CENTER for PRODUCE SAFETY

**CPS 2014 RFP**
**FINAL PROJECT REPORT**

## Project Title
Enteric viruses as new indicators of human and cattle fecal contamination of irrigation waters

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## Objectives
1. *Identify an ideal workflow and protocols for using the "negatively charged membrane method" that optimizes cost and time requirements for surveillance of novel viral indicator organisms.*
2. *To assess the occurrence of bovine polyomavirus (BPyV), bovine adenovirus (BAdV), human adenovirus (AdV), Aichivirus (AiV), pepper mild mottle virus (PMMoV), and the enteroviruses (EV) in irrigation waters of varying quality in Arizona, Georgia, and Wisconsin using qPCR for application as novel viral indicator organisms in relation to traditional cultural methods among and between irrigation districts.*
3. *Based on the presence/absence and relative abundance of novel virus indicators in irrigation waters, we will estimate the amount of fecal contamination present based on the known levels of viral shedding from cow and human fecal contamination sources.*
4. *Provide improvements to exposure data regarding risk associated with fecal contamination on fresh produce from irrigation water.*

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## FINAL REPORT

### Abstract

The use of the current EPA recreational water standard of <126 *E. coli* per 100 ml for evaluating the safety of irrigation water was not intended for—and thus may not be adequate to apply to—risks associated with irrigation management of edible crops. The development of novel approaches to evaluate the presence or absence of fecal contamination in irrigation waters is needed to provide relevant exposure data for risk assessments for fresh produce. Without accurate exposure data, risk-based analyses cannot be conducted and future "risk relevant" standards will be difficult to implement to improve regulatory efforts for produce safety. Recent research has shown that viral targets may provide more conservative and accurate estimates of human and animal fecal contamination. Evaluations of viruses, such as pepper mild mottle virus (PMMoV), Aichivirus, the enteroviruses, and human and bovine adenoviruses, have shown these viruses to be highly prevalent and abundant in wastewater and human and cow fecal samples. In addition, the development of low cost and low sample volume collection methods has yielded efficient and rapid approaches for detecting viruses from environmental water samples. The current study had the following specific objectives: 1) to assess the occurrence of bovine polyomavirus, bovine adenovirus, human adenovirus, Aichivirus, pepper mild mottle virus, and the enteroviruses in irrigation waters of varying quality in Arizona, California, and Georgia; 2) to evaluate these enteric viruses as novel indicators of fecal contamination of water via side-by-side comparisons with the presence of *Salmonella* and *E. coli* (the currently used fecal indicator) in the same water to determine if the presence of these enteric viruses can be correlated with the presence of bacterial pathogens in irrigation waters; and 3) to perform a quantitative microbial risk assessment using this information to determine what levels of fecal contamination from irrigation water pose a public health risk.

It was determined that small volumes (~3 liters) of irrigation waters can be used to detect indicator viruses that are found in high numbers in human and animal feces. The presence of these fecal indicator viruses can point to changes in water quality and may correlate with the presence of pathogens. PMMoV is readily detected in irrigation water samples (between 34% and 63%) and was successfully used to estimate the amount of fecal contamination in the water. This information was then used in a quantitative microbial risk assessment to determine the risks of foodborne illness caused by specific pathogens of interest (*E. coli* O157, *Shigella*, human norovirus, and rotavirus) as a result of consuming fresh produce irrigated with this water. This work should inform future efforts with a more scientifically accurate approach to evaluating the use of irrigation water for fresh produce to better inform industry standards and to mitigate risks.

### Background

Currently, irrigation water quality is being evaluated with the 1973 US EPA recreational water quality standards of geometric mean of <126 *E. coli* per 100 ml as a way of indicating the safety of irrigation water for use on fresh produce. Originally, these criteria were never intended to be applied to risks associated with irrigation management of edible crops as they were developed for evaluating the risk of illness (1 in 10,000) to humans that come into contact with surface waters for recreation purposes (e.g., swimming, boating etc.). However over the last 40 years, there have been few advances in the development of better methods that improve risk-based assessments for evaluating the presence or absence of fecal contamination in irrigation waters for fresh produce. Thus, without being able to improve exposure data and accurately determine the presence/absence or relative level of fecal contamination, more accurate risk-based analysis cannot be conducted, and setting better "risk relevant" standards regarding improved

regulatory efforts of irrigation waters has remained problematic.

The ideal indicator organisms/targets have traditionally been defined as microorganisms a) with an easy method of detection, b) of human or animal origin, c) that survive as long as or longer than pathogenic organisms, d) that are present in densities relevant to the level of fecal contamination, e) that may serve as a surrogate for many different pathogens, and f) that are useful in fresh or saline waters (Yates 2007). When reviewing this ideal definition of an indicator organism, the use of *E. coli* has some major disadvantages that arguably outweigh its "ease of detection" through current cultural methods. The culture-based assays used for the detection of *E. coli* are typically low cost and relatively rapid, but often can yield numerous false positives or overestimate the level of contamination found in the source water, making it difficult to support them under definition (a). "Enteric bacteria" based on this cultural definition are often found as indigenous organisms in the soil, making them poor microbial indicators and in violation of definition (b). These organisms are also well known for their ability to proliferate in the environment outside of their host and their die-off rates can be substantially shorter than many environmental viruses like human norovirus, thus violating definition (d). Without the ability to statistically and accurately evaluate the number of indicator organisms used in the established cultural methods, it is difficult to view the use of enteric bacteria as effective surrogate organisms for pathogenic species under definition (e). For all of the reasons listed above, their effectiveness in fresh or saline waters remains somewhat irrelevant.

In order to develop a better indicator organism(s) and methodologies that go with implementing such a change, we must identify targets that better fit the definition of an effective microbial indicator. Human enteric viruses replicate (i.e., multiply) in the epithelial cells of the gastrointestinal tract and, upon excretion, are able to survive longer outside the host environment than enteric bacteria such as *E. coli*. Enteric viruses include a wide range of human foodborne pathogens that have evolved thermal stability (42–50 °C) and pH stability (pH 3–9) in the extracellular environment. Such stability is largely associated with the small size (30 nm) and robust protein coat called the capsid that enables the virus to bind to the host cells. These features are known to play an important role in the mechanisms of virus survival and transport in the environment (Gerba et al. 2013; Racaniello 2013; Xagoraraki et al. 2014).

