Pulsed Light Inactivation of Murine Norovirus, Tulane virus, *Escherichia coli* O157:H7 and
Salmonella in Suspension and on Berry Surfaces

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Abstract:

Pulsed light (PL) inactivation of two human norovirus (HuNoV) surrogates, murine norovirus (MNV-1) and Tulane virus (TV), and two bacterial pathogens, *Escherichia coli* O157:H7 and *Salmonella*, were evaluated. The viruses and bacteria were suspended in phosphate buffered saline (PBS) to final populations of ~6 log PFU/mL and ~6 log CFU/mL, respectively. Both viral and bacterial suspensions were then irradiated by PL for different durations and the reductions of each microorganisms were determined. MNV-1 and TV were significantly ($P < 0.05$) more resistant to PL treatment than *Salmonella* and *E. coli* O157:H7 in PBS suspension. MNV-1, *Salmonella* and *E. coli* O157:H7 were also inoculated on strawberries and blueberries and the PL inactivation of each microorganism was determined. Lower inactivation of each microorganism was achieved on berry surfaces than in PBS suspension. This study shows that PL can induce rapid inactivation of MNV-1, TV, *Salmonella* and *E. coli* O157:H7 in clear suspension with viruses more resistant to PL treatment than bacteria. The efficacy of PL treatment is substantially influenced by food surface structure.

Key words: pulsed light; human norovirus; *Escherichia coli* O157:H7; *Salmonella*

1. Introduction

Human Noroviruses (HuNoV) are recognized as the leading cause of gastroenteritis. Research on HuNoV has to rely on proper surrogates since HuNoV is non-cultivable. Some common HuNoV surrogates include murine norovirus (MNV), feline calicivirus, and more recently, Tulane virus (TV) (Farkas et al., 2008). MNV shares many biological and molecular properties with HuNoV (Wobus et al., 2006). However, the limitations of MNV are that it differs in several aspects from HuNoV such as clinical manifestations, host receptors, pathogenesis, and infected cell types (Tan and Jiang, 2010). In addition, MNV uses sialic acid as a functional receptor (Taube et al., 2009), whereas HuNoVs use histo-blood group antigens (HBGAs) as cellular receptors and infect digestive epithelial cells *in vivo* (Tan and Jiang, 2005; Tan et al., 2008). Genomic sequence analysis showed that TV was closely related to HuNoV (Farkas et al., 2010). More importantly, similar to HuNoV, TV recognizes type A and B HBGAs as receptors for infection (Farkas et al., 2010). Many groups have studied the resistance of TV to a range of chemical and physical treatments such as chlorine, UV irradiation, ethanol, pH, heat and high pressure (Hirneisen and Kniel, 2013; Li et al., 2013; Tian et al., 2013). However, to our knowledge, study of pulsed light (PL) inactivation of TV has not been reported. PL is effective against a broad spectrum of microorganisms including bacteria (Rowan et al., 1999; Woodling and Moraru, 2007), viruses (Belliot et al., 2013; Lamont et al., 2007; Roberts and Hope, 2003), and spores (Jun et al., 2003). In the US, the use of PL technology was approved by the U.S. Food and Drug Administration in 1996 for decontamination of food or food contact surfaces (21CFR179.41).

Berries have been frequently involved with outbreaks of foodborne pathogens in recent

years. Frozen berries have caused multiple large-scale HuNoV outbreaks in 2005 and 2009 (Friedman et al., 2005; Sarvikivi et al., 2012). In 2012, the largest foodborne outbreak in Germany was associated with HuNoV-contaminated frozen strawberries, which affected more than 11,000 people (Mäde et al., 2013). In 2011, fresh strawberries from a farm in Oregon were linked with an *E. coli* O157:H7 outbreak, which caused at least 15 cases of sicknesses and two deaths (Laidler et al., 2013). In 2009, a multistate outbreak of *Salmonella* Muenchen was reported due to consumption of blueberries, which caused 14 cases of illnesses (CDC, 2009).

The objective of this study was to assess the PL inactivation of TV, MNV-1 and two bacterial pathogens (*Salmonella* and *E. coli* O157:H7) in phosphate buffered saline (PBS) and on strawberries and blueberries. This is the first study describing the use of PL to inactivate TV. The potential application of this nonthermal technology was evaluated.

2. Materials and Methods

2.1. Viruses and cell lines

The detailed information regarding the MNV strain (MNV-1), murine macrophage cell line RAW 264.7, and the growing of MNV-1 was provided in our previous publication (Huang and Chen, 2015). The concentration of virus was performed using the method described by Hwang et al. (2014) with minor modifications. Briefly, the virus was concentrated by centrifugation at $95000 \times g$ for 3 h at 4 °C in a Surespin 630 (36 ml) rotor (Thermo Scientific). The pellet was mixed with 0.5 mL PBS (pH 7.2) and incubated overnight at 4 °C. The pellets were then resuspended in PBS and pooled together. Each tube was rinsed with 0.5 mL PBS and the rinsate was combined with previously pooled

concentrated MNV stock to get a final titer of ~8.7 log PFU/mL.

