similar (Table 1). The mean ratio of MPN with HAP and without HAP was 0.92 (range = 0.77–1.19).

3.2. Combination of HAP filter cake filtration and the Colilert-18 method to quantify E. coli in river water samples

As a feasibility experiment, *E. coli* was quantified in river water samples using a combination of HAP filter cake filtration and the Colilert-18 method (hereafter referred to as the cake filtration/Colilert-18 method), and the results were compared to those obtained using the Colilert-18 method alone. River water samples (100 ml each) were concentrated by HAP filter cake filtration. The entire cake layer, including the residue, was then suspended in 100 ml of sterile deionized water, and a Colilert-18 culture reagent was dissolved in the suspension. The entire mixture, except the supporting filter, was then transferred to a Quanti-Tray/2000 and incubated. As a control method, 100-ml river water samples were directly cultured by Colilert-18. As shown in Fig. 2, a nearly perfect correlation ($r^2 = 0.98$) was obtained between the cake filtration/Colilert-18 method and Colilert-18 method (significant at $P < 0.00001$). On the other hand, the regression slope for the Colilert-18 method versus the cake filtration/Colilert-18 method was slightly < 1.0, indicating that the cake filtration/Colilert-18 method underestimated the amount of *E. coli* with respect to the Colilert-18 method (Fig. 2). However, most of the cake filtration/Colilert-18 data points plotted near $y = x$ (dashed line), and the concentrations of *E. coli* determined using the cake filtration/Colilert-18 method were 90% or more on average (range = 63%–117%) compared to the values obtained using the Colilert-18 method.

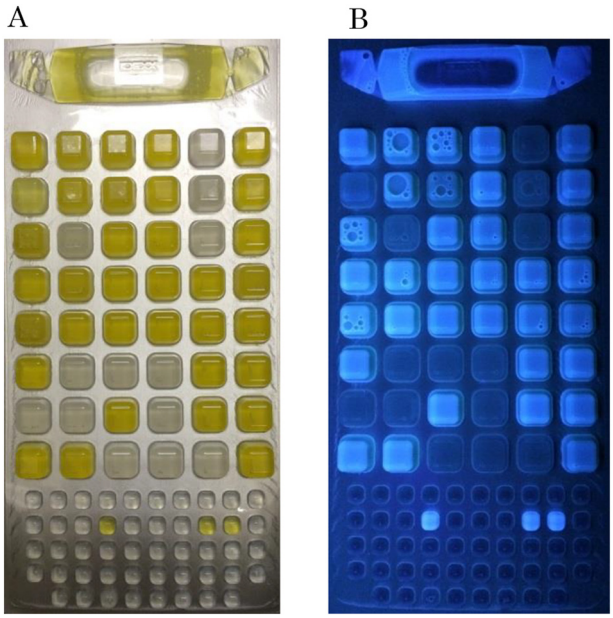

Fig. 1. Development of yellow color (A) and blue-white fluorescence under UV light (B) in Colilert-18 Quanti-Tray/2000 wells with HAP. HAP generated minimal changes in the clarity of the culture medium, and development of both yellow and blue-white fluorescent color was clearly observed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
MPN values of *E. coli* in inoculated samples with and without HAP.

Test no.	*E. coli* (MPN/100 ml) in Colilert-18 medium		Ratio[a]	Mean ratio
	with HAP	without HAP		
1	260.3	325.5	0.80	0.92
2	547.5	461.1	1.19	
3	62.4	80.9	0.77	

[a] with HAP/ without HAP.

3.3. Reduction in false positives for seawater samples by combining the Colilert-18 method with cake filtration

When quantifying *E. coli* in seawater samples with the Colilert-18 method, false positives arise from halophilic bacteria with β-D-galactosidase and/or β-D-glucuronidase activities, such as *Vibrio* spp. (Pisciotta et al., 2002). In this study, nine seawater samples were evaluated using the Colilert-18 method (with and without sample dilution), the cake filtration/Colilert-18 method, and the modified m-TEC

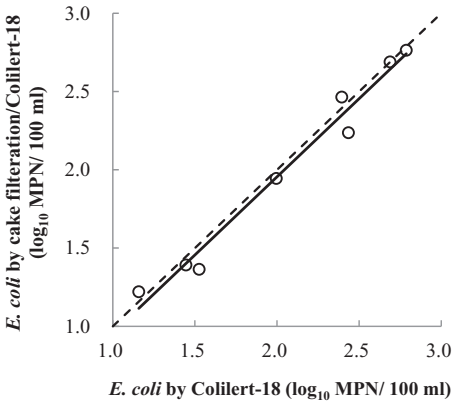

Fig. 2. Simple regression analysis comparing the *E. coli* concentrations determined using the Colilert-18 and cake filtration/Colilert-18 methods for a range of river water samples. The best linear fit is represented by the solid line ($y = 0.9987x - 0.0425$, $r^2 = 0.98$). The dashed line ($y = x$) indicates equal numbers of *E. coli* between both methods.

method as a reference method. The results are shown in Table 2.

