Research Note

Ozone Inactivation of Norovirus Surrogates on Fresh Produce

K. A. HIRNEISEN, S. M. MARKLAND, AND K. E. KNIEL*

Department of Animal and Food Sciences, University of Delaware, Newark, Delaware 19711, USA

ABSTRACT

Preharvest contamination of produce by foodborne viruses can occur through a variety of agents, including animal feces/manures, soil, irrigation water, animals, and human handling. Problems of contamination are magnified by potential countrywide distribution. Postharvest processing of produce can involve spraying, washing, or immersion into water with disinfectants; however, disinfectants, including chlorine, have varying effects on viruses and harmful by-products pose a concern. The use of ozone as a disinfectant in produce washes has shown great promise for bacterial pathogens, but limited research exists on its efficacy on viruses. This study compares ozone inactivation of human norovirus surrogates (feline calicivirus [FCV] and murine norovirus [MNV]) on produce (green onions and lettuce) and in sterile water. Green onions and lettuce inoculated with FCV or MNV were treated with ozone (6.25 ppm) for 0.5- to 10-min time intervals. Infectivity was determined by 50% tissue culture infectious dose ($TCID_{50}$) and plaque assay for FCV and MNV, respectively. After 5 min of ozone treatment, >6 log $TCID_{50}$/ml of FCV was inactivated in water and ~2-log $TCID_{50}$/ml on lettuce and green onions. MNV inoculated onto green onions and lettuce showed a >2-log reduction after 1 min of ozone treatment. The food matrix played the largest role in protection against ozone inactivation. These results indicate that ozone is an alternative method to reduce viral contamination on the surface of fresh produce.

Noroviruses are the number one cause of foodborne illness and are believed to be the cause of >50% of foodborne disease outbreaks *(6)*. Due to the high incidence and nonroutine testing and surveillance of the virus, it is crucial that prevention of product contamination be a high priority for food producers. The foods associated with most norovirus outbreaks include shellfish, ready-to-eat foods, and fresh produce. The low infectious dose of noroviruses, the estimated average being 18 virus particles *(17),* means that even a small amount of contamination has the potential to be a public health threat. An animal or cell culture model for human norovirus does not exist; therefore, norovirus surrogates, including both feline calicivirus (FCV) and murine norovirus (MNV), are currently the best substitutes in inactivation studies. FCV has been used in many studies as a human norovirus surrogate; however, because it is a respiratory virus rather than enteric *(5, 18),* its reliability as a surrogate is questionable. MNV was the first norovirus to be propagated in cell culture and shares more genetic features with human noroviruses than FCV *(25)*. Both MNV and FCV have a size and shape similar to those of human noroviruses *(25)*.

Spraying, washing, or immersion of fruits and vegetables in water is a common practice during postharvest processing. Water is a critical control point in such processes because it can become contaminated easily, and a small amount of contamination can become widely distributed throughout a water source *(16)*. Disinfectants used in food processing reduce microbial populations in process water, prevent the spread of contamination from one surface to another, and further reduce the numbers on the surface of produce. Surface disinfection of produce using chemical disinfectants has been identified as important for produce processors to reduce the risk of enteric pathogens *(3)*. Chlorine-based washes have been the most common disinfectants used on fresh produce, although ozone, a strong broad-spectrum oxidizing agent, is a potential alternative since it is effective against a wide range of bacteria, fungi, spores, viruses, and protozoa. Although a few studies assessing the effects of ozone on bacterially contaminated produce have been conducted, there is a lack of research on the inactivation of viruses by ozone on fresh produce. This study evaluated the ability of ozone to inactivate the common norovirus surrogates, MNV and FCV, on fresh green onions and lettuce submerged in water.

MATERIALS AND METHODS

Virus propagation. FCV (ATCC VR-651) was propagated in Crandell Reese feline kidney cells (CrFK; ATCC CCL-94) using minimum essential medium (Mediatech, Manassas, VA) supplemented with 1% penicillin–streptomycin–amphotericin B, 1% sodium bicarbonate, 1% sodium pyruvate, and 1% minimum essential medium nonessential amino acids. The medium was supplemented with 2% fetal bovine serum (Mediatech) for maintenance and 10% fetal bovine serum for cell growth. FCV viral titers were determined by 50% tissue culture infectious dose ($TCID_{50}$) and calculated using the Reed Muench method *(4)*.

* Author for correspondence. Tel: 302-831-6513; Fax: 302-831-2822; E-mail: kniel@udel.edu.

