Inactivation of human norovirus and Tulane virus in simple media and fresh whole strawberries by ionizing radiation

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Abstract

Human norovirus (NoV) is a major cause of fresh produce-associated outbreaks and human NoV in irrigation water can potentially lead to viral internalization in fresh produce. Therefore, there is a need to develop novel intervention strategies to target internalized viral pathogens while maintaining fresh produce quality. In this study electron beam (E-beam) and gamma radiation were evaluated for efficacy against a human NoV GII.4 strain and Tulane virus (TV). Virus survival following ionizing radiation treatments was determined using direct quantitative reverse transcriptase PCR (RT-qPCR), the porcine gastric mucin magnetic bead (PGM-MB) binding assay followed by RT-qPCR, and plaque assay. In simple media, a high dose of E-beam treatment was required to completely abolish the receptor binding ability of human NoV (35.3 kGy) and TV (19.5-24.1 kGy), as assessed using the PGM-MB binding assay. Both human NoV and TV were more susceptible to gamma irradiation than E-beam, requiring 22.4 kGy to achieve complete inactivation. In whole strawberries, no human NoV or TV RNA was detected following 28.7 kGy of E-beam treatment using the PGM-MB binding assay. Overall, human NoV and TV are highly resistant to ionizing radiation and therefore the technology may not be suitable to eliminate viruses in fresh produce at the currently approved levels. In addition, the PGM-MB binding assay is an improved method to detect viral infectivity compared to direct RT-qPCR.

46 **Keywords:** human norovirus, Tulane virus, ionizing radiation, electron beam, gamma
47 irradiation, viral food-borne illness, strawberries, food safety, food-borne illness

48 **Abbreviations:**

49 CDC – Centers for Disease Control and Prevention

50 CPE – cytopathic effect

51 DMEM – Dulbecco’s modified Eagle medium

52 E-beam – electron beam

53 FBS – fetal bovine serum

54 FCV – feline calicivirus

55 FDA – Food and Drug Administration

56 HBGAs – histo-blood group antigens

57 HBSS – Hank’s balanced salt solution

58 MNV – murine norovirus

59 MOI – multiplicity of infection

60 NoV – norovirus

61 PGM – porcine gastric mucin

62 PGM-MB – porcine gastric mucin conjugated to magnetic beads

63 RT-qPCR – real time reverse transcriptase polymerase chain reaction

64 SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

65 TV – Tulane virus

66 VLPs – virus-like particles

67 VP1 – norovirus viral protein 1 (major capsid protein)

68 cVP1 – the cleaved form of norovirus viral protein 1 (major capsid protein)

1. Introduction

The most recent data from the Centers for Disease Control and Prevention (CDC) estimates that human norovirus (NoV) is responsible for over 21 million cases of illness annually, causing 95% of all non-bacterial gastroenteritis reported each year in the US (Hall et al., 2013). Human NoV is highly infectious, resistant to some common disinfectants, and causes debilitating illness. Over 5 million cases of human NoV food related illness are estimated to occur each year, accounting for an estimated 58% of all food-borne illnesses and leading to 15,000 hospitalizations and nearly 150 deaths (Hall et al., 2012; Hoffmann et al., 2012; Scallan et al., 2011). Human NoV foodborne disease is commonly associated with foods that undergo little or no processing before consumption, such as fresh produce, seafood, and ready-to-eat food. Documented outbreaks have been associated with many types of food including: fresh cut fruit, lettuce, tomatoes, melons, salads, green onions, strawberries, blueberries, raspberries, salsa, oysters, shellfish, clams, as well as many others (Baert et al., 2011; Doyle and Erickson, 2008; Herwaldt et al., 1994; Hjertqvist et al., 2006; Le Guyader et al., 2004; Le Guyader et al., 2009; Li et al., 2012).

Fresh produce can become contaminated with human NoV at any step from farm to fork. Human NoV can enter the fresh produce system during production through fecal contamination of irrigation water, soil, or fertilizers or from infected food handlers during harvesting, processing, and preparation (Baert et al., 2008; Butot et al., 2007; Heaton and Jones, 2008; Seymour and Appleton, 2001; Steele and Odemeru, 2004). Several studies have found that the presence of enteric viruses in irrigation water can lead to subsequent uptake by produce leading to internalization of viruses within the food matrix (Dicaprio et al., 2012; Erickson, 2012; Esseili et al., 2012; Hirneisen and Kniel, 2013; Wei et al., 2010, 2011). These internalized viruses

would be protected from all surface decontamination strategies. Therefore, there is a need to develop novel intervention strategies that have the potential to inactivate internalized viral pathogens while maintaining the quality of fresh produce.

Irradiation is a non-thermal processing technology that is one of the only known techniques that ensures the hygienic quality of raw foods in both the fresh and frozen states (Fan et al., 2008; Farkas, 1998a; Smith and Pillai, 2004; Tauxe, 2001). Ionizing radiation sources approved for food use include gamma rays, electron beams (E-beam), and x-rays (Fan et al., 2008; Farkas, 1998a; Smith and Pillai, 2004; Tauxe, 2001). Gamma irradiation (gamma rays) are released during isotopic negative beta decay of a radioactive isotope and currently both cobalt-60 and cesium-137 are approved sources of gamma irradiation for food use (Farkas, 1998a; Zhou et al., 2011). Gamma irradiation has the ability to penetrate completely through a product, so it has the potential to inactivate both surface pathogens and those found within the food matrix (De Roda Husman et al., 2004; Farkas, 1998a; Feng et al., 2011; House et al., 1990; Smith and Pillai, 2004). E-beams are generated by passing electrons through a high-voltage electrostatic field (Fan et al., 2008; Praveen et al., 2013; Predmore et al., 2015; Preuss et al., 1997; Sanglay et al., 2011; Smith and Pillai, 2004; Zhou et al., 2011). E-beam offers advantages over other sources of ionizing radiation such as 1) being able to be turned off when not in use, 2) it does not use radioactive isotopes, 3) it can be focused directly on a product, and 4) requires a shorter exposure time which results in high throughput processing (Fan et al., 2008; Farkas, 1998a; Smith and Pillai, 2004). However, the penetration depth of E-beam is limited, usually 3-10 cm depending on the food product and the energy of the accelerated electrons, which may limit its ability to inactivate pathogens found within the food matrix (Fan et al., 2008; Farkas, 1998a; Smith and Pillai, 2004).

