Rapid tests for detection and quantitation of *Enterococcus* contamination in recreational waters†

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Presently, growth-based tests are used for the detection and quantitation of microbiological contaminants in the environment. These tests take a minimum of 24 h to generate a result, which compromises the ability to take the most appropriate action. This report describes a rapid test for *Enterococcus* in recreational water as an indicator of faecal contamination. This method involves (1) isolation and lysis of the target organism, (2) purification of ribosomal RNA (rRNA) from the lysate and (3) amplification and detection of the purified rRNA. rRNA is used as the target since, in contrast to DNA, there are hundreds to thousands of copies in the cell. The rRNA is purified from the lysate by target capture onto magnetic microspheres, which removes interfering substances present in the sample. The rRNA is then quantitated using transcription-mediated amplification (TMA) with real-time homogeneous detection of amplicon using a fluorescent oligonucleotide probe. Compared to polymerase chain reaction (PCR) amplification, TMA is isothermal, more rapid, and ideally suited to RNA detection. The test described here demonstrates sensitive detection and quantitation of enterococci over a wide dynamic range with a high level of analytical specificity. The latter is particularly important for accurate and relevant monitoring both for protecting public health and for source tracking. Many conventional microbiological tests are time-consuming, exhibit limited dynamic range and are known to lack specificity. This assay demonstrates the advantages achievable by the application of TMA of rRNA targets to current environmental testing challenges.

Introduction

Routinely used methods for detecting microbiological pollution of the environment rely on growth-based protocols which require at least 24 h to yield results. Accordingly, acute pollution events are always determined retrospectively,[1] which delays detection, action and remediation where required. The monitoring of recreational water for microbiological pollution events is a particularly important example of this, where contamination potentially impacts human health[2] and has potential economic consequences.

Enterococci are frequently recommended and used as indicator organisms for human faecal contamination[3,4] and standards have been established for the maximum acceptable level of enterococci in seawater samples (*e.g.* the California state threshold for a single sample is 104 colony-forming units (CFU) per 100 ml of water).[5] Whilst swimming in water contaminated with faecal material and having elevated enterococci levels has been associated with gastroenteritis,[6] there have also been studies reported in which increased levels of enterococci have not correlated with increased incidence of illness.[7] This contradiction may be due to the fact that existing culture-based methods for the detection and quantitation of enterococci are not specific for this genus. In addition, not all species of enterococci are associated with human faecal material.[8] Moreover, it has been suggested that many faecal indicator bacteria, including enterococci, are capable of significant longevity or even re-growth in certain environmental situations, which confounds their accuracy as indicators of acute pollution events.[9]

It is proposed that a more specific and rapid test for enterococci may be advantageous to its use as an indicator of faecal contamination. Recent advances in molecular biology make it possible to develop test methods of exquisite specificity and sensitivity. Whilst methods based on the polymerase chain reaction (PCR) are probably most familiar, it is a fact that methods based on transcription-mediated amplification (TMA) have been employed with great success for routine detection of microorganisms in the clinical arena.[10] TMA is extremely rapid and, unlike PCR, does not require thermal cycling as it is an isothermal process. Moreover, TMA is ideally suited to the amplification and detection of ribosomal RNA (rRNA) which, unlike DNA conventionally targeted by PCR, exists at copy numbers at least a thousand-fold greater than those of DNA, hence making possible even greater sensitivity of detection. Here we report a rapid, TMA-based method for the quantitation of enterococci in recreational water. The method has been designed not to detect all groups

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of enterococci since many species, in particular those which comprise Group I, are not found extensively in human faeces. Further, the inherent specificity of TMA and rRNA targeting makes it possible to develop even more specific tests if required.

Methods

Seawater samples (100 ml) were collected and filtered as prescribed by USEPA method 1600.[11] This standard method (based on culture on selective mEI agar) was also used to enumerate enterococci when required as a reference. Reference enterococcal cultures were prepared using standard methods with a known strain of *Enterococcus faecalis*. All source organisms for quantitation and cross-reactivity studies were obtained from American Type Culture Collection (ATCC). Standard suspensions of bacteria for filtration were prepared in sterile saline. Purified rRNA from *E. faecalis* was obtained from Gen–Probe Incorporated, San Diego, USA. Oligonucleotides, target capture reagents and amplification reagents were obtained from Gen–Probe. Negative control seawater was produced by autoclaving at 121 °C for 15 min followed by cooling to room temperature.

