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# Inactivation efficiency and mechanism of UV-$TiO_2$ photocatalysis against murine norovirus using a solidified agar matrix

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ABSTRACT

Human norovirus (HuNoV) is the primary cause of viral gastroenteritis worldwide. Fresh blueberries are among high risk foods associated with norovirus related outbreaks. Therefore, it is important to assess intervention strategies to reduce the risk of foodborne illness. The disinfection efficiency of decontamination methods is difficult to evaluate for fruits and vegetables due to an inconsistent degree of contamination and irregular surface characteristics. The inactivation efficiency and mechanism of murine norovirus 1 (MNV-1, a surrogate for HuNoV) was studied on an experimentally prepared solidified agar matrix (SAM) to simulate blueberries using different wavelengths (A, B, C) of UV light both with and without $TiO_2$ photocatalysis (TP). MNV-1 was inoculated on exterior and interior of SAM and inactivation efficiencies of different treatments were investigated using a number of assays. Initial inoculum levels of MNV-1 on the SAM surface and interior were 5.2 log PFU/mL. UVC with $TiO_2$ (UVC-TP) achieved the highest level of viral reduction for both externally inoculated and internalized MNV-1. Externally inoculated MNV-1 was reduced to non-detectable levels after UVC-TP treatment for 5 min while there was still a 0.9 log viral titer after UVC alone. For internalized MNV-1, 3.2 log and 2.7 log reductions were obtained with UVC-TP and UVC alone treatments for 10 min, respectively. The Weibull model was applied to describe the inactivation behavior of MNV-1, and the model showed a good fit to the data. An excellent correlation between the steady-state concentration of OH radicals ($[\bullet OH]_{ss}$) and viral inactivation was quantified using a *para*–chlorobenzoic acid (*p*CBA) probe compound, suggesting that OH radicals produced in the UV-TP reaction were the major species for MNV-1 inactivation. Transmission electron microscopy images showed that the structure of viral particles was completely disrupted with UVC-TP and UVC alone. SDS-PAGE analysis showed that the major capsid protein VP1 was degraded after UVC-TP and UVC alone. Real-time RT-qPCR analysis showed that UVC-TP and UVC alone caused a reduction in the level of viral genomic RNA. Propidium monoazide (PMA) pretreatment RT-qPCR analysis showed that UVC-TP caused damage to the viral capsid protein in addition to viral genomic RNA. UVC both with and without $TiO_2$ was more effective for MNV-1 inactivation than UVB and UVA. Thus, UVC-TP disinfection aimed to reduce levels of food-borne viruses can inactivate viruses present on the surface and internalized in the interior of blueberries.



## 1. Introduction

Human norovirus (HuNoV) is considered to be responsible for >58% of all foodborne infections reported annually (Bouwknegt et al., 2015; Lou et al., 2015). HuNoV is highly contagious and only a few viral particles can cause severe illness (Predmore et al., 2015). Fresh blueberries are among high risk foods associated with norovirus (NoV) related outbreaks (Dicaprio et al., 2015). In Europe, one outbreak of NoV associated with strawberries, and 27 outbreaks of NoV associated with raspberries were reported between 2007 and 2011 (European Food Safety Authority, 2014). HuNoV is the most common food-borne pathogen in South Korea (Lee et al., 2015) and nearly 542 foodborne outbreaks of NoV affecting 19,975 people were reported between 2002 and 2015 (Ministry of Food and Drug Safety, 2015).

Unlike bacteria, viruses are obligatory intracellular parasites that can only replicate inside a living host (Lou et al., 2015). Attempts to culture HuNoV have been hampered due to lack of an *in vitro* cell culture system or a small animal model. A recent attempt at development of an *in vitro* infection model for HuNoV in human B cells was reported as "inconclusive" by Jones et al. (2014). Researchers have to rely on the use of proper surrogates to study HuNoV (Lou et al., 2015). Murine norovirus (MNV), with genetic and pathological features similar to HuNoV, can potentially serve as a surrogate for HuNoV (Wobus et al., 2006). MNV was found to be a better surrogate than feline calicivirus and Tulane virus counterparts for the study of HuNoV inactivation (Yeap et al., 2016).