Unfortunately, determining the efficacy of food processing technologies to inactivate human NoV has been difficult due to the absence of a cell culture system and the lack of a suitable small animal model. Since human NoV cannot be grown in cell culture, most laboratory efforts to study virus survival employ the use of viral surrogates, such as feline calicivirus (FCV), murine norovirus (MNV), and Tulane virus (TV). These surrogate viruses are closely related to human NoV in terms of genetic makeup, size, receptor-binding, pathogenicity, and environmental stability. Other surrogates used for the study of human NoV include virus-like particles (VLPs). Human NoV VLPs are non-infectious because they are composed only of capsid protein and lack the viral genome component of the native virus. While the use of surrogates has aided the understanding of human NoV survival following inactivation treatments, there are limitations in comparing data generated from the use of surrogates to human NoV (Richards, 2012).

Currently, nucleic acid-based detection methods are used to test for human NoV in foods. These methods cannot discriminate between RNA detected from an infectious or non-infectious viral particle, and therefore positive results may not accurately reflect the risk of disease development upon consumption. Recently, porcine gastric mucin conjugated to magnetic beads (PGM-MBs) has been developed to capture human NoV particles capable of binding to their cellular receptor, the histo-blood group antigens (HBGAs) (Dancho et al., 2012; Tian et al., 2005; Tian et al., 2008; Tian et al., 2010). Human NoV bound to PGM-MBs can then be collected, excluding particles which have lost receptor binding ability, and theoretically allow for RNA detection from only intact human NoV particles. This PGM-MB binding assay was found to detect reductions in human NoV GI.1 infectivity in oysters after HPP treatment similar to the results obtained from the human volunteer study (Dancho et al., 2012; Leon et al., 2011).

However, the validity of the PGM-MB binding assay to truly detect infectious virus necessitates further investigation. TV also utilizes HBGAs as cellular receptors (Farkas et al., 2010), so there is the potential to use this cultivatable virus as a side-by-side control to determine the ability of the PGM-MB binding assay to discriminate between infectious and non-infectious virus particles.

The objective of this study was to evaluate the use of ionizing radiation to inactivate a human NoV GII.4 strain and TV in simple media using direct RT-qPCR and the PGM-MB binding assay followed by RT-qPCR, and plaque assay as a comparison. In addition, the use of E-beam to inactivate internalized human NoV GII.4 and TV in strawberries was determined using the same methods.

2. Materials and Methods

2.1. Viruses and cell culture.

Tulane virus (TV) was a generous gift from Dr. Xi Jiang at Cincinnati Children's Hospital. TV was propagated in confluent monolayers of the monkey kidney cell line MK2-LLC (ATCC[®] no. CCL-7[™], Manassas, VA). MK2-LLC cells were cultured in low serum Eagle's minimum essential medium (Opti-MEM, Invitrogen), supplemented with 2% FBS, at 37°C under a 5% CO₂ atmosphere. For growing TV stock, MK2-LLC cells were washed with Hank's balanced salt solution (HBSS) and subsequently infected with TV at an MOI of 0.1. After 1 h incubation at 37°C, 15 ml of Opti-MEM with 2% FBS was added. The virus was harvested 2 days post inoculation and subjected to three freeze-thaw cycles, followed by centrifugation at 1000 × *g* for 30 min.

2.2. *Characterization of a human norovirus GII.4 strain.*

Human NoV clinical isolate 5M was originally isolated from an outbreak of acute gastroenteritis in Ohio. The stool samples were diluted 1:10 in PBS, shaken vigorously for 10 min at 4°C and centrifuged for 10 min at 5,000 × g. The sample was filtered through a 0.45-μm filter, aliquoted and stored at −80°C until use. The entire genomic cDNA of the human NoV strain 5M was amplified by RT-PCR using five to six overlapping fragments. The PCR products were then purified and cloned into a pGEM-T-easy vector (Promega), and sequenced at the Plant Microbe Genetics Facility at The Ohio State University. The full-length genome of the viral isolate was 7558 nt in length and has been deposited into GeneBank at accession number JQ798158. Sequence comparison found that the strain belongs to the norovirus genotype GII.4. The genomic RNA was then quantified by real-time RT-PCR and the GII.4 isolate 5M was found to have 6.7×10^6 genomic RNA copies/ml.

2.3. *Production and purification of human NoV virus-like particles (VLPs) in a baculovirus expression system.*

The capsid VP1 gene of a human NoV GII.4 strain (HS66) was amplified by high fidelity PCR and cloned into a pFast-Bac-Dual expression vector (Invitrogen)—which resulted in construction of the expression vector, pFast-Bac-Dual-VP1. Subsequently, pFast-Bac-Dual-VP1 was transformed into DH10Bac and the baculovirus expressing VP1 protein was generated by transfection of bacmids into *Spodoptera frugiperda* (Sf9) cells (ATCC® no. CRL-1711™, Manassas, VA) using a Cell-fectin Transfection kit (Invitrogen), according to the manufacturer's instructions. Human NoV VLPs were purified from insect cells as previously described with minor modifications (Ma and Li, 2011). Briefly, Sf9 cells were infected with baculovirus at a

MOI of 10, and the infected Sf9 cells and cell culture supernatants were harvested at 6 days post-inoculation. The VLPs were purified from cell culture supernatants and cell lysates by ultracentrifugation through a 40% (w/v) sucrose cushion, followed by CsCl isopycnic gradient (1.28-1.39 g/cm³) ultracentrifugation. Purified VLPs were analyzed by SDS-PAGE, Western blot, and electron microscopy. The protein concentration of the VLPs was determined using Bradford reagent (Sigma Chemical Co., St. Louis, MO).

