CENTER for PRODUCE SAFETY

CPS 2020 RFP
FINAL PROJECT REPORT

Project Title
Survival of infectious human norovirus in water and on leafy greens

Project Period
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Objectives

1. Determine the survival of infectious HuNoV in water and in relation to generic E. coli.

2. Determine the pre-harvest survival of infectious HuNoV on leafy greens.

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FINAL REPORT

Abstract
In the US, the majority of foodborne illnesses (58%) are caused by human norovirus (HuNoV). Lettuce and other leafy greens are most often implicated in the HuNoV outbreaks. Lettuce is the most popular salad vegetable and is almost exclusively used fresh, with minimal chlorine washes postharvest. The presence of HuNoV RNA in river water used for irrigation has been demonstrated in numerous studies worldwide. However, in the US, under the current Produce Safety Rule, agricultural water applied to produce is not required to be tested for viruses, thus overlooking a potential threat to the produce industry. The produce rule requires agricultural water to be tested for generic *E. coli* which may not predict the levels of HuNoV. Because HuNoV is difficult to grow in cell culture, limited data exist on the actual **infectious** viruses in irrigation water, or on pre-and postharvest lettuce. However, recently a new cell culture system for HuNoV, based on human intestinal enteroid (HIE) cells derived from 3D cultures of intestinal stem cells, has been reported. Therefore, the objectives of this study were to determine (i) the survival of infectious HuNoV in spiked river water and its correlation to *E. coli* and (ii) the survival of infectious virus on spiked lettuce under preharvest conditions. Water was sampled from multiple surface freshwater ponds and used to set microcosms in which HuNoV, Tulane virus (TV, a norovirus surrogate), hepatitis A virus (HAV) and *E. coli* were spiked. The microcosms were incubated under conditions similar to preharvest environments and sampled for a period of 14 days (7 days for norovirus). In addition, greenhouse-grown lettuce was spiked with HuNoV, TV and HAV and incubated for 7 days inside an environmental chamber under conditions mimicking preharvest. The infectivity of HuNoV and its RNA titers were determined using HIE and real-time qPCR. The infectivity of HAV and TV was determined using their respective cell lines. The total bacteria as well as the physiochemical properties of water were quantified. Our results indicate that infectious HuNoV was detectable in water for at least 7 days. TV and HAV were still detectable in water on day 14. Human norovirus significantly correlated with total bacteria in the water and percent dissolved oxygen but not with *E. coli* counts. On lettuce, infectious norovirus and HAV were detectable for at least 7 days whereas TV was undetectable on day 7. Therefore, HuNoV in freshwater and on lettuce under preharvest conditions was shown to persist as infectious virus for many days, highlighting the need to safeguard our food and water resources against viral contamination.

Background
Globally, human norovirus (HuNoV) is the leading cause of acute gastroenteritis, causing 1 in 5 of all diarrhea cases. These viruses are small (28-35 nm in diameter), non-enveloped, single stranded RNA viruses. Since the discovery of human norovirus in 1970 until recently, there was a lack of a cell culture system for this virus, which hampered research into anti-virals and vaccine development. Also, investigations of effective measures to prevent and control the virus in food and water were largely performed on surrogate viruses. However, some of these surrogate viruses (such as feline calicivirus and murine norovirus) were found not to mimic HuNoV in many aspects. Therefore, there are many knowledge gaps regarding the survival, transmission, detection and control of infectious norovirus in the environment and in food.

In the US, HuNoV is one of the leading causes of foodborne outbreaks. The majority of these foodborne outbreaks are epidemiologically linked to lettuce. Contamination of lettuce can occur through a number of sources, including irrigation water. Irrigation water can be derived from both surface (rivers, streams, irrigation ditches, canals, reservoirs, and lakes) and ground (well) water. Many studies have shown the presence of HuNoV RNA in river, groundwater, private wells, municipal water and even ice. These water sources can become contaminated with the virus,

resulting in large waterborne norovirus outbreaks. However, the correlation between routine fecal indicator *E. coli* and infectious HuNoV is poorly understood, and therefore standard approaches to asses water contamination might not be sufficient. The produce safety rule requires irrigation water to be tested for generic *E. coli* which can change dramatically from day to day and may not predict the levels of HuNoVs. Also, in the environment many factors might influence the survival of enteric viruses in water, including temperature, light (UV), salts, organic matter, suspended solids, and indigenous microflora. Therefore, there is a knowledge gap in our understanding of the survival of infectious human in surface freshwater and whether *E. coli* can be used as an indicator for the virus.

