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Real-time PCR detection of male-specific coliphages

SFY YONG *PhD*, YF NGEOW* *FRCPath*, YK TONG *BSc*, and JT ONG *BSc*

*School of Arts and Sciences, Monash University Malaysia and *National Public Health Laboratory, Selangor*

Abstract

Male-specific coliphages are often used as indicators of contamination by enteric viruses. These phages can be detected in water samples by plaque assays and by polymerase chain reaction. In this study, the M13 coliphage was used to develop a real-time PCR assay for the detection of male-specific DNA coliphages. The real-time PCR was found to have a reaction efficiency of 1.45 and detection limit of 10^{-3} plaque forming units per reaction mix. Repeated amplification and melting curve analyses demonstrated high specificity and reproducibility of the real-time assay. Quantitative detection with the real-time PCR should allow rapid assessment of the level of viral contamination in water.

Key words: Male-specific coliphage, M13, real-time PCR

INTRODUCTION

Polymerase chain reaction (PCR) is an advanced molecular technique in clinical diagnostic and environmental microbiology that allows multiple copies of a DNA molecule to be generated by *in vitro* enzymatic amplification of a target DNA sequence.[1] This highly effective amplification enables the detection of very low DNA copy numbers in a sample and is particularly useful for the detection of difficult-to-culture or non-culturable organisms.

Following the success of traditional PCR, real-time PCR technology was developed to allow real-time monitoring of the amplification process and quantification of PCR products in each cycle. Oligonucleotide-specific labeled probes or fluorescent molecules like SYBR-Green 1[2] are added into the reaction mixture to detect and quantify the amplified product. Computer software monitors the amount of fluorescence produced at every cycle to generate an amplification plot for each reaction.[3] Compared to traditional PCR, real-time PCR is said to be more sensitive, reproducible and rapid. A further advantage is that, with the real-time PCR, there is no necessity for post-PCR processing such as the use of gel electrophoresis.

In this paper, we describe the development of a real-time PCR assay for the detection of male-specific DNA (FDNA) coliphages, and compare its sensitivity with that of a traditional PCR. Male-specific coliphages are regularly found in sewage contaminated water and are being studied for their potential use as an indication of fecal pollution. A quantitative measurement as that obtained by real-time PCR would enable the assessment of the level of viral contamination in water.

MATERIALS AND METHODS

M13 coliphage

M13, the reference strain of male-specific DNA coliphage used in this study, was kindly provided by Dr. F.C. Hsu, Scientific Methods, Inc., USA. This strain was kept in a 15% glycerol suspension from which 10-fold dilutions were made for DNA amplification to construct the standard curves for the traditional and real-time PCRs

Extraction of genomic nucleic acid from M13 coliphage

Fifty µL of lysis solution containing 0.5 % Nonidet NP-40 (v/v) (Promega, USA), 0.5 % Tween 20 (v/v) (Promega) and 0.1 mg Proteinase K (QIAGEN Inc., USA) per mL was added to 200 µL of M13 coliphage suspension and the mixture was incubated at 60 °C for one hour followed by boiling at 100 °C for 5 minutes to lyse phage particles and release genomic DNA. The nucleic acid suspension was then used directly for PCR.

Address for correspondence and reprint requests: Stacey Yong Foong Yee, School of Arts and Sciences, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, Petaling Jaya 46150, Selangor, Malaysia. E. mail: stacey.yong@artsci.monash.edu.my

Traditional polymerase chain reaction
Primers were designed to amplify a 276 bp conserved region in FDNA coliphages. To do this, the sequence of M13 (Gene Bank: V00604) was aligned with the gene sequences of other FDNA phages like fd and f1.The sequence of the forward primer E is 5'-CTC CAG ACT CTC AGG CAA-3' and that for the reverse primer F is 5'-GCC TCA GAG CAT AAA GCT A-3'.

PCR analysis was carried out using 25 µL volumes of optimized PCR reaction mixture, each containing the following: 2.5 µL of PCR reaction buffer (Promega), 1.5 mM $MgCl_2$, 200 µM dNTP Mix (10 mM of each dNTP), 0.08 µM of each upstream and downstream primer, 20 ng of nucleic acid extract, 1 U of *Taq* polymerase (Promega) and nuclease free water to make up to 25 µL. Amplification was carried out in a PTC-100 programmable thermal cycler. The PCR was performed with one cycle of denaturation at 94 °C for 4 minutes followed by 35 cycles of DNA denaturation at 94 °C for 1 minute, annealing at 61 °C for 1 minute, and extension at 72 °C for 1 minute. This was followed by a final cycle at 72 °C for 10 minutes to finish extending the amplifying DNA sequence. After amplification, PCR products were electrophoresed on a 2.5 % agarose gel (Promega) at 70 V for 40 minutes.

