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Specificity of a defined substrate method used to monitor balneability of tropical coastal waters impacted by polluted stormwater

Oscarina V. Sousa, Norma S. Evangelista-Barreto, Karla M. Catter, Antonio A. Fonteles-Filho, Andrew Macrae and Regine Helena S. Fernandes Vieira

ABSTRACT

Defined substrate (DS) is an alternative technique to monitoring the water quality based on species-specific enzyme activity. Although more sensitive and more specific than traditional media, there is some controversy over use in the warmer waters of tropical and subtropical environments, rich in organic matter and microorganism groups capable of interfering with results. The aim of this study was to test the specificity of DS method (Colilert, IDEXX) for detection of coliforms and *Escherichia coli* in stormwater seawater samples from a coastal city (Fortaleza, Brazil) compared to findings obtained with the multiple tube fermentation (MTF) method. The samples were collected from stormsewers and adjacent seashore locations. The most probable number (MPN) of total coliforms (TC), thermotolerant coliforms (TtC) and *E. coli* was determined and the selectivity of the enzymatic substrate medium in the seawater samples was tested. The MTF method showed samples from sampling points 1, 2 and 3 to be 13.3, 13.3 and 46.7%, respectively, above the legal cut-off value for coastal balneability. With the DS method, the corresponding figures were 60, 53.3 and 80% for *E. coli*. Overall, coliform levels were higher with the DS medium. Vibrios and aeromonads were isolated from *E. coli*-positive DS tubes.

Key words | balneability, coastal waters, coliforms, defined substrate, selectivity, *vibrionaceae*

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INTRODUCTION

The contamination of the marine environment by sewage of both domestic and industrial origin is a major public health concern around the world. Thus, monitoring the microbiological quality of coastal waters is an essential measure to ensure the safety of recreational beach-goers. Sources of contamination of surface water include urban and farm run-off, discharge from wastewater treatment facilities and stormsewers.

Historically, fecal coliforms and *Escherichia coli* have been used as indicators of balneability. The organisms employed for this purpose are rarely pathogenic in themselves but reflect the degree of mixture with untreated wastewater (Lopez-Pila 1998). Coliforms, particularly of the species *E. coli*, have since the late 19th century been used as an indicator of water quality. The multiple-tube fermentation (MTF) technique relies on the ability of coliforms to ferment lactose at 35°C (or at 44.5°C for thermotolerant) (Rompré *et al.* 2002). In spite of divergent views over the use of coliform group to indicate contamination in marine and estuarine environments, these organisms are widely employed as a balneability parameter in marine environments (Gray 1999).

The MTF technique and the membrane filter (MF) technique are traditional methods for determining total

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(TC) and thermotolerant (TtC) coliforms, but require complementary procedures and biochemical tests and the results become available only after 96 h (Cerqueira 1997). Therefore, methods providing results within 24 h are often preferred by monitoring agencies. The methods are based on the exposure of the bacteria to a defined substrate which is the only source of carbon and energy capable of inducing the expression of the enzyme β-D-galactosidase (Fricker & Fricker 1996; Cerqueira 1997; Eckner 1998). However, Tryland & Fiksdal (1998) have pointed out that β-D-galactosidase is produced by a large variety of microorganisms. Because vibrios are major competitors for the defined substrate in marine environments, false-positive results are common during such assessments.

The objective of the study was to test the ability of the defined substrate method (Colilert®, IDEXX Laboratories) to detect coliforms and *E. coli* in tropical seawater and freshwater samples from Fortaleza (Northeastern Brazil) compared to findings obtained with the multiple tube fermentation method.

METHODS

Samples

Bacteriological analyses were performed on freshwater samples (SEW) from three stormwater drain systems located along the seashore east of Fortaleza (Northeastern Brazil) (Figure 1) and on seawater samples (SEA) from the corresponding discharge zones on the beach. The collections were conducted between September to March, a period with a high influx of bathers in coastal zone of Fortaleza. About 1 liter of water samples were collected biweekly during high and low tide, accommodated in sterile amber vials and transported to the laboratory in ice boxes.