When undiluted seawater samples were evaluated directly using the Colilert-18 method, all nine seawater samples were positive for *E. coli*, and the *E. coli* concentrations were higher than those obtained using the other methods. However, the *E. coli* 16S rRNA gene was only detected in three out of the 39 tested YF-positive wells for the nine undiluted samples. Thus, most of the YF-positive wells were considered false positives caused by non-*E. coli* bacteria growth (false positive rate = 92%; Table 3). Furthermore, when the contents of the YF-positive wells were plated on TCBS agar, many yellow and/or green colonies presumed to be *Vibrio* spp. or other halobacteria, which are thought to cause false positives, were observed.

Using the manufacturer-recommended 1:10 sample dilution, the testable water volume was reduced to 10 ml. While false positives can be avoided by dilution, *E. coli* was only detected in two out of the nine diluted seawater samples. The *E. coli* concentrations of samples 1 and 2 were 10 MPN/100 ml (95% confidence interval [CI], 1–55) and 52 MPN/100 ml (95% CI, 23–119), respectively, at near the detection limit (1 MPN/10 ml) with a large 95% CI, (Table 2).

In contrast, the cake filtration/Colilert-18 method produced good results. For the same nine seawater samples, 1000 ml of each sample was filtered via cake filtration, and the whole cakes containing the residues were evaluated using the Colilert-18 method. *E. coli* was detected in eight out of nine seawater samples, with concentrations ranging from 0.41 MPN/100 ml to 17.89 MPN/100 ml (detection limit = 1 MPN/1000 ml). The range of the 95% CI of these MPN values was narrow, indicating highly reliable results. The MPN *E. coli* concentrations of each sample determined using the cake filtration/Colilert-18 method were similar to those obtained using the modified m-TEC method. All 39 YF-positive tested by PCR were positive for *E. coli* 16S rRNA gene (false positive rate = 0%; Table 3), and only a trace number of *Vibrio* colonies grew, if any, when plated on TCBS agar. These data strongly suggest that *E. coli* grew dominantly in the Colilert-18 culture medium.

4. Discussion

The Colilert-18 (Quanti-Tray/2000) quantitative method is less reliable seawater because false positives are often caused by halophilic bacteria such as *Vibrio* spp. (Pisciotta et al., 2002). These false positives can be avoided by diluting samples by > 10 times to lower the salt concentration, but this makes it difficult to detect low levels of *E. coli*. In this study, we attempted to improve the Colilert-18 method's applicability to seawater samples by combining it with cake filtration to decrease salinity without diluting the water sample.

Cake filtration is an effective method for the concentration and recovery of bacteria from water samples, particularly those with high turbidity. Several studies used cake filtration to concentrate bacteria, mainly to detect *Salmonella* (Brezenski and Russomanno, 1969; Cheng et al., 1971). Presnell and Andrews (1976) used cake filtration with diatomaceous earth as a cake material to concentrate and recover total coliforms and *Salmonella*. The authors combined this method with a multiple-tube fermentation technique for quantification. However, no recent reports have combined cake filtration and the Colilert-18 (Quanti-Tray/2000) method for detecting *E. coli*.

In a comparative experiment with river water samples, the *E. coli* concentrations obtained from the cake filtration/Colilert-18 method were 90% or more, on average, compared to those obtained using the Colilert-18 method alone, and the results of both methods were highly correlated. These results demonstrate that the cake filtration method recovered *E. coli* with high yield, indicating that this method can be used as a pre-treatment method for Colilert-18. In conventional membrane filtering, sufficient mixing is required to recover bacteria trapped in/on the membrane filter. For example, Smith et al. (1993) evaluated the *Legionella* recovery rate using multiple types of membrane filters. The authors mixed the membrane filter using a vortex mixer at maximum speed for 2 min. Nevertheless, the highest recovery rate obtained using a polycarbonate filter was 59%, and the maximum recovery achieved by the MCE filter, similar to the one used in this study, was 40%, which is inadequate. In contrast, in the HAP cake filtration method presented here, the bacteria become trapped in/on the cake layer formed on the upper part of the supporting filter. The entire cake layer can then be recovered, along with the supporting filter, and easily re-suspended in liquid by inverting several times. Thus, the bacteria can be recovered with little effort and in high yield compared to the

Table 2
Colilert-18 results for *E. coli* in undiluted, 1:10-diluted, and cake-filtrated seawater samples compared with modified m-TEC agar results.[a]

Seawater sample	Estimated *E. coli* no. by Colilert-18 (MPN/100 ml) for samples processed by:[a]			No. of *E. coli* by modified m-TEC (CFU/100 ml)[c]
	Non-dilution	1:10 dilution	Cake filtration[b]	
1	387.3 (245.9–567.0)	10 (1–55)	17.25 (12.30–23.55)	7
2	307.6 (195.3–471.2)	52 (23–119)	17.89 (12.40–25.78)	13
3	86.2 (63.2–115.4)	< 10 (0–37)	< 0.1 (0.00–0.37)	< 1
4	365.4 (231.9–555.5)	< 10 (0–37)	3.88 (2.61–5.47)	4
5	387.3 (245.9–567.0)	< 10 (0–37)	0.41 (0.17–0.95)	< 1
6	201.4 (135.7–284.0)	< 10 (0–37)	0.52 (0.23–1.19)	< 1
7	325.5 (206.6–498.1)	< 10 (0–37)	2.72 (1.73–4.02)	1
8	224.7 (147.0–343.5)	< 10 (0–37)	1.5 (0.87–2.40)	1
9	152.9 (112.0–206.2)	< 10 (0–37)	2.03 (1.21–3.22)	1

[a] Figures in parentheses represent 95% CI.
[b] Each 1000-ml seawater sample was processed using cake filtration before the quantification of *E. coli* using the Colilert-18 method.
[c] In duplication.