Real-Time PCR
The forward and reverse primers coliDNA-F23 and coliDNA-R214 were designed for FDNA using IDT BioTools OligoAnalyzer 3.0 and Primer Designer 2.01 Their sequences are: 5'-AGC CTT TGT AGA CCT CTC A-3' (coliDNA-F23) and 5'-AGC CTT TAT TTC AAC GCA AG-3' (coliDNA-R214).

A 25 µL volume of optimized PCR reaction mixture was prepared as follows: 12.5 µL of 2x Quantitect SYBR Green PCR master mix containing 2.5 mM $MgCl_2$ (Qiagen), 0.28 µM of each upstream and downstream primer specific for M13, 5 µL of nucleic acid extract, and nuclease-free water to make up to 25 µL. Real-time PCR was carried out with the DNA Engine Opticon I (MJ Instruments, USA). Thermal cycling was performed as follows: hot start at 95ºC for 15 minutes, followed by 38 cycles of denaturation at 95ºC for 1 minute, annealing at 60ºC for 1 minute, and extension at 72ºC for 1 minute. Real-time PCR monitoring was achieved by measuring the fluorescent signal at the end of the extension phase for each cycle. After amplification was complete, a melting curve was generated at 60ºC for 1 second, followed by heating slowly at 0.2ºC/s to 98ºC with continuous collection of fluorescent signals from the reaction mixture. The melting curve and quantitative analysis of data were performed using the built-in software of the real-time PCR instrument. The efficiency of a PCR run was calculated using the formula: $E = 10^{(-1/slope)}-1$.

Enumeration of M13 coliphage
M13 plaque counts were enumerated with the USEPA (United States Environmental Protection Agency) method 1601. A 10-fold serial dilution of M13 coliphage suspension was made in sterile distilled water. One hundred µL from each of the diluted phage suspensions was added into 500 µL of an early-log phase culture of *E. coli* HS(Famp)R (ATCC#700891). The mixture was then transferred into 5 mL of semi-solid tryptic soy agar (TSA) and mixed well before pouring onto a bottom layer of TSA. After the semi-solid agar overlay solidified, the plates were inverted and incubated at 37ºC overnight. Plaques were scored after overnight incubation and recorded as plaque forming units (pfu)/mL.

RESULTS AND DISCUSSION

Ten-fold serial dilutions of the M13 coliphage were made to give 10^6 to 10^{-5} plaque forming units (pfu) per reaction mixture. The limit of detection for the traditional PCR was 10^{-3} pfu (Figure 1) while that for the real-time PCR was 10^{-2} pfu (Figure 2). Hence, the real-time PCR was less sensitive than the traditional assay. This reduced sensitivity could be due to the fact that at low quantities of nucleic acid template, the emission of fluorescence signals is inconsistent and does not allow the real-time PCR to perform quantitative analysis.

With 4 independent real-time PCR assays performed on the same series of M13 dilutions (Figure 2), the standard deviation between runs was found to be less than 0.5 (data not shown), indicating reproducible results. As the plaque count increased, the C(t) value that is defined as the number of cycles to reach the exponential phase of PCR process, decreased correspondingly, giving reaction efficiency and correlation coefficient values of 1.52 and 0.89 respectively (Figure 3).

Specificity for M13 coliphages was demonstrated in the melting curve analysis of PCR products showing the consistency of T_m value at 77.8 °C (Figure 4). The presence of non-specific PCR products is usually indicated