Globally, lettuce is the most popular salad vegetable, and it is almost exclusively used fresh, with minimal washing after harvest. Many studies have shown that irrigation water can be a source of human viral pathogens on produce. In addition, in the US, lettuce and other leafy greens were most often implicated in human norovirus foodborne outbreaks. Contamination of leafy greens with HuNoVs can occur at any stage along the farm-to-fork chain via a number of sources, including fecally contaminated water used for irrigation. Again, there is a knowledge gap in our understanding of survival of infectious human norovirus on lettuce under preharvest conditions.

In 2016, HuNoV was reported to successfully replicate in human intestinal enteroid (HIE) cells derived from human intestinal stem cells. This breakthrough was possible because of the advances in organoid systems where mini-human gut can be grown *in vitro*. In the HIE system, the titer of HuNoV can increase over 1000-fold in 3 days, indicating the presence of infectious virus. Therefore, the objectives of this study were to adapt the use of HIE cell culture of human norovirus to (i) Determine the survival of **infectious** HuNoV in water and in relation to generic *E. coli* and (ii) Determine the pre-harvest survival of **infectious** HuNoV on leafy greens.

Research Methods and Results

Methods:

Mature lettuce plants were inoculated with a human norovirus (GII.4, obtained from our collaborators at the CDC) shown to replicate in the HIE. The virus was spot inoculated on lettuce leaves and allowed to dry. The plants were then incubated in an environmental chamber at 20–15°C and 50–80% relative humidity (day-night) and 12-h photoperiod. Hepatitis A virus (HAV) and Tulane virus (TV), a human norovirus surrogate, were also inoculated on lettuce. HuNoV, HAV and TV were monitored on lettuce leaves on day 0, 1, 3, and 7. On the day of sampling, small areas marked as virus-inoculated on lettuce leaves were aseptically removed from the plants. The lettuce leaves were cut into smaller pieces that were transferred into sterile tubes containing cell culture media supplemented with fetal bovine serum. The lettuce pieces were vortexed to recover the viruses. Then, all the liquid media was collected and centrifuged at 4000 rpm for 10 min at 4°C. The supernatants were transferred to new tubes and stored at -80°C until tested.

HuNoV, HAV, TV and *E. coli*, a water quality indicator, were spiked into separate freshwater microcosms. The *E. coli* strain used is an environmental isolate previously isolated from pond water. All the microcosms were incubated under similar conditions as the lettuce plants. Viruses were recovered from spiked water following centrifugation at 4000 rpm for 10 min at 4°C, filtration through low-binding 0.45-µm filters, and finally ultrafiltration through 100 KDa Amicon ultra centrifugal filter units. The concentrated viruses retained inside the filter units were transferred to new tubes and stored at -80°C until tested.

The water was tested for infectious HuNoV on day 0, 1, 3 and 7, and for TV, HAV, *E. coli* and general bacteria on day 0, 1, 3, 7 and 14. Infectivity for HuNoV was tested on HIE (as described

below) and for HAV and TV on FRhK-4 and LLC-MK2 cell, respectively. In addition, HuNoV RNA was quantified using RNase-treatment followed by qPCR. General bacteria and *E. coli* were tested on R2A agar and RAPID *E. coli* 2 agar. Water quality parameters such as turbidity, dissolved oxygen, total dissolved solids, salinity, and conductivity were monitored using a Hanna multiparameter waterproof meter.

HIE cultures were grown as undifferentiated 3-dimensional (3D) cultures on Matrigel inside 24-well plates in CMGF- media (Advanced DMEM/F12 mixed with 200mM GlutaMax-I, 100X Pencillin/Streptomycin and 1M HEPES) supplemented with 10 µM Y-27632. This media was replaced every 2 days. On day 7, the 3D cultures were split 1:2 and re-cultured. This entire process was repeated for a month before the cells could be used to generate differentiated monolayer for HuNoV infection assay. Specifically, 3D cells were dissociated from Matrigel into single cell suspension using 0.05% Trypsin/0.5 mM EDTA in PBS. The cells were plated at 1-2x 10^5 cells/well as undifferentiated monolayers in 96-well plates pre-coated with collagen. After 24h, the media was replaced with differentiation media made of CMGF- mixed with 1X B27 supplement, 1X N2 supplement, 1mM N-Acetyl-cysteine, 0.05 µg/ml mouse recombinant EGF, 5µM A-83-01, 0.01 µM [Leu15]-Gastrin I and 1x Noggin conditioned media. After 5 days, the monolayers are formed and differentiated and can be used for HuNoV infection assay. Samples to be tested were inoculated on the monolayer (3 wells/sample) and incubated for 1h at 37°C. After 1h, the inoculum was removed and the monolayer washed with CMGF- media, followed by incubation in differentiation media supplemented with 50 mM GCDCA and 50mM Ceramide. A duplicate plate was frozen immediately to determine time zero titer with RT-QPCR and another plate was frozen following 3 days of incubation. Samples positive for infectious HuNoV will show higher RNA titer on day 3 plates as compared to time zero.