The samples were analyzed with the multiple-tube fermentation (MTF) technique and with the defined substrate technique (Colilert®, IDEXX Laboratories), used according to the guidelines in *Standard Methods for the Examination of Water and Wastewater* (APHA 1995). The Colilert medium was hydrated and distributed in the wells according to the manufacturer's instructions. The detection limit for both analyses is 1 Most Probable Number (MPN) per 100 mL of sample.

Selectivity test

To evaluate the selectivity of the defined substrate in marine water, 20 *E. coli*-positive wells, of the same sample, were selected based on the high intensity under UV-light and tested for the presence of vibrios and aeromonads by selective isolation on Thiosulphate-Citrate-Bile-Salts-Sucrose Agar (TCBS, Difco Laboratories) and biochemical identification tests. The latter included Kliger Iron Agar

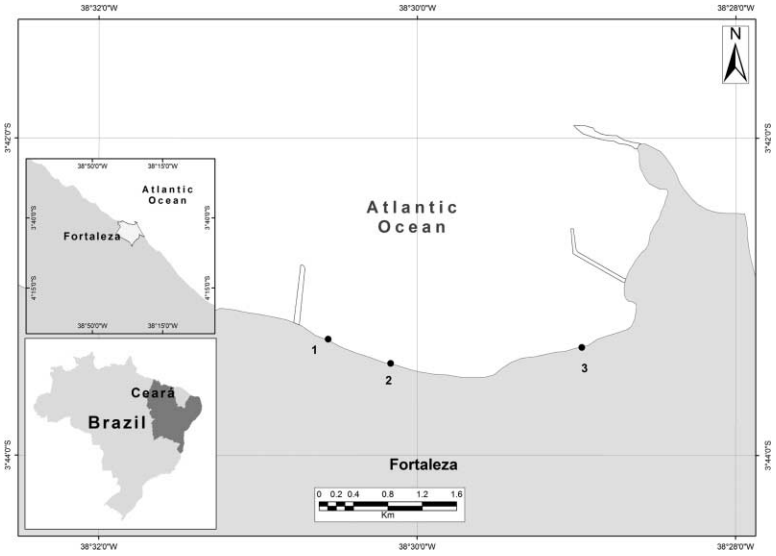

Figure 1 | Map showing the sampling stations close to the Fortaleza stormwater drain systems.

(KIA), Lysine Iron Agar (LIA) and TSA + 1% NaCl, glucose, saccharose, arabinose, mannose, the halophilic test (0, 3, 6, 8 and 10% NaCl), lysine and ornithine decarboxylation, arginine acidification, the glucose fermentation pathway (the VM test and the Voges-Proskauer test) and susceptibility to O/129. Twenty negative wells with Colilert medium were tested for the presence of enterobacteria by culture in Eosin Methylene Blue Agar.

Influence of the choice of detection method on the classification of balneability

In Brazil, the classification of the water bodies according to uses is established by resolution n° 274 of Conselho Nacional do Meio Ambiente (CONAMA). This legislation determine that seawater is appropriate for recreational use when 80% or more of the samples collected at the same location during the preceding five weeks contain on the average less than 1,000 thermotolerant coliforms or 800 *E. coli* or 100 enterococcus per 100 mL. Seawater is deemed unsuitable if this requirement is not met or if the last sample collected contains over 2,500 thermotolerant coliforms or 2,000 *E. coli* or 400 enterococcus per 100 mL (Brasil 2000). However, since the sampling was biweekly (and not weekly as determined by the law), the influence of the choice of detection method on the classification of balneability was assessed using the cut-off values 2,500 MPN of TC and 2,000 MPN of *E. coli*.

Table 1 | Logarithm of average values of the Most Probable Number (MPN/100 mL) of TC, TtC and *E. coli* in water samples from stormwater drain systems and from the corresponding discharge zones on the beach

			Multiple-tube fermentation		Defined substrate	
Sample type	**Point**	***n***	**TC**	**TtC**	**TC**	***E. coli***
Stormwater	P1	14	4.27	3.63	4.94	4.25
	P2	13	5.39	4.97	5.83	5.62
	P3	15	5.00	4.48	5.55	5.45
Seawater	P1	15	2.79	2.63	3.93	3.49
	P2	15	2.66	2.59	3.95	3.46
	P3	15	3.69	3.31	4.77	4.20

n = number of samples.