The detailed information regarding the TV strain (MNV-1), monkey kidney cell line LLC-MK2, and the growing of TV was provided in our previous publication (Li et al., 2013). TV was concentrated following the methods described by Farkas et al. (2008) with minor modifications. Briefly, TV was precipitated by mixing equal volume of 20% polyethylene glycol 8000 (PEG) (Fisher Scientific) and 0.6 M NaCl stock solution with viral suspension and incubated at 4 °C overnight. Polyethylene glycol precipitate was collected by centrifugation at $10,000 \times g$ for 45 min and resuspended in 1/30 of the original culture volume of PBS to obtain a viral titer of ~6.7 log PFU/mL.

2.2. Bacterial strains and inoculum preparation

E. coli O157:H7 strain 251 (lettuce outbreak isolate) and *Salmonella enterica* serotype Newport H1275 (sprout outbreak isolate) were used. The preparation and growing of nalidixic acid-resistant mutants of the strains were described in our previous publication (Huang et al., 2013). Bacterial cells were harvested by centrifugation at $3000 \times g$ for 15 min (Sorvall ST16 R, Thermo Scientific). The supernatant was discarded and the pellet was resuspended in 1 mL of sterile PBS to yield a final concentration of $\sim 10^8$ CFU/mL.

2.3. PL treatment

The PL treatments were performed with a laboratory scale PL system (Steripulse-XL RS-3000, Xenon Corp., Wilmington, MA). Information about the PL system and measurement of PL energy received by samples was provide in our previous publication (Huang and Chen, 2014). For PL treatment in suspension, virus stocks and bacterial suspensions were diluted in PBS to final populations of ~6.0 log PFU/mL and ~6.0 log

CFU/mL for viruses and bacteria, respectively. Viral or bacterial suspensions (2 mL) were placed in a 35-mm sterile petri-dish, which was centered at the bottom of the PL chamber. The samples were irradiated with PL for 1-16 pulses at distances of 16 cm from the quartz window. After PL treatments, populations of the viral and bacterial samples were determined as described in our previous publications (Huang and Chen, 2015; Li et al., 2013). For PL treatments on berry surfaces, fresh strawberries and blueberries were illuminated with UV-C (254 nm) in a biosafety hood for at least 10 min to lower the load of natural background microbiota. Blueberries (~5g/sample) and strawberry halves (~10g/sample) were then spot-inoculated in multiple small droplets on the skin (avoiding the calyx) with 150 μ L suspension of *Salmonella*, *E. coli* O157:H7 or MNV-1. Samples were then air-dried in a biosafety hood for 2 h at 22 ± 2 °C to facilitate bacterial and viral attachment. Inoculated berries were irradiated by PL for 6, 12 or 24 s.

2.4.Extraction of MNV-1 from berries

MNV-1 was extracted from the berries as described by Dubois et al. (2002) and Kingsley et al. (2005) with some modifications. Individual berry samples were transferred into a 50 mL conical tube containing 10 mL of vegetable extraction buffer (Huang and Chen, 2015) and 50 μ L pectinase (Sigma-Aldrich, St Louis, MO). To release viruses from berries, each tube was vortexed for 5 times and each pulse lasted for 5 s on a Vortex Genie 2 vortexer (Scientific industries, Bohemia, NY) with speed set at 8-9. The liquid portion was transferred to a 15 mL tube and centrifuged at $3000 \times g$ for 10 min at 4 °C. The supernatant was serially diluted in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies Corporation) and used for plaque assays as described by Huang and Chen (2015).

2.5. Bacterial recovery and enumeration

After PL treatment, berry samples were transferred into sterile filter bags (Whirl-Pak, Nasco, USA) containing 40 mL (for blueberry samples) or 45 mL (for strawberry sample) of Dey-Engley (D/E) neutralizing broth (Difco). The samples were pummeled in a laboratory stomacher (Seward 400, Seward, London, U.K.) for 2 min at 260 rpm. The homogenate was serially diluted in sterile 0.1% peptone water and surface-plated on tryptic soy agar (Difco Laboratories, Sparks, MD) supplemented with 0.6% yeast extract (Difco) and 50 µg/mL of nalidixic acid (Fisher Scientific) (TSAYE-N). Presumptive colonies of *E. coli* O157:H7 or *Salmonella* formed on the plates were counted after 72-h incubation at 35 °C.

