Application of real-time polymerase chain reaction (PCR) coupled with ethidium monoazide treatment for selective quantification of viable bacteria in aquatic environment

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ABSTRACT

Ethidium monoazide (EMA) was used to quantify DNA selectively from viable cells with healthy membrane/cell wall system, but not from dead cells, of a target bacterium in the aquatic environment using real-time PCR. Spiking experiments to determine the EMA treatment conditions showed that EMA treatment with EMA at 10–25 μg/ml and subsequent halogen light exposure for 2 min was suitable for selective quantification of DNA from viable cells in an aquatic sample using real-time PCR coupled with EMA treatment (real-time EMA-PCR). Optimized real-time EMA-PCR was applied in combination with culture-based method and conventional real-time PCR without EMA treatment to elucidate the behavior of an Escherichia coli strain inoculated into a pond water microcosm. Quantification results obtained using real-time EMA-PCR were lower than those by conventional real-time PCR without EMA treatment and higher than those by culture-based method. The results suggest that quantification by real-time EMA-PCR seemed to represent the viable population, which would partly include viable but non-culturable state bacteria. Real-time EMA-PCR optimized here can be a useful tool for selective monitoring of the viable population of a target bacterium in the aquatic environment, and thereby contribute to assessment of potential microbial risks generated from waterborne pathogenic bacteria.

Key words | aquatic environment, real-time EMA-PCR, viable but non-culturable state, viable population

INTRODUCTION

To date, PCR-based quantification methods such as real-time PCR have been applied widely and intensively for microbial monitoring for various environmental purposes because of the methods’ extremely high sensitivity and specificity. However, conventional PCR has a salient disadvantage: it cannot discriminate DNA derived from viable and dead cells. The DNA that can be amplified by PCR persists in the environment for a long period after their original cells have died (Josephson et al. 1993). Therefore, PCR-based diagnostics engender overestimation of target microorganisms or even false-positive results in the absence of viable cells. The lack of viable/dead differentiation has remained as a serious impediment to wider implementation of PCR-based diagnostics in a broad range of applications.

Among the strategies developed to overcome the difficulty of PCR-based diagnostics, ethidium monoazide (EMA)-PCR developed by Nogva et al. (2003) is a promising and easy-to-use alternative to amplify DNA selectively from viable cells. Actually, EMA is a DNA intercalating agent that penetrates only into dead cells with compromised membrane/cell wall systems. Once photolyzed, EMA covalently links to DNA and prevents PCR amplification of DNA derived from dead cells. Consequently, only DNA from viable cells can be amplified (Figure 1). To date, EMA-PCR has been applied for food safety control and clinical...
analysis (Rudi et al. 2005; Lee & Levin 2006; Nocker & Camper 2006; Wang & Levin 2006). However, to our knowledge, no study has applied the method to microbial monitoring in an aquatic environment.

In this study, we aimed at optimizing real-time PCR coupled with EMA treatment (real-time EMA-PCR) to monitor the behavior of the viable population of a target bacterium in an aquatic environment. Optimal EMA concentration and halogen light exposure time were determined to quantify the target DNA selectively from viable cells in the aquatic sample. Optimized real-time EMA-PCR was then applied in combination with culture-based method and conventional real-time PCR without EMA treatment to monitor, differentially, culturable (using culture-based method), viable (using real-time EMA-PCR) and total (using conventional real-time PCR) populations of the target bacterium inoculated into a pond water microcosm. In this study, viable bacteria, the target of real-time EMA-PCR, were defined as those with healthy membrane/cell wall system, regardless of their metabolic activity. An *Escherichia coli* strain was selected as the target bacterium because it is representative of the fecal indicator bacteria used most widely for monitoring and predicting potential pathogenic microorganisms in aquatic environments.

