An evaluation of membrane faecal coliform agar and Colilert-18® for the enumeration of *E. coli* bacteria in surface water samples

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ABSTRACT

Aims: To evaluate and review two methods for enumeration of *E. coli* bacteria in surface water samples.

Methods and results: Filtration using membrane faecal coliform (mFC) agar and a defined substrate technology™ method (Colilert-18®) was evaluated. *E. coli* BioBall™ bacteria were seeded into autoclaved surface water samples. In addition, 266 surface water samples from South East Queensland were analysed in parallel using both mFC and Colilert-18®.

Conclusions: *E. coli* is the bacterium of choice when analysing water samples for faecal contamination. An overall lower mean recovery was demonstrated using mFC agar than Colilert for both seeded surface water and in parallel testing of surface water samples. There was a statistically significant difference between BioBall bacteria on mFC agar and values on the QA certificate ($p \leq 0.0001$) but there was no significance difference between Colilert for *E. coli* using the BioBall ($p = 0.8488$). There was a significant difference between mFC agar and Colilert in parallel testing of surface water samples ($p \leq 0.0001$) with greater sensitivity demonstrated by the Colilert procedure.

This data confirms the conclusions of other researchers that Colilert is a suitable substitute for membrane filtration for surface water analysis for the detection of *E. coli* as faecal indicator bacteria.

Key words | BioBall, Colilert, *E. coli*, mFC

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INTRODUCTION

Indicator bacteria have been used for many years to determine the quality and safety of water for human consumption. Coliform and *E. coli* bacteria may be present where there has been faecal contamination originating from warm-blooded animals (Chao *et al.* 2003). There is an ongoing debate among Australian water authorities regarding the ideal choice of method and organism to target, to analyse water samples for faecal indicator bacteria. Some bacteria in the coliform group are present in the environment and therefore are not true indicators of faecal contamination. *Enterobacter*, *Klebsiella* and *Citrobacter* include species which are normal inhabitants of plants and the environment, and their detection in water samples may be misleading when assessing potential faecal pollution (Tortorello 2003). Faecal coliforms (more correctly known as thermotolerant coliforms) include the bacterium *E. coli*, which is a more specific indicator of faecal contamination than thermotolerant coliforms (Krewski *et al.* 2004).

The presence of faecal indicator bacteria in a fully treated (i.e. chlorinated) drinking water supply may indicate contamination due to a failure in the treatment process (Tortorello 2003). However, the absence of these bacteria in water does not necessarily guarantee the absence of pathogens (Krewski *et al.* 2004). Thurman *et al.* (1998)

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analysed several Australian reservoirs and creeks for conductivity, pH, *Giardia*, *Cryptosporidium*, *E. coli*, coliforms, total number of bacteria, turbidity and rainfall. No relationship was found between the detection of indicator bacteria and *Cryptosporidium* and/or *Giardia*.

Analysis for indicator bacteria is still the only means of routinely screening water for faecal pollution. In comparison to coliforms, *E. coli* has been deemed the most selective bacterium to indicate faecal contamination by many researchers (Clark *et al.* 1991; Hamilton *et al.* 2005; Kinzelman *et al.* 2005). Coliforms can readily grow in drinking water systems, be normal inhabitants of soil, water and plants, and not always be present during waterborne outbreaks of disease. However, coliforms should not be detected immediately after a disinfection process, which may be useful to treatment plant operators by providing early warning of a failure in the treatment process. The *Australian Drinking Water Guidelines* (National Health and Medical Research Council 2004) has removed reference to coliforms, due to the likelihood that coliforms detected could be from a non-faecal source, and states that no sample should contain any thermotolerant coliforms or alternatively *E. coli* in a 100 ml water sample.