Several novel viruses have only recently become realized as a potential improvement on traditional indicator organisms. These viruses are advantageous for several reasons. First of all, viruses require a host to replicate and therefore are in no danger of re-growth once released into the environment, making them ideal static target organisms that can be more easily quantified and correlated with the amount of fecal contamination present in a sample. They are almost always found in an equal or higher abundance than pathogenic organisms, providing a more conservative estimate of the level of fecal contamination that improves our level of detection and provides a more quantifiable range of fecal contamination. This will allow for an improvement of exposure assessments for risk analyses. Viruses are also generally host-specific, allowing for the specific detection of fecal contamination for both cattle and humans that commonly contaminate source water through either runoff events or direct sewage infiltration. This makes them ideal as both a microbial source tracker and sensitive microbial indicator (Wong and Xagoraraki 2010). Additionally, previous research has shown that the level of virus shedding for the model organisms proposed (see Methods section) shows little seasonal variability and is highly abundant in fecal materials (Hamza et al. 2011; Han et al. 2013; Wong et al. 2012). Aichivirus has been detected in high numbers in both raw and treated sewage in the U.S., Japan, Venezuela, and Tunisia, and in shellfish in France. Pepper mild mottle virus (PMMoV) is the most abundant virus in fecally contaminated waters worldwide; enteroviruses are also commonly found in contaminated waters. Adenovirus is one of the longest-lived viruses in environmental waters because of its high level of resistance to ultraviolet light.

Nevertheless, only recently have methodologies improved enough for the realization of low cost and rapid detection methods for viruses from environmental source waters. Traditional

virus absorption–elution methods (or VirAdEl) were developed for concentrating viruses from water for culture-based applications, and relied on large volumes (>100 liters), expensive filter cartridges (>$200), and multiple-day, labor-intensive procedures (Ikner et al. 2011, 2012; USEPA 2010). The high cost, long turn-around times, and poor concentration efficiencies associated with these methods have, in the past, prohibited their widespread use as an effective tool for environmental virus monitoring. The recent development of new methodologies like the "negatively charged membrane method" (Haramoto et al. 2007) have allowed for vast improvements in the concentration and detection of viruses for detection using molecular methods. This new concentration technique requires smaller water volumes of only a few liters, utilizes an inexpensive filter media (<$5), and can be conducted in less than one day. These improvements have now made it possible to apply environment virus detection as an effective tool for evaluating irrigation water quality.

## Research Methods and Results

***Objective 1.*** *Identify an ideal workflow and protocols for using the "negatively charged membrane method" that optimizes cost and time requirements for surveillance of novel viral indicator organisms.*

The virus concentration method proposed can quickly and efficiently concentrate small volumes of water using basic laboratory equipment and supplies. There are several approaches to how the sample can be treated. Two to three-liter water samples can be shipped back to the lab for concentration and analysis, which may be logistically difficult; alternatively, the concentrates can be prepared in the field. However, it is unclear if viral integrity and recovery can be maintained if field concentration is done. In theory, the binding of viral particles to the HA filter membrane used in this study could allow for the rapid sampling and easy shipping of small, lightweight 90-mm filter membranes for later virus elution at the testing facility. Sample collection methodologies were evaluated under these two scenarios for the recovery and overall feasibility using model organisms under field and laboratory conditions.

Initially, ten 4-liter irrigation water samples were collected from various canals in Yuma, AZ, and brought back to the University of Arizona on ice for processing via concentration using HA filters. For nine of the 10 samples, 3 liters were successfully passed through an HA filter to concentrate the viruses in the sample. For the $10^{th}$ sample, only 2.5 liters was passed through the filter before it clogged. Based on these preliminary tests, it appeared that a volume of 3 liters was optimal for this method with these surface irrigation waters (2 to 3 liters was proposed). All samples with less than 3 liters filtered were recorded so that this lower volume could be taken into consideration in any subsequent quantification of viral genome copy numbers.

Subsequently, an additional thirty 4-liter samples were collected in Yuma and in the Imperial Valley, CA. Unlike the ten previous samples, many of these remaining 3-liter samples could not be successfully filtered using the HA filters alone. Due to this issue, a number of pre-filter types were evaluated for their ability to remove water components that were clogging the filters while still successfully allowing smaller particles, such as viruses, to pass through. After numerous tests, a filter with a pore size of 20–25 microns in diameter (Whatman 41; 90-mm diameter) was determined to be optimal. The filter was pre-soaked in 3% beef extract as a blocking buffer to take up available sites on the filter that could otherwise cause indiscriminate binding of viruses to the filter.

In addition to the appropriate volume to be filtered, it was also necessary to determine if filters could be shipped on ice from collaborators in other regions without a loss in virus integrity. To determine this, four 3-liter volumes of dechlorinated tap water were seeded with a known amount of poliovirus (an enterovirus) and the water was concentrated using the established HA filtration method. Following this, half of the samples were placed in petri dishes wrapped in

parafilm and stored on ice in a cooler for two days to simulate overnight shipment from other regions. The other half of the samples were processed/eluted immediately. The recovery efficiency of the virus was compared for the two methods. No significant differences were observed and it was determined that collaborators in other regions could send already concentrated samples on filters on ice through the mail overnight without any appreciable loss of virus integrity. This resulted in a significant savings in shipping costs for the samples since it was not necessary to ship multiple liters of water per sample.

