CENTER for PRODUCE SAFETY

CPS 2013 RFP
FINAL PROJECT REPORT

Project Title
Effect of physicochemical and biological parameters on survival, persistence and transmission of norovirus in water and on produce

Project Period
January 1, 2014 – December 31, 2015

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Objectives

1. Determine the effects of specific physical, chemical and biological factors on the survival and persistence of NoVs in water.

2. Determine the effects of specific chemical and biological factors on the survival and persistence of NoVs on tomatoes under common transport and storage conditions.

3. Determine the impact of specific environmental conditions and produce contact time on tomato-associated NoV transmission to a host.

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FINAL REPORT

Abstract
Human noroviruses are the leading cause of non-bacterial gastroenteritis worldwide and are responsible for over half of foodborne infections in the United States annually. Outbreaks from this pathogen are widespread and long lasting which are in part due to the extreme environmental stability of noroviruses. In particular, these viruses are able to survive months to years in water and soil and their presence in irrigation water has been previously linked to outbreaks of disease. This project evaluated the impact of physical, chemical and biological factors on norovirus survival in water and on produce and attempted to assess how these factors influenced transmission to the natural host. Results showed that temperature, UV light, ammonium chloride and often sodium phosphate could result in more rapid loss of infectious virus compared to untreated samples. Interestingly, it was also discovered that sodium phosphate (under specific conditions), bacteria and bacterial supernatants could enhance murine norovirus stability. Additionally, it was discovered that >90% of norovirus in sample will attach to bacteria, potentially shedding light into a mechanism by which these bacteria persist in the natural environment.

Background
Human noroviruses (HuNoVs) are responsible for a significant number of foodborne diarrheal cases each year, causing greater than 58% of the ca. 9 million total cases (1). These viruses are estimated to cause nearly 15,000 hospitalizations and 150 deaths each year in the United States as a result of food and waterborne transmission alone (1, 2). Produce has been widely implicated in HuNoV outbreaks, particularly leafy greens (3–5) and soft red fruits (6–9). While some produce outbreaks have resulted from handler contamination at the food preparation level, HuNoVs have been detected on market-ready produce and shown to be infectious (10–12). One recent study demonstrated that HuNoVs can persist on strawberries and raspberries at common storage conditions well past the shelf life of the fruits (13). Overall, HuNoVs are estimated to account for 40% of produce-related outbreaks (14).

Contamination of produce commonly occurs through the application of contaminated irrigation waters (6, 15, 16), and HuNoVs have been detected in water used for irrigation (17, 18). Contamination of ground and surface waters with enteric pathogens is a major problem in the United States and can occur in several ways, including discharge of treated and untreated wastewater, illegal dumping of human excrements (feces or vomit), and unintentional discharges due to urban, rural and agricultural run-off (19–21). Documented gastroenteritis outbreaks linked to contaminated surface and groundwater confirm the public health risk caused by the presence of enteric viruses in the environment (22). Moreover, once in the environment these viruses are able to persist for long periods of time. Human NoVs are stable in groundwater, river water, mineral water, and tap water for months (23–25). Remarkably, a recent study demonstrated that a HuNoV can persist in groundwater for years (23).

Collectively, there is strong evidence indicating that HuNoVs can survive for prolonged periods of time (weeks to months) on produce as well as in water that can itself contaminate the produce. We currently lack key knowledge regarding the factors that enhance or reduce norovirus survival in water and on produce.

Noroviruses are extremely hardy and resistant to desiccation, low pH, and common disinfectants, including dilute chlorine bleach (26–29). Relatively few environmental conditions (e.g., temperature, relative humidity) have been evaluated for their impact on NoV survival (26, 30, 31), and discrepancies between *in vitro* and epidemiological data suggest additional factors are also involved. Specifically, while laboratory experiments have demonstrated that high relative humidity and high temperatures lead to viral degradation (26, 30, 31), this is in stark contrast to epidemiological studies that reveal HuNoV disease is virtually endemic in tropical regions where humidity and temperatures are continually elevated (32, 33). Based on these conflicting observations, we predict that other as-yet-undefined factors contribute to both the environmental stability of NoVs and their transmission to a host. These knowledge gaps prevent food safety experts from adequately addressing issues critical to the development of virus removal/inactivation methods. Such critical issues include (i) the ability to remove or inactivate virus below the threshold level of infectivity considering the extremely low infectious dose of NoVs; (ii) the relationship between NoVs and other environmental microorganisms; and (iii) the impact of environmental factors on the transmission of NoVs to a host. The effects of the food matrix and naturally occurring microorganisms on NoV transmission to a host are largely unknown.