The National Health and Medical Research Council's *Guidelines for Managing Risks in Recreational Water* (2005) states that the use of coliform bacteria as an indicator for assessing the quality of recreational water is inadequate, as they are not specific to faecal pollution, and *E. coli* is the recommended bacteria for demonstrating water quality for this use. The *Queensland Water Recycling Guidelines* (2005), produced by the Environmental Protection Agency, also specifies *E. coli* as the bacterial indicator for water quality assessments for A+ to D class recycled water. Finally, the United Kingdom Standing Committee of Analysts (2002) recommends that *E. coli* be used as the indicator bacterium for water quality monitoring and assessment. In the United States, the National Academies of Science has also called for the removal of the faecal coliform assay as an indicator of faecal pollution, due to the possible misinterpretation of the results (Doyle & Erickson 2006).

The most commonly used methods in Australian laboratories to detect faecal indicator bacteria include membrane filtration (Eckner 1998), using membrane faecal coliform (mFC) agar and membrane lauryl sulfate (mLS) agar, most probable number (MPN) techniques (Grasso *et al.* 2000) and the commercial kit Colilert® (McFeters *et al.* 1993). This latter procedure utilises a defined substrate technology system (DST) with indicators to simultaneously detect total coliforms and *E. coli*.

The technology of Colilert is based on the fact that *E. coli* produces the enzyme β-glucuronidase, which produces a fluorescent product from the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG). If used in combination with ortho-nitrophenyl-β-D-galactopyranoside (ONPG) as a substrate for β-galactosidase, the kit also identifies the presence of coliforms by the development of a yellow colour (Tortorello 2003). Membrane filtration methods are based on the ability of *E. coli* to grow at 44°C, ferment lactose and produce indole from tryptophan (Eaton *et al.* 2005) and the number of *E. coli* strains that are negative for one or more of these tests is higher than the number of strains that are negative by enzyme detection (Niemela *et al.* 2003). Membrane filtration requires more labour and materials, and requires a high level of technical proficiency to read, interpret and confirm results (Buckalew *et al.* 2006).

Colilert is able to detect bacteria in the coliform group that may not be detectable by the other methods. Hence higher counts of coliforms may be detected using the Colilert procedure when compared with traditional lactose fermentation methods. Fricker *et al.* (1997) compared Colilert to membrane filtration for surface and drinking water samples and found that Colilert detected more coliforms than membrane filtration but there was no significant difference between methods for the detection of *E. coli*. Table 1, taken from the National Health and Medical Research Council's Review of Coliforms (2003), lists the coliform bacteria identifiable by lactose fermentation compared with the bacteria detected by enzyme-based procedures.

The Colilert system is easy to use and produces conclusive results within 18–22 h of testing. Membrane filtration requires initial incubation of samples, assessment of plates for typical colony morphology, followed by subculturing presumptive target organisms for confirmation using a biochemical test, producing results in approx. 72 h after initial sample analysis. Operator judgement in selecting the presumptive target organisms for further

Table 1 | Coliform bacteria identifiable by lactose fermentation and enzyme-based detection procedures (Source: National Health & Medical Research Council 2003)

Produce acid from lactose	Enzyme-based detection
Escherichia	*Escherichia*
Klebsiella	***Klebsiella***
Enterobacter	***Enterobacter***
Citrobacter	***Citrobacter***
Yersinia	***Yersinia***
Serratia	***Serratia***
Hafnia	***Hafnia***
Pantoea	***Pantoea***
Kluyvera	***Kluyvera***
	Cedecea
	Ewingella
	Moellerella
	Leclercia
	Rahnella
	Yokenella

Bold type = coliforms which can be from both human and environmental sources.
Bold and underline = coliforms which are primarily environmental.

confirmation plays an important role in confirmation of coliform and *E. coli* bacteria using membrane filtration. The counting range of the Colilert using the 51-well tray is typically up to 200 organisms per 100 ml. The acceptable range for membrane filtration is up to 80 organisms per 100 ml (Fricker *et al.* 1997). Use of a 97-well tray for Colilert can increase the counting range to 2419. Background growth typically found on membrane filtration samples is not a problem with Colilert samples. However, diluting samples is required for both methods if high numbers of bacteria are expected.