***Objective 2.*** *To assess the occurrence of bovine polyomavirus, bovine adenovirus, human adenovirus, Aichivirus, pepper mild mottle virus, and the enteroviruses in irrigation waters of varying quality in Arizona and Georgia using qPCR for application as novel viral indicator organisms in relation to traditional cultural methods among and between irrigation districts.*

To evaluate the use of novel viral indicator organisms for human and cattle fecal contamination, four irrigation regions were chosen that specialize in fresh produce from around the country (Yuma, AZ; Maricopa, AZ; Imperial Valley, CA; and an area near Atlanta, GA). Samples were collected between November and March for a period of two years. A small set of samples was also collected during the middle of summer (August) in Yuma to look at seasonal variations. Sampling locations were chosen that represented a variety of canal characteristics such as main canals and lateral canals, cement lined and unlined canals, urban and rural canals, and irrigation ponds. In addition, data collected from previous studies regarding the occurrence of fecal contamination and the presence of pathogens in the irrigation water in these regions were studied to ensure that areas with historically low contamination and higher contamination levels were included. This included 30 different locations from irrigation canals in Yuma, 30 locations from canals in Maricopa, 30 locations from irrigation canals in the Imperial Valley, and 4 large irrigation ponds in Georgia. The irrigation ponds in Georgia included one pond next to a large dairy farm and another in which cattle had direct access to the water. All of the ponds could be directly accessed by local wildlife.

A total of 330 samples were collected. This included 190 samples collected from Yuma AZ (150 in the winter, 40 in the summer), 60 samples from Maricopa AZ, 60 samples from California, and 20 samples from Georgia. Bacterial sampling for both indicator organisms (total coliforms and generic *E. coli*) and pathogens (*E. coli* and *Salmonella*) also was conducted for comparison purposes. Physical/chemical data were collected for metadata purposes.

Water quality analyses: Water quality parameters including the air temperature, water temperature, relative humidity, pH, electro-conductivity, and the amount of total dissolved solids were recorded in the field using a multi-parametric sensor. Upon arrival in the laboratory, the turbidity (cloudiness) was also measured using a turbidimeter. To determine the number of indicator organisms present, a 100-ml volume was quantified for total coliforms and *E. coli* using the Colilert Quanti-Tray 2000 system (IDEXX, Westbrook, ME). The most probable number (MPN) (a statistical estimate of the number of coliforms/*E. coli* in the sample) was obtained following the manufacturer's procedure.

Cultural analyses: For *E. coli* and *Salmonella* spp., a 1-liter grab sample was concentrated using membrane filtration (0.45-μm pore size; 47-mm diameter) and the filter was placed in tryptic soy broth (TSB) overnight at 37°C for the enrichment of injured cells. This method reduces the number of false-negative results by up to ten-fold compared to direct selective enrichment (Blackburn and McCarthy 2000).

For *E. coli,* the enriched culture was used to inoculate test tubes containing 10 ml of EC broth with an inverted Durham tube. Following incubation at 44.5°C for 24 hours (or at 48 hours if negative after 24 hours), the tubes that exhibited both bacterial growth (turbidity) and the production of gas (bubbles in the Durham tube) were subcultured onto mEndo agar plates with

overnight incubation at 44.5°C. Darkly pigmented colonies were transferred via the streak for isolation method onto tryptic soy agar (TSA) plates and incubated at 37°C overnight. Colonies were tested for the presence of an oxidase enzyme and the production of indole. These purified presumptive *E. coli* cultures (oxidase-negative, indole-positive) were then tested using API 20E biochemical strips to confirm this identification. Isolates confirmed as *E. coli* were tested for the presence of *E. coli* virulence genes using primers described by Wahl et al. (2011) that recognize the Shiga-toxin genes (*stx1, stx2*) and the membrane protein intimin gene (*eae*). The presence of either of the Shiga toxin genes indicates that an isolate is a Shiga toxin–producing strain of *E. coli* (i.e., STEC). The Wahl et al. (2011) method was modified for conventional polymerase chain reaction (PCR) using a 25-µl PCR mixture containing 0.4 µM of each primer, 200 µM of each dNTP, 1 X PCR buffer (20 mM Tris-HCl, 50 mM KCl), 1.5 mM $MgCl_2$, and 0.75 U of Taq polymerase. The following conditions were used during the *stx1* and *stx2* PCR assays: a) 95°C for 1 minute; b) 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds; c) a final step at 72°C for 4 minutes. The PCR mixture and conditions were similar for the *eae* gene, with the following exceptions: a) annealing temperature of 50°C instead of 58°C; and b) 45 cycles used instead of 40 cycles.

For *Salmonella* species, bacteria from the TSB were further enriched in tetrathionate broth for 24 hours at 37°C. A subsequent enrichment was performed in Rappaport Vassiliadis broth for 24 hours at 37°C and samples streaked for isolation onto xylose lysine deoxycholate (XLD) agar plates for the selection of *Salmonella* spp. Suspect colonies (black centers) were transferred to TSA plates, and oxidase-negative cultures were further confirmed as *Salmonella* spp. through API 20E biochemical strips.

Processing of samples for viruses: Using the optimized HA filtration method, 3 liters of each sample were passed through stacked pre-filter and HA filters to concentrate the viruses. The viruses were eluted using the methods described by Katayama et al. (2002). Following elution from these filters (which results in the primary concentration of the samples from 3 liters down to 10 ml), the samples also underwent a secondary concentration step in which the volume was further reduced to ~0.65 ml using Centriprep centrifugal ultrafilters. These secondary concentrates were frozen and stored at −80°C pending further evaluation by quantitative PCR (qPCR).

