Short Communication

Comparison of Ethidium Monoazide and Propidium Monoazide for the Selective Detection of Viable Legionella Cells

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SUMMARY: Ethidium monoazide (EMA) and propidium monoazide (PMA) have been utilized for selective PCR amplification of DNA from viable bacterial cells. In this study, we compared the abilities of EMA and PMA, together with real-time PCR, to specifically distinguish dead Legionella cells from viable cells. Several experiments showed that PMA or EMA treatment could specifically prevent the PCR amplification of DNA from dead Legionella cells in water samples. However, a 4-fold higher concentration of PMA than EMA was required to achieve this effect. EMA may therefore be more useful for practical environmental investigations of Legionella.

Legionella is a Gram-negative, rod-shaped bacterium that replicates as an intracellular parasite of protozoa existing in aquatic environments and moist soil (1–3). The bacterium causes Legionnaires’ pneumonia and Pontiac fever in humans (4,5). Man-made water systems, such as cooling towers, hot springs, and public baths, are common sources of legionellosis. The monitoring and removal of Legionella from waters that come into contact with humans, particularly water from distribution systems, are effective ways to prevent infections caused by Legionella; however, it usually takes 4–7 days to isolate viable Legionella organisms from environmental samples. In order to identify the sources and routes of infections caused by Legionella at an early stage, it is important to develop rapid detection methods.

Recently, ethidium monoazide (EMA) and propidium monoazide (PMA) have been used together with PCR or real-time PCR for the selective detection of viable bacterial cells by excluding dead cells (6–10). EMA and PMA are DNA-intercalating dyes and can selectively enter those cells that have compromised cell walls and membranes. Within these cells, they covalently link to DNA (11–13); the linked DNA then cannot be amplified by PCR/real-time PCR (10,12). A combination of EMA or PMA treatment and PCR or real-time PCR may distinguish the DNA of damaged and/or dead bacterial cells, which cannot be amplified by PCR, from that of viable cells, which can be amplified (6,7,9,12). The combination of EMA treatment and PCR/real-time PCR has been used for the rapid detection of viable Legionella cells from environmental water samples (6,7). However, in some of the water samples examined, the number of Legionella cells estimated by real-time PCR after treatment with 10–20 μg/ml of EMA was less than that determined by plating (6). Although the exact reasons for this are not known, it was postulated that it may be due to the penetration into and/ or lethal effect of EMA on culturable Legionella cells (8,9).

Another possibility is that EMA entered into and inhibited PCR amplification of DNA from damaged cells, which then recovered after incubation on nutritious plates. PMA shows lower penetration and is not considered to have a lethal effect on bacterial cells; therefore, it is superior to EMA for viable cell detection of several bacterial species such as Listeria monocytogenes and Escherichia coli (8,9). In this study, we compared the effect and utility of EMA/ real-time PCR and PMA/real-time PCR on the selective detection of viable Legionella cells from water samples.

Stock solutions of EMA (Sigma–Aldrich Chemical Co., St. Louis, Mo., USA) and PMA (Biotium, Inc., Hayward, Calif., USA) were prepared at concentrations of 20 mM or 100 mM. Legionella pneumophila serogroup 1 strain 80-045 (14) incubated at 37°C on BCYE agar (Becton Dickinson, Sparks, Md., USA) was used for analysis. The minimum inhibitory concentration (MIC) of EMA and PMA to 80-045 cells was determined by the microdilution method (15). The 80-045 cells were suspended in [N-(2-acetamido)-2-aminoethanesulfonic acid]-buffered yeast extract (BYE) broth at approximately 1×106 colony-forming units (CFU)/ml. After 50 μl of BYE containing serial 2-fold dilutions from 3,200 to 1.5 μM of EMA or PMA were added, 50 μl of the 80-045 solution was inoculated in a 96-well microplate. MIC was defined as the minimum concentration of the dyes that inhibited visible bacterial growth after culturing at 35°C for 2 days. The MIC of EMA to 80-045 cells was 50 μM and that of PMA was 200 μM.