Clark *et al.* (1991) found no significant difference between Colilert and mFC agar in untreated surface water. This earlier research found that chlorine-injured cells present in treated water supplies may require longer incubation times to produce fluorescence, resulting in a possible lower *E. coli* count using Colilert when compared with mFC agar (Clark *et al.* 1991; Edgerg & Edgerg 1998). This has not been the case in more recent studies and has been alleviated by a new formulation of Colilert called Colilert-18® (McFeters *et al.* 1993; Chao *et al.* 2004; Kinzelman *et al.* 2005). Hamilton *et al.* (2005) state that bacteria subjected to thermal stress (i.e. 44°C) and inhibitory culture media (i.e. mFC) may fail to ferment lactose, while Colilert incubated at 37°C improves recovery of stressed organisms. Hamilton *et al.* (2005) evaluated mFC agar and Colilert for the detection of *E. coli* in surface water collected from urban streams and found an improved recovery for *E. coli* when using Colilert. They also found that the greater proportion of thermotolerant coliforms detected was confirmed as *E. coli*.

To some degree, the varying results from comparative studies using duplicate samples of surface and drinking waters can be blamed on the lack of a standard inoculum (i.e. seeding dose) for these comparisons. It cannot always be guaranteed that equal numbers of bacteria are divided between these duplicate samples. The use of freeze-dried cultures has been suggested as an alternative method to overcome this problem.

The BioBall™ product is a freeze-dried sphere (3 mm in diameter) of viable bacteria which dissolves upon contact with solution. The number of bacteria is determined by a cell-sorting flow cytometer, programmed to count 30 organisms and deposit them into liquid nitrogen. The frozen spheres are then freeze-dried and stored in a vial ready for transport and storage at −20°C for up to two years (Morgan *et al.* 2004). The efficacy, detection and enumeration of faecal indicator bacteria in water samples can be easily evaluated using BioBall.

This is the first time the *E. coli* BioBall has been used to compare the accuracy and precision of Colilert and membrane filtration using mFC agar for the enumeration of bacteria in seeded surface water samples. We also performed a parallel study using both detection methods analysing raw surface water samples (Australian/New Zealand *Standard Methods* 1995; Eaton *et al.* 2005).

MATERIALS AND METHODS

Organisms and inoculation procedure

BioBall vials containing freeze-dried *E. coli* (ATCC 11775/NCTC 9001) culture were obtained (BTF Pty Ltd, NSW, Australia). A minimum of 10% of each batch had been quality checked by the manufacturer using randomly selected vials. The quoted acceptance criterion on the QA

certificate supplied for the batch used was a mean of 30.1 colony-forming units (cfu) and a standard deviation (SD) of 2.7. These BioBall cultures are obtained from known reference strains, are four passages from the original strain and are a certified Reference Material according to the International Standard Organisation (ISO) Guide 34 and are tested in an accredited laboratory to ISO/IEC 17025 requirements. All methods were tested at least 10 times using the BioBall culture for *E. coli* and the mean number ± SD of all readings was calculated.

Sampling and seeding

Twenty surface water samples were obtained and autoclaved to inactivate background flora. Each sample was mixed well before 100 ml aliquots were aseptically dispensed into two sterile sample containers. A total of 40 sample containers were prepared. Parallel samples were analysed using both mFC agar and Colilert (i.e. 20 samples for mFC agar and 20 samples for Colilert).

Between 8 February and 6 September 2006, 266 raw surface water samples from rivers, creeks and dams in the South East Queensland region of Australia were analysed in parallel using both mFC and Colilert.

