Investigation of transcription-mediated amplification as a rapid test method for *Enterococci* in recreational water

Neal Walkley,[a] Douglas Moore,[b] Charles McGee,[c] Daniel Kacian[d] and Ian Weeks*[a]

Received 17th June 2008, Accepted 25th July 2008
First published as an Advance Article on the web 6th August 2008
DOI: 10.1039/b810284h

Growth-based methods for detecting faecal contamination of recreational water require at least 24 hours to yield results, which can delay detection, action and remediation, if required. Such delays can put human health at risk while closing beaches inappropriately can cause unnecessary economic losses. There is a requirement for more rapid methods to facilitate the management of pollution events. In this study, undertaken in our respective laboratories, a molecular assay based on transcription-mediated amplification (TMA) technology was compared with established growth-based methods. The assays were used to quantitate enterococcal faecal indicator bacteria (relative to the legislative threshold of 104 colony forming units/100 mL) in 138 water samples collected from 41 different locations, representative of different recreational water types routinely sampled in Southern California. The results demonstrate the efficacy of the TMA assay for the detection of *Enterococcus* species in recreational water as a rapid alternative to traditional methods.

Introduction

The currently approved methods for detecting faecal contamination of recreational water rely on growth-based protocols, which require at least 24 hours to yield results. Accordingly, acute contamination events are always detected retrospectively[1] which delays detection, action, and remediation, if required. Such contamination potentially impacts human health[2] and has potential economic consequences.

Enterococci are presently used as the indicator organisms of choice for monitoring human faecal contamination[3] and standards have been established for the maximum acceptable level of *Enterococci* in seawater samples. In California, as set forth in the October 1997 state legislation AB411, the threshold for a single sample is 104 colony-forming units (CFU) per 100 mL of water.[4] It has been established that swimming in water contaminated with faecal material and having elevated *Enterococci* levels has been associated with gastroenteritis.[5] However, other studies reported that increased levels of *Enterococci* were not correlated with increased incidence of illness.[6,7] Such findings may reflect the fact that existing culture-based methods for the detection and quantitation of *Enterococci* are not specific for this genus and/or that not all species of *Enterococci* are associated with human faecal material.[8] A recent study[9] confirmed that, in certain samples, up to 43% of *Enterococci* "positive" isolates from growth-based methods were not *Enterococcus* species.

[a]*Molecular Light Technology Research Ltd., 5 Chiltern Close, Cardiff Industrial Park, CF14 5DL, Wales, UK. E-mail: ian.weeks@mltresearch.com; Fax: +44 29 20747118; Tel: +44 29 20747033*
[b]*Orange County Public Health Laboratories, 700 Shellmaker Road, Newport Beach, CA, 92660, USA*
[c]*Orange County Sanitation District, 10844 Ellis Avenue, Fountain Valley, CA, 92708, USA*
[d]*Gen-Probe Incorporated, 10210 Genetic Center Drive, San Diego, CA, 92121, USA*

Additionally, it has been reported that faecal indicator bacteria, such as *Enterococci*, are capable of significant longevity or even re-growth in certain environments such that elevated levels are not necessarily representative of a current contamination incident. This also detracts from the ability of *Enterococcus* species to act as an accurate indicator of acute pollution events.[10] More specific and rapid tests for *Enterococcus* species may improve its efficacy as an indicator of faecal contamination. Recent advances in molecular biology permit the development of test methods of exquisite specificity and sensitivity. Amplification methods based on the quantitative polymerase chain reaction (qPCR) are probably most familiar and have previously been described for the detection of *Enterococci*.[11] An alternative amplification method, transcription-mediated amplification (TMA), has been employed with great success for routine detection of microorganisms in the clinical laboratory.[12] TMA technology is extremely rapid and, unlike PCR, does not require thermal cycling since it is an isothermal process. Bacteria, such as *Enterococci*, contain many thousands of copies of ribosomal RNA (rRNA) in a single cell yet conventional PCR uses DNA as a target, which is nominally present as a single copy. In contrast, TMA is ideally suited to the amplification of rRNA, hence, making possible even greater sensitivity. Moreover, the TMA method used here has been designed for minimal cross-reactivity with non-enterococcal species and also designed not to detect all groups of *Enterococci* since many species, in particular those which comprise Group I, are not found extensively in human faeces.