Microbial risk analysis has indicated that even low levels of viral contamination in irrigation waters can result in a significant level of risk to consumers (34), but factors that contribute to virus survival on produce and ultimately transmission to a host are almost completely uncharacterized. While methods to reduce virus levels on contaminated produce have been discovered (35), the incidence of diarrheal disease upon consumption of virally contaminated produce is on the rise (36). Therefore, understanding agricultural and environmental properties that influence virus stability is necessary in order to prevent viral transmission and thus protect public health.

In this study, we investigated the ecology of virus:water and virus:produce interactions. These data are necessary to develop rational and effective strategies to prevent viral contamination of produce. Specifically, we proposed that NoV interaction with bacteria in the environment enhances persistence of NoVs in water and on produce, leading to enhanced transmission to a host. Biological parameters, such as enteric bacterial concentration, have been suggested to contribute to viral persistence but have not been investigated in detail (18). Moreover, bacteria have been shown to enhance infectivity of another enteric virus *in vitro* and *in vivo* and so may also aid in their persistence and stability (38). There is also conflicting data that has shown *P. aeroginosa* can negatively impact the viability of some viruses, presumably due to the secretion of specific bacterial enzymes (37), but this theory has not been investigated in detail or in relation to enteric viruses. Taken together, a thorough investigation into the role naturally occurring bacteria play in the survival and transmission of noroviruses is necessary.

Research Methods and Results

The impact of ammonium, phosphate and solar radiation (in the form of ultraviolet (UV) light) on the survival of norovirus were assessed during this project. Specifically, murine norovirus (MuNoV) was incubated at various temperatures (4, 22, 37 and 65°C) in a range of chemical (ammonium chloride or sodium phosphate) concentrations. These mixtures were sampled periodically over the course of 45–60 days, and viral loads were quantified using both qRT-PCR and 50% Tissue Culture Infectious Dose ($TCID_{50}$) assays. For UV light treatment, two concentrations of MuNoV (10^4 and 10^7 $TCID_{50}$/ml) were exposed to a range of UV doses. Two

independent methods of analysis were used to discriminate between total viral RNA present (which is currently the only technique available for detection of human noroviruses (HuNoVs) and actual infectious virus).

Experiments evaluating the effect of UV light demonstrated that doses of 100,000 µJ/cm2 and higher completely inactivated MuNoV, while a dose as low as 10,000 µJ/cm2 did not reduce the levels of infectious virus compared with untreated samples (Figure 2). Results from initial experiments determining virus survival at the various temperatures revealed (as expected) that virus survival in the untreated control samples was increasingly stable with decreasing temperature (Figure 1). At 65°C, viral titers decreased rapidly and were undetectable by 3 days post infection (dpi). Surprisingly, at 37°C the virus remained detectable until 10 dpi, but concentrations did steadily decline over time, as expected. MuNoV was the most stable at 22 and 4°C, surviving past 10 and 60 days, respectively. Based on these control studies, 4°C provides and optimal condition for evaluating the ability of the aforementioned compounds to decrease viral stability, while 37 and 22°C provide an environment in which stability enhancement of MuNoV by these compounds can be assessed.