Virus detection using qPCR: In the detection of viruses by qPCR, inhibitory substances can reduce viral nucleic acid extraction efficiency and interfere with cDNA synthesis and/or polymerase activity that strongly affect the molecular detection and quantification of viruses in environmental samples (Hata et al. 2011). To monitor the extraction-qPCR efficiency, our team used murine norovirus as an internal process control for the processing of all samples, as described previously (Hata et al. 2013). We believe this was necessary as some environmental samples have inhibitory substances that can reduce the extraction and qPCR efficiency. The viral nucleic acid was extracted from 200 µl of each sample using a MO BIO PowerViral™ Environmental RNA/DNA Isolation Kit (MO BIO, San Diego, CA) to obtain 100 µl of extracted RNA or DNA. In a previous study, we found this kit to be more efficient in the extraction and purification of viral nucleic acids from environmental water concentrates than two other widely used commercial kits (Iker et al. 2013). Virus data was collected using qPCR and reverse transcription (RT)-qPCR assays to detect and quantify viral genomes from bovine polyomavirus and bovine adenovirus (Wong and Xagoraraki 2011), human adenoviruses (Ko et al. 2005), Aichivirus and enteroviruses (Hata et al. 2014), PMMoV (Haramoto et al. 2013), and murine norovirus (Kitajima et al. 2010) using specific Taqman probe-based assays. qPCR assays were performed with a LightCycler® 480 Real-Time PCR Instrument II (Roche Diagnostics, Mannheim, Germany). Basically, reaction mixtures (25 µl) consisted of 12.5 µl of LightCycler® 480 Probes Master Mix (Roche Diagnostics), forward and reverse primers, probe, and 5 µl of DNA/cDNA template. Serial ten-fold dilutions of the standard plasmid DNA containing inserts of

the amplification region were used to generate a standard curve, and thus quantitative data on cDNA copy numbers were obtained. qPCR fluorescence readings were collected and analyzed with LightCycler® 480 Software version 1.5 (Roche Diagnostics).

Results:

General water quality parameters: The results for the various irrigation water quality parameters and the traditional indicator organisms (total coliforms and generic *E. coli*) are shown in **Table 1** (see Appendices). All three regions differed significantly ($P \leq 0.05$) in the following parameters: conductivity, total dissolved solids, air temperature, and relative humidity. The pH of the samples collected from California was significantly higher than the samples collected from both regions in Arizona. The water temperature in Yuma AZ was significantly lower in the winter than the temperatures in Maricopa AZ and California. And finally, the turbidity of the samples collected from Yuma AZ was significantly higher than the samples collected from California. These statistical differences suggest that the physical/chemical characteristics of the irrigation water can vary greatly between regions.

The numbers of coliforms and *E. coli* also varied between regions. The number of total coliforms was significantly different from all four regions with only one exception – the levels of total coliforms found in samples collected from California and Georgia were not statistically different. A similar result was found for *E. coli* levels – all the regions had significantly different levels of *E. coli*, with two exceptions (Georgia *E. coli* levels were not statistically different than the levels found in Yuma AZ and California). This also suggests that the microbial quality of the water can vary greatly between regions.

In addition, there can be significant seasonal variation between samples. The subset of 40 samples that were collected from the Yuma AZ region in the summer varied in several water quality parameters from the samples collected during the winter. The summer samples had higher water and air temperatures as well as being more turbid than the winter samples. In addition, significantly greater numbers of coliforms were found in the summer samples than in the winter samples. Nevertheless, the levels of generic *E. coli* as measured by Colilert were only slightly higher in the summer.

Occurrence of Shiga toxin–producing *E. coli* in irrigation waters: The occurrence of generic *E. coli* in irrigation waters as measured by the enrichment/selection method described previously is shown in **Figure 1**. Although generic *E. coli* was isolated from nearly all the samples in Maricopa AZ, California, and Georgia, and the majority of samples in Yuma AZ, none of the *E. coli* isolates tested positive for any of the three virulence genes (*stx1, stx2, eae*). Therefore, no Shiga toxin–producing *E. coli* (STEC) were identified in any of the samples.

Occurrence of *Salmonella* species in irrigation waters: The occurrence of *Salmonella* species as measured by the enrichment/selection method described previously is shown in **Table 2**. Since these samples were all enriched for *Salmonella*, no quantitative data are available. However, despite the occurrence of *Salmonella* being fairly high (between 15–40%), the *Salmonella* levels are likely low in these samples, given the absence of illness outbreaks linked to these regions.

Occurrence of virus indicators/pathogens in irrigation waters: The results for the viruses are shown in **Table 3**. Of the 330 samples collected, 320 (not including the first 10 samples collected from Yuma during the winter) were evaluated for the presence of indicator and pathogenic viruses. Neither of the bovine species was found in any of the irrigation water samples from any of the four regions, including from irrigation ponds with direct inputs from cattle. This result could be due to the efficiency of the method and the limit of detection of the assays. The bovine viruses could be present in these waters, but not at high enough levels to be detected in a 3-liter sample. Aichiviruses were detected in only 3 samples (0.9%; range of $3.9\times10^{1}$ to $3.7\times10^{5}$ genome copies/liter) and human adenoviruses from 5 samples (1.6%; range

of $2.5\times10^1$ to $2.4\times10^6$ genome copies/liter). The enteroviruses were detected in 27 samples (8.4%; range of $2.4\times10^1$ to $1.1\times10^9$ genome copies/liter) from two regions. Notably, all 20 samples collected from Georgia were positive for enteroviruses. The primers used to detect the enteroviruses via qPCR will detect numerous animal enteroviruses as well as human enteroviruses.

By far the most commonly detected virus was PMMoV, which was found routinely in all four regions in 36% to 63% of samples, with a geometric mean of $2.0\times10^3$ genome copies per liter (range of $3.1\times10^1$ to $1.2\times10^6$ genome copies/liter).

Overall for all of the samples, weak correlations were observed between the following parameters and microbial concentrations: Air and water temperatures (r = 0.83), air temperature and the relative humidity (r = −0.44), water temperature and the relative humidity (r = −0.29), total coliforms and *E. coli* (r = 0.32), *Salmonella* and total coliforms (r = 0.47), water temperature and total coliforms (r = 0.41), and the turbidity and total coliforms (r = 0.33).

In the Yuma samples, the occurrence of *Salmonella* was weakly correlated with the water temperature (r = 0.38) and total coliforms (r = 0.44). In the Maricopa samples, both *Salmonella* and *E. coli* could be correlated with the relative humidity (r = 0.30 and 0.36, respectively). Interestingly, in the California samples, *Salmonella* was negatively correlated with the water temperature (r = −0.44), but positively correlated with conductivity (r = 0.37), total dissolved solids (r = 0.34), and turbidity (r = 0.34). None of the viruses measured could be correlated with any of the other measured parameters. This was most likely due to the low levels of occurrence of most of the viruses examined.