Existing culture-based methods for detection and quantitation of *Enterococcus* species are approved by the USEPA, although the methods are known to exhibit cross-reactivity with non-enterococcal organisms. A recent, limited study[9] compared USEPA-approved culture methods with several rapid methods including a variety of qPCR and TMA tests. The study demonstrated the feasibility of the use of TMA in this context. Here we

present a comprehensive comparison of TMA with EPA-approved membrane filtration and defined substrate culture methods for the detection and quantitation of *Enterococcus* species in marine coastal recreational water.

Experimental

Sample collection and reference testing

A total of 138 water samples were included in the study. The samples were collected from a range of 41 different geographical locations representative of water bodies routinely sampled in Southern California and included samples collected during a period of wet weather, which is known to give rise to increased numbers of *Enterococci* in coastal water. The types and numbers of water samples included enclosed beach ($n = 22$), open beach ($n = 44$), open beach near storm drain ($n = 18$), storm drain ($n = 29$), and wet weather ($n = 25$) samples. Each sample was collected into two 500 mL bottles. The contents of the two bottles were pooled and then aliquotted into 100 mL samples. Samples were stored at 5 °C to 10 °C until testing (within 6 hours of collection). Samples were tested in duplicate by TMA, membrane filtration, and Enterolert methods. USEPA Method 1600 was utilized for membrane filtration.[13] The sample was filtered through a 47 mm, 0.45 micron filter, which was placed on an mEI agar plate and incubated for 22 to 24 h at 41 °C before enumerating colonies possessing blue halos. The Enterolert method (Idexx Laboratories, Inc., Westbrook, Maine) was performed according to the manufacturer's instructions. Concentrations were reported as colony forming units (CFU) or most probable number (MPN) per 100 mL, for the USEPA and Enterolert methods, respectively. For the purposes of the present study, CFU and MPN were regarded as being sufficiently similar to permit comparison of results.

Since the reference methods themselves are not completely accurate, the "true" status of the samples was based on a composite reference result using duplicate test results from both reference methods (four results total). An unambiguous, confirmed, qualitative result is only obtained when all of the USEPA Method 1600 and Enterolert replicate results are in qualitative agreement, *i.e.* positive or negative, relative to the AB411 standard of 104 CFU/100 mL. Samples with discordant reference results were classified as "ambiguous". Any sample with less than four reference results (or two results when comparing individual methods) was regarded as "unconfirmed".

Sample processing and TMA

The method of analysis was broadly as described previously.[14] Briefly, to determine *Enterococcus* levels, water samples (100 mL) were processed within 6 h of collection. All water samples were filtered on a vacuum manifold using 47 mm, 0.45 micron mixed cellulose ester filters. Once filtration of the sample was complete, the filters were rinsed with a 3% polyvinylpyrrolidone solution. The filters were transferred, grid-side up, onto the base of a sterile container. Lysis reagent was added to release the rRNA from any *Enterococci* present. The lysates were recovered into micro-centrifuge tubes and were either processed immediately or stored below −60 °C until ready to use.

Volumes of the lysates (1 mL) were processed using a magnetic particle separation apparatus (Target Capture System [TCS], Gen-Probe Inc., San Diego, California). Target Capture Reagent, containing both magnetic microspheres coated with oligo-dT and soluble poly-A tail *Enterococcus* capture probe, was added and mixed thoroughly taking care to avoid splashing. Reaction mixtures were incubated at 60 °C for 5 min to permit annealing of the capture probe to enterococcal rRNA and then allowed to stand for 10 min at room temperature to anneal the capture probe/target complex to the magnetic particles. The magnetic particles were then separated on the TCS magnetic rack for 5 min. The liquid was aspirated under a vacuum and the particles were washed with TCS wash solution and separated three times before elution from the particles into 60 μL of sterile water.

Aliquots of sample eluate and assay positive and negative controls (10 μL each), respectively, were added to 20 μL of amplification reagent containing the required amplification oligonucleotides, fluorescent molecular torch specific for amplicon detection, NTPs and dNTPs in the wells of a 96 well plate (BioRad Laboratories, Hercules, California) and heated in a Chromo4 fluorescence-reading thermocycler (BioRad Laboratories, Hercules, California) at 60 °C for 10 min. The temperature was then reduced to 42 °C and the TMA enzymes (10 μL) were added to each reaction well while taking care to avoid splashing. The plate was sealed with a clear sealing card, removed from the thermocycler heated block, and vortex-mixed briefly before the plate was returned to the thermocycler heated block set at 42 °C. The measurement cycle was started and the resulting fluorescence from each well was recorded every 30 s for a period of 35 min and displayed as a plot of intensity against reaction time. The emergence time (C(T)) above a pre-determined fluorescence intensity threshold was calculated for each reaction well. A linear calibration curve was constructed using the mean C(T) value obtained from the duplicate measurements of known solutions of rRNA (purified from laboratory cultured *E. faecalis* by standard methods) in lysis reagent and which had previously been characterized in terms of "CFU equivalents" by reference assays against known numbers of *E. faecalis* organisms. In this way, the results for unknown samples were expressed as CFU and assessed as being positive or negative relative to the AB411 standard of 104 CFU/100 mL.