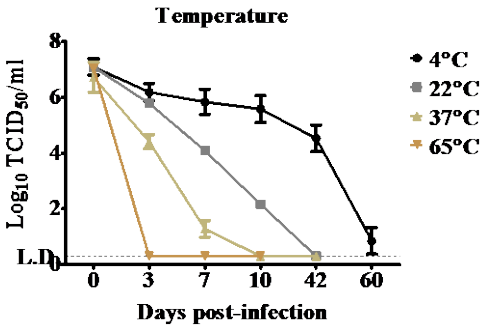

Figure 1

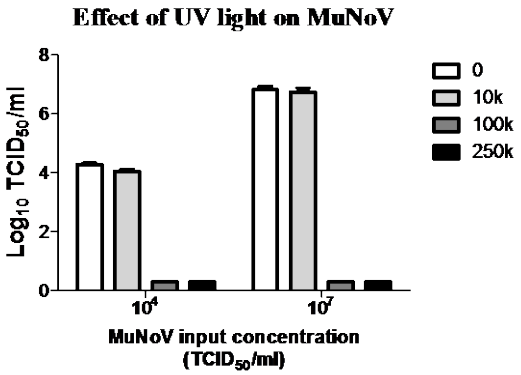

Figure 2

When evaluating the impact of chemical compounds at 4°C, higher concentrations (800 mg/ml) of ammonium chloride (NH_4Cl) resulted in a significant ($p < 0.05$) loss of infectious MuNoV after 3 days of incubation compared with untreated controls (Figure 3A). By 7 dpi, both lower (80 mg/ml) and higher doses of the compound resulted in significantly ($p < 0.01$) lower concentrations of MuNoV and this trend continued out through 42 dpi. Exposure of MuNoV to sodium phosphate ($NaPO_4$) did not result in significant reductions in viral titers compared with untreated controls until 7 dpi, at which time the highest concentration of compound (5 mg/ml) resulted in a significant ($p < 0.001$) 1.3-log reduction in infectious viral particles (Figure 4A). By 10 dpi, even low amounts (1 mg/ml) of $NaPO_4$ were able to significantly ($p < 0.001$) reduce viral titers, and this trend continued through 42 days for both compound concentrations.