**MATERIALS AND METHODS**

**Bacterial strain and culture condition**

As the target strain for monitoring in the aquatic samples, *E. coli* HB101(pJP4) was used. The plasmid pJP4 was introduced into *E. coli* HB101 (Boyer & Roulland-Dussoix 1969) by broth mating with *Cupriavidus necator* JMP134 (pJP4) (formerly *Ralstonia eutropha* JMP134(pJP4); Don & Pemberton 1981) purchased from the German Collection of Microorganisms and Cell Cultures (DMS, Germany) to give the selective marker (mercury resistance). Transconjugant colonies appeared on deoxycholate agar (Eiken Chemical Co. Ltd.) that had been supplemented with streptomycin at 20 mg/l; also, HgCl₂ at 5 mg-Hg/l confirmed the presence of plasmid pJP4 by agarose gel electrophoresis and *tfdB* gene-targeting PCR. Consequently, *E. coli* HB101(pJP4) with mercury resistance was obtained.

The *E. coli* HB101(pJP4) was routinely maintained in L medium (bacto peptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, pH 7.2) supplemented with HgCl₂ at 5 mg-Hg/l. In microcosm experiments, the strain was enumerated selectively using a basal salt medium (K₂HPO₄ 1 g/l, (NH₄)₂SO₄ 1 g/l, MgSO₄·7H₂O 0.2 g/l, FeCl₃·6H₂O 0.02 g/l, NaCl 0.1 g/l, CaCl₂·2H₂O 0.1 g/l, pH 7.2) supplemented with D(+) glucose at 1 g/l, leucine, proline and thiamine at 50 mg/l, streptomycin at 20 mg/l, and HgCl₂ at 5 mg-Hg/l. Agar was used at 1.7% (w/v) for solid media.

**Spiking and monitoring experiments**

An aquatic sample (dissolved organic carbon 7.2 mg/l, pH 7.2) collected from Zuion pond in Suita, Osaka, Japan was used in this study. Sterilized and intact microcosms consisted of 500-ml Erlenmeyer flask containing 100 ml of the pond water sample with and without filtration with...
0.22-μm pore size membrane filters (Nihon Millipore Ltd., Tokyo, Japan), respectively. An overnight culture of *E. coli* HB101(pJP4) was harvested by centrifugation (8,500 × g, 4°C, 10 min), washed twice with sterilized 50 mM potassium phosphate buffer (pH 7.5), and inoculated into microcosms at a concentration of approximately 1 × 10^7 CFU/ml. For spiking experiments to determine EMA treatment conditions, heat-killed cells (90°C, 10 min) were also used as the inoculum. In monitoring experiments, microcosms inoculated with *E. coli* HB101(pJP4) were incubated at 28°C on a rotary shaker at 120 rpm. Then culturable, viable and total cells of the inoculated bacterium were monitored periodically. Culturable cells were counted using the selective medium described above. Viable and total cells were quantified respectively using real-time PCR with and without EMA treatment.

**EMA treatment**

Before storage at −20°C in the dark, EMA bromide (Molecular Probes Inc., Eugene, Oregon, USA) was dissolved in water to a stock concentration of 2 mg/ml. This EMA stock solution was added to 0.5 ml of aquatic samples in microcentrifuge tubes to make final concentrations of 1, 2.5, 5, 10, 25, 50, 100, and 200 μg/ml. Samples were incubated for 5 min on ice in the dark, and were subsequently exposed for an appropriate period of 0–3 min to a 650-W halogen light source (Ushio Lighting Inc., Tokyo, Japan) located approximately 20 cm from the tube. During light exposure, samples were kept on ice to avoid excessive heating.

**DNA extraction and real-time PCR quantification**

DNA templates were prepared from samples with and without EMA treatment through a combination of cell lysis with proteinase K and phenolchloroform extraction as described previously (Sei *et al.* 2000).