Statistical methods

Method comparisons were carried out using only those samples that yielded concordant results for the individual replicates returned for the reference methods. Of these samples, the result was regarded as unambiguously positive or negative relative to the regulatory threshold of 104 CFU/100 mL. The result for the TMA was classified as correct, false positive, or false negative relative to the composite result. The proportion of false positive and false negative results relative to the reference methods have been considered both in terms of individual replicate qualitative determinations and also qualitative determinations derived from quantitative replicate means of results in CFU/100 mL. Correlation analysis of the methods was performed using Microsoft Excel. The sensitivity of detection of all the methods used and referenced herein has previously been quoted as <10 CFU/100 mL.

Table 1 Results of duplicate testing of samples with reference and TMA methods

Method	Positive (%)[a]	Negative (%)[a]	Ambiguous (%)[a]	Unconfirmed (%)[b]
mEI	29.6	67.4	3.0	2.2
Enterolert	26.0	68.7	5.3	5.1
TMA	20.3	75.4	4.3	0
Reference[c]	24.2	64.8	10.9	7.2

[a] Calculated as a percentage of the total number of samples ($n = 138$) minus the number of samples with incomplete analyses (unconfirmed results).[b] Incomplete analyses (unconfirmed results) calculated as a percentage of the 138 samples submitted in the study.[c] Composite result from testing with the USEPA Method 1600 (mEI) and Enterolert methods.

Results and discussion

Qualitative considerations

Results from duplicate testing of samples with the USEPA Method 1600 (mEI), Enterolert, and TMA methods are shown in Table 1.

The results from the composite analysis using the two reference methods are also shown. Of the 138 unique samples, 114 had concordant positive or concordant negative reference method results. Table 2 shows the percentage of false positive and false negative results for the individual replicate results of the TMA test relative to the composite reference results.

Similarly, the individual replicate results for a given reference method are compared with the corresponding unambiguous result (if available) of the alternative reference method. Table 3 shows the result of direct comparison of all method means as opposed to comparison of individual replicates for a given test with reference means. The proportion of apparently false negative results is higher for this TMA than is the corresponding figure for the reference methods relative to each other irrespective of whether the individual replicates or duplicate means are used for comparison. Table 4 shows the comparison of the two growth-based reference method results stratified by water sample type. It can be seen that the two reference methods yield a greater proportion of discordant results for those samples derived from drains. Table 5 compares the TMA assay results with the unambiguous composite reference results.

Table 2 Comparison of TMA results with reference results

Method (no. of replicates)	Positive (%) [false positive (%)]	Negative (%) [false negative (%)]
TMA (228)[a]	23.5 (3.9)	76.5 (7.5)
mEI (245)[b]	28.2 (2.4)	71.8 (2.0)
Enterolert (252)[c]	29.0 (2.8)	71.0 (2.8)

[a] Unambiguous results from the composite reference result used as reference.[b] Unambiguous Enterolert results used as reference.[c] Unambiguous USEPA Method 1600 (mEI) results used as reference.

Table 3 Comparison of TMA results with reference results for sample means

Method (no. of samples)	False positive (%)	False negative (%)	Overall accuracy (%)
TMA (114)[a]	4.4	7.0	94.3
mEI (128)[b]	3.9	3.1	96.5
Enterolert (128)[c]	3.1	3.9	96.5

[a] Unambiguous results from both reference methods.[b] Unambiguous Enterolert results used as reference.[c] Unambiguous USEPA Method 1600 (mEI) results used as reference.

Table 4 Results of reference methods stratified by water sample type

Sample	n	Median[a]	Positive[b]	Negative[b]	Ambig.[b]	Acc.[bc]
WW[d]	22	400	54.5	40.9	4.5	95.5
OB[e]	37	16	13.5	81.1	5.4	94.6
EB[f]	22	85	4.5	90.9	4.5	95.5
SD[g]	29	9	31.0	41.4	27.6	72.4
ND[h]	18	22	22.2	66.7	11.1	88.9

[a] Median number of *Enterococci* per 100 mL.[b] Ambiguous result expressed as percentage relative to total samples with complete, unambiguous results (two reference results per method per water type).[c] Accuracy expressed as a percentage relative to corresponding reference method.[d] WW = wet weather.[e] OB = open beach.[f] EB = enclosed beach.[g] SD = storm drain.[h] ND = open beach near drain.