TABLE 1. *Reduction by ozone of FCV and MNV suspended in water or on lettuce and green onions*

| | Amt (mean ± SD) of: | | | | | |
| | FCV | | | MNV | | |
Time (min)	Water (log $TCID_{50}$/ml)	Lettuce (log $TCID_{50}$/g)	Green onions (log $TCID_{50}$/g)	Water (log PFU/ml)	Lettuce (log PFU/g)	Green onions (log PFU/g)
0.5	1.39 ± 0.08	1.77 ± 0.12	0.17 ± 0.02	2 ± 1.18	0.97 ± 0.33	0.50 ± 0.28
1	2.70 ± 2.72	1.22 ± 0.94	0.5 ± 0.41	3.89 ± 0.82	2.48 ± 0.43	2.08 ± 0.73
5	6.79 ± 0.06[a]	2.09 ± 0.37	2.07 ± 1.09	4.69 ± 1.01	2.91 ± 0.12	1.61 ± 0.74
10	6.79 ± 0.06[a]	3.08 ± 1.50	2.02 ± 1.21	5.31 ± 0.81[a]	3.09 ± 1.11	3.78 ± 0.68

[a] Virus was inactivated below the limit of detection (100 infectious viral units).

MNV-1 (generously provided by H. Virgin, Washington University, St. Louis, MO) was propagated in the RAW 264.7 cell line cultured in Dulbecco's modified Eagle's medium (Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% glutamine, 1% HEPES buffer, and 1% glutamate. MNV infectivity was determined by plaque assay as previously described *(22)*. In brief, samples were diluted and inoculated onto confluent monolayers of RAW 264.7 cells grown in 12-well plates. After overnight incubation at 37°C, the inoculum was aspirated and the cells were overlaid with 1 ml of 1.5% Sea Plaque (Lonza, Rockland, ME) agarose in Dulbecco's modified Eagle's medium. After 1 day of additional incubation at 37°C, the plaques were visualized by staining with neutral red for 6 to 8 h.

Food sample preparation. Lettuce and green onions (5-g samples) were cut into pieces measuring 1 cm by 1 cm and then exposed to UV for 10 min to reduce the natural microflora of the produce that could interfere with viral analysis *(7)*. The produce was then spot inoculated with 0.5 ml of FCV (final concentration of 10^7 $TCID_{50}$/ml) or 0.5 ml of MNV (final concentration of 10^6 PFU/ml), dried for no longer than 30 min until samples were visibly dry, and placed into 45 ml of sterile double-distilled water (pH 7.0) contained within a 100-ml borosilicate glass flask.

Ozone treatment. The ozone treatment of the lettuce and green onions was similar to the procedure described by Williams et al. *(23)*. Lettuce and green onion samples in sterile water were treated with bubbling gaseous ozone from an ozone generator (Golden Buffalo, Orange, CA) designed to produce 0.9 g of ozone/h at a flow rate of 2.4 liters/min (6.25 ppm). The ozone gas was delivered directly to samples through nonreactive plastic tubing into the flask on a magnetic stirrer. Samples were ozonated for 0.5, 1, 5, and 10 min, during which the contents of the beaker were stirred to ensure dispersal of the ozone. Ozone was produced and delivered throughout the duration of the treatment times. At the end of each treatment time, the residual ozone was quenched with 2 ml of 5% sodium thiosulfate. The residual ozone was measured with a HACH ozone test kit (HACH Company, Loveland, CO) and was determined to be 0.15 ppm for all time points. Inoculated lettuce and green onions placed in 45 ml of sterile water without applied ozone (0 min) served as the controls. To further compare differences between MNV and FCV, 45 ml of sterile water was inoculated with pure virus (approximately 10^6 $TCID_{50}$ or PFU/ml) and ozonated. After ozone treatment, green onion and lettuce samples were stomached for 2 min in sterile pouches (VWR Technologies, Mississauga, Ontario, Canada). Infectivity was determined by $TCID_{50}$ and plaque assay for FCV and MNV, respectively, as previously described. Reductions in viral titer were determined by comparing treated samples with untreated controls.

Viral loss due to recovery was estimated to be ≤1 log. The limit of detection was 100 infectious viral units for both FCV and MNV.

Statistics. Experiments were performed in triplicate on different days, and the results are reported as the means and standard deviations. Difference of means t tests and chi-square analysis were performed with JMP (SAS Institute, Inc., Cary, NC), and P values of <0.05 were considered significant.

RESULTS AND DISCUSSION

MNV and FCV were first treated with ozone (6.25 ppm) at various time intervals (0.5 to 10 min) in sterile water (Table 1). Overall, viral inactivation increased as a factor of time. For FCV and MNV, there was no significant difference in inactivation after ozone treatment for 5 and 1 min ($P \leq 0.05$), respectively. For ≥5 min, treatment of FCV resulted in inactivation levels beyond the limit of detection (100 virus particles). Lettuce and green onions were chosen for use in this study because of their association with previous viral outbreaks and also because of their varying surface morphology. The strongest variable in ozone inactivation was the presence of the food matrix (Table 1) versus other factors such as surrogate type and treatment time. This is probably due to the increased organic load provided by the food matrix. Greater treatment times of ozone were needed in produce to achieve the same level of inactivation of FCV and MNV in water. To achieve a 2- to 3-log inactivation of FCV on green onions and lettuce, 5 to 10 min of ozone (6.25 ppm) was needed, whereas in water, 1 min of ozone treatment was required for similar inactivation of FCV. The same was observed for MNV, where after 0.5 min of ozone in water, approximately 2 log MNV was inactivated. To achieve a similar reduction of MNV on lettuce and green onions, a 1-min treatment was necessary. As seen with pure virus, the inactivation of both viruses on green onions and lettuce increased with increasing time of treatment (Table 1), with the exception that there were no significant differences between 5- and 10-min treatments of either virus in any matrix.

