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Pretreatment with propidium monoazide/sodium lauroyl sarcosinate improves discrimination of infectious waterborne virus by RT-qPCR combined with magnetic separation☆

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ABSTRACT

RT-qPCR allows sensitive detection of viral particles of both infectious and noninfectious viruses in water environments, but cannot discriminate non-infectious from infectious viruses. In this study, we aimed to optimize RT-qPCR-based detection of chlorine-inactivated human norovirus (NoV) and pepper mild mottle virus (PMMoV) in suspension by pretreatment with an optimal combination of a monoazide and a detergent that can efficiently penetrate damaged viral capsids. Four methods were compared to determine the efficacy of chlorine disinfection (at 1, 3, and 5 min mg/L): (A) RT-qPCR alone, (B) RT-qPCR assay preceded by magnetic bead separation for enrichment of viral particles (MBS-RT-qPCR), (C) MBS-RT-qPCR assay with pretreatment with propidium monoazide (PMA-MBS-RT-qPCR), and (D) PMA-MBS-RT-qPCR assay with pretreatment with sodium lauroyl sarcosinate (INCI-PMA-MBS-RT-qPCR). On the basis of a PMA optimization assay, 200 and 300 μM PMA were used in subsequent experiments for NoV GII.4 and PMMoV, respectively. Optimal INCI concentrations, having minimal influence on NoV GII.4 and PMMoV, were found to be 0.5% and 0.2% INCI, respectively. For NoV GII.4, there were significant differences ($P < 0.05$) in $\log_{10}$ genome copies between the PMA-treated and the INCI + PMA-treated samples ($\log_{10}$ genome copies differed by 1.11 and 0.59 $\log_{10}$ for 3 and 5 min mg/L of chlorine, respectively). For PMMoV, INCI induced differences in $\log_{10}$ genome copies of 0.92, 1.18, and 1.86, for 1, 3, and 5 min mg/L of chlorine, respectively. Overall, the results of this study indicate that an optimal combination of PMA and INCI could be very useful for evaluating disinfection methods in water treatment strategies.

1. Introduction

Among the pathogenic viruses involved in waterborne outbreaks, human noroviruses (NoVs) belonging to genogroups I and II are major disease-causing pathogens (Hall et al., 2013). NoV are typically transmitted through the fecal-oral route either by person-to-person contact or by ingestion of contaminated water (Kotwal and Cannon, 2014; Matthews et al., 2012). Waterborne transmission of NoV is also suspected to be an important cause of endemic gastroenteritis through fecal contamination of drinking water (Payment et al., 1991). The Safe Drinking Water Act requires the U.S. Environmental Protection Agency to identify and publish a drinking water Contaminant Candidate List (CCL) periodically, which consists of unregulated and emerging contaminants (Karim et al., 2015). Because of the frequent outbreaks caused by NoVs in contaminated water and their potential public health risks, NoVs classified in the *Caliciviridae* family were recently nominated on CCL 4 (Federal Register, 2016). Vegetable wash water, in particular chlorination-treated water, is increasingly being recognized as an important vehicle for transmission of human pathogens (Lynch et al., 2009). Therefore, substantial research has focused on the search for an appropriate water chlorination treatment to inactivate waterborne pathogens with the aim of ensuring water quality and safety (Gil et al., 2009).

Pepper mild mottle virus (PMMoV), which is a defined group of non-enveloped, rod-shaped, positive-sense, and single-stranded plant RNA viruses belonging to the genus *Tobamovirus* in the family *Virgaviridae*, has been recently proposed as a novel indicator of

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human fecal pollution in water environments (Cheng et al., 2011; King et al., 2011; Rosario et al., 2009). According to Zhang et al. (2006), PMMoV was the most abundantly detected in human feces, with titers of up to 10^9 virions/g. Furthermore, although there is no conclusive evidence that plant viruses cause disease in humans and animals, several studies have reported the potential pathogenicity of PMMoV in humans and have linked the presence of PMMoV RNA in human feces with a specific immune response and clinical symptoms (Colson et al., 2010; Hamza et al., 2011; Zhang et al., 2006).