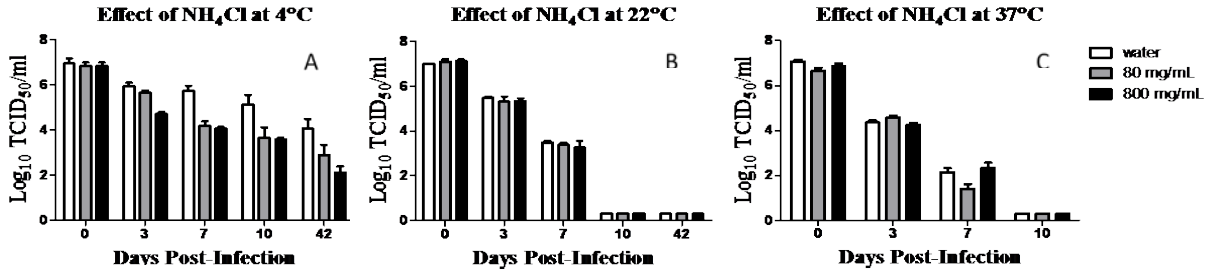

Figure 3

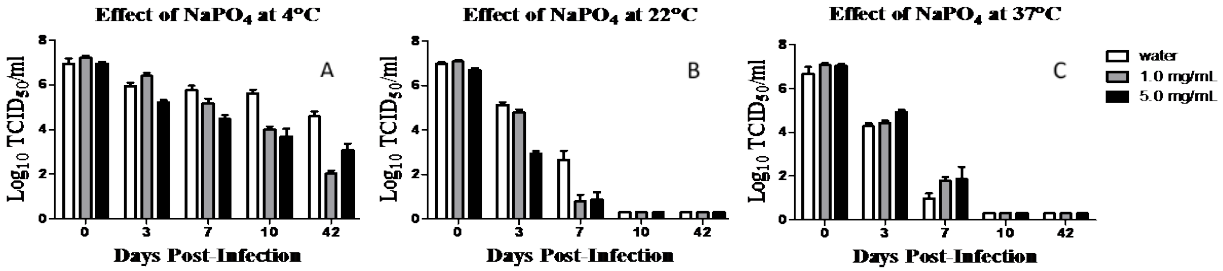

Figure 4

Unlike the significant effects reported above, ammonium chloride had no effect on MuNoV concentrations at 22 or 37°C compared with untreated controls throughout the time when detectable virus was present (7 dpi, Figure 3B and C). However, sodium phosphate negatively impacted survival of MuNoV at 22°C (Figure 4B). By 3 dpi, the highest concentration of $NaPO_4$ had significantly ($p < 0.001$) lowered MuNoV concentrations compared with the untreated control; by 7 dpi, both concentrations of the compound resulted in significant ($p < 0.001$) virus reductions. At 37°C, $NaPO_4$ had little impact on virus survival through 3 dpi (Figure 4C). By 7 dpi, the virus concentrations had decreased in all samples, but the virus concentration in both phosphate-treated samples were significantly higher ($p < 0.05$ and $p < 0.01$ for 1 and 5 mg/ml, respectively) compared with the untreated control. These results were complicated somewhat by the slightly lower input in the control samples; however, even taking this into consideration, the higher viral loads in the sample containing 5 mg/ml of $NaPO_4$ are still significant ($p < 0.05$). None of the treatments impacted virus survival positively or negatively at 65°C (data not shown).

This project also evaluated the survival of MuNoV on the surface of tomatoes. Green tomatoes were acquired from local growers immediately after harvest, prior to waxing. Tomato surfaces were spotted 10 times with 10-µl aliquots of MuNoV at a concentration of either 10^4 or 10^7 $TCID_{50}$/ml. The spots were allowed to dry and tomatoes were incubated for 24, 48 or 72 h. After incubation the tomatoes were manually scrubbed in sterile bags containing 50 ml of phosphate buffered saline (PBS), and the concentration of virus in the wash was determined using qRT-PCR and $TCID_{50}$. These studies demonstrated the ability of MuNoV to remain stable and infectious on the surface of the tomato even after 72 h of incubation, regardless of input concentration (Figure 5). Decreases in concentration were observed over time, but are consistent with the effect of room temperature incubation on viral stability. Interestingly, the concentration of virus removed from the tomatoes at every time point was similar to the amount applied to the tomatoes, suggesting this virus does not form a strong attachment to the surface of tomatoes and

can therefore be easily removed even after prolonged incubation. A second interesting observation revealed during data analysis of these samples was the comparison of infectious virus concentrations as determined by $TCID_{50}$ and viral concentrations as determined by qRT-PCR. For high amounts of virus, there was no difference in viral titer between these two types of analysis; however, for lower viral concentrations, the amount of virus determined by qPCR was always higher than the amount of infectious virus present (Figure 6).