2.4. Virus enumeration by plaque assay.

TV was quantified by plaque assay in LLC-MK2 cells. Briefly, cells were seeded into six-well plates (Corning Life Sciences, Wilkes-Barre, PA) at a density of 2×10^6 cells per well. After 24 h incubation, MK2-LLC cell monolayers were infected with 400 μ l of a 10-fold dilution series of TV and the plates were incubated for 1 h at 37°C with gentle agitation every 10 min. The cells were overlaid with 3 ml of Eagle minimum essential medium (MEM) containing 1% agarose, 2% FBS, 1% sodium bicarbonate, 0.1 mg of kanamycin/ml, 0.05 mg of gentamicin/ml, 15 mM HEPES (pH 7.7), and 2 mM L-glutamine. After incubation at 37 °C and 5% CO₂ for 2 days, the plates were fixed in 10% formaldehyde. The plaques were visualized by staining with 0.05% (w/v) crystal violet after removal of overlay. Viral titer was expressed as mean log₁₀ plaque forming unit (PFU)/ml $\pm$ standard deviation.

2.5. Enumeration of viral genomic RNA by real time reverse transcriptase PCR (RT-qPCR).

Since human NoV cannot be grown in cell culture, real-time RT-PCR was used to quantify viral genomic RNA copies. Briefly, total RNA was extracted from samples using an RNeasy Kit (Qiagen), followed by reverse transcription and real-time PCR. All primers and

probes were custom designed. First strand cDNA was synthesized by SuperScriptase III (Invitrogen) using the primer VP1-P1 (5'- TTATAATACACGTCTGCGCCC-3'), which targets the VP1 gene of human NoV. The VP1 gene was then quantified by real-time PCR using custom Taqman primers and probes (Forward primer: 5'-CACCGCCGGGAAAATCA-3') (Reverse primer: 5'-GCCTTCAGTTGGGAAATTTGG-3') (Reporter: 5'-FAM-ATTTGCAGCAGTCCC-NFQ-3') on a StepOne real-time PCR machine (Applied Biosystems, Foster City, CA). PCR reaction and cycling parameters followed the manufacturer's protocol (Invitrogen). Briefly, TaqMan Fast Universal Master Mix was used for all reactions. For cycling parameters, a holding stage at 95°C was maintained for 20 s prior to cycling, followed by 50 cycles of 95°C for denaturation, 1 s for annealing, and 60°C for 20 s for extension. Standard curves and StepOne Software v2.1 were used to quantify genomic RNA copies. Viral RNA was expressed as mean log₁₀ genomic RNA copies/ml ± standard deviation. TV RNA was also quantified by RT-qPCR using custom designed primers and probes. First strand cDNA was synthesized by SuperScriptase III (Invitrogen) using the primer TVRT (5'-AATTCCACCTTCAACCCAAGTG-3'), which targets the VP1 gene of Tulane virus. The VP1 gene was then quantified by real-time PCR using custom Taqman primers and probes (Forward primer: 5'-TTGCAGGAGGGTTTCAAGATG-3') (Reverse primer: 5'-CACGGTTTCATTGTCCCCATA-3') (Probe: 5'-FAM-TGATGCACACATGTGGGA-NFQ-3') on a StepOne real-time PCR machine (Applied Biosystems). PCR reaction, cycling parameters, and quantification method were identical to those used with human NoV.

2.6. Porcine gastric mucin conjugated magnetic bead binding assay.

Type III porcine gastric mucin (PGM) (Sigma, St. Louis, MI) was cross-linked to MagnaBind carboxyl derivatized beads (MBs) (Pierce Biotechnology, Rockford, IL) following the manufacturer's instructions. Briefly, 1 ml of MBs was washed 3 times with PBS using a magnetic separation rack (New England Biolabs, Ipswich, MA) for MB separation. Subsequently, 1 ml of 10 mg/ml Type III PGM in 0.1M 2-(N-morpholino) ethanesulfonic acid (MES) buffer (0.9% NaCl, pH 4.7) and 0.1 ml of 10 mg/ml 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) in MES buffer were added to the MBs and incubated for 30 min at room temperature rotating at 8 rpm. Following incubation, beads were isolated from the PGM solution using magnetic separation followed by washing 3 times with PBS. The PGM-MBs were re-suspended in 1 ml of PBS containing 0.05% sodium azide and stored at 4°C until use. The level of PGM binding to MBs was determined by measuring the initial protein concentration (absorbance at 280 nm) in the 10 mg/mL PGM solution and the protein concentration in the PGM solution removed from beads prior to washing. Following ionizing radiation treatment, 100 µl of untreated or treated viruses were combined with 100 µl of PGM-MBs and 800 µl PBS was added for a final volume of 1 ml. The virus-PGM-MB solution was incubated for 30 min at room temperature rotating at 8 rpm. The beads were separated and washed 3 times with PBS, and finally re-suspended in 100 µl of PBS for RNA extraction using the RNeasy kit (Qiagen). Human NoV and TV RNA levels were determined by RT-qPCR.

2.7. Transmission electron microscopy (TEM).

Twenty µl of ionizing radiation-treated or untreated VLPs were pipetted onto the surface of a square of parafilm. Next formvar film coated copper grids (Electron Microscopy Sciences, Inc., Hatfield, PA) were floated on top of the droplet for 10 min. The grids were then removed

from the droplet with fine tipped forceps and excess liquid was removed by gently tapping the edge of the grid on filter paper. The grid was then applied to a droplet of water for 10 s followed by floating on 1% ammonium molybdate (for negative staining) for 10 s. The samples were delivered to the Microscopy and Imaging Facility at The Ohio State University and visualized by a FEI Tecnai G2 Spirit transmission electron microscope (TEM) at 80 kV. Images were captured using a MegaView III side-mounted CCD camera (Soft Imaging Systems, Lakewood, CO) and figures were processed using Adobe Photoshop software (Adobe Systems, San Jose, CA).