Statistical analysis

The observed numbers of TC, TtC and *E. coli* were converted to $\log_{10}$ values and expressed as $\log_{10}$ MPN per 100 mL water. The degree of linearity between TC values was determined with Pearson's correlation coefficients (r) ($\alpha = 0.01$). Variance analysis (ANOVA) and MacNemar's non-parametric test were used to compare TC values for both types of samples obtained with the two methods and to compare findings for TtC and *E. coli*. The level of statistical significance was set at $p = 0.05$.

RESULTS

Eighty-seven water samples were analyzed. Of these, 42 came from stormsewers and 45 came from the corresponding discharge zones on the beach (Table 1). The numbers of TC, TtC and *E. coli* were invariably greater in samples from stormsewers than in seawater samples. Values were higher with the DS method than with MTF method for both types of samples. However, the detection methods employed yielded discrepant results: using the DS method the number of *E. coli*–a subgroup of thermotolerant coliforms–was greater than the number of TtC obtained with the MTF method.

No statistically significant correlation was observed between stormsewer samples and seawater samples with regard to TC. Thus, with the MTF method the coefficients were: $r = 0.455$, $r = 0.356$ and $r = 0.224$. The corresponding coefficients for the DS method were $r = -0.029$, $r = 0.124$ and $r = 0.506$. Due to the absence of correlation, variance analysis was used to compare the two methods for the two types of sample separately, with the following results: (a) the method significantly influenced TC detection in stormsewer samples ($F = 28.790$; $p < 0.01$) and seawater samples ($F = 6.255$; $p < 0.01$); (b) TC values varied significantly between sampling points in both stormsewers ($F = 7.610$; $p < 0.01$) and seawater ($F = 9.855$; $p < 0.01$); and (c) there was no significant association between detection method and sampling points in either stormsewers ($F = 0.090$; $p > 0.05$) or seawater ($F = 0.298$; $p > 0.05$), showing that the TC detection method provided consistent readings across the sampling points.

Table 2 | Comparison between the DS method and the MFT method (McNemar's test)

	Stormwater			Seawater		
	P1	P2	P3	P1	P2	P3
Number of samples *(n)*	14	13	15	15	15	15
Highest recovery by DS method	9	7	10	14	13	14
Highest recovery by MFT method	5	4	1	1	0	4

When comparing the number of TtC and *E. coli* in the two types of samples, the following was observed: (a) TtC levels differed significantly between stormsewer samples and seawater samples ($F = 94.163$; $p < 0.01$) and between sampling points ($F = 9.643$; $p < 0.01$), indicating an association between the factors ($F = 15.945$; $p < 0.01$); (b) *E. coli* concentrations differed significantly between stormsewer samples and seawater samples ($F = 43.689$; $p < 0.01$) and between sampling points ($F = 7.438$; $p < 0.01$), again indicating an association between the factors ($F = 8.259$; $p < 0.01$). The associations observed between the two environments (stormsewer and sea) and the sampling points indicate that the concentrations of TtC and *E. coli* must have differed due to variations in the amounts of sewage discharged into the sea by the stormsewers.

The TC patterns obtained with the two methods are shown in Table 2. Compared to MTF, findings obtained with the DS method were 2.6 times greater in stormsewer samples and up to 8.2 times greater in seawater samples.

The MTF method showed samples from points 1, 2 and 3 to be 13.3, 13.3 and 46.7%, respectively, above the cut-off value of 2,500 MPN of TtC/100 mL. With the DS method the corresponding figures were 60, 53.3 and 80% above the cut-off value of 2000 MPN of *E. coli*/100 mL (Table 3).

Table 3 | Number of seawater samples with TtC and *E. coli* concentrations above legal cut-off values for coastal balneability

	Indicator bacteria	
Sample site	Thermotolerant coliforms >2,500 MPN/100 mL*	*E. coli* >2,000 MPN/100 mL*
P1	2 (13.3%)	9 (60%)
P2	2 (13.3%)	8 (53.3%)
P3	7 (46.7%)	12 (80%)

*Brazilian legal cut-off values for coastal balneability (CONAMA, Brazil 2000).