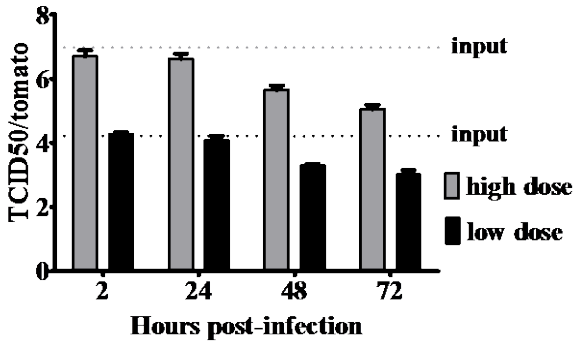

Figure 5

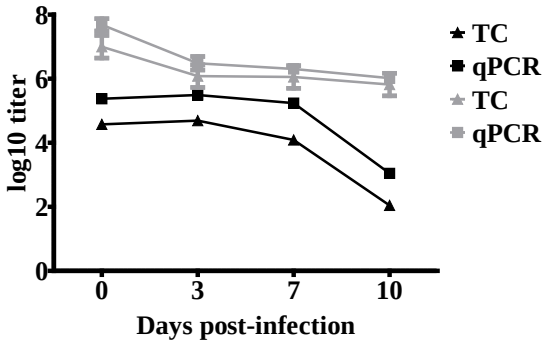

Figure 6

Virus from chemical/UV treatments and tomato washes were used to infect mice in order to assess the ability of the virus to transmit to its natural host after exposure to the various conditions. C57BL/6 mice (n=3 for each strain/time point/condition) were infected orally with a maximum volume of virus containing sample from these conditions. After 24 h the mice were euthanized and pertinent organs (specifically, the distal ileum [small intestine], colon [large intestine] and mesenteric lymph nodes) in which MuNoV replicated were harvested. The tissues were homogenized and viral titers determined using plaque assay. Infection studies using UV-treated virus mirrored results from tissue culture infection where the highest levels of virus replication in the host occurred with samples that were untreated, and viral concentrations in the tissues were only slightly less in samples where virus had been treated with 10,000 µJ/cm2 prior to infection **(**Figure 7**)**. These reductions in tissue titers are likely due to the slightly lower amount of infectious virus present after UV treatment. As with tissue culture, samples treated with 100,000 or 250,000 µJ/cm2 were not infectious to the host. Therefore, exposure to low amounts of UV light did not significantly reduce the transmissibility of the virus, however high doses of UV light did completely inactivate the virus and hinder transmission. For samples that were exposed to either ammonium chloride or sodium phosphate, a reduction in the ability of the virus to transmit to the host was observed after only 3 days of incubation prior to infection (Figures 8 and 9). However, this was observed under all conditions, including the untreated virus samples, suggesting that the incubation alone, and exposure to the compounds, was responsible for the decreased ability of the virus to transmit. Infections in mice were also performed using the washes from tomato attachment studies described above. There was no detectable virus in the harvested tissues at one day post infection, indicating that infection by the virus did not occur (data not shown).

