Enumeration of viable *Escherichia coli* by real-time PCR with propidium monoazide

N. Yokomachi and J. Yaguchi

**ABSTRACT**

A photo-inducible DNA-binding dye, propidium monoazide (PMA), was used to distinguish viable and dead *Escherichia coli* cells. Microscopic observations using a combination of the dyes 4',6-diamidino-2-phenylindole and PMA indicated that PMA stained only dead cells, with membrane damage, red. Mixtures of viable and heat-treated *E. coli* cells were subjected to real-time polymerase chain reaction (PCR) with PMA treatment. Viable cell counts were linearly related to real-time PCR threshold cycle values for PMA-treated cells in the mixtures of viable and heat-treated cells, as long as the ratio of dead cells to viable cells was no greater than 10. In the wastewater treatment plants, total, viable and culturable *E. coli* were enumerated by real-time PCR, real-time PCR coupled with PMA treatment and the most probable number method using EC-MUG medium, respectively. The concentrations of viable *E. coli* in the wastewater treatment plants were much higher than those of culturable cells. In addition, viable cells were even more chlorine resistant than culturable ones.

**Key words** | DNA, *Escherichia coli*, propidium monoazide, real-time PCR, viable but non-culturable

**INTRODUCTION**

Enumeration of viable or active pathogens in aquatic environments has relied on fecal indicator bacteria culture methods using selective media. However, many researchers have reported that various bacteria including some pathogenic species, such as *Salmonella*, *Shigella*, *Vibrio* spp. and *Escherichia coli*, could enter a viable but non-culturable (VBNC) state when subjected to various environmental stresses and starvation (Colwell et al. 1985; Roszak & Colwell 1987; Byrd et al. 1991). In addition, retention of virulence in the VBNC state has been reported frequently (Rahman et al. 1996; Kahla-Nakbi et al. 2007). Consequently, conventional enumeration may underestimate the total number of viable or active fecal indicator bacteria in water supplies and wastewater treatment systems. We previously applied direct viable count methods and fluorescent staining using BacLight™ kit and CTC reagent to determine the total number of viable bacteria in the water environment (Sawaya et al. 2008). Although a great number of bacteria were found to remain in the VBNC state, these methods could not distinguish between specific pathogens or fecal indicator bacteria and other bacteria. Other currently applied enumeration methods are based on polymerase chain reaction (PCR) amplification of DNA such as real-time PCR (RT-PCR). PCR-based methods have been developed widely and intensively because of their high sensitivity and specificity. Due to the relatively long persistence of DNA after cell death, however, these methods tend to overestimate the number of viable bacteria (Josephson et al. 1993; Sheridan et al. 1999). Moreover, mRNA detection using real-time PCR has been developed as a viability test for VBNC bacteria (Del Mar Lleo et al. 2000; Coutard et al. 2005). In the past few years, ethidium monoazide (EMA) and propidium monoazide (PMA) have been proposed to distinguish between viable and dead bacterial cells as useful alternatives to conventional culture and PCR methods (Nogva et al. 2003; Rudi et al. 2005; Nocker et al. 2006; Inoue et al. 2008). These intercalating DNA-binding chemicals penetrate only dead cells with membrane damage and then irreversibly cross-link to the nucleic acids following photoactivation, thereby preventing PCR amplification of DNA derived from dead cells. However, EMA has been found to penetrate into a part of viable cells of some bacteria (Nocker et al. 2006; Flekna et al. 2007).

The objectives of this research were to develop a method to estimate the total number of viable *E. coli* including the VBNC state cells and determine the real proportion of viable *E. coli* in wastewater treatment plants.
MATERIALS AND METHODS

Bacterial strain and culture condition

E. coli JCM1649T obtained from the RIKEN BioSource Center (Saitama, Japan) was used in this study. An overnight culture of E. coli in LB broth was repeatedly grown to log phase (ca. 10^8 colony forming units (CFU)/ml) for a few hours at 37°C with shaking at 120 rpm. One millilitre aliquots of the culture were vacuum-filtered onto black polycarbonate membranes (Advantec; pore size 0.20 μm) and stained with 1 ml of 4',6-diamidino-2-phenylindole (DAPI; Wako Pure Chemical Industries Ltd, Osaka, Japan) for determination of the concentration of E. coli cells by epifluorescence microscopic counting. Plate counting on LB agar medium was also used to determine the number of E. coli (CFU). The CFU concentration was confirmed to be equivalent to the direct counts microscopically determined after staining with DAPI. Heat-killed E. coli cells were prepared by exposure at 80°C for 10 min. Loss of viability was examined by culturing cell suspensions on LB agar medium at 20°C for a week.