In order to investigate the effects of EMA and PMA on the specific detection of viable Legionella cells, mock water samples were prepared by adding the equivalent of approximately 1×105 CFU of viable or killed 80-045 cells into tap water. Dead Legionella cells were prepared by heat or sodium hypochlorite (Sigma–Aldrich) treatment as described previously (6). Furthermore, a model spa system was prepared as described by Sugiyama et al. (16) and paired water samples were collected from the bathtub (odd-numbered samples) and the filter tank (even-numbered samples) at the same time points. After men bathed, the bath water was kept for 6 days without chlorine disinfection for bacterial growth. Water samples No. 1–2 and No. 3–4 (see Table 1) were collected on days 4 and 6, respectively, after bathing.
samples No. 3 and 4 were obtained, chlorine was swiftly added and circulated in reverse throughout the filter tank to prepare chlorine-treated bacterial cells (16). Water samples No. 5–6, 7–8, and 9–10 were collected at 1, 3, and 8 h, respectively, after the addition of chlorine (see Table 1). The residual free chlorine concentration was assayed using DPD liquid (Kanto Chemical Co., Tokyo, Japan).

After the samples of mock water and spa water were collected, the chlorine in the water samples was immediately inactivated with sodium thiosulfate. Collection of bacterial cells by centrifugation and treatment of the cells with 0.2 M KCl-HCl buffer (pH 2.2) were performed as described previously (10). The actual number of cultivable Legionella cells in the water samples was determined by plating the concentrated solution on BCYE or GYPC agar plates (Sysmex bioMérieux, Co., Tokyo, Japan). Similar Legionella suspensions were maintained at 4°C for 5 min in the dark after various concentrations of EMA or PMA were added. Each sample was then set on ice and exposed to visible light for 5 min. After photoactivation, the bacteria were collected by centrifugation and their genomic DNAs were purified using a MonoFas® genome for Legionella Column (GL Sciences, Inc., Tokyo, Japan). PCR amplifications were carried out with Ex Taq polymerase (Takara Bio, Otsu, Japan). PCR amplifications were carried out with Premix Ex Taq polymerase (Takara Bio) using the primers LEG427F and LEG880R, which target the 16S rRNA gene of Legionella, and the PCR products were separated in 2% agarose gels (Takara Bio). Real-time PCR was performed with Premix Ex Taq polymerase (Takara Bio) using the primers LEG427F and LEG880R and molecular beacon probe P1 (6) or a CycleavePCR Legionella (5S rRNA) Detection Kit (Takara Bio) in an ABI-Prism 7000 (Applied Biosystems, Foster City, Calif., USA). The external standard, negative control (PCR-grade water), positive control, and test samples were prepared and run as described previously (6).

We previously showed that the amplified PCR band of the DNA from EMA-treated dead Legionella cells targeting the 16S rRNA gene cannot be observed (6). In the present study, we first examined whether the combined use of PMA treatment and PCR could specifically detect DNA from viable Legionella cells. Viable, heat-killed, and chlorine-killed 80-045 cells were treated with 100, 150, or 200 μM of PMA. Cells without PMA treatment were used as a control. The results of the PMA-PCR assay on viable and chlorine-treated 80-045 cells are shown in Fig. 1. The PCR products of viable cells showed similar electrophoretic patterns on the agarose gel with or without PMA treatment (Fig. 1, lanes 1–4). We also detected an amplified fragment of genomic DNA from the chlorine-killed 80-045 cells without PMA treatment (Fig. 1, lanes 5) at a similar density to that from viable cells. However, the intensity of the amplified fragments from the PMA-treated killed cells gradually decreased with increasing concentrations of PMA (Fig. 1, lanes 6–8). No visible PCR band was observed under 200 μM PMA (Fig. 1, lane 8). Similar results were obtained when heat-treated 80-045 cells were used (data not shown). These results indicate that PMA treatment effectively inhibits the PCR amplification of DNA from killed Legionella cells, but not that from viable cells.

In order to compare the utility of EMA/real-time PCR and PMA/real-time PCR for the detection of viable Legionella cells from environmental samples, we prepared mock water samples (6). The numbers of 80-045 cells detected in 100 ml water are shown in Fig. 2. Approximately 6.78 ± 0.48 log10 CFU of viable 80-045 cells were detected by plating on BCYE.
agar. The number of culturable *Legionella* cells in the heat-treated samples was below the limit of detection (10 CFU/100 ml); that is, no colonies were detected on the agar after plating 1/10 volume of the concentrated bacterial solution.