Table 4 | Identification of strains grown on TCBS agar isolated from *Escherichia coli*-positive Colilert tubes

Family	Species	Number (%)
Vibrionaceae (9)	*Vibrio* spp.	4 (14.8)
	Vibrio fluvialis	3 (11.2)
	Vibrio alginoliticus	2 (7.4)
Aeromondaceae (6)	*Aeromonas* spp.	2 (7.4)
	Aeromonas veronii biotype sobria	2 (7.4)
	Aeromonas eucrenophila	1 (3.7)
	Aeromonas caviae	1 (3.7)
Strains not identified		12 (44.4)
Total		27 (100)

In this study the selectivity of the DS medium to seawater was also tested. From 20 wells analyzed, 13 (65%) presented growth on TCBS agar and 27 strains with different colony morphology were isolated from the TCBS plates. Nine of the isolated strains were identified as vibrios while 6 were aeromonads. Twelve strains (44.4%) tested were *Vibrio* and *Aeromonas*-negative (Table 4). EMB agar plates inoculated from wells with negative DS medium showed no growth.

DISCUSSION

The absence of correlation in TC concentrations between SEW and SEA samples may be due to (a) the lethal effect of saltwater on the bacteria, especially on thermotolerant coliforms and/or (b) the dilution of the discharged sewage in the seawater in the tidal zone. Together these effects would rapidly decrease the concentration of coliforms in the water along the shore.

The estimated values obtained with DS were invariably higher than those obtained with traditional media for both sample types. The present results disagree with findings published by Gale & Broberg (1993) and by Grasso *et al.* (2000) whose counts were higher when using the traditional MTF technique with freshwater samples. In this case, the discrepancy between the results may be due to differences in sample type, considering that our study was based on stormsewer and seawater samples. The presence of high

levels of suspended solids related to clandestine connections to domestic and industrial outlets could also have interfered with our results. The role of stormsewers as a vector of microbial pollution in coastal cities in Northeastern Brazil was established by Vieira *et al.* (2002) and Cardonha *et al.* (2004).

The *E. coli* concentrations detected by DS medium were also invariably higher than TtC concentrations in both sample types. In their analysis of seawater, Palmer *et al.* (1993) compared Colilert to other techniques and reached similar results. As described by Niemela *et al.* (2003), DS detects coliforms based on the presence of the enzyme β-D-galactosidase. Many organisms, which possess this enzyme, do not ferment lactose on primary isolation when inhibitory agents are present. Thus, the presence of coliforms unable to ferment lactose but testing positive with ortho-nitrophenyl-β-galactoside (ONPG) assay could explain why the enzymatic method detected a greater number of *E. coli*.

Pommepuy *et al.* (1996) tested the hypothesis that the β-D-galactosidase activity of *E. coli* would be increased by induction during exposure to seawater. However, their findings suggest that the rise in enzyme levels was better accounted for by the inhibition of enzymatic activity of β-D-galactosidase by potentially viable but non-culturable *E. coli*. According to George *et al.* (2000), the presence of coliforms may be underestimated when evaluated by non-enzymatic methods because a greater proportion of the bacteria has detectable β-D-galactosidase and β-D-glucoronidase activity without growing in the culture media employed.

Importantly, the two methods are based upon the measurement of different bacterial growth products. MTF measures metabolic activity as determined by fermentation and the production of gas. Chromogenic methods measure the ability of organisms to metabolize a specific labeled substrate, thereby releasing a chromogen. Not surprisingly, differences in analytical endpoint often lead to discrepant results between methods (Noble *et al.* 2003).

The presence of viable but non-culturable (VNC) bacteria could explain the fact that the β-D-galactosidase and β-D-glucoronidase activity calculated per organism (TC and *E. coli*) in samples from the environment is greater than that observed for pure culture. False-negative results produced by other bacteria may also play an important role (Fiksdal *et al.* 1997; Tryland & Fiksdal 1998).