Table 3
False-positive rates for Colilert-18 YF-positive wells from undiluted and cake-filtrated seawater samples.

Seawater sample no.	Non-dilution				Cake filtration			
	n[a]	No. of wells with PCR results of:[b]		False-positive rate (%)[c]	n	No. of wells with PCR results of:		False-positive rate (%)
		positive	negative			positive	negative	
1	5	0	5		5	5	0	
2	5	2	3		5	5	0	
3	0	–	–		0	–	–	
4	5	0	5		5	5	0	
5	4	0	4		4	4	0	
6	5	0	5		5	5	0	
7	5	1	4		5	5	0	
8	5	0	5		5	5	0	
9	5	0	5		5	5	0	
total	39	3	36	92	39	39	0	0

[a] Number of YF wells tested.
[b] Positive, *E. coli* 16S rRNA gene was detected in the YF well culture; Negative, *E. coli* 16S rRNA gene was not detected in the YF well culture.
[c] False-positive wells were defined as those that developed yellow color and blue-white fluorescence in the absence of *E. coli* 16S rRNA gene.

conventional membrane filter method.

The cake filtration/Colilert-18 method was applicable for evaluating seawater samples. Although the *E. coli* concentration when measuring undiluted seawater samples using Colilert-18 was 10–100 times higher than that for the reference method (modified m-TEC method), the *E. coli* concentrations by the cake filtration/Colilert-18 method were similar to those determined by the reference method. In addition, the *E. coli* gene was detected in all YF wells obtained using the cake filtration/Colilert-18 method, and the growth of *Vibrio* was effectively inhibited in these wells, indicating zero false positives. Most bacteria causing false positives in seawater are halophilic, such as *Vibrio* (Baudart et al., 2009; Sercu et al., 2011). Except for *Vibrio cholerae* and *V. mimicus*, *Vibrio* require Na^+ for growth (Farmer III et al., 2005). *V. cholera* and *V. mimicus* are ONPG-positive (Davis et al., 1981) but MUG-negative (i.e., 1% positive according to the percent chart in BD BBL Crystal MIND software, available from: http://www.bd.com/en-us/support/bd-bbl-crystal-mind-software). Our results demonstrate that the cake filtration/Colilert-18 method can completely remove salinity during the filtration step and is effective for suppressing false positives.

Baudart et al. (2009) evaluated the strength of β-D-glucuronidase activity in *Vibrio* spp. predominantly isolated from a French bathing beach. They found that the β-D-glucuronidase activity of *Vibrio* depended on the species and strain, and the activities of some strains of *V. harveyi* were notably higher than those of *E. coli*. The abundance of *Vibrio* spp. changes depending on the seawater temperature (Thompson et al., 2004; Blackwell and Oliver, 2008), and the abundance and distribution of *Vibrio* spp. may also vary by sea area. While the present study and Pisciotta et al. (2002) demonstrate that false positives caused by halophilic bacteria can be reduced by decreasing the salt concentration in seawater samples, further studies may be needed in geographically diverse coastal locations.

Another advantage of the cake filtration/Colilert-18 method is its flexible sample volume, which enables the use of large sample volumes and increases sensitivity. Tiwari et al. (2016) evaluated using Colilert-18 as an alternative to the miniaturized most probable number (MMPN) method (ISO 9308-3, 1998), which is used to measure *E. coli* in seawater based on the European bathing water directive (2006/7/EC). The MMPN method has a long culture time (36–72 h), a test water volume of < 10 ml, and a detection limit of 15 culturable *E. coli* per 100 ml. The European Microbiology Expert Group (2016) suggested that the low *E. coli* numbers measured by the MMPN method using a small volume of test water have high variability due to the Poisson distribution characteristics. This may lead to misclassification of seawater quality based on percentile calculations. Diluting seawater samples for Colilert-18 analysis causes similar problems with sensitivity and precision. However, combining Colilert-18 with the cake filtration method does not require a lower test water volume and provides quantitative results with a narrow confidence interval (i.e., increased precision). Thus, this combined method could be a powerful tool that can solve the above-mentioned issues.

Thus, the new combined method proposed is expected to greatly improve the applicability to the measurement of *E. coli* in seawater samples using the Colilert-18. However, to ensure a higher reliability, it is necessary to demonstrate with a large number and wide range of seawater samples and to conduct statistical analysis.

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