2.8. E-beam treatment of human NoV VLPs.

Thirty μ l of human NoV VLPs was placed in a sterile polyethylene stomacher bag (Fisher Scientific, Pittsburg, PA) and heat sealed with an AIE-200 impulse sealer (American International Electric, Whittier, CA). A Dynamitron E-beam accelerator (Radiation Dynamics, Inc., Edgewood, NY) with a 120-cm exit aperture at 100Hz was used for treatments and was located at NEO Beam facility in Middlefield, OH. Samples were treated with 12 and 28 kGy under the conditions of 5.0 MeV, 7.5 kW, and 5.0 mA. Initial and final sample temperatures were measured with thermocouple attached to a portable data logger (model OM-3001, Omega Technologies Co., Stamford, CT) and to measure the absorbed irradiation dose, BioMax alanine dosimeter films (Eastman Kodak Co., Rochester NY) were placed on the top and bottom of sample bags. The concentration of free radicals produced by the electron irradiation in the alanine dosimeters was measured by ESR using a suitably calibrated Bruker e-Scan spectrometer. To calculate the absorbed dose in each sample, the average dose absorbed on both the top and bottom of the sample bag was used. The average measured dosages were 11.8 and 28.3 kGy. Following treatment, VLPs were visualized for structural damage using TEM and

assayed for receptor binding ability using PGM-MBs. Briefly, 6 µl of VLPs were added to 10 µl PGM-MB and 34 µl H₂O and incubated at room temperature for 30 min with gentle agitation to allow for binding. Next, PGM-MBs and bound VLPs were collected using a magnetic separator and washed 3 times with PBS. The PGM-MBs were then re-suspended in 10 µl PBS and the level of VLPs bound to the PGM-MBs was determined using SDS-PAGE.

2.9. E-beam treatment of viruses.

Human NoV and TV were diluted in sterile phosphate buffered saline (PBS) or Dulbecco's modified Eagle medium (DMEM) to achieve a titer of 1×10^6 RNA copy/ml. 500 µl of either human NoV or TV was placed in a sterile polyethylene stomacher bag (Fisher Scientific, Pittsburg, PA) and heat sealed with an AIE-200 impulse sealer (American International Electric, Whittier, CA). Samples were treated with 0, 2, 4, 6, and 8 kGy under the conditions of 5.0 MeV, 7.5 kW, and 1.5 mA. Treatments of 12, 16, 20, 24, and 28 kGy were under the conditions of 5.0 MeV, 7.5 kW, and 5.0 mA. Initial and final sample temperatures and absorbed E-beam dose were determined as described above. The actual measured dosages for target dosages of 2, 4, 6, 8, 12, 16, 20, 24, and 28 kGy were 2.3, 4.5, 6.8, 10.3, 11.9, 14.7, 19.5, 24.1, and 35.3 kGy, respectively. Following treatment, 100 µl of each virus was used for direct RNA extraction and the PGM-MB binding assay followed by RNA extraction, respectively. In addition 50 µl of TV was used to determine the infectious virus titer using plaque assay.

2.10. E-beam treatment of viruses in whole strawberry.

In order to make the dose as uniform as possible in whole strawberries two-sided irradiation was used. To determine the appropriate E-beam energy to deliver the best dose

uniformity ratio (DUR) to a whole strawberry fruit, a simulation was performed at different electron energies using the Monte Carlo Code PENELOPE. The strawberry was simulated as a solid sphere with a radius of 1.67 cm, an average mass of 19.7 g, and an average volume of 0.758 g/cm³, for which measurements were taken from experimental strawberries. The elemental composition of the strawberries used for the simulation was obtained from USDA nutritional composition database, with the composition of the strawberries being approximately 31.50% oxygen, 63.02% hydrogen, 0.11% nitrogen, and 1.23% carbon. The geometry used for the simulation was for a strawberry on a sheet of cardboard on top of an aluminum tray sitting 25 cm away from the titanium window of an electron accelerator. The parameters for the simulation included a point electron source with an aperture of 0.92° traveling in vacuum a distance of 187 cm and then traveling through the titanium window of an electron accelerator with a thickness of 60 µm. The simulation was performed to get one million showers of electrons for E-beam energies of 3.5, 4.0, and 4.5 MeV. The information collected from the simulations was the depth dose through the central axis of the E-beam trajectory, from the top surface of the strawberry to its bottom surface. Following the simulation, the depth dose data obtained was used to generate the depth-dose curve. A mirror image of the one-sided depth-dose curve was added to the original depth-dose curve to simulate a two sided irradiation. Then the maximum and minimum dose values from these graphs were used to determine the DUR. The appropriate E-beam energy was selected from the DUR value closest to a value of 1 for a whole strawberry fruit.

To determine the best initial energy for irradiating the strawberry, the DUR was obtained by using the equation:

$$DUR = \frac{D_{\max}}{D_{\min}}$$

Where $D_{\max}$ is the maximum dose and $D_{\min}$ is the minimum dose obtained from the depth-dose curve. Initial energies of 3.5, 4.0, and 4.5 MeV were used in the simulation and resulted in DURs of 4.34, 1.87, and 2.75, respectively. Based on the results from these simulations the required energy to treat whole strawberries with a diameter of 3.34 cm is 4 MeV. The dose uniformity ratio obtained at this energy from the simulation was 1.87. The simulation was followed by an experiment in which samples of strawberries were irradiated with a Dynamitron[®] electron accelerator at the NEO Beam Facility using the following parameters: 4.0 MeV, 1.5 and 5 mA to deliver doses to the strawberries of 4, 8, 12, 16, and 28 kGy.

Whole strawberries were purchased from a local grocery store in Columbus, OH. Strawberries were weighed and then placed in a biosafety cabinet under UV light for 15 min. Human NoV and TV were diluted in sterile phosphate buffered saline (PBS) to achieve a titer of 1×10^6 RNA copies/ml. 500 μ l of either human NoV or TV was injected into whole strawberries using a 1 ml syringe and needle. Alanine films were placed on top of the strawberries to measure the top surface dose. Strawberries were treated on one side at half target dosage and following first treatment turned over and new dosimeters were placed on top of the strawberries and were then treated again at half the target dosage. The dose administered to each side was measured using a Bruker e-scan alanine film dosimetry system and the final dosage was calculated by adding the dosages applied to each side of the strawberry. The actual measured dosages applied to the strawberries were determined to be 4.7, 9.8, 12.2, 16.3, and 28.7 kGy. Following treatment, the level of human NoV RNA was determined using the RT-qPCR and the PGM-MB binding assay followed by RT-qPCR. TV levels were determined using the same methods as human NoV in addition to standard plaque assay.