Wastewater samples

Wastewater samples were collected from the college wastewater treatment facility at Hachinohe, Aomori, and the sewage treatment plant in the southeast of Aomori Prefecture. Raw wastewater, primary, secondary and chlorinated effluents were collected to measure the concentration of E. coli cells from August to December in 2010. Table 1 shows the characteristics of two wastewater treatment plants during the period of sampling.

<table>
<thead>
<tr>
<th>Treatment type</th>
<th>College wastewater treatment plant</th>
<th>Sewage treatment plant</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Expanded aeration</td>
<td>Activated sludge</td>
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<td>Flow rate (m³/day)</td>
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<td>50,000</td>
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<tr>
<td>Aeration tank volume (m³)</td>
<td>330</td>
<td>19,384</td>
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<td>Water quality (mg/l)</td>
<td>Biochemical oxygen demand</td>
<td>Total suspended solids</td>
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<td>Influent</td>
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<tr>
<td>Primary effluent</td>
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<td>128–175</td>
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<tr>
<td>Secondary effluent</td>
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<td>0–6</td>
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<tr>
<td>Final effluent</td>
<td>4.5–5.1</td>
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</table>

DNA extraction and real-time PCR

Five hundred microlitre aliquots of E. coli culture, with or without PMA treatment, were harvested by centrifugation at 10,000 × g for 5 min. To the cell pellets in 500 μl tubes, PMA treatment

PMA (Biotium, Inc., CA, USA) was dissolved in 20% dimethyl sulfoxide to obtain 50 mM stock solutions. PMA stock solution was added to water samples in light transparent 500 μl microcentrifuge tubes to give a final concentration of 50 μM. The sample tubes were incubated for 5 min in the dark at room temperature and then exposed to a 500 W halogen lamp (Arcland Sakamoto, Niigata, Japan; GTHT500S) for 5 min at a distance of approximately 10 cm. During exposure, the tubes were set into chipped ice to avoid excessive heating. The concentration and the incubation time of PMA used in this study have been reported to be optimal for enumeration of viable cells (Nocker et al. 2006; Pan & Breidt 2007).

PMA staining

Intact viable and heat-treated E. coli cultures grown in LB broth were stained with a combination of PMA and DAPI dyes for direct visualization by fluorescence microscopy. Dilutions of the cultures were treated with PMA at a final concentration of 50 μM for 5 min and 1 ml aliquots were filtered onto black polycarbonate membranes, followed by staining with 1 ml of DAPI (10 mg/l) for 5 min. A BX41 epi-fluorescence microscope (Olympus, Tokyo, Japan) equipped with a mercury burner was used for observation. DAPI-stained cells were observed with a U-MWU2 excitation filter, and PMA-stained cells were viewed with a U-MNIB2 excitation filter.
180 μl of Instagene Matrix (Bio-Rad Laboratories, Tokyo, Japan) were added, followed by heating at 56°C for 30 min. After vortexing, the tubes were heated at 100°C for 8 min and centrifuged. The supernatants were used in real-time PCR reactions as DNA templates.

Cell lysis of wastewater samples was achieved by bead beating using a mini-bead beater (Ieda Trading Co., Tokyo, Japan; Model3110BX) at 4,800 rpm for 30 s. Genomic DNA was extracted from the college wastewater treatment facility and the sewage treatment plant using the FastDNA® SPIN Kit for soil (MP Biomedicals, CA, USA) and DNA Isolation Kit ISOIL for Beads Beating (Nippon Gene, Tokyo, Japan), respectively. The volume of sample used for each extraction was 500 μl for influent and primary effluents, and 50–100 ml for secondary and chlorinated effluents. DNA extraction was performed according to the manufacturer’s instructions.