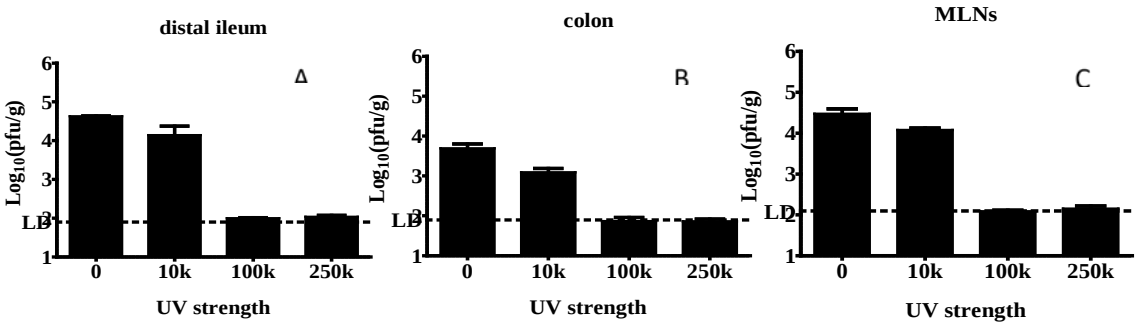

Figure 7

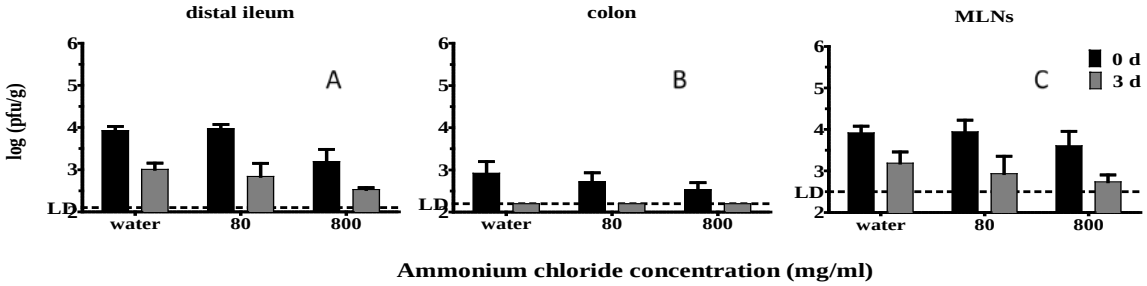

Figure 8

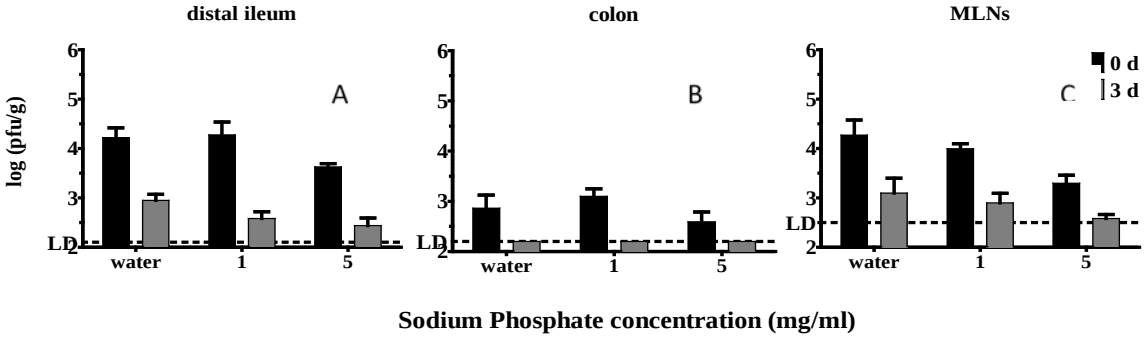

Figure 9

In addition to assessing the ability of physical and chemical factors to impact norovirus survival, the ability of bacteria and their secreted products to impact MuNoV survival were also assessed. For these experiments, high doses (10^7 $TCID_{50}$/ml) of MuNoV were incubated with either *Psuedomonas aeruginosa*, *E. coli*, *Enterobacter cloacaie* or *Citrobacter fruendii* or the cell free supernatants of these bacteria for 10 days, and the concentration of the virus monitored over time using RT-qPCR and $TCID_{50}$ assays. Results showed that MuNoV (MNV) binds to bacteria at a high rate at various temperatures. At 4, 22 and 37°C, greater than 95% of the virus bound to bacteria (Figure 10).

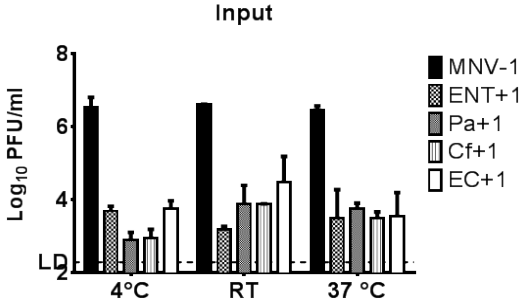

Figure 10

This attachment hindered the ability to analyze virus stability using tissue culture assays, but given the strong correlation demonstrated between tissue culture and molecular detection (Figure 6), qPCR was used for assessing the impact of bacteria on MuNoV survival. Results showed that incubation with live bacteria stabilized MNV during room temperature incubation (Figure 11). However, the degree of stabilization was dependent on virus concentration. High virus inoculums demonstrated significant decline over the 10-day incubation while low virus inoculums were much more stable. This pattern was seen for all three bacteria, and while there was variability among the bacterial concentrations (low concentrations appeared to stabilize better than high concentrations for some samples) that were tested, those differences were not significantly different (Figure 11). These findings are particularly exciting in light of the known instability of MNV at room temperature. Room temperature incubation of MNV has been repeatedly demonstrated (by us and others) to be detrimental to virus stability, leading to a total loss of detectable bacteria by 7–10 days. In addition, considering that environmental concentrations of both virus and bacteria are most similar to the lower concentrations used in these studies, these data may point to a strategy employed by the noroviruses to aid in their stability when outside the natural host.