The number of *Legionella* cells was first estimated by real-time PCR targeting the 16S rRNA gene (Fig. 2A). EMA and PMA were used at doses of 50, 100, 200, or 400 μM. The numbers of viable and heat-killed cells without EMA or PMA treatment were 7.30 ± 0.32 log_{10} and 6.67 ± 0.85 log_{10} cells, respectively. The lower numbers of heat-killed cells suggests that some of the genomic DNA of the killed cells was destroyed by the heat treatment. After the viable cells were treated with 50, 100, 200, and 400 μM of EMA, 6.48 ± 0.45 log_{10}, 6.03 ± 0.54 log_{10}, 6.93 ± 0.31 log_{10}, and 5.47 ± 0.21 log_{10} cells, respectively, were detected by real-time PCR. There was no significant difference (P > 0.05) in the number of viable 80-045 cells as determined by plating and estimated by real-time PCR after treatment with EMA at 50, 100, or 200 μM. However, a significant decrease (P < 0.01) in the PCR-estimated cell number was detected after treatment with EMA at 400 μM. When the heat-treated *Legionella* suspension described above was treated with EMA at 50, 100, 200, and 400 μM, 1.53 ± 0.48 log_{10}, 1.13 ± 0.47 log_{10}, 1.10 ± 0.17 log_{10}, and 1.47 ± 0.45 log_{10} cells, respectively, were detected by real-time PCR. The PCR-estimated cell number of heat-killed cells treated with EMA was approximately 5 log_{10} lower than the number estimated for the same sample without EMA treatment. There was no significant difference (P > 0.05) in the numbers of heat-killed 80-045 cells as estimated by real-time PCR after treatment with EMA at 50, 100, 200, or 400 μM. These results indicate that 50 μM of EMA was sufficient to inhibit the PCR amplification of DNA from 5 log_{10} dead cells and that high concentration (400 μM) of EMA could inhibit the PCR amplification of DNA from viable *Legionella* cells.

When viable *Legionella* cells were treated with PMA at 50, 100, 200, and 400 μM, 6.87 ± 0.23 log_{10}, 7.00 ± 0.26 log_{10}, 6.73 ± 0.23 log_{10}, and 6.63 ± 0.31 log_{10} *Legionella* cells, respectively, were detected by real-time PCR (Fig. 2A). The number of bacterial cells estimated by real-time PCR was almost the same as that determined by plating even when the concentration of PMA was at 400 μM. When the same samples were heat-killed before treatment with 50, 100, 200, and 400 μM of PMA, the number of cells was estimated to be 2.53 ± 1.01 log_{10}, 2.13 ± 0.72 log_{10}, 1.47 ± 0.93 log_{10}, and 1.57 ± 0.31 log_{10}, respectively, by real-time PCR (Fig. 2A). These results indicate that at least 200 μM of PMA is necessary to inhibit the PCR amplification of DNA from 5 log_{10} heat-treated 80-045 cells; the same result was achieved with 50 μM of EMA. On the other hand, there was no obvious inhibition of the DNA from viable cells even when PMA was used at 400 μM. Similar results were obtained when chlorine-treated 80-045 cells were used (data not shown). Thus, a 4-fold higher concentration of PMA than EMA is required to obtain a similar effect with dead *Legionella* cells.