Real-time PCR was performed in 48-well optical plates using the Mini Opticon System (Bio-Rad). Each reaction was run in triplicate for quality assurance and statistical analysis. The E. coli uidA gene sequence, which encoded for the β-D-glucuronidase enzyme, was amplified using the primers and probe developed by Frahm & Obst (2003). The presence of this enzyme has been used for detection of E. coli in culture-based methods (Bej et al. 1991). The reaction mixtures (25 μl) consisted of the primers (500 nM), the probe (200 nM), 2.0 μl of DNA template and 12.5 μl of iQ Supermix (Bio-Rad). PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 45 s, 60°C for 1 min. Each plate included no template controls with 2.0 μl of PCR-grade water instead of DNA template. Threshold cycle (Ct) values were automatically generated by the MyiQ (Bio-Rad) software.

Detection of E. coli in wastewater sample

Concentrations of culturable E. coli and fecal coliform indicator bacteria were determined by the most probable number (MPN) method using EC-MUG medium (Difco) and the membrane filter method on m-FC medium (Difco), respectively (JSWA 1997; Becton, Dickinson and Company 2009). The MPN method with EC-MUG medium could evaluate the presence of β-D-glucuronidase, an enzyme possessed by E. coli. Both media were incubated for 24 ± 2 h at 44.5 ± 0.2°C. Total and viable E. coli were enumerated in triplicate by real-time PCR and real-time PCR coupled with PMA treatment, respectively. For real-time PCR of E. coli, secondary and chlorinated effluents were concentrated 100–200 times by centrifugation (Tomy Seiko Co., Tokyo, Japan; CAX-370). PCR inhibition by the wastewater samples was not tested.

RESULTS AND DISCUSSION

Application of real-time PCR to the detection of E. coli

A real-time PCR method was applied to the detection of E. coli based on the uidA gene sequence. The standard curve for the real-time PCR assay is shown in Figure 1. A linear relationship was observed between the log cell concentration and the C_T values, ranging from 1 × 10^1 to 1 × 10^6 CFU/tube. It could be shown that the detection limit of the assay was around 1 × 10^1 CFU/tube. The correlation coefficient, R^2, of the standard curve was 0.993, and the PCR amplification efficiency calculated from the slope −3.15 was 107%. The standard curve should be theoretically characterized by a slope of −3.33 and an efficiency of 100%. The efficiency of this study is closer to 100% than that obtained from the previous study of Frahm & Obst (2003).

PMA staining

A DNA-binding dye, PMA, was able to distinguish between viable and dead cells, as shown by microscopic observation using a combination of the dyes DAPI and PMA. DAPI is generally used to stain all cells blue, both with intact and compromised cell membranes, whereas PMA stains them red only if the dye can penetrate the cell membranes. Figure 2 shows microphotographs of heat-treated E. coli cells stained with a combination of DAPI and PMA. DAPI-stained cells were observed with a UV-light excitation filter (Figure 2(a)), and PMA-stained cells were

Figure 1 | Standard curve of real-time PCR for the detection of E. coli.
observed with a blue light excitation filter (Figure 2(b)). Heat-treated cells were efficiently stained both blue with DAPI and red with PMA. Figure 3 shows microphotographs of mixtures containing viable and heat-treated *E. coli* cells in an equivalent cell concentration treated with a combination of DAPI and PMA. Although DAPI stained all cells blue (Figure 3(a)), PMA stained just a half of cells red (Figure 3(b)). No red cell was observed when only viable cells were examined microscopically. The images obtained from viable cells treated with a combination of DAPI and PMA showed PMA did not penetrate viable cells. These microscopic observations confirmed that PMA stained only dead *E. coli* cells with compromised membranes red, not viable cells with intact membranes.

**Effect of PMA on real-time PCR**

To elucidate the effect of PMA on DNA amplification, aliquots of viable or heat-treated cell suspensions of *E. coli* were treated with PMA and then exposed to the halogen light source for 0–10 min followed by real-time PCR. Figure 4 illustrates the effect of light exposure time on inhibition of PCR amplifications of DNA derived from viable and heat-killed cells. The C_T values for heat-treated cells increased as the light exposure time was raised from 1 to 5 min, meaning that more cycles were necessary to detect a real-time PCR signal. This increase in C_T values indicated increasing inhibition of the PCR amplification of DNA from heat-killed cells after PMA treatment. However, there was no significant difference in C_T values for viable cells. Because the inhibition curve flattened off with exposure times of 5 min and longer, light exposure for 5 min was selected in this study.