Membrane filtration (MF)

Each water sample was filtered onto a 0.45 μm mixed cellulose membrane (Millipore Australia Pty Ltd, NSW, Australia) and filters were placed onto membrane faecal coliform (mFC) agar (Oxoid) and incubated at 44–44.5°C for 22–24 h according to the APHA *Standard Methods for the Examination of Water and Wastewater* (9222.D). *E. coli* was identified by the presence of typical blue colonies, followed by confirmation using biochemical testing. Seeded samples were not confirmed by biochemical testing.

Colilert

The Colilert-18 reagent (IDEXX Laboratories, NSW, Australia) was added to each sample and mixed well to dissolve it. Samples were poured into a 51-well Quanti-tray (IDEXX) providing counts from 1 to 200 organisms per 100 ml of sample, sealed and incubated at 37°C for 22 h. Colilert simultaneously detects total coliforms by counting the number of yellow wells and *E. coli* by counting yellow wells that are also fluorescent at 365 nm. The MPN was calculated using the chart provided by IDEXX.

Statistical analysis

For seeded surface water samples, a paired *t*-test was used to compare mean and SD between methods with the expected values for the BioBall bacteria as supplied on the QA certificate.

For comparison of raw surface water samples, the Wilcoxon matched-pairs signed-ranks test was performed. Statistical analysis was performed using GraphPad InStat version 3.06 for Windows 95 (GraphPad Software, San Diego CA, www.graphpad.com).

RESULTS

The *E. coli* BioBall batch used in the seeding experiment had a specified mean of 30.1 organisms with a SD of 2.7. In this study, the mean recovery of *E. coli* in seeded surface water samples was 29.9 ± 3.79 using Colilert and 20.6 ± 6.3 using mFC agar (Table 2).

For the seeded surface water samples, the two-tailed paired *t*-test comparing Colilert to mFC agar is considered extremely significant ($p \leq 0.0001$). There was no significant

Table 2 | Mean recovery and SD of *E. coli* BioBall using Colilert and mFC for seeded surface water samples

	E. coli BioBall*	Colilert†	mFC‡
Mean	30.1	29.9	20.6
n	20	20	20
SD	2.7	3.79	6.3
Std. error	N/A	0.8	1.4
Min	N/A	24	3
Max	N/A	36	30
Median	N/A	31	23
Lower 95% CI	29	28	17
Upper 95% CI	31	32	24

*Expected values as supplied on the BioBall QA certificate.
†Colilert vs BioBall is considered not significant ($p = 0.8488$).
‡MF vs BioBall is considered extremely significant ($p = 0.0001$).
N/A Not Applicable, insufficient data supplied.

difference when comparing Colilert to the BioBall bacteria ($p = 0.8448$). But there was an extremely significant difference when comparing mFC to the BioBall ($p \leq 0.0001$).

The bias for a specific sample type using the difference between a true value (i.e. BioBall) and the related mean of the sample test can provide an estimation of the average systemic error (i.e. bias of the test method) (ASTM D2777-03) (ASTM 2003). Using the data obtained for the samples seeded with the BioBall cultures, it was possible to determine the bias of each test method using the following calculation:

$$\text{Bias}\,(\%) = 100(x - b - c)/c$$

x = the mean of usable data for that matrix
b = the mean background concentration reported if necessary
c = the true concentration added

mFC Bias(%) = 100 (20.6 − 30.1)/30.1
Bias (%) = −31%

Colilert Bias (%) = 100 (29.9 − 30.1)/30.1
Bias (%) = −0.66%.

For parallel testing of surface water samples using Colilert and mFC agar, the Wilcoxon matched-pairs signed-ranks test showed a significant difference between these methods ($p \leq 0.0001$). Data was excluded from the calculation when both values were equal. The mean for Colilert (77.8) was higher than the mean obtained for mFC agar (70.6) (Table 3).