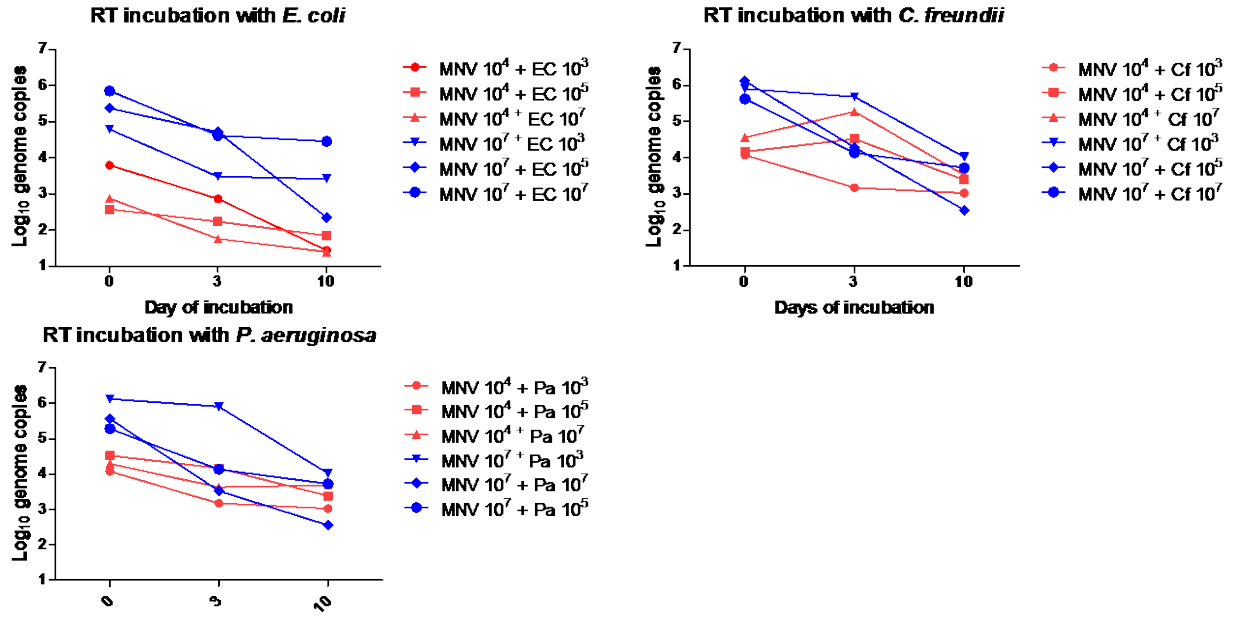

Figure 11

We also evaluated samples where MNV was incubated with the cell free supernatant (CFS) of the three bacteria. Results from these studies demonstrated that both high and low concentrations of the virus can be stabilized by the factors secreted from *Pseudomonas aeruginosa* (Figure 12). In fact, when compared to incubation with the live bacterium (Figure 11), it appears stability is better when only the secreted factors are present.

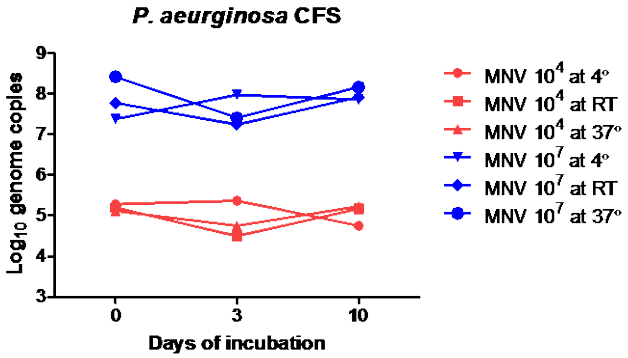

Figure 12

One manuscript entitled "Physical and chemical factors impact norovirus in vitro survival and host transmission" has been completed and submitted to Environmental Microbiology. The team is completing final experiments for one other manuscript, entitled "Bacterial enhancement of norovirus survival," which will be submitted in late 2016 or early 2017.