2.11. Gamma irradiation treatment of viruses.

Human NoV and TV were diluted in sterile phosphate buffered saline (PBS) to achieve a titer of 1×10^6 RNA copies/ml. 500 µl of either human NoV or TV was placed in a sterile polyethylene stomacher bag (Fisher Scientific, Pittsburg, PA) and heat sealed with an AIE-200 impulse sealer (American International Electric, Whittier, CA). Samples were treated at the Nuclear Reactor Laboratory (NRL) at The Ohio State University in a Cobalt-60 irradiator. Samples were irradiated at dosages of 2.8, 4.2, 11.2, 15.4, and 22.4 kGy. 100 µl of each virus was used for direct RNA extraction and PGM-MB binding assay followed by RNA extraction. 50 µl of TV was used for plaque assay.

2.12. Statistical analysis.

All experiments were performed in triplicate. Statistical analysis was performed by one-way ANOVA analysis using Tukey's family comparisons using Minitab 16 statistical analysis software (Minitab Inc., State College, PA). A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Effect of E-beam treatment on human NoV VLPs.

We first determined if E-beam treatment can damage virus particles using VLPs as a model because VLPs can be generated in large quantity *in vitro* and are structurally and antigenically similar to native human NoV. As shown in Fig. 1, untreated VLPs had a normal round structure and two different sizes of VLPs were observed, which has previously been described (Lou et al., 2012). Following 11.8 kGy of treatment, less intact VLP particles were

observed and the VLPs appeared aggregated (Fig. 1). 28.3 kGy of treatment showed mostly small particles and also an increase in debris (Fig. 1).

Using SDS-PAGE analysis, the VLP protein [viral protein 1 (VP1), cleaved form of viral protein 1 (cVP1)] detected decreased with E-beam treatment, however VP1 and cVP1 were still observed on the gel following 28.3 kGy of treatment (Fig. 2). Gel analysis averaging the band intensity of results from three independent experiments, found that treatment with 11.8 kGy of E-beam lead to an 84% reduction in the detection of VLP protein and increasing treatment to 28.3 kGy lead to a 90% reduction. The PGM-MB binding assay was used to determine the receptor binding ability of the remaining VLPs following E-beam treatment. All remaining VLPs were found to bind to PGM-MBs, with no significant difference in band intensity between the input (VLPs directly loaded to gel) and bound (VLPs bound to PGM-MBs and loaded to gel) VLPs following E-beam treatment (Fig. 2).

3.2. Human NoV inactivation by E-beam in simple media.

Using direct RT-qPCR the level of human NoV RNA detected in untreated control samples was $5.9 \pm 0.06 \log_{10}$ RNA copies/ml in PBS and $5.60 \pm 0.10 \log_{10}$ RNA copies/ml in DMEM (Fig. 3). In PBS, the level of human NoV RNA was reduced by 1 log following 10.3 kGy of treatment (Fig. 3). After 10.3 kGy of treatment, human NoV suspended in DMEM had a $<1\text{-log}_{10}$ reduction (Fig. 3). The level of RNA for human NoV in PBS remained relatively stable until 24.1 kGy of treatment where the level of viral RNA was reduced by 1.9-log_{10} RNA copies/ml (Fig. 3). Human NoV in DMEM was relatively stable at E-beam dosages between 11.9-24.1 kGy with approximately 5 logs of RNA being detected (Fig. 3). Following 35.3 kGy of E-beam treatment, the highest dosage applied, a 4-log_{10} reduction in RNA copies was detected

for human NoV suspended in PBS and a 2.32- $\log_{10}$ reduction in RNA copies was detected when the virus was suspended in DMEM at this treatment level (Fig. 3).

Because direct RT-qPCR can detect all viral RNA regardless of whether it is from infectious or non-infectious human NoV, we used the PGM-MB binding assay coupled with RT-qPCR which together detects RNA from receptor-binding human NoV particles. Human NoV RNA levels detected in untreated controls were $5.78 \pm 0.35 \log_{10}$ RNA copies/ml in PBS and $5.84 \pm 0.14 \log_{10}$ RNA copies/ml in DMEM (Fig. 3), similar to what has been observed for direct RT-qPCR ($p > 0.05$). This suggests that untreated human NoV particles bind efficiently to PGM-MB.

Only minimal reductions in human NoV receptor binding was detected at low dosages of E-beam (< 10.3 kGy) (Fig. 3). Following 11.9 kGy of E-beam treatment a 1.51- $\log_{10}$ RNA copy reduction was detected when the virus was suspended in PBS (Fig. 3). Significantly lower levels ($p < 0.05$) of human NoV RNA were detected when the virus was suspended in PBS at 19.5 and 24.1 kGy, with a 3.94- $\log_{10}$ RNA copy reduction and a 4.21- $\log_{10}$ RNA copy reduction detected following each treatment, respectively (Fig. 3). At 19.5 kGy of treatment the level of human NoV RNA detected in DMEM by the PGM-MB binding assay was reduced by 2.52- $\log_{10}$ RNA copies and at 24.1 kGy a 4.18- $\log_{10}$ RNA copy reduction was detected (Fig. 3). No human NoV RNA was detected using the PGM-MB binding assay followed by RT-qPCR at 35.3 kGy in either PBS or DMEM (Fig. 3).

3.3. TV inactivation by E-beam in simple medium.

Using plaque assay as the method of detection, the initial titer of TV suspended in PBS was $5.85 \pm 0.27 \log_{10}$ PFU/ml and in DMEM was $5.84 \pm 0.14 \log_{10}$ PFU/ml (Fig. 4A). At low

dosages a 0.5-2.5 $\log_{10}$ reduction in infectious TV was detected (Fig. 4A). At 10.3 kGy in PBS a 3.29- $\log_{10}$ PFU reduction in TV was detected and in DMEM a 3.08- $\log_{10}$ PFU reduction was detected (Fig. 4A). After 11.9 kGy of treatment of TV in PBS a 2.92- $\log_{10}$ PFU reduction was detected and in DMEM there was a 4.25 $\log_{10}$ PFU reduction in infectious virus (Fig. 4A). No infectious TV was recovered in either PBS or DMEM at treatment levels of 14.7-35.3 kGy (Fig. 4A).