The produce type was also observed to be the major factor for enteric viral inactivation in UV treatment *(7)*. While the difference was not significant, the overall ozone inactivation of both MNV and FCV was lowest on green onions (Table 1). This inactivation difference between lettuce and green onion could be due to differences in the organic composition of the two produce items, because the

ozone reacts with the complex organic compounds in foods due to the high oxidation potential *(10, 11)*. It is also possible that if any virus particles were able to internalize into the lettuce or green onion tissue at the cut edge, as was demonstrated for MNV in romaine lettuce *(22),* they were then inaccessible to the ozone, since ozone is more effective at the surface of the produce *(12)*.

Viral sensitivity to ozone has been shown to vary by type and strain. In this study, both human norovirus surrogates (MNV and FCV) were compared. While MNV is generally recognized as the more suitable surrogate for human norovirus *(1, 5, 9),* MNV was only introduced as a surrogate in 2004 *(24)*. Before MNV, FCV was used for inactivation studies. It is of interest to compare the behavior of these two surrogates under different processing technologies. Ozone treatment of MNV was recently documented by Lim et al. *(13),* where more than 2 log MNV was inactivated at 1 ppm of ozone in 2 min at pH 7 and 5°C. Under the same conditions (1 ppm of ozone at pH 7 and 5°C) in a different study, >4 log FCV was inactivated in 15 s *(19)*. In comparing these two studies, MNV shows greater resistance to aqueous ozone inactivation in ozone-demand-free buffer; however, our results conclude that there is no significant difference ($P > 0.05$) between MNV and FCV inactivation by ozone in water or on lettuce and green onions. In this study, ozone treatment was conducted at room temperature (approximately 20°C); as temperature increases, ozone becomes less soluble in water but the reaction rate increases *(11)*. The inactivation differences observed for the two norovirus surrogates in this and previous studies *(13, 19)* may be due to differences in the temperatures at which treatment occurred; however, in the Lim et al. *(13)* study, there was no significant difference in MNV inactivation at 5 and 20°C. The similar inactivation of both MNV and FCV may be due to the mechanism of viral inactivation by ozone, which is believed to occur through the destruction of both the capsid proteins and the viral genome *(20, 21)*.

Aqueous ozone is applicable on low-ozone-demand products that have smooth and intact surfaces, such as fruits and vegetables *(12)*. In the produce industry, ozone can be used to treat process water, as a produce wash, in storage, and in recycled water *(26)* and can reduce bacterial populations in flume and wash water; typical ozone concentrations for disinfection of postharvest water are 2 to 3 ppm *(15)*. The parameters used in this study (6.25 ppm, 20°C, and 0.5 to 10 min) have proved to be effective at inactivating a variety of foodborne pathogens and common surrogates, including *Bacillus cereus, Escherichia coli, Pseudomonas fluorescens, Salmonell*a Enteritidis, *Salmonella* Typhimurium, *Staphylococcus aureus,* bacteriophage f2, hepatitis A virus, poliovirus, *Cryptosporidium parvum,* and *Giardia lamblia* (reviewed by Kim et al. *(12)*). It is important to consider the common industrial practice of reusing wash water, which can lead to the build-up of field soil. This can decrease the efficacy of the disinfectant used, as was shown with hepatitis A virus–contaminated strawberries in a ClO$_2$ wash *(14)*.

In addition to viral inactivation, studies using ozonated wash water on iceberg lettuce showed that ozonated water maintained the visual appearance of fresh-cut lettuce and controlled browning during storage, and there was no detrimental reduction in the polyphenol and vitamin C levels *(2)*. In our study, physical changes, including browning, loss of structure, or color changes, were not visually observed in either lettuce or green onion pieces after ozone treatment. Ozone treatment is probably more effective for viral inactivation on precut/preshredded produce items because the surface area is increased and, therefore, the area for ozone to interact on produce is increased.

Ozone is an attractive disinfectant for the produce industry due to the fast decomposition into simple oxygen with no safety concerns of residual ozone in the treated food products *(8)*. This decomposition also prevents the accumulation of waste products in the environment *(12)*. The results of this study lead to the conclusion that ozone inactivation of human norovirus surrogates is dependent upon the presence of a food matrix and the time of treatment, and for both FCV and MNV, an average of 3 log was inactivated by ozone.

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