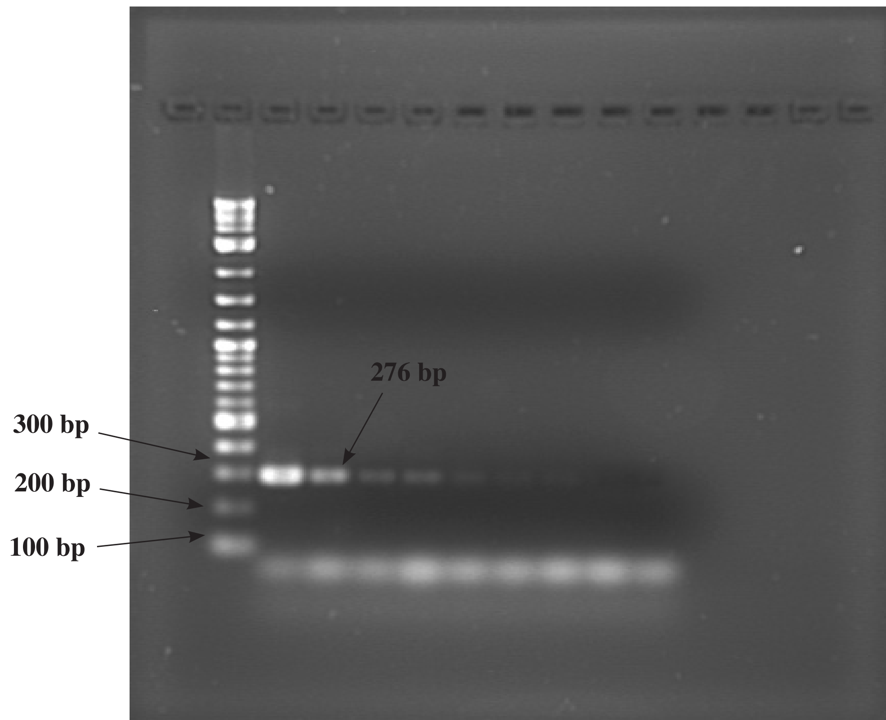

FIG. 1: Gel electrophoresis of PCR products (276bp) in traditional PCR assay for M13 FDNA coliphage. Lane 1: 2 Log DNA ladder; 2: 1.6 x 10^{2} pfu; 3: 1.6 x 10^{1} pfu; 4: 1.6 x 10^{0} pfu; 5: 1.6 x 10^{-1} pfu; 6: 1.6 x 10^{-2} pfu; 7: 1.6 x 10^{-3} pfu; 8: 1.6 x 10^{-4} pfu; 9: 1.6 x 10^{-5} pfu; 10: negative control without DNA samples.

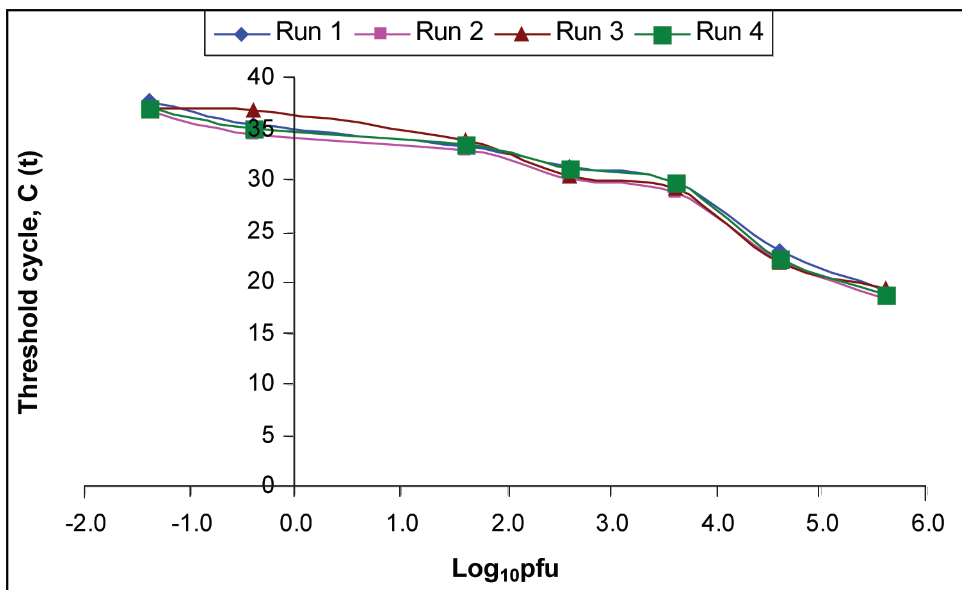

FIG. 2: The detection ranges of the four independent real-time PCR assays for 10-fold serial dilutions of M13 coliphage and indication of the threshold cycle C(t).