2.6. Statistical analysis

At least three independent trials were conducted for all the experiments. Colony counts and plaque counts were converted to log reductions and standard deviations were calculated. Statistical analyses were conducted using JMP (SAS Cary, NC, USA). One-way analysis of variance and Tukey's one-way multiple comparisons were used to determine significant differences among treatments ($P < 0.05$).

3. Results

3.1. Inactivation of MNV-1, TV, *E. coli* O157:H7 and *Salmonella* in PBS by PL

An increasing reduction of MNV-1, TV, *E. coli* O157:H7 and *Salmonella* in PBS was observed as the treatment duration or fluence of PL increased (Table 1). The *E. coli* O157:H7 strain was significantly more sensitive to PL treatment than the *Salmonella* strain ($P < 0.05$). No significant difference ($P > 0.05$) in the PL resistance of MNV-1 and TV was observed after 1-pulse of PL treatment; however, TV showed significantly higher ($P < 0.05$) resistance

to PL treatment during longer PL treatments (2-8 pulses). Overall, comparing to the two pathogenic bacteria, the two viruses showed significantly higher resistance to PL treatments ($P < 0.05$).

3.2. Inactivation of MNV-1, *E. coli* O157:H7 and *Salmonella* on berry surfaces by PL

The titer of MNV-1 on strawberry was reduced by 0.7-0.9 log units after 6-24 s of PL treatments (Table 2). Increasing PL fluence from 5.9 to 22.5 J/cm² did not significantly induced higher reduction of MNV-1 on strawberries ($P > 0.05$). Similar results were also observed for the inactivation of *Salmonella* and *E. coli* O157:H7 on strawberries as there was no significant difference ($P > 0.05$) between PL treatments at different fluence levels (5.9-22.5 J/cm²). Compared with *Salmonella* and *E. coli* O157:H7, MNV-1 showed significantly higher resistance to PL treatment on strawberries ($P < 0.05$). On blueberries, MNV-1 was reduced by 3.1-3.8 log units after PL treatments at fluence of 5.9-22.5 J/cm², which were significantly higher ($P < 0.05$) than those on strawberries. Similarly, the reductions of *E. coli* O157:H7 and *Salmonella* were significantly higher ($P < 0.05$) on blueberries than strawberries. On blueberries, no significant difference ($P > 0.05$) in inactivation was observed between MNV-1 and *Salmonella*; however, MNV-1 was more resistant ($P < 0.05$) to PL treatment than *E. coli* O157:H7 at PL fluence of 11.4 and 22.5 J/cm².

4. Discussion

In the present study, both *E. coli* O157:H and *Salmonella* could be effectively inactivated by > 5.8 log CFU/mL after only two light pulses (0.62 J/cm²) in clear PBS suspension. Sauer and Moraru (2009) reported that PL could effectively inactivate *E. coli* O157:H7 from liquids

with different levels of clarity. In their study, *E. coli* O157:H7 was reduced by 5.76 and 7.15 log in turbulent apple cider and juice, respectively, after a PL treatment at 12 J/cm². It should be noted that in general, PL shows higher efficacy in inactivating bacterial cells in clear media than in opaque media.

At fluence of 2.47 J/cm² and 4.94 J/cm², PL treatments resulted in > 5.8-log reduction of MNV-1 and > 6-log reduction of TV, respectively. The inactivation of enveloped and nonenveloped viruses in PBS by PL treatment was investigated by Roberts and Hope (2003). A total dose of 1 J/cm² is sufficient to inactivate 4.8-7.2 log units of viruses. Tian et al. (2013) reported a > 4 log reduction in TCID₅₀ of TV when the virus was exposed to 60 and 70 mJ/cm² of UV-C radiation. The authors also reported that TV was seemingly more resistant than MNV, which was in agreement with our result. Since the actual inactivation of HuNoV by PL is unknown, it is impossible to know which virus, MNV-1 or TV, would be a better surrogate for HuNoV in PL inactivation studies. Since it was difficult to grow TV in cell culture to a high titer (> 7 log PFU/mL), MNV-1 was used for the challenge study in berries and compared with *Salmonella* and *E. coli* O157:H7.