Outcomes and Accomplishments

Established Goals	Accomplishments
To evaluate survival of MuNoV in response to physical stressors, i.e., temperature and UV light	The physical stressors of increased temperature and UV light are detrimental to norovirus survival.
To evaluate survival of MuNoV in chemically adjusted waters at 4, 22, 37 and 65°C using high and low doses of chemical compounds	Chemical compounds, such as ammonium chloride and sodium phosphate, can also reduce the levels of infectious virus but their impact is temperature and time dependent. Specifically at 37°C, high concentrations of sodium phosphate can stabilize the virus after extended incubation.
Evaluate viral attachment and persistence on tomatoes	MuNoV does not strongly adhere to the surface of tomatoes but does survive and remain infectious through 72 h of incubation.
To evaluate the transmission of virus exposed to physical or chemical treatments to natural murine host	Infections from ammonium chloride treated samples and UV treated samples were completed. Plaque assay analysis for UV treated samples was completed.
To evaluate the transmission of virus exposed to tomato surface to natural murine host	MuNoV removed from tomatoes did not cause infection in the natural host.
To assess the effect of tomato matrix on MuNoV infection of host	The tomato matrix was found to be detrimental to cell culture assays and thus unusable for determining viral concentrations necessary to cause infection.
To evaluate the effects of bacteria on viral persistence	Under conditions where virus decay occurs, the presence of bacteria aids in stabilizing MuNoV.

To evaluate the effects of bacterial secreted factors on virus survival	Through molecular detection it was demonstrated that bacteria enhance the survival of MuNoV either through direct contact with the virus or indirectly through the secretion of protective factors into the supernatant.
To evaluate the effect of bacteria on viral attachment and persistence on tomatoes	The presence of bacteria did not negatively impact MuNoV survival on tomatoes. Due to high levels of virus survival on tomatoes without bacteria it could not be determined if the presence of bacteria enhanced MuNoV survival on produce.
To evaluate the effect of biological parameters on MNV infection of a host	High levels of MuNoV attachment to live bacteria significantly reduced their concentrations so that virus levels in filtered inoculum were too low to cause infection in the host.

Summary of Findings and Recommendations

This project confirmed that noroviruses are extremely stable and that factors that contribute to norovirus stability can be multi-faceted. Only UV light and very high temperatures (67°C) were able to quickly eliminate infectious virus particles. All other treatments resulted in slower declines in virus stability. However, the decline in infectious virus can be accelerated by the addition of high concentrations of ammonium chloride or potassium phosphate. Thus, the addition of these compounds to irrigation water systems may prove useful in controlling and lowering norovirus in water when contamination events occur. Sodium phosphate also demonstrated a slight protective effect for murine norovirus under certain conditions, but bacteria provided the biggest stabilizing force of all the conditions tested. Furthermore, it seems the presence of the bacteria themselves is not necessarily required for enhancing viral stability. Enhanced viral stability can be achieved through products secreted by the bacterium. These observations may help explain the stability of norovirus in water sources and particularly in wastewater treatment systems.

During this project period we also learned a great deal regarding the difficulty in working with poly-microbial systems and the challenges that arise when trying to analyze experiments and data. These complications extended to evaluating transmission of this virus, which also proved to be difficult. However, through these challenges much was learned and techniques were modified or developed to aid follow-up studies.

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APPENDICES

Publications and Presentations

Two publications are in progress from this project. The first, entitled "The effect of physical and chemical factor on norovirus survival" (Zhu et al.) is under revision for subsequent re-submission. The second publication, "Bacterial enhancement of norovirus survival in vitro" (Zhu et al.) is in preparation for first submission to Applied and Environmental Microbiology. Final experiments are underway and submission is planned for Spring of 2016.

Portions of this work were presented at the Annual CPS Symposia in 2014 (poster) and 2015 (oral presentation). Another presentation of data will also be held at the Annual CPS Symposium in Seattle, WA (June 2016).

Budget Summary

For PI-Jones, the supply budget for the entire project period was spent, but there was approximately $1,000 left in salary/fringe costs. This small amount left was due to efforts to avoid overspending the account. The travel budget has a balance of $6,000, a portion of which will cover costs for attending the Annual CPS symposium in Seattle, WA, in June 2016. For Co-PI-Schneider, there was also approximately $7,100 left on the sub-award. This excess was due to unexpectedly low travel and supply costs.