Table 3 | Mean recovery and SD of Colilert and mFC for parallel testing of surface water samples

	Colilert	mFC
Mean	77.8	70.6
n	241	241
SD	331	318.3
Std. Error	21	21
Min	0	0
Max	2,800	3,200
Median	7	5
Lower 95% CI	36	30
Upper 95% CI	119	110

DISCUSSION

A problem with similar studies to this one is the lack of a standard reference inoculum to compare methods, with bacterial inocula obtained by serial dilution being variable. We used a precise reference standard to obtain a highly reproducible inoculum size and this enabled a better assessment of these methods for enumerating bacteria.

A previous study (Wohlsen *et al.* 2006) was undertaken to evaluate eight commonly used methods for the isolation and enumeration of indicator bacteria in treated water samples including membrane filtration (MF) using mFC and membrane lauryl sulfate (mLS) agars, standard plate count (PCA-P pour plates and PCA-S spread plates), Colilert, Colisure, MPN and Petrifilm. BioBall cultures of *E. coli* and *Enterobacter aerogenes* (to represent coliforms) were selected and procedures were performed in accordance with standard methods (AS 4276.6-1995, Australian/New Zealand Standards 1995; AS 4276.21-2005, Australian/New Zealand Standards 2005; Eaton *et al.* 2005). The study concluded that, for the methods specifically targeted to isolate coliform bacteria from 100 ml water samples (i.e. MF, Colilert, Colisure and MPN), Colisure and mFC were found to yield the lowest recovery rate for *E. coli* compared with other methods. Colilert produced the most consistent results with the lowest coefficient of variation for both bacteria (Wohlsen *et al.* 2006).

Buckalew *et al.* (2006) compared Colilert to membrane filtration using mFC agar to assess the detection of faecal coliforms and *E. coli* from surface water samples over a three year period. These researchers found that counts obtained by Colilert compared equally with those obtained by membrane filtration for all samples by year, stream and season and concluded that Colilert was an improved method for assessing *E. coli* for freshwater stream samples compared with membrane filtration. Kinzelman *et al.* (2005) also assessed the suitability of using Colilert for freshwater samples over a two year period and determined that Colilert was a suitable alternative to traditional membrane filtration techniques.

Previous researchers have shown that mFC agar underestimates the true levels of *E. coli* present and that significantly more *E. coli* was detected in media containing MUG than with the mFC method (Clark *et al.* 1991).

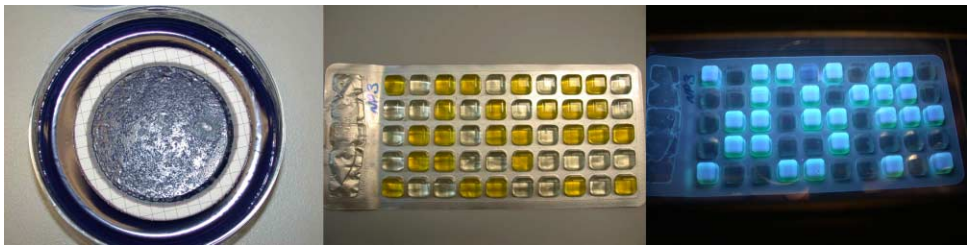

Figure 1 | Surface water sample filtered using mFC agar (left) and the same sample using Colilert (middle and right) with an *E. coli* MPN of 34 bacteria (i.e. yellow + fluorescent wells) in the 100 ml sample.

McFeters *et al.* (1993) supports the use of Colilert for the detection of coliforms and *E. coli* for source and treated water samples and demonstrated that Colilert produced comparable results to MF and MPN procedures when using chlorine-stressed organisms.

Chao *et al.* (2003) showed that mFC agar yielded lower counts of *E. coli* than Colilert and that 18% of samples failed to produce the "typical" blue colonies in waters from sub-tropical areas. We also obtained low average counts using mFC agar and possible reasons for this could be the high selectivity of the mFC agar and the higher incubation temperature (i.e. 44°C) compared with other methods (i.e. 37°C).

Our experience with mFC agar is that background growth of non-target organisms can inhibit the isolation and confirmation of *E. coli* (Figure 1).