***Objective 3.*** *Based on the presence/absence and relative abundance of novel virus indicators in irrigation waters, we will estimate the amount of fecal contamination present based on the known levels of viral shedding from cow and human fecal contamination sources.*

One of the advantages of using qPCR for the detection of viral genomes is the ability to have a broad range of detection without the need for dilution. A standard qPCR assay can reliably and accurately detect as few as 100 genomes per liter to well over 10 million. The static nature of viruses in the environment (i.e., no growth or re-growth) allows for a better estimate of the level of contamination for improvements in exposure data. Recent research has shown that viruses like PMMoV and Aichivirus are consistently present in human fecal pollution and maintain concentrations of ~$10^6$ to $10^7$ log copies per liter of sewage. Similarly, viruses like bovine polyomavirus and bovine adenovirus can consistently be found at high concentrations of $10^5$ to $10^6$ copies per ml of bovine fecal material. In this manner, a relative level of fecal contamination can be obtained by quantifying the number of virus genomes present and estimating the amount of fecal material present in a given water sample. This quantitative approach might allow for better estimates and thus more accurate exposure data to improve risk relevant levels of contamination with regards to human exposure to fecal contamination on fresh produce.

Estimated fecal contamination and calculated pathogen of interest concentrations: PMMoV, enteroviruses, Aichivirus, and human adenoviruses were detected in some of the 320 irrigation water samples collected from Arizona, California, and Georgia. Viral content of 2.8 ± 0.52 liters of each sample were concentrated using a negatively charged membrane filtration method and a secondary concentration step to produce a final volume of 0.636 ± 0.053 ml. DNA or RNA was extracted and viral copies were identified and quantified using qPCR.

Samples displaying no viral detection were assigned a limit of detection value. The limit of detection of the qPCR methods was determined first by assigning a limit of quantification of one viral genome copy per qPCR reaction. This limit of quantification was used to calculate a limit of detection per liter for each sample showing no viral detection. This resulted in a limit of detection for each sample in units of viral genome copies per liter. The limit of detection values were combined to determine an average limit of detection per liter for each virus tested. Limits

of detection for human adenoviruses, bovine adenovirus, and bovine polyomavirus were 22 genome copies per liter, while the limits of detection for Aichivirus, enteroviruses, and PMMoV were 44 genome copies per liter.

Fecal contamination levels were estimated based on quantitative values of an indicator virus, PMMoV, which was found in the greatest number of irrigation water samples. PMMoV concentrations were related to recorded PMMoV concentrations of $1.5\times10^6$ to $2.2\times10^7$ virus copies/ml raw sewage and human feces (Rosario et al. 2009) to estimate the levels of human fecal presence in the sample using the equation shown below. This equation was applied to each sample individually. The PMMoV genome copies per ml of raw sewage value were randomly generated from the range given above, assuming a uniform distribution across the range. Samples that were below the detection limit of the assay for PMMoV were assigned the value of $1\times10^{-5}$ rather than the limit of detection, to indicate that no fecal contamination had been detected.

$$\frac{\boldsymbol{estimated\ volume\ (ml)\ raw\ sewage}}{\boldsymbol{volume\ (ml)\ irrigation\ water}} = \frac{measured\ copies\ PMMoV}{1\ ml\ irrigation\ water} \times \frac{1\ ml\ raw\ sewage}{copies\ PMMoV}$$

Using the estimated quantity of fecal contamination for each sample and known concentrations of pathogens in human sewage and cattle feces, the theoretical quantity of four pathogens of interest (human norovirus, rotavirus, *E. coli* O157, and *Shigella*) within each sample was calculated using recorded levels of these pathogens in sewage/feces (**Table 4**) and the following equation:

$$\frac{\boldsymbol{y\ pathogens}}{\boldsymbol{mL\ irrigation\ water}} = \frac{mL\ fecal\ matter}{mL\ irrigation\ water} \times \frac{x\ pathogens}{mL\ fecal\ matter}$$

These estimates of the amount of each pathogen of interest in irrigation waters were used in the quantitative microbial risk assessment (QMRA) conducted under objective 4 (see below).

***Objective 4.*** *Provide improvements to exposure data regarding risk associated with fecal contamination on fresh produce from irrigation water.*

Using standard risk-based models and the exposure data that were obtained from this research, new models were developed for determining the risk to human health associated with the consumption of fresh produce from potentially contaminated irrigation water. Quantitative microbial risk assessment (QMRA) is a valuable tool to predict risk associated with exposure to microbial pathogens. Predicting this risk can help identify large scale trends and predict the impact of water quality on food safety. This study aimed to assess the risk associated with the consumption of fresh lettuce contaminated with enteric pathogens via irrigation water. Irrigation water samples from Arizona, California, and Georgia were screened for human adenovirus and enteroviruses and the proposed fecal indicator, PMMoV. The following pathogens of interest were also considered important for this risk assessment: Human norovirus, rotavirus, *E. coli* O157, and *Shigella*. Concentrations of pathogens of interest were estimated using measured PMMoV concentrations and known concentrations of PMMoV and the pathogens of interest that are shed in human or cattle feces. These values were then incorporated into known risk assessment models to further evaluate the potential risk posed by the consumption of contaminated fresh produce at a specified point along the farm-to-fork pathway. The following assumptions were made during the conduct of the QMRA:

1. This risk assessment stops at the point of harvest and does not account for microbial decay post-harvest, microbial removal due to washing or sanitization, or other methods

of microbial removal or decay post-harvest. As such, this is an extremely conservative approach.