In an attempt to correlate the infectivity and receptor binding capability, we also compared the RNA detection by two methods: PGM-MB binding assay coupled with RT-qPCR method and direct RT-qPCR. Using direct RT-qPCR $6.49 \pm 0.18 \log_{10}$ RNA copies/ml of TV in PBS was detected and $6.56 \pm 0.14 \log_{10}$ RNA copies/ml of TV in DMEM (Fig. 4B). Only after 10.3 kGy treatment was a 1-log reduction in TV RNA achieved when the virus was suspended in PBS (Fig. 4B). In DMEM, a 1-log reduction in TV RNA levels was achieved after 11.9 kGy of treatment (Fig. 4B). In PBS, the level of TV RNA following 24.1 kGy was reduced by 2.11- $\log_{10}$ PFU and in DMEM a 2.66- $\log_{10}$ reduction was detected (Fig. 4B). After the highest dosage of 35.3 kGy, 4.13- and 3.41- $\log_{10}$ RNA reductions were detected in TV suspended in PBS and DMEM, respectively (Fig. 4B).

Using the PGM-MB binding assay prior to RT-qPCR $6.19 \pm 0.15 \log_{10}$ RNA copies/ml was detected in PBS and $6.25 \pm 0.08 \log_{10}$ RNA copies/ml was detected in DMEM (Fig. 4B). After 6.8 kGy of treatment the RNA detected for TV suspended in PBS was reduced by 2.01- $\log_{10}$ RNA copies and in DMEM the level of RNA detected was reduced by 1.17- $\log_{10}$ RNA copies (Fig. 4B). The level of RNA detected using the PGM-MB binding assay was further reduced following 14.7 kGy of E-beam treatment by 3.59- $\log_{10}$ RNA copies and by 3.1- $\log_{10}$ RNA copies in PBS and DMEM, respectively (Fig. 4B). For TV in PBS following 19.5 kGy of

E-beam treatment a 4.57- $\log_{10}$ reduction of RNA was detected using the PGM-MB binding assay (Fig. 4B). After 19.5 kGy of treatment, no TV RNA was detected using the PGM-MB binding assay when the virus was suspended in DMEM (Fig. 4B). No TV RNA was detected using the PGM-MB binding assay at treatments of 24.1 and 35.3 kGy in either PBS or DMEM (Fig. 4B).

3.4. Human NoV inactivation in whole strawberries by E-beam.

The level of human NoV RNA detected in strawberries using direct RT-qPCR in untreated control samples was $5.78 \pm 0.19 \log_{10}$ RNA copies/g (Fig. 5A). There was no significant decrease in human NoV RNA detected following E-beam treatment in the strawberries using direct RT-qPCR and after 28.7 kGy of E-beam treatment (Fig. 5A). Using the PGM-MB binding assay prior to RNA detection with RT-qPCR, the level of human NoV RNA detected in the control strawberry was $5.53 \pm 0.39 \log_{10}$ RNA copies/g (Fig. 5A). Following 12.2 kGy of E-beam treatment, the level of human NoV RNA decreased by over 1 $\log_{10}$ RNA copy/g (Fig. 5A). E-beam treatment of 16.3 kGy resulted in a 2.46- $\log_{10}$ reduction in strawberries (Fig. 5A). Following 28.7 kGy of E-beam treatment, no human NoV RNA was detected in the strawberries using the PGM-MB binding assay prior to RT-qPCR (Fig. 5A).

3.5. TV inactivation in whole strawberries by E-beam.

In untreated strawberries the average titer of TV was $4.85 \pm 0.15 \log_{10}$ PFU/g (Fig. 5B). Following 9.8 kGy of treatment the titer was reduced by approximately 1 $\log_{10}$ PFU/g (Fig. 5B). Treatment with 12.2 kGy of E-beam resulted in a 2.09- $\log_{10}$ PFU reduction (Fig. 5B). No infectious virus was detected after a treatment level of 16.3kGy (Fig. 5B).

The average TV RNA level detected in untreated control strawberries was 5.57 ± 0.09 $\log_{10}$ RNA copies/g (Fig. 5C). No significant decrease in TV RNA was detected after any E-beam treatment dosage using direct RT-qPCR (Fig. 5C). Using the PGM-MB binding assay prior to RNA detection, the level of TV RNA detected in the control samples was 5.27 ± 0.14 $\log_{10}$ RNA copies/g (Fig. 5C). The level of RNA detected decreased by approximately 2 logs following both 12.2 kGy and 16.3 kGy of E-beam treatment (Fig. 5C). No TV RNA was detected in the strawberries using the PGM-MB binding assay prior to RT-qPCR after 28.7 kGy of E-beam treatment (Fig. 5B).

3.6. Human NoV inactivation by gamma irradiation.

Using direct RT-qPCR as the method of detection, 6.31 ± 0.30 $\log_{10}$ RNA copies/ml of human NoV RNA was detected in the untreated samples (Fig. 6A). The RNA level detected after gamma irradiation treatment was similar to the level of RNA in the untreated samples, even at the highest dosage of gamma irradiation applied (Fig. 6A). Using the PGM-MB binding assay prior to RT-qPCR, the level of human NoV RNA detected in the untreated samples was 5.02 ± 0.68 $\log_{10}$ RNA copies/ml (Fig. 6A). The level of RNA detected at 2.8 and 4.2 kGy was similar to the untreated samples (Fig. 6A). The level of RNA detected at 11.2 kGy was 1.58 ± 0.76 $\log_{10}$ RNA copies/ml and at 15.4 kGy was 1.51 ± 0.66 $\log_{10}$ RNA copies/ml, which was an approximate 3.5-log reduction, compared to the untreated samples (Fig. 6A). At 22.4 kGy of gamma irradiation treatment no human NoV RNA was detected using the PGM-MB binding assay (Fig. 6A).

3.7. TV inactivation by gamma irradiation.

Using plaque assay as the method of detection, at 0 kGy of treatment the viral titer was $6.41 \pm 0.17 \log_{10}$ PFU/ml (Fig. 6B). After 2.8 kGy of gamma irradiation treatment the viral titer decreased by 2 $\log_{10}$ PFU (Fig. 6B). After 11.2 kGy of treatment, only 1 replicate had positive detection of infectious virus at a low titer resulting in an average of $0.13 \pm 0.23 \log_{10}$ PFU/ml (Fig. 6B). At 15.4 and 22.4 kGy of treatment, no infectious virus was detected (Fig. 6B).