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Most food and water-borne viruses are more resistant to heat, acidic pH, and disinfectants than bacteria. Current procedures for controlling bacterial infections in foods may not be useful against viral pathogens (Predmore et al., 2015). The highest commercially permitted level of 200 ppm chlorine was ineffective for removal of HuNoV from fresh produce (Dicaprio et al., 2015). Chlorine spray achieved lower viral inactivation levels in green onions than other treatments (Hirneisen and Kniel, 2013). Moreover, there is a global trend for elimination of chlorine from fresh produce disinfection processes due to the production of carcinogenic by-products. The use of chlorine with minimally processed products has already been prohibited in the European countries of Belgium, Denmark, Germany, and the Netherlands (Meireles et al., 2016).

A limited number of methods have been reported for prevention of viral contamination in berries (Huang et al., 2016). There is a need for the development of innovative technologies for elimination of viruses from berries and other fruits (DiCaprio et al., 2016; Predmore et al., 2015). Nonthermal processing technologies provide effective microbial inactivation while maintaining safety, quality, and freshness of foods (Adhikari et al., 2015). UV-assisted $TiO_2$ photocatalysis (UV-TP) is an advanced nonthermal technology that inactivates microbial pathogens under aqueous conditions via generation of strong oxidizing agents from a $TiO_2$-coated surface when illuminated with UV light (Yoo et al., 2015; Foster et al., 2011; Thiruvenkatachari et al., 2008; Srinivasan and Somasundaram, 2003).

Disinfection efficiencies of decontamination methods are difficult to evaluate for fruits and vegetables due to an inconsistent degree of contamination and irregular surface characteristics. Furthermore, for UV irradiation, the physical surface structure of fruits may affect light penetration, causing a shadowing effect (Adhikari et al., 2015). Hence, development of a standard food model surface is useful for evaluation of the effectiveness of disinfection methods (Yoo et al., 2015). In this study, an artificially prepared matrix was used to demonstrate the virucidal effects of different disinfection treatments.

The objectives of this study included (i) development of a solidified agar matrix (SAM) for simulation of blueberries, (ii) determination of whether UV-TP disinfection under A, B and C wavelengths of UV light can inactivate MNV-1 on exterior and interior of SAM, and (iii) evaluation of viral inactivation mechanisms of different assays.

## 2. Materials and methods

### 2.1. Viral cell culture

Strain MNV-1 of the murine norovirus was supplied by Herbert W. Virgin, Washington University School of Medicine, St. Louis, MO, USA. MNV-1 was propagated in the RAW 264.7 (KCLB, Seoul, Korea) mouse leukemic monocyte macrophage cell line. RAW 264.7 cells were grown in Dulbecco's minimum essential medium (Gibco, NY) supplemented with 10% fetal bovine serum (Gibco, NY) and 1% antibiotics–antimycotics (Penicillin–streptomycin; Gibco, NY) in 75 $cm^2$ tissue culture flasks, and incubated at 37 °C in a humidified 5% $CO_2$ incubator. The MNV-1 stock was prepared according to the procedure described by D'Souza and Su (2010) with slight modification. When monolayers of RAW 264.7 cells in culture dishes were 90% confluent, the growth medium was removed via aspiration. An aliquot of 1 mL of a viral inoculum at a multiplicity of infection of 1 was added to flasks, followed by incubation at 37 °C in a 5% $CO_2$ atmosphere for 1 h. Viruses were then cultivated on confluent RAW 264.7 cell monolayers for 3 to 4 days. When cytopathic effects were observed in >90% of cells, infected cells were subjected to freezing and thawing three times to release viral particles, followed by centrifugation at 6000 ×*g* for 2 min at 4 °C to remove cell debris, supernatants were then harvested. To further concentrate MNV-1, the supernatant was subjected to ultrafiltration (AmiconUltra-15; Millipore, MA) at 4500 ×*g* for 30 min at 4 °C. The supernatant from the ultrafiltration unit was recovered and kept frozen at −80 °C for later use.