To ensure water safety, chlorination is widely used for disinfection because of its high effectiveness, low cost, and residual biocidal effect (Rodriguez and Serodes, 2001). Chlorine in water includes Cl_2, OCl^-, and HOCl, of which HOCl is the most effective and strongest disinfection agent (McDonnell, 2007). A number of studies have evaluated the effectiveness of chlorine disinfection against NoVs in water (Cromeans et al., 2010; Lim et al., 2010; Urakami et al., 2007). However, quantitative, sensitive, and reliable detection of NoVs on the basis of their infectivity is difficult, although attempts have been made to culture NoV (Ettayebi et al., 2016; Guix et al., 2007). As for PMMoV, cell-culture-based detection can be difficult and time-consuming (Reddy, 2014). Thus, in recent decades, molecular methods such as quantitative reverse transcriptase PCR (RT-qPCR) have been used widely for viral detection and quantification owing to their specificity, sensitivity, and short assay time. Nevertheless, there are two major obstacles to the use of nucleic acid-based detection techniques for the detection of viruses in water and food samples: (1) the presence of PCR inhibitors in the test samples, and (2) their inability to discriminate non-infectious from infectious viruses. One approach to PCR inhibitor elimination is the use of specific magnetic bead separation (MBS). The MBS technique has been used successfully to replace enrichment in selective media (Coleman et al., 1995) and, more recently, has been combined with RT-qPCR (MBS-RT-qPCR) to concentrate enter(ic viruses and minimize inhibitors from food (Tian et al., 2008). To discriminate viral integrity, several reports suggested that samples be treated with an intercalating dye, such as propidium monoazide (PMA) or ethidium monoazide (EMA), before nucleic acid extraction and amplification (Coudray-Meunier et al., 2013; Karim et al., 2015). The monoazide agents can penetrate damaged viral capsids and then covalently link to viral nucleic acids under visible-light exposure, thereby inhibiting RT-qPCR (Coudray-Meunier et al., 2013; Escudero-Abarca et al., 2014; Jeong et al., 2017; Leifels et al., 2015; Moreno et al., 2015; Parshionikar et al., 2010; Randazzo et al., 2016; Sánchez et al., 2012; Sangsanont et al., 2014). Therefore, PMA-RT-qPCR has been used to evaluate the effectiveness of chemical or physical treatments for inactivating microorganisms. In particular, PMA-RT-qPCR assay was effective in determining the viability of non-culturable or fastidious virus (Parshionikar et al., 2010). Kim and Ko (2012) reported that the Ct values obtained by PMA-RT-qPCR were significantly higher than those of RT-qPCR after heat treatment. Furthermore, detergents, including sodium deoxycholate (Lee and Levin, 2009; Yang et al., 2011) and sodium lauroyl sarcosinate (INCI) (Wang et al., 2014), have been used; these anionic detergents help PMA to penetrate damaged cells and enhance the discrimination between dead and live cells. For hepatitis A virus, surfactants can be useful to enhance the penetration of monoazide into slightly-damaged capsids (Coudray-Meunier et al., 2013; Fuster et al., 2016; Moreno et al., 2015). Although EMA/PMA-RT-qPCR has been used to discriminate non-infectious viruses from infectious viral particles, these studies directly performed RT-qPCR, without MBS.

Therefore, this study aimed to investigate the applicability of MBS-PMA-RT-qPCR to test the efficacy of chlorine treatment against NoV and PMMoV in water. Additionally, we examined the efficacy of INCI to discriminate between infectious and non-infectious viral particles of NoV and PMMoV to further optimize the detection method.

2. Materials and methods

2.1. Virus stocks

NoV-positive stool samples containing genogroup II genotype 4 (GII.4) were kindly provided by the laboratory of Dr. Changsun Choi (Chung-Ang University, Kyounggi-do, South Korea). Stool samples were diluted in RNase-free water (Quanta Biosciences, Gaithersburg, MD, USA) and vortexed briefly, clarified by centrifugation at 550*g* for 2 min to remove the solids, and then serially filtered through sterile Millex-HV 0.45-μm and Millex-GV 0.22-μm Teflon, low protein-binding syringe filters (Millipore; Billerica, MA, USA). The supernatant was stored in 1-mL aliquots at −80°C. PMMoV-positive control stock samples were kindly provided by Dr. Kwak (Crop Protection Division, National Institute of Agricultural Science, Korea).

2.2. Virus inactivation by chlorine treatment

A free chlorine stock solution (100 mg/L) of sodium hypochlorite (Sigma Aldrich, St. Louis, MO, USA) was prepared with chlorine demand free water. Free and total chlorine concentrations were measured by the *N*,*N*-diethyl-*p*-phenylenediamine colorimetric method with a DR2700 spectrophotometer (Hach, Loveland, CO, USA). The efficacy of the test chlorine treatment was estimated using a modified European CEN EN 1276 method (dilution-neutralization method) based on quantitative suspension testing (Koivunen and Heinonen, 2005). In this study, 200 μL of NoV GII.4 and PMMoV stock suspensions (containing approximately 10^6 NoV GII.4 and 10^8 PMMoV genome copies, respectively) were suspended in each concentration of chlorine. Briefly, 200 μL of NoV GII.4 or PMMoV stock suspension was mixed with 1800 μL of either a test chlorine solution or PBS (negative control). The virus disinfectant (or negative control) mixture was quickly vortexed and incubated at room temperature as indicated in the CEN EN 1276 procedure. Each virus-inoculated sample was treated with 1.0 mg/L free chlorine and then incubated for 1, 3, or 5 min at room temperature. The results of chlorine treatment (min·mg/L) were expressed as $\log_{10}$ genome copies/μL or a threshold cycle (Ct) value, which corresponded with the time (min) of treatment multiplied by disinfectant concentration (mg/L). After disinfection, the number of NoV and PMMoV genome copies was determined by RT-qPCR. Suspension tests of the virucidal activity of the chlorine disinfection were conducted as described previously by Liu et al. (2010). Immediately following the exposure period, 40 mL of PBS with sodium thiosulfate (4 mg/L final concentration) was added to the mixture to neutralize the disinfectant reaction.

2.3. Sample processing for concentration and quantification of viruses

Fig. 1 represents a flow diagram of the analytical methods. Three methods for the concentration and detection of viral RNA from chlorine-disinfected samples were examined in comparison to reference a method: (A) ISO 15216 (2017) reference method; (B) MBS-RT-qPCR assay; (C) MBS-RT-qPCR assay with PMA pretreatment (MBS-PMA-RT-qPCR); (D) MBS/RT-qPCR assay with pretreatment combining PMA and INCI (MBS-PMA-INCI-RT-qPCR).

For method (A), the concentration procedure was described in ISO 15216 (2017). Filtration of chlorine-disinfected viral particles with positively charged ultrafiltration membranes was used to