The use of the PGM-MB binding assay prior to RT-qPCR resulted in the detection of $5.58 \pm 0.37 \log_{10}$ RNA copies/ml of TV RNA in the untreated samples (Fig. 6C). There was an approximate 1-log reduction in RNA detected after 4.2 kGy of gamma irradiation treatment using the PGM-MB binding assay (Fig. 6C). After 11.2 and 15.4 kGy of treatment, the level of RNA detected was reduced by over 3 $\log_{10}$ RNA copies (Fig. 6C). After 22.4 kGy of treatment, no TV viral RNA was detected using the PGM-MB binding assay (Fig. 6C). Similar to the results for human NoV, direct RT-qPCR detected higher levels of TV RNA following gamma irradiation treatment (Fig. 6C).

4. Discussion

4.1. Comparison of TV infectivity as detected by plaque assay and TV receptor binding ability detected by the PGM-MB assay following ionizing radiation treatments.

PGM-MBs were initially used for the purpose of extracting human NoV from complex matrices such as foods and sewage in order to remove PCR inhibitors and enhance RT-qPCR detection (Tian et al., 2008; Tian et al., 2011; Tian et al., 2012). The idea that these PGM-MBs could be used to discriminate between infectious and non-infectious human NoV was demonstrated following HPP, UV, and thermal treatments (Dancho et al., 2012) and subsequently for treatments with chlorine, chlorine dioxide, peroxyacetic acid, hydrogen

peroxide, and trisodium phosphate (Kingsley et al., 2014). The results obtained using the PGM-MB binding assay to detect human NoV GI.1 inactivation by HPP was similar to results from the human volunteer study showing that HPP treatment at 600 MPa at 6°C for 5 min was sufficient to inactivate human NoV GI.1 in oysters and protect subjects from disease development (Dancho et al., 2012; Leon et al., 2011).

The mechanism of viral inactivation by ionizing radiation has been shown to include the disruption of virion structure (Feng et al., 2011; Predmore et al., 2015). Using E-beam treated human NoV VLPs as a model, it was found that increasing dosages of E-beam reduced the ability of human NoV VLPs to bind to PGM-MBs. Since E-beam impaired the receptor binding ability of human NoV VLPs, we used the PGM-MB binding assay prior to RT-qPCR detection of viral RNA to more accurately estimate human NoV infectivity following ionizing radiation treatment.

The PGM-MB binding assay followed by RT-qPCR was used to estimate the loss of infectivity of TV in comparison to plaque assay. Following E-beam treatment, the infectivity of TV suspended in PBS and DMEM was lost at 14.7 kGy, while approximately 3 logs of viral RNA was still detectable using the PGM-MB binding assay. However, at E-beam dosages of 2.3 and 4.5 kGy, there was good correlation between the log reductions observed using plaque assay and the PGM-MB binding assay. Similar results were observed for gamma irradiation-treated TV and E-beam-treated TV in whole strawberries, with TV RNA still detected using the PGM-MB assay at a condition sufficient to inactivate TV as detected by plaque assay. This correlation between TV infectivity and PGM-MB binding has also been observed when the virus was treated by HPP, in which there was good correlation between the two assays when the viral reduction was approximately 2 logs but above this level the results started to diverge (Li and Chen, 2015).

This may be due to the fact that ionizing radiation treatment damaged other portions of the capsid not involved in receptor binding, which leads to a loss of infectivity but not a loss of receptor binding. It is also possible that viral protein/RNA complexes interacted in a specific or non-specific manner with other components of the PGM which lead to increased detection of viral RNA using the PGM-MB assay compared to the infectivity detected by plaque assay. Finally, it is possible that the regions of the RNA genome damaged by ionizing radiation which rendered the virus non-infectious were not within the region of RNA amplified by RT-qPCR. This would also lead to an over estimation of viral survival using the RNA detection method. However, it can be stated that overall the PGM-MB binding assay is an improved method to estimate virus survival compared to direct RT-qPCR alone.

4.2. Human NoV GII.4 and TV inactivation by ionizing radiation.

Irradiation improves the shelf-life of raw foods and also the safety of foods as most microorganisms in their vegetative form are sensitive to low dosages of ionizing radiation (Fan et al., 2008; Farkas, 1998a; Smith and Pillai, 2004; Tauxe, 2001). E-beam treatment of a human NoV GII.4 strain at 35.3 kGy led to loss of detectable RNA following the PGM-MB binding assay, however 2-4 log₁₀ RNA copies/ml were detected using direct RT-qPCR when the virus was suspended in PBS or DMEM at this dosage. More significant reductions in human NoV RNA were detected using the PGM-MB binding assay compared to direct RNA detection in both medias, PBS starting at 10.3 kGy and in DMEM at 14.7 kGy (p<0.05). Human NoV and TV inactivation by E-beam was enhanced in PBS compared to DMEM. It has been demonstrated that more complex samples matrices impair the anti-viral effects of irradiation (Predmore et al., 2015; Sanglay et al., 2011).

In contrast to E-beam, gamma irradiation was able to abolish human NoV and TV receptor binding ability at 22.4 kGy using the PGM-MB binding assay, a lower dosage than required for E-beam. Treatment of 15.4 kGy of gamma irradiation was sufficient to inactivate TV as detected by plaque assay. Previously it was shown that MNV-1 suspended in DMEM required 24 kGy of gamma irradiation treatment for complete loss of infectivity (Feng et al., 2011). MNV-1 susceptibility to E-beam treatment has also been tested and 12 kGy lead to a 6.4-log reduction in infectious MNV-1 suspended in PBS and a 3.59-log reduction in DMEM (Sanglay et al., 2011). In an independent study, the titer of MNV-1 in PBS was reduced from $7.2 \pm 0.1 \log_{10}$ PFU/ml to $0.5 \pm 0.5 \log_{10}$ PFU following 16.9 kGy of E-beam treatment (Predmore et al., 2015). In another study, the D_{10} value for E-beam treatment of MNV-1 suspended in PBS was found to be 2.55 ± 0.45 kGy (Praveen et al., 2013).

