International Journal of Food Microbiology 210 (2015) 143–148

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Evaluation of propidium monoazide-quantitative PCR to detect viable *Mycobacterium fortuitum* after chlorine, ozone, and ultraviolet disinfection

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ARTICLE INFO

Article history:
Received 16 October 2014
Received in revised form 16 June 2015
Accepted 22 June 2015
Available online 24 June 2015

Keywords:
Chlorine
Mycobacterium fortuitum
Ozone
Propidium monoazide
Real-time quantitative PCR
Ultraviolet

ABSTRACT

We evaluated whether propidium monoazide (PMA) combined with real-time quantitative PCR (qPCR) is suitable for detecting viable *Mycobacterium fortuitum* after chlorine, ozone, and ultraviolet (UV) disinfection. PMA-qPCR was effective in determining the viability of *M. fortuitum* compared with qPCR based on the membrane integrity. However, with a mild chlorine concentration, PMA-qPCR as an alternative method was not applicable due to a large gap between loss of culturability and membrane integrity damage. In ozonation, PMA-qPCR was able to differentiate between viable and injured mycobacteria, and the results were similar to those obtained by the culture method. Interestingly, PMA-qPCR was successful in monitoring the viability after UV disinfection due to the long UV exposure needed to effectively inactivate *M. fortuitum*. The findings of the present study suggested that the characteristics of disinfectants and the *M. fortuitum* resistance to disinfectants play critical roles in determining the suitability of PMA-qPCR for evaluating the efficacy of disinfection methods.

1. Introduction

Nontuberculous mycobacteria (NTM) or environmental mycobacteria can be found in diverse environments, and some species are capable of infecting humans and animals (WHO, 2004). Particularly, drinking water has been shown as a potential source of NTM infections due to the high resistance of NTM to disinfectants (Covert et al., 1999; Le Dantec et al., 2002; Taylor et al., 2000). In chlorine and ozone disinfection, the Ct values for 3-log inactivation of *Mycobacterium avium* are at least 580- and 50-times greater, respectively, than those for *Escherichia coli* (Taylor et al., 2000). *Mycobacterium fortuitum* is 10-fold more resistant to ultraviolet (UV) than *E. coli* for 3-log inactivation (Lee et al., 2010).

The culture method requires a long incubation time due to the slow growth rate of mycobacteria and their inefficacy to detect viable but not culturable cells under stressful conditions. Moreover, decontamination agents used to suppress the growth of other microorganisms in the media may kill mycobacteria (Brooks et al., 1984).

Real-time quantitative PCR (qPCR) has been used to overcome these disadvantages of culture methods, but it is not able to differentiate between live and dead cells. Recently, propidium monoazide (PMA) has been used with qPCR for selective detection of viable bacterial cells by excluding dead cells due to the ability of PMA to penetrate into membrane compromised cells, intercalate into DNA, and prevent PCR amplification (Chang et al., 2010; Nocker et al., 2006). PMA-qPCR has been applied to detect various bacteria including *Listeria monocytogenes* (Nocker et al., 2006; Pan and Breidt, 2007), *E. coli* O157:H7 (Nocker et al., 2006), *Campylobacter jejuni* (Josefsen et al., 2010), and *Legionella pneumophila* (Delgado-Viscogliosi et al., 2009). Furthermore, several studies have investigated the effect of disinfectants on several pathogens using the PMA-qPCR method (Delgado-Viscogliosi et al., 2009; Nocker et al., 2007).

However, to our knowledge, few studies have assessed the suitability of PMA-qPCR to measure viability of mycobacteria after various disinfection processes compared with the culture method. However, PMA-qPCR has been applied to investigate the viability of heated mycobacteria (Kralik et al., 2010; Nocker et al., 2007). Application of PMA-qPCR to detect viable mycobacteria after disinfection used in water treatment might be useful due to the high resistance to disinfectants and long culture periods of mycobacteria.

Therefore, we evaluated whether PMA-qPCR is useful to efficiently monitor viability after the use of disinfection technologies of chlorine, ozone, and UV against *M. fortuitum*. Moreover, we investigated the effect of chlorine, ozone, and UV on the viability of *M. fortuitum* by comparing the PMA-qPCR, qPCR, and cultivation results.

2. Materials and methods

2.1. Microorganisms

M. fortuitum, a type of nontuberculous mycobacteria, was utilized in this study. Pure cultures of *M. fortuitum* (ATCC 23010) were cultured in Middlebrook 7H9 broth (Difco, Sparks, USA) containing 10% (vol/vol) oleic acid albumin enrichment (BBL, Sparks, USA) at 37 °C for 7 days.

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http://dx.doi.org/10.1016/j.ijfoodmicro.2015.06.019
0168-1605/

The microorganism was then centrifuged at 3000 ×*g* for 20 min. The pellet was suspended in sterilized phosphate buffered saline (PBS; pH 7.0) and mixed with sterilized glass beads (1 mm; Sigma-Aldrich, St. Louis, USA) to reduce bacterial aggregation. The concentration of the suspension was approximately 10^6 CFU/mL, and the cell suspension was used as a sample for the experiment.

2.2. PMA treatment

PMA (phenanthridium, 3-amino-8-azide-5-[3-(diethymethylammonio)propyl]-6-phenyl dichloride; Biotum, CA, USA) was dissolved in 20% dimethyl sulfoxide (DMSO; Sigma-Aldrich) to obtain a 20 mM stock solution. The stock solution was added to 500 μL samples in light transparent tubes (Abgene, Rochester, USA) to reach final concentrations ranging from 15 to 200 μM. The samples were incubated at room temperature in the dark for 5 min with occasional mixing, and they were exposed to light for 2 min using a 500 W halogen lamp at a distance of 20 cm. During the light exposure, samples were placed on ice to avoid being excessively heated. Total genomic DNA from samples was extracted by the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's manual.