2. Incorporates aspects of two irrigation systems. The volume of irrigation water that clings to one gram of lettuce was derived from dunking lettuce into water and assessing the volume it retains. This is somewhat representative of spray irrigation; however, it should be considered the most conservative approach of water exposure during spray irrigation. To incorporate parameters of a more realistic irrigation method for lettuce, the recorded viral transfer rates of a viral and bacterial surrogate during furrow irrigation were applied to the concentrations of pathogens within the irrigation water, whether observed or estimated. For example, the viral transfer rate recorded was 1.5% (Stine et al. 2005). Therefore, 1.5% of the observed adenovirus or enterovirus concentrations were applied to the model.
3. Assumes that all observed viral genome copies detected by qPCR are infectious.
4. Applies known indicator/pathogen concentration ratios in sewage to estimate non-measured pathogen concentrations in irrigation water. Assumes the same die-off rates (and all other relevant parameters) within the canal and irrigation system between indicator and pathogens.
5. Assumes a certain consumption volume per day, not annually.
6. Assumes a single irrigation event with the contaminated water as the only potential input of pathogens to the produce surface.

Indicator organism selection: Because of its prevalence in irrigation waters in the current study, PMMoV was used as an indicator virus to estimate human fecal levels in the irrigation samples. PMMoV is abundant in human feces and is non-detectable in most animal feces, lending to its host specificity properties (Haramoto et al. 2013). It is nonpathogenic to humans, demonstrates weak seasonality, and is more resistant to environmental factors than bacterial indicators (Colson et al. 2010; Haramoto et al. 2013; Kitajima et al. 2014).

Pathogen selection and hazard identification: Enterovirus and adenovirus were selected for this risk assessment based on the hazard they pose to human health. Human adenovirus is pathogenic and can cause enteric, respiratory, and eye infections in humans (Jiang 2006). Enteroviruses can cause hand, foot, and mouth disease in humans (Cabrerizo et al. 2014) as well as a variety of other diseases ranging from rashes to polio-like illness. The pathogens of interest, norovirus, rotavirus, *E. coli* O157, and *Shigella*, were selected based on their negative impact on human health and annual cost of treatment; each of these pathogens poses a unique threat to human health. The high impact of each pathogen warrants further investigation into the risks posed by direct exposure to irrigation water contaminated at estimated levels.

Exposure assessment: This risk assessment assumed an adult consuming lettuce irrigated with water contaminated at the levels observed or estimated for either the observed pathogens (adenoviruses and enteroviruses) or the estimated pathogen of interest. The daily dose (λ) received was calculated using the following equation, modified from a dose calculation previously recorded by Hamilton et al. (2006) to include a pathogenic transfer rate recorded by Stine et al. (2005):

$$\lambda = M((p * c_{iw})V_{prod}e^{(-k_{Viral\,decay}t)})$$

where *M* is the mass of lettuce consumption per person per day (g/person per day), *p* is the percent pathogen transfer from the irrigation water to the surface of lettuce, $c_{iw}$ is the concentration of the target virus in the irrigation water (PFU/ml), $V_{prod}$ is the volume of irrigation

water that clings to the produce surface (based on prior studies: Stine et al. 2005; Hamilton et al. 2006), *k* is the general kinetic virus decay constant, and *t* is the time between the irrigation event and harvest. This equation is for a single irrigation event, with the contaminated water as the only input of pathogens to the produce surfaces. The information for each of these variables for the exposure assessment calculation is provided in **Table 5**.

Dose-response assessment: Each pathogen, measured and estimated, was recommended to best fit either the exponential model or the beta-Poisson model (**Table 6**). Norovirus was assigned the exponential model as previously recorded due to limited knowledge of norovirus dose-response behavior (Masago et al. 2006). Adenovirus and enterovirus was assigned the exponential model using specific parameters (Table 6):

Exponential response model:

$$P(response) = 1 - \exp(-k \times \lambda)$$

Beta-Poisson response model:

$$P(response) = 1 - \left[1 + \lambda \frac{(2^{\frac{1}{\alpha}} - 1)}{N_{50}}^{-\alpha}\right]$$

Risk model: The model incorporated the previously described dose calculation into the best-fit dose response model (e.g., the exponential model). The final exponential model is shown below. The model was applied to both human adenovirus and enterovirus with viral species-specific parameters.

**Final risk model:**

$$P(response) = 1 - \exp(-k_{virus} \times M(p * c_{iw)} V_{prod} e^{(-k_{Viral\ decay} t)})$$

Using this equation, the model was run with viral species-specific parameters for both enteroviruses and adenoviruses using the entire datasets. Models were tested using a Monte Carlo technique, with a total of 10,000 iterations, using the @RISK software (Palisade Corp., Ithaca, NY). The datasets were assigned a lognormal distribution within the modeling software and the distribution was defined using lognormal distribution parameters (mean and standard deviation of the $\log_{10}$ values) input into the @RISK program. The distribution was truncated at zero during the simulation to prevent illogical negative concentration values. The distributions assigned to each estimated pathogen are shown in **Table 7**.

Results:

This risk assessment does not account for microbial decay or removal at any subsequent point on the farm-to-fork pathway beyond harvest. It assumes that the microbial concentration estimated to be on the produce surface immediately before harvest will eventually reach the consumer. This assumption ignores the removal of the outer leaves of head lettuce during harvest, continued microbial decay post-harvest, microbial removal via washing sanitization, and other factors that would cause subsequent microbial reduction on the produce surfaces. There are numerous such circumstances and conditions that would result in a decrease in pathogen levels on produce prior to it being consumed. As such, this should be approached as a conservative estimate of risk. The actual risk of illness to the consumer would be much lower.

Observed viral pathogen levels and associated risk: Adenovirus was detected in 1.5% of samples, with a geometric mean of $1.0\times10^4$ genome copies per liter of the original water sample. Enteroviruses were detected in 8.2% of samples, with a geometric mean of $2.6\times10^5$ genome copies per liter of the original water sample.

When assessing the entire datasets, the mean predicted risk for adenoviruses and enteroviruses was $3.1\times10^{-5}$ and $1.2\times10^{-5}$, respectively, which translates to a risk of 3.1 adenovirus infections per 100,000 exposures and 2.1 enterovirus infections per 100,000 exposures (**Table 8**).

Fecal matter concentration in irrigation water and estimated risk for pathogens of interest: The amount of fecal concentration across all samples was $7.6\times10^{-7} \pm 6.1\times10^{-6}$ ml raw sewage per ml of irrigation water, assuming a normal distribution of values. This level of fecal contamination was used to predict the contamination levels for the pathogens of interest: norovirus, rotavirus, *E. coli* O157, and *Shigella* (**Table 8**). The estimated levels of risk associated with these pathogens based on these contamination levels in irrigation water are also indicated.