Following filtration of the sample, the filter was washed through with polyvinylpyrrolidone solution (50 ml, 3% w/v) and the disc transferred to a shallow screw-top container slightly wider in diameter than the filter. The gram-positive enterococci were lysed using sequential treatment with lytic enzymes and detergent. The lysate containing the released rRNA was transferred to a 4 ml polystyrene test tube and hybridised with a capture oligonucleotide consisting of a sequence of oligo dA nucleotides attached to a sequence complementary to part of the *Enterococcus* rRNA. Immobilisation of the resulting hybrid was facilitated by incubation with a suspension of magnetic microspheres coated with an oligo dT nucleotide sequence to form an oligo dA · dT hybrid. The magnetic particles were then washed using target capture wash solution on a magnetic rack-based target capture system (Gen–Probe) and the enterococcal rRNA eluted using sterile, RNAse-free water (60 μl).

A TMA method[12] with molecular torch detection[13] was set up as described by Becker *et al*. Oligonucleotides used in the test method had been designed not to hybridise with rRNA from Group I enterococci. Briefly, amplification reagent containing the oligonucleotides required for amplification was pipetted into the requisite number of wells of a 96-well amplification plate. For each original seawater sample, 2 μl of the eluate prepared above was also pipetted into a well. The plate was incubated at 60 °C for 10 min and then cooled to 42 °C. Amplification enzyme reagent was then added and the plate transferred to a variable temperature plate-reading fluorimeter (Chromo4, Bio–Rad Laboratories Inc.) set at 42 °C. Each well was measured sequentially for 30 s until 70 measurements had been taken for each well. Each well was assessed for the time taken for the fluorescent signal to reach a pre-determined intensity, since this time (the emergence time, $C(T)$) is inversely proportional to the original concentration of rRNA (and hence the number of enterococci) present in the original sample. Quantitation was achieved by contemporaneously measuring the assay response to known concentrations of purified rRNA in the amplification reaction and constructing a calibration curve. This in turn was related to the number of enterococci present in the sample from the knowledge of the amount of rRNA present in enterococcal cells. The time taken to perform the assay on 40 filtered samples was about 4 h.

The assay was characterised in terms of its analytical sensitivity, dynamic range, analytical specificity and reproducibility.

Results and discussion

Fig. 1 shows an example of kinetic plots obtained with the assay and demonstrates how the emergence time is related to the concentration of *Enterococcus* rRNA in the sample.

Regulations exist which specify the maximum allowable level of *Enterococcus*, beyond which exposure is likely to lead to illness. For example, in California the limits are 104 CFU per 100 ml for a single sample and 35 CFU per 100 ml for the monthly average of samples. The TMA exhibited a limit of detection of at least 10 CFU per 100 ml. This was verified by assaying suspensions of bacteria at this level relative to a 0 CFU control. Fig. 2 shows that 10 CFU per 100 ml is distinguishable from zero in every assay performed.

For monitoring recreational water quality, quantitation of *Enterococcus* up to 10 000 CFU per 100 ml is adequate. Fig. 3 shows the assay response to rRNA concentrations over a range of four decades and demonstrates that the method is effective at quantitation up to this level, though inherently TMA is capable of exhibiting a dynamic range of at least six orders of magnitude.

The specificity of the method was studied by assaying suspensions of various enterococcal and non-enterococcal organisms at a level of 10^6 CFU per 100 ml. Table 1 shows the response of the assay and demonstrates its lack of reactivity with Group I enterococci and selected non-enterococcal species, which are largely irrelevant to human faecal contamination.

To study the reproducibility of this method, known numbers of enterococci were spiked into negative control seawater

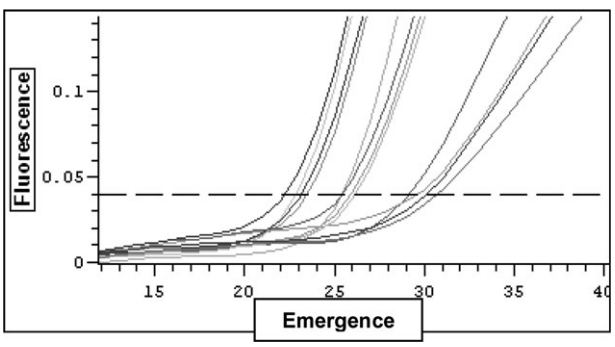

Fig. 1 Typical emergence curves for the TMA reaction in response to three distinct levels (ten-fold differences) in rRNA concentration (each level $n = 4$). Units of emergence are (min × 2), units of fluorescence are arbitary relative fluorescence units. The emergence is inversely proportional to the rRNA concentration.