Table 5 Comparison of TMA results with unambiguous results of reference methods stratified by water sample type

Sample	n	Positive[b]	Negative[b]	FP[a tab5fnb]	FN[a b]	Acc.[b]
WW[c]	21	61.9	38.1	4.8	0.0	97.6
OB[d]	35	11.4	88.6	5.7	8.6	92.9
EB[e]	21	0.0	100.0	0.0	4.8	97.6
SD[f]	21	47.6	52.4	9.5	4.8	92.9
ND[g]	16	6.3	93.8	0.0	18.8	90.6

[a] FN = false negative, FP = false positive.[b] Accuracy expressed as percentage relative to total samples with complete, unambiguous results (two reference results per method per water type).[c] WW = wet weather.[d] OB = open beach.[e] EB = enclosed beach.[f] SD = storm drain.[g] ND = open beach near drain.

Quantitative considerations

Table 6 shows the regression parameters for Enterolert relative to the USEPA Method 1600 (mEI), and the TMA assay relative to the individual reference methods and to the composite reference method result. The results of this TMA correlate more strongly with USEPA Method 1600 than they do with Enterolert.

Table 6 Linear regression correlation analysis of test methods[a]

	Enterolert/ Method 1600	TMA/ Method 1600	TMA/ Enterolert	TMA/ Ref.
Slope	0.31	0.81	0.95	1.12
Intercept	329	73	106	−65
r^2	0.5959	0.9698	0.6040	0.9329

[a] Results returned as "greater than" were not included in the quantitative analysis ($n = 4$) and all results expressed as "less then 10 CFU" were assigned a value of 10 CFU in order to align all methods with the stated limit of quantitation of Enterolert of 10 CFU.

The aim of the studies reported here was to establish comparisons between this novel TMA method for determination of *Enterococci* in recreational water and the currently established culture methods. However, though widely-used, these latter methods themselves are acknowledged to have certain method-dependent disadvantages and in certain circumstances, the results of such tests do not always represent the true number of human faecal derived *Enterococci*.

The California state single sample standard (AB411) for the presence of *Enterococci* is 104 CFU/100 mL and samples yielding results above or below this are regarded as being qualitatively positive or negative, respectively. Ideally, a "reference method" would yield unequivocal results but, in reality, the established methods yield ambiguous results in a proportion of the samples tested, a fact which is confirmed in the present study. Since both reference methods are approved by the USEPA and routinely used, it is reasonable to combine the results of the methods to yield a composite "reference" result, which is theoretically not subject to bias from any one of the particular reference methods used. It can be seen from Table 1 that 10.9% of the qualitative results of the reference methods were ambiguous and therefore cannot be regarded as definitive results with which TMA can be compared. Accordingly, only unambiguous reference results have been used here for comparison purposes.

It can be seen from Table 2 that though the apparent proportion of false positive results for this TMA method is only slightly higher than the reference methods, the apparent proportion of false negative results is substantially higher. This is confirmed in Table 3, which is based on the quantitative means of each method. The overall accuracy of this TMA method is 94.3% relative to the combined result of the reference methods, which is similar to the 96.5% mutual accuracy of the reference methods themselves. Consideration needs to be given to the apparent 7% false negative results of TMA since it is known that the reference methods themselves "cross-react" with irrelevant organisms and therefore cannot be used to derive a "true" proportion of false negative results for TMA. Several studies[8,9,10] have demonstrated that not all "positive" isolates from growth-based methods are *Enterococci* and that such methods are positive for all *Enterococcus* species. By contrast, it is known that this TMA method is more specific[15] and therefore it might be expected that it would yield a lower rate of positive results than less specific methods in situations where a wide spectrum of microorganisms exists. It is known that storm drains discharge a complex mixture of chemicals and microorganisms and one might anticipate greater variability in the results of such samples. Indeed, the highest percentage of ambiguous reference results occurred in samples deriving from or having a significant storm drain component (Table 4). It is unsurprising, therefore, that the accuracy of TMA relative to the reference methods (Table 5) appears to be lower in this situation since the reference methods do not always yield consistent results in this class of sample. Interestingly, the ratio of negative to positive results for this TMA method correlates well with the corresponding ratio for the reference methods themselves when the ambiguous reference sample results are accounted for (data not shown).