Real-time PCR was performed using a sequence detection system (ABI PRISM® 7000; Applied Biosystems, Tokyo, Japan), targeting the *tbpA* and *tfdB* genes on the chromosome and plasmid pJP4, respectively, of *E. coli* HB101(pJP4). Primers for the target genes were designed using software (Primer Express® ver. 2.0; Applied Biosystems, Tokyo, Japan). The PCR mixtures (20 μl) contained 1 × SYBR® Green PCR Master Mix (Applied Biosystems, Tokyo, Japan), 200 nM of forward and reverse primers for the *tbpA* gene (tbp-F 5'-CGC CAC AAA GCC TGA AAG AA-3' and tubp-R 5'-GCA TCC ATA GCA ACA GAC CCA-3') or *tfdB* gene (tfd-F 5'-CCA TCC AGG ACA GCT TCA ATC-3' and tfd-R 5'-AAT CCT CAA GCG ACT TGT TCG-3') and 4 μl of DNA template. Amplification was performed using shuttle PCR in 40 cycles under the thermal profile of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Employing DNA extracted from pure culture of *E. coli* HB101(pJP4) as the standard, quantification results for each target gene were calculated as CFU equivalents per milliliter (CFU-eq/ml).

In the monitoring experiments, the quantification results based on the detection of the *tfdB* gene on plasmid pJP4 and the *tbpA* gene on the chromosome showed no significant difference, suggesting that segregation of plasmid pJP4 from the inoculated bacterium and horizontal transfer of plasmid pJP4 and its partial sequence to indigenous bacteria by conjugation and natural transformation was negligible. Therefore, only the results on the *tbpA* gene were described in Results and Discussion.

**RESULTS AND DISCUSSION**

**Optimization of EMA treatment conditions**

**EMA concentration**

We treated viable and heat-killed cells of *E. coli* HB101(pJP4) with varying concentrations (1, 2.5, 5, 10, 25, 50, 100, 200 μg/ml) of EMA to determine the optimal EMA concentration that enables selective amplification of DNA from viable cells with inhibition of amplification of DNA from dead cells in an aquatic sample. In the experiments, cell suspensions after adding EMA were exposed to halogen light for 2 min. Inhibition of amplification of target DNA derived from viable cells did not occur in real-time EMA-PCR when viable cells were treated with EMA at a concentration of 25 μg/ml or less (Figure 2A). Notable reductions in amplification of target DNA from viable cells were observed when the EMA concentration was greater than 25 μg/ml. Quantification results decreased by four orders of magnitude from the control without EMA.
treatment when the EMA concentrations were 100 μg/ml and 200 μg/ml. The decrease in quantification results at EMA concentrations above 25 μg/ml is attributable to the interference of DNA extraction and/or PCR amplification by excessive EMA. The amplification of target DNA derived from heat-killed cells was inhibited with increasing concentrations of EMA of 1–10 μg/ml (Figure 2B). Treatment with EMA at 10 μg/ml resulted in the reduction of amplification of target DNA by four orders of magnitude. No notable reduction in the quantification result was observed when heat-killed cells were treated with EMA at 10 μg/ml or higher. These results show that EMA at 10–25 μg/ml seemed suitable for selective quantification of DNA from viable cells in an aquatic sample using real-time EMA-PCR. Consequently, we applied EMA at 10 μg/ml, the lowest concentration among the preferable concentration range, in subsequent experiments. Although EMA at varying concentrations of 1–100 μg/ml was applied for different bacteria such as Campylobacter jejuni, E. coli O157:H7, Listeria monocytogenes, Salmonella spp., and Vibrio vulnificus, no clear relationship was observed between bacterial species and the EMA concentration used (Nogva et al. 2003; Rudi et al. 2005; Lee & Levin 2006; Nocker & Camper 2006; Wang & Levin 2006). Furthermore, EMA at 100 μg/ml, which is 10 times higher than the optimal concentration in this study, was applied for EMA treatment of E. coli O157:H7 strains in liquid cultures (Nogva et al. 2003) and drinking water biofilm (Nocker & Camper 2006). Thus, the optimal EMA concentration might be determined by the combination of the target bacterial species and the sample type to be treated. However, further study to clarify the critical factors determining the optimal EMA concentration should be performed to make EMA-PCR a routine microbial monitoring method in the aquatic environment.