### 2.2. Preparation of a solidified agar matrix (SAM)

SAM were prepared following the procedure of Yoo et al. (2015) with slight modification. Briefly, a 2.5% Bacto™ agar solution, a routinely used microbial medium, was cast in a mold after UVC irradiation for 30 min for elimination of existing microorganisms. The agar solution in the mold was rapidly solidified in a deep freezer at −80 °C for 20 min to achieve hardening. SAM was prepared in order to investigate the efficacy of UV-TP for inactivation of MNV-1 on a fruit surface and within a fruit matrix. Therefore, SAM was prepared in a spherical shape of 10 mm in diameter to resemble blueberries. Morphological characterization of SAM was carried out using scanning electron microscopic analysis (Fig. 1). Briefly, blueberry fruit and SAM samples were prepared and photo images were recorded using scanning electron microscopy (FE-SEM S-800, Hitachi Ltd., Tokyo, Japan) (Shahbaz et al., 2016).

### 2.3. Inoculation of MNV-1 on the SAM exterior and interior

A solid agar surface showed more stable adhesion characteristics for bacterial cells than surfaces prepared using other materials (Yoo et al., 2015). Herein, SAM was immersed in an MNV-1 inoculum of 7.8 log PFU/mL for 10 min for surface inoculation. Contaminated SAM was allowed to dry on a clean bench for 10 min at 22 °C for viral attachment (VS-1400LS, Vision Scientific, Daejeon, Korea). The initial levels of MNV-1 fixed at the SAM surface were 5.2 log PFU/mL. MNV-1 was also injected into SAM in another experiment for evaluation of whether UV-TP was effective against internalized viral particles present within the matrix. Six microliters of a 7.8 PFU/mL viral stock was injected into the SAM center via pipetting. The initial viral population inside the SAM was 5.2 log PFU/mL. Three SAM samples were independently used for each treatment.

### 2.4. Inactivation treatments for MNV-1 in SAM

A small scale photocatalytic reactor was customized following a previous study (Yoo et al., 2015). The reactor consisted of a $TiO_2$-coated (0.7 to 0.9 mm thickness; Taekyeong UV Co., Namyangju, Korea) UV lamp (8.0 W, Sankyo Denki Co., Tokyo, Japan), and non-coated quartz tubes of 25 mm diameter and 50 mm height. The lamp could be changed to non-$TiO_2$ coated UV quartz tubes for UV irradiation without $TiO_2$. The UV intensity was measured before treatment using the sensor of a UV radiometer (UVC: ST-512, Sentry Optronics Corp., New Taipei City, Taiwan; UVA and UVB: UV Spectroradiometer, UV-340A, Lutron, Taiwan). An air pump was used for agitation of SAM samples with turbulent flow. Three contaminated SAM were immersed in the quartz tube containing 5 mL of distilled water, and UV (A, B, C) both with and without $TiO_2$ treatments were applied for 0, 0.5, 1, 2, 3, 5, 7, and 10 min. A water washing treatment with air bubbles (WB) was carried out in the same reactor using disconnected UV lamps.

### 2.5. Analysis

#### 2.5.1. Viral enumeration based on a plaque assay

A plaque assay was performed to determine the viability of treated MNV-1 following the protocol of Gonzalez-Hernandez et al. (2012) with minor modification. Surviving viruses from SAM were extracted via homogenization in a stomacher (MIX 2, AES Laboratories, Combourg, France) using a cold phosphate-buffered saline solution, followed by serial dilution with saline (Predmore et al., 2015). RAW 264.7 cells were seeded in a 6-well cell culture plate at a density of $2 \times 10^6$ viable cells/mL in each well, and incubated at 37 °C in a 5% $CO_2$ atmosphere for 24 h. When cells were 60–80% confluent in wells, cell monolayers were infected with 0.5 mL of a 10-fold diluted series of treated viruses and incubated for 1 h with agitation every 15 min. After incubation, viruses were removed and monolayers were covered with a mixture of Eagle minimum essential medium and 3% SeaPlaque