2.3. Killing conditions

During the experiment to optimize the PMA treatment, *M. fortuitum* suspended in PBS were killed by heating at 85 °C for 15 min using a standard laboratory heat block as previously described (Nocker et al., 2006).

2.4. Chlorine disinfection

A chlorinated solution prepared by diluting 10% sodium hypochlorite (Sigma-Aldrich) was added to 100 mL of oxidant demand-free buffered water to reach final concentrations of 2 and 40 mg/L at pH 7.0 and 20 °C. The residual free chlorine was detected with a DPD pocket colorimeter (HACH, Loveland, USA). Once *M. fortuitum* was added to the chlorine solutions, 10 mL samples were taken after 5, 10, 30, 60, and 120 min to 2 mg/L free chlorine treatments and after 2, 4, 6, 8, and 10 min to 40 mg/L free chlorine treatments. Chlorine residuals were quenched with sterile 10% sodium thiosulfate.

2.5. Ozone disinfection

Ozone was produced by a laboratory scale ozone generator (OZONE TECH, Daejeon, Republic of Korea), and the ozone concentration was considered as the displayed value. The final concentrations of ozone were 0.75, 1.0, 1.25, and 1.5 mg/L, and the different ozone concentrations were mixed with the *M. fortuitum* suspension. After a 20 min exposure to the ozone concentrations of 0.75, 1.0, and 1.25 mg/L as well as a 120 min exposure to the ozone concentration of 1.5 mg/L, the samples were centrifuged and re-suspended in PBS before being treated with PMA.

2.6. UV disinfection

UV experiments were conducted at room temperature using a bench-scale collimated beam apparatus (Calgon Carbon Corp.). Low pressure UV irradiance was measured at 254 nm with a radiometer (International light IL1400A, detector SED 240, International Light Inc.) equipped with a SED 240 UV detector. The *M. fortuitum* suspension (10 mL) was placed in a 60 mm Petri dish and irradiated with UV doses of 100 mJ/cm^2 for a 3.5-log reduction of *M. fortuitum* as described previously (Lee et al., 2010).

2.7. Cultivation

After treatment with disinfectants, all samples were serially diluted in PBS and cultured on Middlebrook 7H10 agar (Difco). The number of mycobacteria was counted after 7 days at 37 °C.

2.8. Real-time quantitative PCR

We applied two sets of primers and probes, which targeted the 16S rRNA gene of two different sizes to detect *Mycobacteria* spp. The following primers and probe were used as one set to detect *Mycobacteria*: primers, 5′-GATGCAACGCGAAGAACCTT-3′ (forward) and 5′-TGCACC ACCTGCACACAGG-3′ (reverse); and probe, 5′-FAM-CCTGGGTTTGACAT GCACAGGACG-TAMRA-3′ (Torvinen et al., 2010). The real-time PCR mixture was prepared by combining 400 nM primers (forward and reverse), 200 nM probe, 5 μL of DNA template, and 25 μL of iQ supermix (Bio-rad, Hercules, USA) to yield a final volume of 50 μL. The PCR program was as follows: one cycle of 50 °C for 2 min and 95 °C for 10 min; and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The length of the PCR product was 90 bp.

The other primers were 5′-CCTGGGAAACTGGGTCTAAT-3′ (forward) and 5′-CGCACGCTCACAGTTA-3′ (reverse), which were used with the 5′-FAM-TTTCACGAACAACGCGACA-BHQ1-3′ TaqMan probe (Radomski et al., 2010). The real-time PCR mixture was prepared by combining 300 nM primers (forward and reverse), 100 nM probe, 5 μL of DNA template, and 25 μL of iQ supermix (Bio-rad) to yield a final volume of 50 μL. The PCR program was as follows: one cycle at 95 °C for 10 min; and 40 cycles at 95 °C for 15 s, 55 °C for 20 s, and 72 °C for 40 s. The length of the PCR product was 442 bp, and PCR amplification was performed using an Icycler (Bio-Rad).

2.9. Data analysis

All samples were tested in triplicate. The results are presented as the gene copies (log values) calculated by standard curve after qPCR. Moreover, the cell count according to the culture method was transformed as log values to compare with the qPCR results. Statistical analysis was performed using the SAS program (SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) was applied to compare results obtained by the PMA-qPCR, qPCR, and culture methods. *P* values lower than 0.05 were considered to be statistically significant.

3. Results

3.1. Optimization of PMA treatment

PMA was added to 500 μL aliquots of viable and heat-killed cells at final concentrations of 0, 15, 30, 50, 100, and 200 μM to determine the optimal PMA concentration. The effect of PMA on heat-killed cells increased with increasing PMA concentrations, and the greatest difference in gene copies between PMA-treated and untreated dead cells was observed at 200 μM PMA (Fig. 1). However, higher PMA concentrations also resulted in decreased gene copies for viable cells compared with untreated viable controls (Fig. 1a). The highest DNA loss in dead cells with minimal PMA effects on viable cells occurred at 30 μM PMA.

The PMA-qPCR results were different according to the size of PCR product. When using both sets of primers and probes of different sizes for mycobacterial detection, PMA discriminated better between viable and injured mycobacteria using the 442 bp fragment compared with the 90 bp fragment (Fig. 1b).

Therefore, 30 μM PMA in combination with the primers and probe that generated a 442 bp PCR product were selected for the subsequent experiments on mycobacteria.