Subsequently, real-time PCR targeting the 5S rRNA gene of *Legionella* was also performed (Fig. 2B). The PCR-estimated numbers of viable and heat-killed 80-045 cells without EMA or PMA treatment were 6.98 ± 0.26 and 5.77 ± 0.21 log_{10} CFU, respectively. After treatment with EMA at 50 and 100 μM, 6.45 ± 0.29 log_{10} and 6.13 ± 0.19 log_{10} viable cells, and 3.30 ± 0.17 log_{10} and 3.20 ± 0.17 log_{10} killed cells were estimated by real-time PCR, indicating that there was no obvious difference between the estimated number of heat-killed cells treated with 50 and 100 μM of EMA. When similar water samples were treated with PMA at 50, 100, 200, and 400 μM, 6.68 ± 0.43 log_{10}, 6.40 ± 0.37 log_{10}, 6.25 ± 0.37 log_{10}, and 6.00 ± 0.37 log_{10} viable cells and 4.23 ± 0.23 log_{10}, 3.97 ± 0.06 log_{10}, 3.80 ± 0.17 log_{10}, and 3.27 ± 0.21 log_{10} killed cells were detected. Although there was a slight difference in the PCR-estimated numbers of dead cells treated with the various concentrations of PMA, the number of detected cells under the treatment with 400 μM of PMA was most similar to that under the treatment with 50 μM of EMA. Even though the killed cells were treated with 100 μM EMA or 400 μM PMA, 3.20 ± 0.17 log_{10} and 3.27 ± 0.21 log_{10} Legionella cells were also detected by real-time PCR. By comparison with the estimated numbers of killed cells treated with 100 μM of EMA (1.13 ± 0.47 log_{10} cells) or 400 μM of PMA (1.57 ± 0.31 log_{10} cells) by real-time PCR targeting the 16S rRNA gene, we found that PCR amplification of DNA corresponding to approximately 1.5 log_{10} killed cells could not be inhibited when real-time PCR targeting the 5S rRNA gene was performed. These results show that the combined use of EMA and real-time PCR targeting the 16S rRNA gene was appropriate to specifically detect viable *Legionella* cells from the mock samples.

We also compared the utility of EMA/real-time PCR and PMA/real-time PCR assays of water samples from a model spa system. EMA was used at 12.5, 25, or 50 μM and PMA at 50, 100, or 200 μM. Samples without either treatment were used as controls. The numbers of *Legionella* cells determined by plating on GVPC plates or estimated by real-time PCR targeting the 16S rRNA or SS rRNA gene are shown in Table 1.

Water samples No. 1–4 were collected from the bathtub or filter tank of the model system on days 4 and 6 after bathing. The concentration of free chlorine in the 4 samples was 0.02 ppm. We expected almost all of the *Legionella* cells to be viable under these conditions. At a low concentration of EMA (12.5 μM) or PMA (50 μM), the numbers of cultivable cells estimated by the PCR method targeting both the 16S rRNA and 5S rRNA genes were almost identical to those determined by culturing (Table 1, No. 1–4). On the other hand, the number of viable *Legionella* cells estimated by real-time PCR targeting the 16S rRNA gene decreased as the concentrations of EMA (25 and 50 μM) and PMA (200 μM) increased (Table 1, No. 1–4), suggesting that, similar to EMA, a higher concentration of PMA could inhibit the PCR amplification of DNA from the culturable *Legionella* cells in the water samples. On day 6 after bathing, chlorine was added to kill *Legionella* cells. Water samples No. 5–10 were collected from the bathtub or filter tank at 1, 3, and 8 h after the addition of chlorine, when a large number of viable bacterial cells had been killed. In these samples, a higher concentration of EMA (50 μM) or PMA (200 μM) was required to obtain the same number of cells by direct plating and by PCR targeting the 16S rRNA gene (Table 1, No. 5–10). The required concentrations of EMA and PMA was found to depend on the number of dead cells in these samples: a higher concentration of EMA was needed in water samples with a larger number of dead *Legionella* cells; we reported a similar observation in our previous study (6). The required concentration of PMA was 4 times higher than that of EMA in order to obtain a similar effect.