Mixtures with defined ratios of viable and heat-treated *E. coli* cells as shown in Table 2 were subjected to PMA treatment followed by real-time PCR in replicate (Run 1 and Run 2). There was no sample containing equal concentrations of viable and heat-treated *E. coli* cells in the second run. The effect of PMA treatment on PCR amplification of the mixtures is shown in Figure 5. Increasing viable cell suspension volumes and decreasing heat-treated cells led to a decrease in C_T values from real-time PCR after PMA treatment. There were enough differences between sample 1 containing only viable *E. coli* cells and sample 6 containing only heat-killed cells to quantify viable cells with a combination of PMA and real-time PCR. These differences are as great as that of the PMA–PCR analysis using *E. coli* O157: H7 reported by Nocker et al. (2006) and are much greater than the result of the EMA–PCR assay of *Campylobacter jejuni* examined by Rudi et al. (2005).
Quantification of viable cells of *E. coli* in the presence of dead cells

To determine the relationship between viable *E. coli* cell counts and CT values, three replicate mixtures of viable cells and heat-treated cells (Figure 6; Table 3) were used. A linear relationship between the number of viable cells and mean CT value was observed in spite of the presence of dead cells (Figure 6). The correlation coefficients and the amplification efficiencies are summarized in Table 3. The addition of low concentrations of heat-treated *E. coli* cells resulted in the amplification efficiencies sufficiently close to the theoretically calculated value. In the case of Run 4 and 5, in which the higher concentrations of killed cells were added to viable ones, however, the efficiencies significantly departed from the theoretical value. If the points in parentheses in Figure 6, at which the ratio of dead cells to viable cells was greater than 10, were excluded, five standard curves closely overlapped and the efficiencies calculated from the remaining data were close to 100%. Therefore, as long as the ratio of dead cells to viable cells was no greater than 10, viable cell counts were linearly related to CT values for PMA-treated cells in the mixtures of viable and heat-treated cells. This ratio is less than the results (10^4) of the PMA–PCR analysis of *Listeria monocytogenes* reported by Pan & Breidt (2007). Figure 7 illustrates these remaining data from the PMA–PCR amplification in comparison with the data points from PCR amplification without PMA shown in Figure 2. Five linear relationship curves between the concentrations of viable *E. coli* and CT values, obtained from real-time PCR with PMA treatment of mixed viable-heat killed cells, almost overlapped with the standard curve obtained from real-time PCR.
without PMA treatment of viable cells with no dead cells. These results demonstrated that a real-time PCR coupled with PMA treatment could quantify only viable *E. coli* in the mixtures of viable and heat-treated cells. A new standard curve was calculated from all these data between real-time PCR with and without PMA treatment. The correlation coefficient, $R^2$, of the standard curve was 0.952, and the PCR amplification efficiency calculated from the slope $-3.19$ was 106%.

**Application of PMA–PCR in two wastewater treatment plants**

Real-time PCR with PMA treatment was used to determine the real proportion of viable *E. coli* in environmental waters. In the water samples from two wastewater treatment plants, total, viable and culturable *E. coli* were enumerated by real-time PCR, real-time PCR coupled with PMA treatment and the MPN method using EC-MUG medium, respectively. The detection limits of the *E. coli* quantification based on real-time PCR and MPN method in environmental waters were calculated to be 3 cells/ml and $3 \times 10^{-2}$ MPN/ml, respectively. Culturable concentration of fecal coliform indicator bacteria was also measured by the membrane filter method to the detection limit of $1 \times 10^{-2}$ cells/ml.