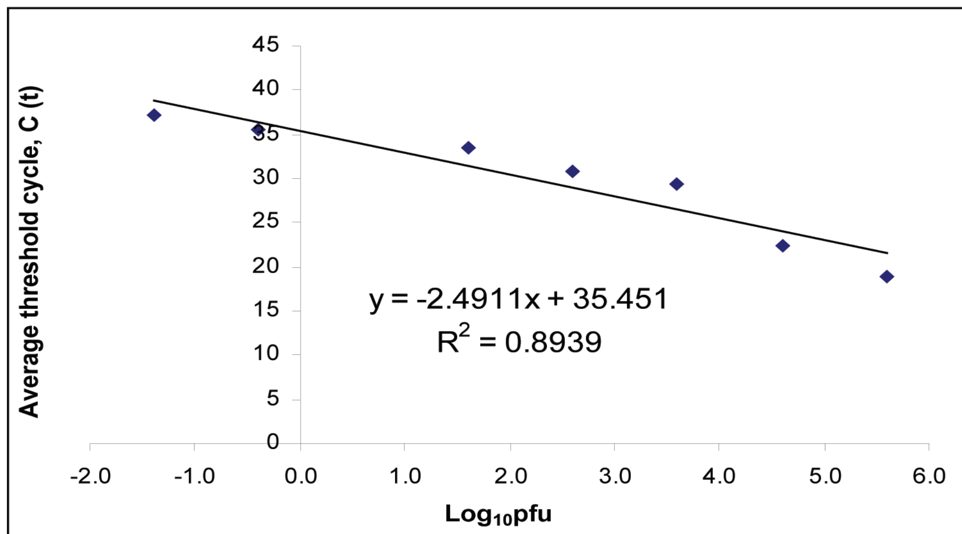

FIG. 3: Standard calibration curve for real-time PCR detection of 10-fold dilutions of M13 coliphage using SYBR Green 1.

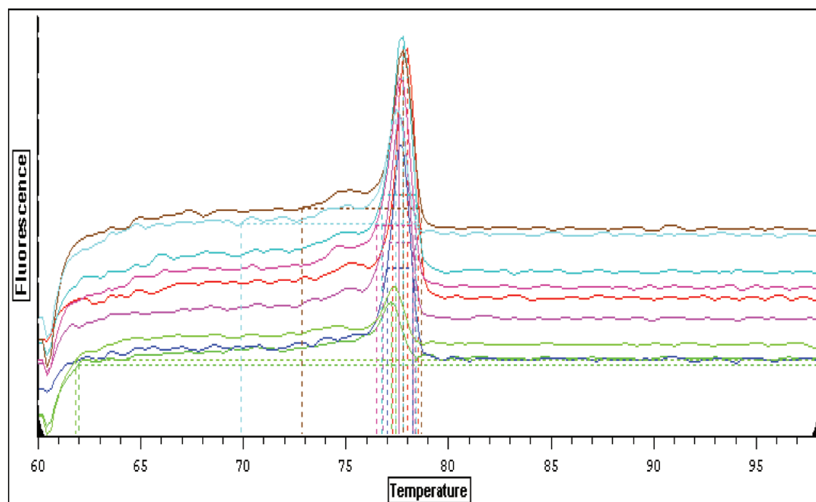

FIG. 4: Melting curve analysis on amplicons generated in the real-time PCR detection of M13 coliphage, showing the T_m value of the amplicons at 77.8 ºC.

by the appearance of different or broader peaks in the melt curve.[3]

In summary, the real-time PCR assay for the detection of M13 coliphage appeared to be reliable, and suitable for further development into a method for high-throughput quantitative detection of FDNA coliphages in the assessment of water for fecal contamination.

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REFERENCES

1. Brown TA. Gene Cloning: An Introduction. 3rd ed. Stanley Thornes Publishers Ltd. 1998, p. 233-236
2. Litaker RW, Tester PA. Molecular Methods for Detecting and Characterizing Harmful Phytoplankton. In: Hurst CJ, Crawford RL, Knudsen GR, McInerney MJ, Stetzenback LD editors. Manual of Environmental Microbiology. 2nd ed. American Society for Microbiology. 2002. p. 342 – 6
3. Nolte FS. Quantitative Molecular Techniques. In: Specter S, Hodinka RL, Young SA, editors. Clinical Virology Manual. 3rd ed. American Society for Microbiology; 2000. p. 198 - 200