Vibrios have already been implicated as a source of β-D-galactosidase activity in seawater (Geissler *et al.* 2000). The ability of aeromonads to mediate false-positive reactions in Colilert has also been demonstrated (Landre *et al.* 1998). Since twelve of the isolates did not belong to the families *Vibrionaceae* or *Aeromonadaceae*, it is clear that the medium may allow for and be influenced by bacterial growth associated with other groups. The presence of ONPG-positive strains in seawater capable of interfering with the enzyme substrate has been reported by a number of researchers (Kaspar *et al.* 1987; Geissler *et al.* 2000; Pisciotta *et al.* 2002). According to Hartman (1989), the enzyme beta-D-glucuronidase(GUS) is present in 94–96% of *E. coli* strains, but may also be observed in *Salmonella*, *Shigella* and *Yersinia* spp. Other authors have reported GUS-positive strains of *Citrobacter freundii, Klebsiella oxytoca*, *Serratia fonticola* and *Yersinia intermedia* (Gauthier *et al.* 1991; Alonso *et al.* 1996).

Thus, a deceptively high number of coliforms may be produced depending on the concentration of the interfering organisms. Microorganisms belonging to the natural microbiota of coastal waters display competitive advantages over coliforms. This advantage may be reverted by rehydrating the Colilert medium with distilled water because marine vibrios do not survive for long in fresh water. However, even when the salinity is reduced by dilution, *Vibrio vulnificus* may survive well enough to yield a positive response to β-D-galactosidase in Colilert-MW (Davies *et al.* 1995; Palmer *et al.* 1993).

The combination of β-D-galactosidase and β-D-glucuronidase activity of different concentrations of non-coliform bacteria in seaweeds and aquatic plants may also lead to false-positive results or make it harder to interpret the test (Davies *et al.* 1994). Some authors assume that 4% of *E. coli* strains are β-D-glucuronidase-negative (Moberg 1985; Peterson *et al.* 1987). In this study no false-negative results were registered. Schets *et al.* (1993) found that Colilert gave false-negative results in water samples with low numbers of *E. coli* or total coliforms, indicating that Colilert does not always support the growth of these environmental indicators. In a study involving human feces, 34% of the strains were negative to hydrolyse

4-methylumbelliferyl-beta-D-glucuronide (MUG test), even in the presence of the gene *uid*A, which is responsible for the expression of this enzyme (Chang *et al.* 1989; Feng *et al.* 1991). The false-negative responses would have been determined by the genetic and physiological conditions of the cells (Martins *et al.* 1993; Alonso *et al.* 1996). When analyzing tropical freshwater samples with Colilert-18, Chao (2006) had 36.4% false-positive and 11% false-negative results for *E. coli*. The false-positive rate was 10.3% for coliform detection.

Several authors have tested the ability of the DS method to detect different strains of *E. coli* and total coliforms compared with pure cultures. Maheux *et al.* (2008) found that Colilert detected β-D-glucuronidase production only in 51.4% of 74 *E. coli* strains of different geographic origins and serotypes encountered in fecal and environmental settings.

The choice of bacterial detection method was shown to have a strong influence on the classification of balneability suitability of seawater, as demanded by Brazilian Law (CONAMA, Brasil 2000). When using the MTF method, 4.5 times fewer samples were found to be above the limits established for thermotolerant coliforms than when employing the DS method for *E. coli*.

Alternatively, the use of fluorogenic or chromogenic media to quantify microorganisms in water may constitute a specific, sensitive and time-saving method. Furthermore, the presence of a specific enzyme using suitable substrates could eliminate the need for subculture and further biochemical testing to identify microorganisms (Manafi 1998).

On the other hand, although enzymatic tests can make evaluation work both swifter and simpler, values obtained from analyses of untreated water should be interpreted with caution. Tropical aquatic environments, especially seawater, contain potentially interfering microorganisms. Thus, further study is required to establish the applicability and limitations of this technique.

CONCLUSIONS

Although both the MTF method and the DS method have been approved by APHA for monitoring coastal balneability, the values obtained in our analyses of freshwater and seawater samples differed significantly between the methods. The presence of vibrios in wells with luminescence under UV radiation suggests low bacterial selectivity and interference with quantitative estimates since vibrios may also produce β-D-galactosidase. The choice of detection method was shown to directly influence the classification of balneability of tropical coastal areas. The defined substrate method and analysis should therefore be used with caution.

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