This observation has been confirmed by other researchers who have also noted that overgrowth of background bacteria obtained by membrane filtration methods occur frequently, resulting in difficultly in reading the membranes and non-target organisms could inhibit the growth and confirmation of coliform and *E. coli* bacteria. (Niemela *et al.* 2003).

Coliform detection in Colilert is based on possession of the enzyme β-D-galactosidase. There are more coliform bacteria that possess this enzyme than are able to ferment lactose on inhibitory media (Niemela *et al.* 2003). Thus Colilert is able to detect a greater range of coliforms than methods based primarily on lactose fermentation. Colilert is capable of identifying anaerogenic and indole negative *E. coli* (Ostensvik 2000). *Aeromonas* species in surface waters may ferment lactose and could be falsely identified as a coliform in traditional membrane filtration methods. Colilert does not support the growth of lactose-fermenting non-coliforms (Chao *et al.* 2004).

Fricker *et al.* (1997) also concluded that Colilert could detect more coliforms in water samples than membrane filtration. Colilert is also less harsh to bacteria than membrane filtration where organisms may be stressed due to drying of the surface of the filter and Colilert also reduces the risk of nutrient shock as it contains minimal nutrients (Niemela *et al.* 2003).

The Colilert system is one of the recommended methods for the analysis of drinking and source water samples by the American Public Health Association (*Enzyme Substrate Coliform Test 9223*) for the detection of both coliforms and *E. coli* (Eaton *et al.* 2005). Colilert is approved in 28 countries including Brazil, Italy, Denmark, Germany, Korea, the United Kingdom, the World Health Organisation and is approved by the USEPA for drinking water, recreational waters, surface waters and wastewaters (http://www.idexx.com/water/colilert18/moreinfo.jsp#42). Standards Australia (2005) have also issued *Method 21: Examination for coliforms and Escherichia coli–Determination of most probable number (MPN) using enzyme hydrolysable substrates* (AS/NZ 4276.21-2005) for water analysis, with no limitations regarding matrix type, except where estuarine or brackish water samples are expected to contain ONPG-positive non-target microorganisms, in particular *Vibrio* species. The UK Standard Committee of Analysts (previously Report 71) has also approved Colilert as a suitable test for the examination of drinking waters and source waters of moderate to high turbidity (United Kingdom Standard Committee of Analysts 2002).

CONCLUSION

There is an ongoing debate among Australian water authorities regarding the ideal method and target organism

to analyse water samples for faecal indicator bacteria. Previous research as reviewed in this paper support the use of *E. coli* as the bacterium of choice for the analysis of water samples. This is the first study utilising the BioBall bacteria to seed surface water samples for comparative testing. This study demonstrated an overall lower mean recovery using mFC agar than Colilert, for both seeded surface water samples and in parallel testing of raw surface water samples. For parallel testing of surface water samples using Colilert and mFC agar, there was significant difference between these methods ($p \leq 0.0001$) with a overall higher mean for Colilert (77.8) than for mFC agar (70.6), indicating a higher sensitivity for the Colilert procedure.

The standard deviation calculation is regarded as a universal measure of random sources of error (ASTM D2777-03) (ASTM 2003) and these results demonstrate that a higher SD was obtained with mFC agar (SD 6.3) than was obtained with Colilert (SD 3.79) when analysing seeded surface water samples. The large bias of -31% for mFC agar compared with -0.66% for Colilert also confirms a lower systematic error (or lower bias of the procedure) for the Colilert method. Although there was a statistically significant difference when comparing BioBall bacterial recovery on mFC agar with the expected values on the QA certificate ($p = 0.0001$), there was no significant difference between the BioBall bacterial recovery and Colilert.

This data confirms the conclusions of other researchers (Fricker *et al.* 1997; Niemela *et al.* 2003; Chao *et al.* 2004) that Colilert is a suitable, if not better, substitute for membrane filtration for the analysis of surface water sample for the detection of *E. coli* as faecal indicator bacteria.

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