**College wastewater treatment plant**

Figure 8 shows the concentrations of *E. coli* and fecal coliform bacteria at the three stages in the college wastewater treatment plant. The measurements estimated by the four methods generally decreased through the successive treatment stages. The reductions in total and viable *E. coli* cells from the primary effluent to the secondary effluent were lower than that of culturable cells based on MPN method by one to two orders of magnitude. All total *E. coli* counts estimated by real-time PCR were higher than viable counts determined by PMA–PCR method in the college wastewater treatment plant, but the differences in counts were always less than 10-fold. Viable *E. coli* cells in the primary and secondary effluents were intermediate between total and culturable cells. These results indicated that several VBNC *E. coli* were present in the primary and secondary effluents. In the final effluents after chlorine disinfection, all the measurements were below the detection limit on 4 August 2010. No fecal coliform determined by membrane filtration method was detectable, even though 100 ml of the final effluent was filtered and incubated. On 21 December 2010, total and viable *E. coli* were detectable using PCR and PMA–PCR, respectively, while two indicators based on culture assay were below the detection limit. This implied that all viable *E. coli* cells remained in the VBNC state and they were not disinfected completely. The measurements of total and culturable counts are similar to the previous studies reported by He & Jiang (2005) in which chlorination...
inactivated *Enterococcus* cells but did not completely degrade the target DNA.

**Sewage treatment plant**

Figure 9 shows the measurements of *E. coli* and fecal coliform bacteria concentrations at the four stages in the sewage treatment plant. The numbers of *E. coli* and fecal coliforms determined by the four methods ranged from $10^4$ to $10^5$ cells/ml in the raw wastewater. The reductions of approximately two orders of magnitude occurred in these two indicators from the influent to the secondary effluent. All total *E. coli* counts based on real-time PCR were also <10 times greater than the viable counts estimated by PMA-PCR method in the sewage treatment plant. Viable *E. coli* counts were always intermediate between total and culturable counts in the raw wastewater and primary and secondary effluents, implying that most of the *E. coli* cells remained in the VBNC state. The effect of chlorine disinfection was significantly different between the sampling dates. In the final chlorinated effluent, the numbers of *E. coli* and fecal coliforms were below the detection limit on 15 November 2010. On the other two days, however, two indicators were detectable. Fecal coliforms and culturable *E. coli* concentrations declined below $10^6$ cells/ml, but total and viable *E. coli* concentrations remained from $10^2$ to $10^3$ cells/ml on 29 October and 8 December. Chlorine disinfection treatments were not as effective in reducing viable *E. coli* containing VBNC cells as they were in reducing culturable cells. The results showing lower reductions in total counts based on real-time PCR than those in culturable ones are consistent with the previous studies on *Enterococcus* reported by He & Jiang (2005). Contrary to the studies on *Enterococcus* and Bacteroides (Varma et al. 2009), however, an obvious trend toward greater reductions in viable counts estimated by PMA-PCR than in total counts was not observed.

The differences in chlorine effect between the sampling dates observed in two treatment plants are most likely due to plant operations. On 4 August 2010, chlorine contact time in the disinfection tank of the college wastewater treatment facility was probably kept long enough to diminish the target DNA below the detection limit because of low loading during the summer vacation (Table 1). As the inactivation rate coefficients of viable *E. coli* were reported to be a half to one-third those of culturable cells (Sawaya et al. 2008), further increases in chlorine concentration and contact time will be required to reduce VBNC *E. coli* concentration in the chlorinated effluent.

**CONCLUSIONS**

In this study, a combination of PMA and real-time PCR was developed to enumerate viable *E. coli* in the presence of dead cells. We found that real-time PCR with PMA
treatment could be used for quantification of viable *E. coli*, as long as the ratio of dead cells to viable cells was no greater than 10. The application of PMA–PCR in two wastewater treatment plants indicated that a great number of *E. coli* in the influents and primary and secondary effluents remained in the VBNC state, and VBNC cells were even more chlorine resistant than culturable ones. This study demonstrated that real-time PCR coupled with PMA could be a useful tool for comprehensive elucidation of microbial behaviour in wastewater treatment plants. The development of the method is very likely to allow a real evaluation of health risks from viable pathogenic bacteria. However, the PMA–PCR method would not seem useful for the enumeration of viable *E. coli* cells in environmental waters such as river and marine water where the number of viable *E. coli* is much lower than that of dead cells. Further studies on PMA treatment are needed to achieve more efficient differentiation between viable and dead *E. coli* cells.

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