## Outcomes and Accomplishments

Beneficiaries of this project are growers, irrigation district managers, produce processing companies, consumers, and government agencies. The current study resulted in the development and evaluation of a novel sampling method for viruses in irrigation waters using small volumes that are feasible for collection by growers.

In addition, the development of a more accurate and quantitative method for the detection of fecal contamination in irrigation water will provide the industry with a necessary tool to evaluate water quality through a more scientifically based approach targeting a novel indicator organism that better fits the definition of an effective indicator organism. With this information, growers and government agencies are provided a statically driven approach to setting limits of fecal contamination that is acceptable for irrigation purposes. From the results of this study, pepper mild mottle virus (PMMoV) appears to be a good candidate for a new indicator organism for fecal contamination of irrigation waters.

By obtaining quantitative data regarding the level of fecal contamination present in irrigation water through these new viral targets, useful estimates for the amount of fecal contamination and the presence of various pathogens in irrigation water could be made. This data could be used for even more improved exposure assessments of fecal contamination found in the various irrigation waters by including post-harvest outcomes for pathogens on produce such as removal through washing/sanitization or microbial die-off during processing and transport prior to the produce reaching the consumer. With such information, major information gaps regarding exposure will be available for more sophisticated risk analyses to be conducted. This study resulted in a QMRA using observed PMMoV levels in irrigation waters from four regions in the United States to estimate the levels of pathogens of interest and the risks to consumers of fresh produce associated with their presence in waters used to irrigate such crops.

At least two publications related to this work are being drafted; these are related to the occurrence and relative abundance of the proposed viral indicators in this study and the use of PMMoV to estimate the amount of fecal contamination and thus the predicted amount of pathogens present in irrigation waters.

## Summary of Findings and Recommendations

The findings and recommendations resulting from this study include the following:

- Physical/chemical and microbial parameters can vary significantly between different regions
- It is important to concentrate samples in order to detect viruses
- It is feasible to sample small volumes (~3 liters) of irrigation waters to detect indicator viruses that are found in high numbers in human and animal feces. These viruses could be used for the routine monitoring of irrigation water quality
- The presence of fecal indicator viruses can point to changes in water quality and may correlate with the presence of pathogens
- PMMoV is readily detected in irrigation water samples (between 36% and 63%) and can be used to estimate the amount of fecal contamination in the water
- Cattle viruses do not appear to be good fecal indicators in irrigation waters in the regions included in the current study
- Estimates of fecal contamination obtained with PMMoV can be used to perform a quantitative microbial risk assessment to determine the risks of foodborne illness caused by various pathogens as a result of consuming fresh produce irrigated with contaminated water
- The QMRA performed in the current study resulted in a conservative estimate of risk. Future studies should try to develop data to fill the gaps between an irrigation event using contaminated water and the survival/persistence of specific pathogens between this event and subsequent harvest, processing, transport, etc.

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## APPENDICES

### Publications and Presentations

Two publications are currently being drafted to submit to peer-reviewed scientific journals.

### Budget Summary

Funding has been expended to date that includes approximately $58,648 in materials and supplies, $6,873 in travel expenses (gasoline, hotels, per diem), $148,399 in personnel costs, and $7,420 in indirect costs for a total of $221,340 spent on the project. All grant funds were utilized during the course of the project.

### Figure and Tables

**Figure 1.** The occurrence of generic *E. coli* in irrigation waters in four regions in the U.S.

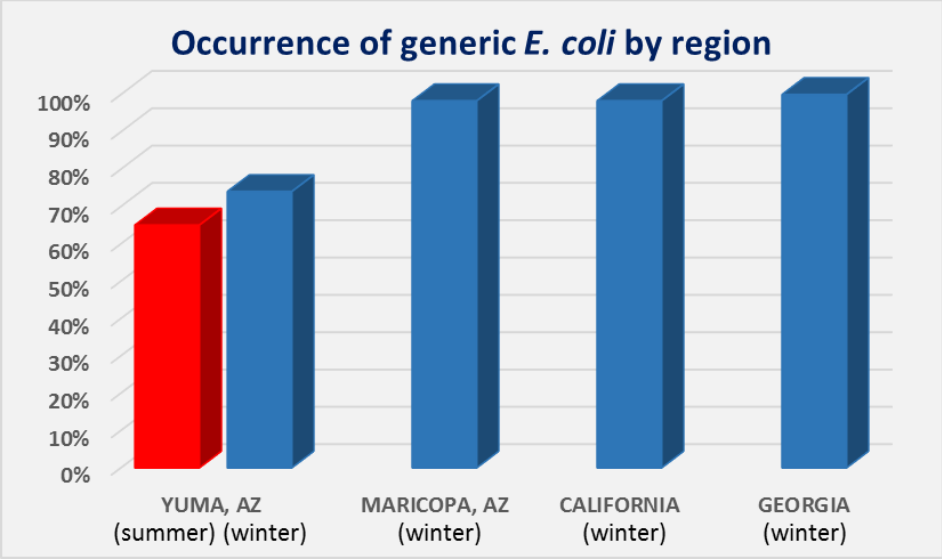

**Table 1.** Irrigation water quality in each region.

| Irrigation water quality by region (geometric means) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Region | Season | Physical / Chemical Parameters | | | | | | | Colilert | |
| | | $H_2O$ Temp ($^oF$) | Air Temp ($^oF$) | Relative Humidity (%) | pH | Conduct. | TDS (ppm) | Turbidity (NTU) | Coliforms (MPN/100 ml) | *E. coli* (MPN/100 ml) |
| YUMA, AZ | summer | 84 | 95 | 23 | 8.7 | 1209 | 765 | 4.0 | 1396 | 5 |
| | winter | 57 | 66 | 30 | 8.8 | 1236 | 920 | 2.2 | 268 | 4 |
| MARICOPA, AZ | winter | 71 | 85 | 12 | 8.5 | 1415 | 1000 | 2.5 | 683 | 27 |
| CALIFORNIA | winter | 66 | 77 | 22 | 8.9 | 1115 | 792 | 3.0 | 1663 | 10 |
| GEORGIA | winter | N/A | N/A | N/A | N/A | N/A | N/A | N/A | 1474 | 9 |