When real-time PCR targeting the 5S rRNA gene was performed, ≥1.0 log_{10} uncultivable cells were still estimated from water samples No. 5–10, even under treatment with 50
Therefore, we investigated the effect of temperature and decreased by preconditioning the bacterial suspension at 4°C is temperature- and photoactivation-dependent and can be reported that the lethal effect of EMA on the viable effect on viable cells of the two dyes. It has been shown that the lethal effect of EMA and PMA on viable Legionella cells prepared at 4°C, 25, or 37°C for 2 h, the bacterial cells were not detectable in the samples preconditioned at 4, 25, and 37°C, respectively. There were no obvious differences between the numbers of viable Legionella cells from dead cells in environmental water samples, possibly because of the lethal effect on viable cells of the two dyes. It has been reported that the lethal effect of EMA on L. monocytogenes is temperature- and photoactivation-dependent and can be decreased by preconditioning the bacterial suspension at 4°C (9). Therefore, we investigated the effect of temperature and photoactivation on the EMA and PMA treatment of Legionella cells. A total of 8.13 ± 0.17 log_{10} CFU/ml of 80-045 cells were suspended in normal saline solution. After preconditioning at 4, 25, or 37°C for 2 h, the bacterial cells were treated with either 50 μM of EMA or 200 μM of PMA. Following incubation of these samples in the dark for 5 min, the number of 80-045 cells coincubated with EMA were 8.12 ± 0.15 log_{10}, 8.11 ± 0.07 log_{10}, and 8.10 ± 0.18 log_{10} CFU/ml, respectively, and those of 80-045 cells coincubated with PMA were 8.03 ± 0.16 log_{10}, 8.09 ± 0.05 log_{10}, and 8.14 ± 0.12 log_{10} CFU/ml. After further exposure to light for 5 min, approximately 8.00 ± 0.14 log_{10}, 8.04 ± 0.06 log_{10}, and 8.08 ± 0.17 log_{10} CFU/ml of EMA-treated cells and 8.04 ± 0.11 log_{10}, 7.99 ± 0.10 log_{10}, and 8.00 ± 0.07 log_{10} CFU/ml of PMA-treated cells were killed. We were not able to kill legionella cells in the samples preconditioned at 4, 25, and 37°C, respectively. There were no obvious differences between the numbers of Legionella cells prepared under the different preconditions. These results show that the lethal effect of EMA and PMA on viable Legionella cells does not change by modifying the experimental conditions. Lastly, in order to clarify the difference in the detection of viable Legionella cells between EMA and PMA, penetration of the two dyes into 80-045 cells was compared following the method described by Nocker et al. (8). After treatment of 500 μl1 bacterial suspension with mixtures of 1.25 μl of SYTO9 (3.34 mM; BacLight LIVE/DEAD Bacterial Viability Kit; Molecular Probes, Leiden, The Netherlands) and 1.25 μl of EMA or PMA (20 mM) for 5 min, the viable and heat-killed 80-045 cells stained with SYTO9, EMA/PMA, or both dyes were observed under fluorescence microscope (Olympus Co., Tokyo, Japan). All of the viable 80-045 cells could be stained with green (SYTO9) but not with red (EMA or PMA), while the dead cells were stained with both green and red (data not shown). Thus, no obvious difference in penetration into viable 80-045 cells could be detected between EMA and PMA.

In conclusion, the combination of EMA/PMA treatment and real-time PCR targeting the 16S rRNA gene was able to specifically distinguish DNA of dead Legionella cells from that of viable cells. However, EMA was effective at a lower dose and therefore costs for EMA would be less than those for PMA to produce a similar effect. The specific reasons why EMA was more effective than PMA could not be completely elucidated by the assays performed in the present study, however differences in the molecular sizes of EMA (MW = 420.3) and PMA (MW = 512.5), the absolute quantity that enters the cells, and/or the effects of DNA-intercalating between the two dyes might be responsible. Therefore, we suggest that EMA is more practically useful and superior to PMA for the rapid detection and investigation of Legionella in environmental samples.

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REFERENCES

4. Fraser, D.W., Tsai, T.R., Orenstein, W., et al. (1977): Legionnaires’ disease:

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Table 1. Comparison of results of plating and real-time PCR for water samples from a model spa

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>No. of Legionella by plating (log_{10} CFU/100 ml)</th>
<th>No. of Legionella estimated by real-time PCR target 16S rRNA gene (log_{10}/100 ml)</th>
<th>No. of Legionella estimated by real-time PCR target SS rRNA gene (log_{10}/100 ml)</th>
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1: Samples No. 1, 3, 5, 7, and 9 were obtained from the bathtub, and No. 2, 4, 6, 8, and 10 were from the filter tank of a model spa.
2: The number of bacteria was determined by plating cells on GVPC agar plates.
3: –, Not detected.