Although gamma irradiation and E-beam are both sources of ionizing radiation, they differ in the way they are produced and the time it takes to achieve the desired dosage. For example, E-beam treatment typically takes seconds to achieve the required dosage, while gamma irradiation may take minutes to hours (Farkas, 1998b; Smith and Pillai, 2004). In this experiment, E-beam treatment of samples was conducted in a matter of seconds, whereas several hours (>24 h) was required to achieve the highest dosage of gamma irradiation (24 kGy). Therefore, the treatment duration may be contributing to the increase in efficacy of gamma irradiation to inactivate human NoV and TV compared to E-beam. This could lead to prolonged exposure of the virus to secondary products of radiolysis, such as hydroxyl (-OH) and oxygen free radicals, which may increase virus inactivation. Due to these differences, it is important to evaluate E-beam and gamma irradiation technologies independently as methods to inactivate viruses.

In previous studies, a protective effect of the food matrix on viral inactivation due to ionizing radiation has been observed (Feng et al., 2011; Praveen et al., 2013; Predmore et al., 2015; Sanglay et al., 2011). Different types of produce vary widely in their composition and structure. Data indicates that ionizing irradiation's inactivation of viruses in different types of produce varies depending on the type or variety of produce being treated and the source of ionizing radiation. In this study, the elemental composition, mass, density, and size of the strawberry were used in a computational simulation to determine the optimal energy for a dose uniformity ratio close to 1 using a two sided E-beam treatment of whole strawberries. Using this two sided irradiation would ensure that the E-beam dosage was more uniformly distributed throughout the whole strawberry as compared with a single side irradiation. It was found that the loss of infectivity and loss of receptor binding of human NoV and TV detected in strawberries following E-beam treatment was similar to those detected in simple media, indicating that using the two-sided irradiation and determining the DUR (dose uniformity ratio) improved the efficacy of E-beam treatment in whole strawberries.

The use of ionizing radiation in foods is regulated by FDA and is approved at different maximum dosages depending on the food product. The maximum dose for fresh lettuce and spinach is 4.0 kGy and for most other fruits and vegetables the maximum dose of 3.0 kGy (Fan et al., 2008; Farkas, 1998a; Smith and Pillai, 2004; Tauxe, 2001). Based on our findings, both TV and human NoV GII.4 are highly resistant to ionizing radiation, and in the food matrix a maximum of 1-log reduction in both viruses was detected at the approved level. Therefore, ionizing radiation is not a practical method to eliminate human NoV in soft fruits. It is likely that the small size and simple structure of human NoV makes it resistant to ionizing radiation. Also, at dosages higher than approved levels there were visual negative changes in the

strawberry, although no quantitative methods were used to determine sensory changes in the strawberry after E-beam treatment in this study.

Overall, our results indicate that (i) the PGM-MB binding assay coupled with RT-qPCR is an improved method for detecting inactivation of human NoV by ionizing radiation compared to direct RT-qPCR alone; (ii) a human NoV GII.4 strain and TV were resistant to E-beam and gamma irradiation treatment, and dosages well above the allowable limit for fresh produce were required for inactivation.

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FIGURE LEGENDS

Figure 1. Effect of E-beam treatment on human NoV VLPs evaluated by transmission electron microscopy (TEM). 30 µl of human NoV VLPs were treated with 12 and 28 kGy of E-beam. 10 µl of E-beam treated or untreated VLPs were fixed to copper grids and subsequently negatively stained with 1% ammonium molybdate. The samples were visualized by a FEI Tecnai G2 Spirit transmission electron microscope (TEM) at 80 kV at the Microscopy and Imaging Facility at The Ohio State University.

Figure 2. PGM-MB binding of human NoV VLPs following E-beam treatment analyzed by SDS-PAGE. 6 µl of VLPs were added to 10 µl PGM-MB and 34 µl H₂O and incubated at room temperature for 30 min with gentle agitation to allow for binding. PGM-MBs and bound VLPs were collected and re-suspended in 10 µl PBS. 6 µl of treated VLPs + 4 µl PBS (input), 10 µl PGM-MB conjugated VLPs (bound), and 10 µl of supernatant from collected PGM-MBs (unbound) were loaded to an SDS-PAGE gel.

Figure 3. Human NoV GII.4 inactivation by E-beam in PBS and DMEM. Human NoV was diluted in PBS (A) or DMEM (B). 500 µl of human NoV (1×10^6 RNA copies/ml) was treated

with 0, 2.3, 4.5, 6.8, 10.3, 11.9, 14.7, 19.5, 24.1, and 35.3 kGy. Following treatment, 100 µl of human NoV was used for direct RNA extraction and the PGM-MB binding assay followed by RNA extraction, respectively. Each point represents the mean of 3 replicates $\pm$ 1 standard deviation. Data points marked * were below the assay detection limit.

Figure 4. TV inactivation by E-beam in PBS and DMEM. TV was diluted in sterile PBS or DMEM. 500 µl of TV (1×10^6 RNA copies/ml) was treated with 0, 2.3, 4.5, 6.8, 10.3, 11.9, 14.7, 19.5, 24.1, and 35.3 kGy. (A) 50 µl of TV was used to determine the infectious virus titer using plaque assay. (B) Following treatment, 100 µl of TV was used for direct RNA extraction and the PGM-MB binding assay followed by RNA extraction, respectively. Each point represents 3 replicates $\pm$ 1 standard deviation. Data points marked * were below the assay detection limit.

Figure 5. Human NoV GII.4 (A) and TV (B, C) inactivation in whole strawberries by E-beam. Whole strawberries injected with human NoV (A) or TV (B,C) were treated with E-beam at dosages of 4.7, 9.8, 12.2, 16.3, and 28.7 kGy. 100 µl of each virus was used for direct RNA extraction and PGM-MB binding assay followed by RNA extraction. 50 µl of TV was used for plaque assay (B). Each point represents the mean of 3 replicates $\pm$ 1 standard deviation. Data points marked * were below the assay detection limit.

Figure 6. Human NoV GII.4 and TV inactivation by gamma irradiation. Human NoV (A) and TV (B,C) were diluted in sterile PBS to achieve a titer of 1×10^6 RNA copies/ml. 500 µl of either human NoV or TV were treated in a Cobalt-60 irradiator at dosages of 2.8, 4.2, 11.2, 15.4, and 22.4 kGy. 100 µl of each virus was used for direct RNA extraction and PGM-MB binding

777 assay followed by RNA extraction (A and C). 50 µl of TV was used for plaque assay (B). Each
778 point represents the mean of 3 replicates $\pm$ 1 standard deviation. Data points marked * were
779 below the assay detection limit.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6