While PL was highly effective at reducing both viruses and bacteria in PBS suspension, its inactivation effectiveness varied with different topographies of berry surface and microorganisms (Table 2). In general, PL treatment at fluence of 5.9, 11.4 and 22.5 J/cm² were not significantly different in reducing microorganisms on strawberries, which suggested that shielding effect is the main factor that limit the inactivation. For blueberries, significantly higher reductions were only observed for one of the three microorganisms, *Salmonella*, when PL fluence was increased from 5.9 J/cm² to 22.5 J/cm². Similar to results shown in Table 1,

MNV-1 showed higher resistance to PL treatment than *Salmonella* and *E.coli* O157:H7 on strawberries for PL treatments at 11.4 and 22.5 J/cm² . However, the inactivations of MNV-1 and *Salmonella* were not significant different ($P > 0.05$) on blueberries. In addition, higher reductions of microorganisms were observed in blueberries than strawberries. Compared to the smooth skin of blueberries, the presence of achenes of strawberries could potentially shield microorganisms from the highly directional, coherent PL beam from reaching its target, leading to partial decontamination. Similar results have also been observed in some previous studies (Huang and Chen, 2014, 2015). Kim and Hung (2012) observed a persistent higher population of *E. coli* O157:H7 recovered from the blueberry calyx than from the skin after UV treatment. Woodling and Moraru (2005) investigated the effect of surface topography on PL inactivation using stainless steel of different surface roughness. Their results showed that the inactivation levels for the two smoothest surfaces were significantly higher than those for the two roughest surfaces.

In conclusion, our results indicated that PL was a powerful tool for rapid decontamination of viruses and bacteria. Complete inactivation of TV, MNV-1, *Salmonella* and *E. coli* O157:H7 were achieved in clear liquid within seconds. As a nonthermal technology, PL can be used as a promising alternative to thermal treatment for processing of clear liquids. In PBS suspension, MNV-1 and TV were more resistant to PL treatment than *Salmonella* and *E. coli* H157:H7. Food matrices reduced the effectiveness of PL treatment for both bacteria and viruses. At the current legal fluence level (21CFR179.41), PL treatment cannot completely eliminate MNV-1, *Salmonella* and *E. coli* O157:H7 on berries. Further assessments are required for the application of PL technology for decontamination of foods..

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Table 1. Inactivation of MNV-1, TV, *E.coli* O157:H7 and *Salmonella* in PBS by PL. The viruses and bacteria suspended in PBS were treated by PL for up to 16 pulses. Data represent mean of at least three replicates $\pm$ one standard deviation.

Treatment (number of pulses)	Fluence (J/cm ²)	Log reduction			
		MNV-1	TV	<i>E.coli</i> O157:H7	<i>Salmonella</i>
1	0.31	1.3 $\pm$ 0.1Dc	1.0 $\pm$ 0.1Dc	5.1 $\pm$ 0.3Ba	3.2 $\pm$ 0.0Bb
2	0.62	2.4 $\pm$ 0.1Cb	1.7 $\pm$ 0.2Dc	>6.2Aa	>5.8Aa
4	1.23	4.4 $\pm$ 0.3Ba	3.0 $\pm$ 0.5Cb	N/A	N/A
8	2.47	>5.8Aa	4.9 $\pm$ 0.1Bb	N/A	N/A
16	4.94	>5.8A	>6.0A	N/A	N/A

Data in the same column followed by the same uppercase letter are not significantly different ($P > 0.05$).

Data in the same row followed by the same lowercase letter are not significantly different ($P > 0.05$).

Detection limit for the bacterial plating assay is 1 CFU/mL.

Detection limit for the virus plaque assay is 1.25 PFU/mL.

Table 2. Inactivation of MNV-1, *E.coli* O157:H7 and *Salmonella* on strawberries and blueberries by PL. Berries spot-inoculated with one of the microorganisms were treated by PL for up to 24 s. Data represent mean of at least three replicates $\pm$ one standard deviation.

Microorganism	Treatment (s)	Fluence (J/cm ²)	Log reduction	
			Strawberry	Blueberry
MNV-1	6	5.9	0.7 $\pm$ 0.0Aa	3.1 $\pm$ 0.3Ab
	12	11.4	0.9 $\pm$ 0.2Aa	3.2 $\pm$ 0.3Ab
	24	22.5	0.9 $\pm$ 0.2Aa	3.8 $\pm$ 0.6Ab
<i>E. coli</i> O157:H7	6	5.9	1.5 $\pm$ 0.6Aa	5.4 $\pm$ 1.7Ab
	12	11.4	1.6 $\pm$ 0.3Aa	5.6 $\pm$ 1.4Ab
	24	22.5	1.9 $\pm$ 0.3Aa	5.7 $\pm$ 1.2Ab
<i>Salmonella</i>	6	5.9	1.6 $\pm$ 0.5Aa	3.4 $\pm$ 0.3Bb
	12	11.4	1.8 $\pm$ 0.2Aa	3.8 $\pm$ 0.1ABb
	24	22.5	2.1 $\pm$ 0.4Aa	4.2 $\pm$ 0.2Ab

Data in the same column and same microorganism category followed by the same uppercase letter are not significantly different ($P > 0.05$).

Data in the same row followed by the same lowercase letter are not significantly different ($P > 0.05$).

Detection limit for the bacterial plating assay is 50 CFU/sample.

Detection limit for the virus plaque assay is 12.5 PFU/sample.