From a quantitative standpoint, correlation analysis (Table 6) of the mean results of each method shows a relatively low correlation coefficient between the two reference methods. By contrast, the correlation coefficient between TMA and the USEPA Method 1600 is substantially larger. Overall, the relatively low correlation coefficient between the Enterolert method and the USEPA Method 1600 and TMA individually results in a slightly lower correlation coefficient between TMA and the combined reference results as compared with the corresponding result between TMA and the USEPA Method 1600 alone.

The performance of the TMA should next be confirmed in an appropriate multi-centre study. However, the comparison of TMA with existing culture-based methods is not an ideal way of establishing the effectiveness of the assay since the established methods are not without their problems and any differences seen may be a facet of either method (as seen in the differences between the reference methods themselves). The ultimate aim must therefore be to establish the presence or absence of a link between the result of the TMA method and swimming related human ill-health in an appropriate epidemiology study.

Conclusion

The present study demonstrates the efficacy of TMA as a basis for the rapid detection and quantitation of *Enterococci* in recreational water. TMA is a high-gain, isothermal amplification system and is ideally suited to amplification of rRNA of which there are many thousands of copies in *Enterococci*, thus further increasing the sensitivity of detection. The rRNA extracted from the bacterium is isolated from potential inhibitors in the sample using specific target magnetic capture. The use of a molecular torch for amplicon detection further enhances the specificity of the method and minimises the risk of false positive results characteristic of culture-based methods.

Acknowledgements

The authors would like to extend their appreciation to the following for their valuable contribution to this study: Mariam Zhowandi, Reza Mahallati, Martin Getrich, Sharon Murathodzic, Jacqueline Marsh, Victoria Nixon and Ceri Morris.

This research was funded by a grant from Molecular Light Technology Research Limited, Cardiff, Wales, UK. All oligonucleotides and reagents were generously donated by Gen-Probe Incorporated, San Diego, CA 92121, USA.

References

1 A. B. Boehm, S. B. Grant, J. H. Kim, S. L. Mowbray, C. D. McGee and C. D. Clark, *Environ. Sci. Technol.*, 2002, **36**, 3885–3892.
2 A. Pruss, *Int. J. Epidemiol.*, 1998, **27**, 1–9.
3 *Bacteriological ambient quality criteria for marine and freshwater recreational waters*, U.S. Environmental Protection Agency, Springfield, VA, PB86-158-045, 1986.
4 California Department of Health Services, Regulation AB411, 1999.
5 V. J. Cabelli, A. P. Dufour, L. J. McCabe and M. A. Levin, *Am. J. Epidemiol.*, 1982, **115**, 606–616.
6 I. A. van Asperen, G. Medema, M. W. Borgdorff, M. J. Sprenger and A. H. Havelaar, *Int. J. Epidemiol.*, 1998, **27**, 309–315.
7 J. M. Colford, T. J. Wade, K. C. Schiff, C. Wright, J. F. Griffith, K. S. Sukhminder and S. B. Weisberg, *Southern California Coastal Water Research Project Technical Report 449*, Costa Mesa, CA, 2005.
8 M. H. Zhowandai, D. M. Ferguson, M. A. Getrich, D. F. Moore, and A. Lissner, Poster Q-487, *American Society for Microbiology General Meeting 2003*, Washington, DC, 2003.
9 J. F. Griffith, and S. B. Weisberg, *Southern California Coastal Water Research Project Technical Report 485*, Costa Mesa, CA, 2006.

10 D. M. Ferguson, D. F. Moore, M. A. Getrich and M. H. Zhowandai, *J. Appl. Microbiol.*, 2005, **99**, 598–608.
11 R. A. Haughland, S. C. Siefring, L. J. Wymer, K. P. Brenner and A. P. Dufour, *Water Res.*, 2005, **39**, 559–568.
12 M. Chernesky, D. Jang, K. Luinstra, S. Chong, M. Smieja, W. Cai, B. Hayhoe, E. Portillo, C. MacRitchie, C. Main and R. Ewart, *J. Clin. Microbiol.*, 2006, **44**, 400–405.
13 *Method 1600: Enterococci in Water by Membrane Filtration Using Membrane-Enterococcus Indoxyl-D-Glucoside Agar (mEI)*, EPA-821-R-02-022, Office of Water, U.S. Environmental Protection Agency, Washington, DC, 2002.
14 U.S. Pat. Appl., 20070264658, 2007.
15 R. Morgan, C. Morris, K. Livzey, J. Hogan, N. Buttigieg, R. Pollner, D. Kacian and I. Weeks, *J. Environ. Monit.*, 2007, **9**, 424–426.