Halogen light exposure time

To optimize the halogen light exposure time, viable and heat-killed cells of E. coli HB101(pJP4) were treated with EMA at 10 μg/ml; they were then exposed to the halogen light source for 0–3 min. Inactivation of free EMA, which might interfere with the DNA extraction and/or PCR amplification, was achieved with light exposure of 1–3 min, which was reflected by a slight increase of PCR amplification compared to amplification without light exposure (Figure 3A). Although target DNA was amplified without light exposure after adding EMA, light exposure of heat-killed cells similarly treated with EMA for 1 min or longer prevented amplification (Figure 3B). Results clarified that the halogen light exposure led to the covalent linkage of EMA to DNA in dead cells; consequently, the amplification was prevented. Comparing the light exposure times examined, light exposure for 2 min was best for prevention of PCR amplification of DNA from heat-killed cells (Figure 3B).

Monitoring behavior of culturable, viable and total populations of an E. coli strain in aquatic microcosm

Optimized real-time EMA-PCR was applied in combination with the culture-based method and conventional real-time
PCR to elucidate the behavior of *E. coli* HB101(pJP4) that had been inoculated into sterilized and intact microcosms. Quantification results obtained using the culture-based method, real-time EMA-PCR, and conventional real-time PCR respectively reflected the culturable, viable, and total populations of the inoculated bacterium.

In the sterilized microcosm, the numbers of culturable, viable, and total populations of *E. coli* HB101(pJP4) increased slightly between day 0 and day 1 and decreased rapidly by three orders of magnitude between day 1 and day 3 (Figure 4A). Thereafter, the culturable population rapidly decreased to below the detection limit by day 6, whereas viable and total populations gradually declined to below the detection limit by day 10. In the intact microcosm, the behaviors of culturable, viable and total populations closely resembled those in the sterilized microcosm between day 0 and day 3 (Figure 4B). Thereafter, all populations had declined gradually below the detection limit by day 10. Similar behavior of the inoculated bacterium in both sterilized and intact microcosms suggested that not biotic factors, but abiotic factors such as a shortage of carbon sources and other nutrients, mainly affected the population decline.

Although general behaviors of culturable, viable, and total populations were similar, the respective numbers of viable and total populations were higher than those of culturable and viable populations during the period when the inoculated bacterium decreased. Culturable and viable populations were, respectively, <0.33% and 12% of the total population on day 6 in the sterilized microcosm, although they were 8.7% and 39% on day 3 and 3.1% and 47% on day 6 in the intact microcosm (Figure 4). These results suggest that exogenous bacteria lose viability after exposure to an environmental stress, but that some non-viable, dead population persists in the environment for a certain period. Large differences between the numbers of culturable and viable populations suggest that most of the remaining viable population loses the colony-forming ability before disappearance. Therefore, culture-based method and conventional real-time PCR seem to engender underestimation and overestimation of the viable population, respectively. Viable population measured by real-time EMA-PCR would partly include viable but non-culturable (VBNC) state bacteria although it could not be confirmed in our experimental system. Because VBNC state bacteria can resuscitate to their vegetative state according to the transformation of environmental conditions, they should be considered for estimation of potential microbial health risks. In this light, real-time EMA-PCR can be a suitable monitoring method for microbial risk assessment. Further study should be conducted to confirm the availability of real-time EMA-PCR for monitoring the behavior of VBNC state bacteria in the aquatic environment.

![Figure 3](https://iwaponline.com/wst/article-pdf/58/5/1107/436245/1107.pdf)

**Figure 3** | Effect of light exposure time on EMA inactivation and inhibition of PCR amplification of DNA from dead cells. Aquatic samples inoculated with viable (A) and heat-killed (B) cells of *E. coli* HB101(pJP4) were treated with EMA at 10 μg/ml and subsequently exposed to the halogen light for 0–3 min. Quantification results without EMA treatment are shown as the control. Error bars indicate data ranges for 2–3 replicate measurements.
CONCLUSION

In this study, EMA treatment conditions were optimized for selective detection of DNA from viable cells in the aquatic environment using real-time PCR. Our results demonstrate that real-time EMA-PCR is applicable for selectively monitoring the behavior of the viable population, which would partly include VBNC state bacteria, of a target bacterium in the aquatic environment. Consequently, it can be concluded that real-time EMA-PCR can be a powerful tool for comprehensive elucidation of microbial behavior in the aquatic environment. Application of the method engenders the correct estimation of potential health risks originated from total viable cells of waterborne pathogenic bacteria.

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