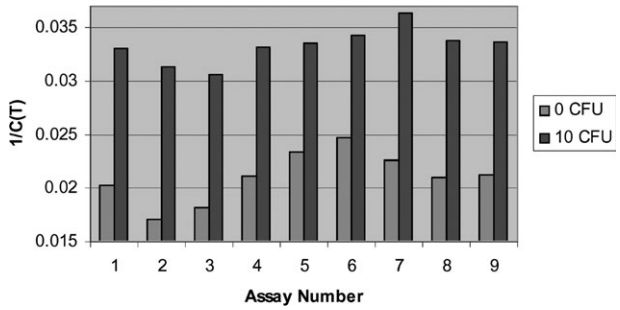

Fig. 2 Assay response, expressed as the reciprocal of the emergence time, $1/C(T)$, for 0 CFU and 10 CFU of *E. faecalis* filtered from 100 ml saline. Each determination represents a single filter measurement. Data were obtained from three separate assays (three filters per assay). Note that the reciprocal response has been used here to transform the inherent inverse relationship between rRNA target concentration and emergence to a direct relationship.

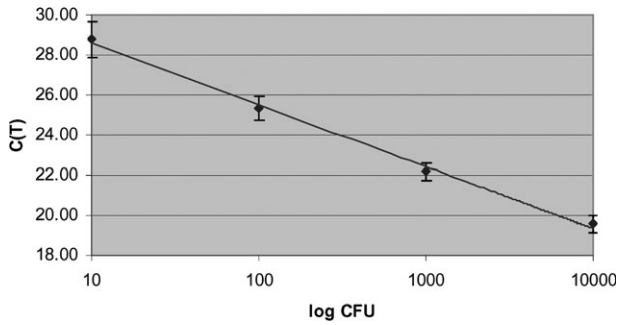

Fig. 3 Assay response, expressed as emergence time, $C(T)$, to rRNA from *E. faecalis* (CFU equivalents). Data points are derived from the inter-assay mean and standard deviation of six determinations run over three separate assays.

and measured in replicate over multiple assays. The reproducibility is shown in Fig. 4.

Contamination of recreational water with human sewage has implications both for human health and local economies. Although culture-based microbiological tests for such contamination are widely used, they suffer from difficulties due to long time to result and lack of specificity. These methods recognise many species of enterococci and several non-enterococcal species not relevant to human faecal contamination. It is possible that these limitations contribute to the lack of correlation between levels of enterococci and human illness reported in some epidemiological studies. A rapid, sensitive and specific test is therefore required in order to establish the safety status of beaches in a timely manner. The method described here is rapid, sensitive and has been designed to target rRNA from a limited range of enterococci. The test does not recognise the irrelevant Group I enterococci or selected non-enterococcal bacteria studied here, and so is inherently more specific than culture methods.

Table 1 Cross-reactivity of the *Enterococcus* TMA. Comparison of emergence times, $C(T)$, for 10^6 CFU. $C(T)$ values of >30 correspond to <10 CFU *E. faecalis*

Enterococcal		Non-enterococcal	
Species	$C(T)$	Species	$C(T)$
Non-group I			
E. faecalis	10.42	*Listeria monocytogenes*	>70
E. gallinarum	10.02	*Carnobacterium pisciola*	>70
E. faecium	9.81	*Lactobacillus caseii*	>70
E. casseliflavis	10.36	*Aerococcus viridans*	>70
E. mundtii	9.25	*Streptococcus bovis*	>70
E. durans	10.39	*Streptococcus equi*	>70
E. hirae	10.09		
E. columbae	13.50		
Group I			
E. avium	>70		
E. pseudoavium	>70		
E. malodoratus	>70		
E. raffinosus	>70		
E. saccharolyticus	>70		

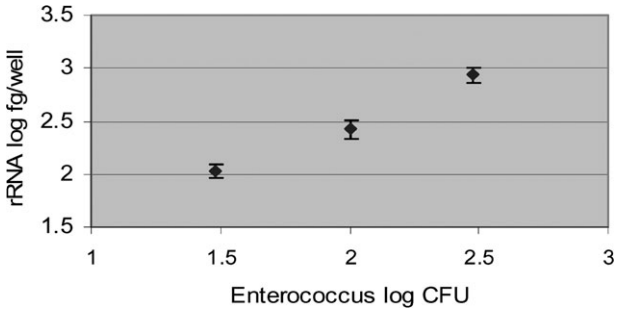

Fig. 4 Discrimination over the range of interest associated with the Southern California permitted level of 104 CFU (per 100 ml sample). $n = 6$, mean $\pm$ sd.

Conclusion

The data presented here show that TMA offers a convenient and powerful basis for the development of rapid tests for microbiological contamination of the environment.

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