All experiments were independently repeated two times with three technical replicates. The entire data set was transformed to $\log_{10}$. Means and standard errors were calculated from all technical replicates. The $\log_{10}$ reductions were calculated by subtracting virus titers of all technical replicates for a specific microorganism from the average titer on day 0. GraphPad Prism version 5 (Graph Pad Software, USA) was used for all statistical analyses.

Results:
HuNoV recovered from lettuce leaves on day 1, 3 and 7 was found to replicate in HIE, indicating the presence of infectious viruses. On day 0, infectious TV was recovered at ~5 log from lettuce and showed ~1.7 and 3.5 log reduction in infectivity on day 1 and day 3, respectively. TV was not detectable on or beyond day 7. In contrast, infectious HAV was recovered at ~5.5 log from lettuce on day 0 and showed ~0.7 and 2 log reduction in infectivity on day 1 and day 3, respectively. Infectious HAV was detectable on lettuce on day 7 but showed a 4-log reduction in infectivity.

In water, the recovered HuNoV was found to replicate in HIE on day 0, 1, 3 and 7, indicating the presence of infectious viruses. HuNoV RNA showed <1 log decrease by day 14, as compared to day 0. Both TV And HAV were still detectable on day 14. Specifically, on day 0, infectious TV was recovered at ~6.2 log from water but the titer was reduced by 3.5 log on day 14. In contrast, HAV was recovered at ~6.4 log from water but the titer was reduced by only 2.2 log at day 14. The general bacteria (~5.5 log CFU/100ml on day 0) started to increase on day 3 and by day 14 showed 1 log increase. In contrast, *E. coli* showed gradual decline from 2 log to 5 log on day 3 and day 14, respectively. Significant negative correlations were found between HuNoV and the total bacteria count in water. Significant positive correlations were found between HuNoV and the percent dissolved oxygen. Also, significant correlations were found between log reduction in *E. coli* count and Tulane virus, but no other correlations were found between *E. coli* and HuNoV or HAV over a period of 14 days.

Outcomes and Accomplishments
The human intestinal enteroid (HIE) model is a sophisticated cell culture system for human norovirus that is resource and labor intensive, however, we were able to successfully implement this cell culture model. This model is still dependent on the availability of human fecal samples positive for human norovirus. We were successful in obtaining multiple fecal samples to screen on HIE from various collaborators in the US and abroad.

A protocol for the recovery of infectious human norovirus from lettuce and from surface freshwater containing natural microflora was successfully optimized using the HIE model.

Infectious human norovirus was found to be present for at least 7 days in water and on lettuce under preharvest conditions. However, the limited duration of this study is due to limitations in the availability of fecal samples positive for HuNoV and that show successful replication in HIE.

E. coli counts were not found to correlate with human norovirus in freshwater over the duration of the study. However, human norovirus was found to correlate with total bacteria count and with % dissolved oxygen in freshwater.

Summary of Findings and Recommendations
This is the first study to show that HIE can be used to recover infectious HuNoV from lettuce and freshwater. Infectious HuNoV persisted for at least 7 days on preharvest lettuce and in freshwater. *E. coli* did not correlate with HuNoV or HAV in water over a period of 14 days. The naturally occurring freshwater bacteria can negatively influence the survival of human norovirus.

Our results highlight the need to identify another fecal indicator that correlates better with human norovirus. Also, more efforts should be placed on preventing water from being contaminated with HuNoV, as the virus may survive long enough in water to contaminate irrigated crops, such as lettuce. Lettuce contaminated in the field with human norovirus poses a risk to consumers as the virus may survive through the duration of this product's shelf life.

APPENDICES

Publications and Presentations
Two manuscripts describing the results from HuNoV survival experiments on lettuce and in water are currently in preparation.

One poster as well as a 3-minute thesis presentation was done by Revati Narwankar at the 30th Center for Food Safety annual conference at UGA. This poster will also be presented in April at the 2023 Southern Section AOAC Annual Meeting. Also, one abstract was accepted for poster presentation at the international calicivirus conference. This conference happens every three years and covers all aspects of human norovirus from molecular virology to ecology.

Budget Summary
This research project was awarded $199,570 in grant funds. To date, most funds have been spent and the remaining funds will be used to cover manuscript publication costs and travel to the annual CPS Research Symposium in June 2023.

Suggestions to CPS
Thank you for your support through the duration of this project, especially that the start of this project coincided with the pandemic.