**Table 2.** The occurrence of Salmonella species in irrigation waters in four different U.S. regions

| Region | # positives / # samples | % positive |
|---|---|---|
| YUMA, AZ | 28 / 190 | 15% |
| MARICOPA, AZ | 13 / 60 | 22% |
| CALIFORNIA | 21 / 60 | 35% |
| GEORGIA | 8 / 20 | 40% |

**Table 3.** Occurrence of viruses in irrigation waters from four different regions in the U.S.

| Agricultural Region | Bovine Polyomavirus | Bovine Adenovirus | Aichivirus | Pepper Mild Mottle Virus | Adenoviruses | Enteroviruses |
|---|---|---|---|---|---|---|
| Yuma, AZ | 0/180 (0%) | 0/180 (0%) | 3/180 **(2%)** | 65/180 **(36%)** | 3/180 **(2%)** | 7/180 **(4%)** |
| Maricopa, AZ | 0/60 (0%) | 0/60 (0%) | 0/60 (0%) | 38/60 **(63%)** | 0/60 (0%) | 0/60 (0%) |
| California | 0/60 (0%) | 0/60 (0%) | 0/60 (0%) | 28/60 **(47%)** | 2/60 **(3%)** | 0/60 (0%) |
| Georgia | 0/20 (0%) | 0/20 (0%) | 0/20 (0%) | 12/20 **(60%)** | 0/20 (0%) | 20/20 **(100%)** |

**Table 4.** Recorded levels of pathogens of interest in sewage.

| Pathogen | Concentration in raw sewage | Assumption |
|---|---|---|
| *E. coli* O157 | 0.1–1 CFU/ml (Nwachuku & Gerba 2008) | Uniform distribution |
| *Shigella* | $1x10^3$ CFU/ml (National Resource Council) | Point-estimate |
| Rotavirus | 0.0098 FFU/ml (Hejkal et al. 1984) | Point-estimate |
| Norovirus | $2×10^2$ GC/ml (Lodder & de Roda Husman 2005) | Point-estimate |

* CFU = colony forming units; FFU = fluorescent focus forming units; GC = genome copies

**Table 5.** Values for variables used in the exposure assessment.

| Variable | Variable description | Value | Reference |
|---|---|---|---|
| $M$ | Lettuce consumed daily per capita (g/person per day) | 12.1 | USEPA 2011b |
| $c_{iw}$ | Concentration of viruses in irrigation water (copies/ml) | Observed or estimated for this study | |
| $V_{prod}$ | Volume of irrigation water captured by the produce (ml/g) | 0.108 | Hamilton et al. 2006 |
| $p$ | Pathogen transfer from the water to the surface of the lettuce (%) | 1.5 (viruses)<br>0.007 (bacteria) | Stine et al. 2005 |
| $k_{Virus}$ | Kinetic virus decay constant (% per day) | 0.69 (viruses)<br>0.35 (bacteria) | Hamilton et al. 2006<br>Stine et al. 2005 |
| $t$ | Time between last irrigation and harvest (days) | 4 | Recommended for water of poor quality |

**Table 6.** Models and best-fit parameters for each pathogen. All parameter and best-fit models were based on the QMRAwiki (http://qmrawiki.canr.msu.edu).

| Pathogen | Best-fit model | Optimized parameters | $LD_{50}/ID_{50}$* | Response |
|---|---|---|---|---|
| *E. coli* EHEC | Exponential | $k=2.18\times10^{-4}$ | $3.18\times10^{3}$ CFU | shedding in feces |
| *Shigella* | Beta-Poisson | $\alpha=2.65\times10^{-1}$, $n=1.48\times10^{3}$ | $1.4\times10^{3}$ CFU | positive stool isolation |
| *Rotavirus* | Beta-Poisson | $\alpha=2.53\times10^{-1}$, n=6.17 | 6.17 FFU | infection |
| Norovirus | Exponential | $k=-\log 0.5/ID_{50}$ $ID_{50}$ was set to 10 (10 was selected to lower the $ID_{50}$ and create a more conservative estimate of risk) | 10 | infection |
| Adenovirus | Exponential | $k=6.07\times10^{-1}$ | $1.14\times10^{0}$ | infection |
| Enterovirus | Exponential | $k=1.85\times10^{2}$ | $1.85\times10^{2}$ | infection |

* $LD_{50}$ = lethal dose 50%; $ID_{50}$ = infectious dose 50%

**Table 7.** Distribution assigned to each estimated pathogen.

| Pathogen | Distribution |
|---|---|
| *Shigella* | Normal, truncated at P1 and P99 |
| *E. coli* O157 | Normal |
| Rotavirus | Normal, truncated at P1 and P99 |
| Norovirus | Normal |

**Table 8.** Estimated or measured pathogen levels in irrigation water samples and the associated risk of illness following one irrigation event.

| Pathogen of interest | Estimated/measured concentration* | Risk of illness (per # of exposures) |
|---|---|---|
| *E. coli* O157 | $9.9\times10^{-7}$ CFU/ml | 5.6 cases per $10^{14}$ |
| *Shigella* | $1.6\times10^{-3}$ CFU/ml | 8.3 cases per $10^{10}$ |
| Human Norovirus | $3.2\times10^{-4}$ GC/ml | 1.2 cases per $10^{7}$ |
| Rotavirus | $1.6\times10^{-8}$ FFU/ml | 1.2 cases per $10^{10}$ |
| Human Adenoviruses | $2.6\times10^{-2}$ GC/ml | 3.1 cases per $10^{5}$ |
| Enteroviruses | $9.2\times10^{-2}$ GC/ml | 1.2 cases per $10^{5}$ |

* CFU = colony forming units; FFU = fluorescent focus forming units; GC = genome copies