CA Lettuce Research Board 2007-08 Research Report Summary

Project Title: Assessment and Validation of Improved Methods for Irrigation Water Quality Compliance Monitoring and Detection of *Escherichia coli* O157:H7

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Objectives:

- To assess the seasonal variation in water quality indicators associated with selected irrigation reservoirs and the associated irrigated Romaine lettuce crop.
- To evaluate improved methods to re-assess irrigation water as a potential risk factor for *E. coli* O157:H7 or other enteric pathogen contamination in leafy greens production environments.

Summary of Findings:

- Population levels of nonpathogenic *E. coli* in irrigation reservoirs were not found to be predictive of populations on the irrigated Romaine lettuce crop. *E. coli* populations on all three overhead irrigated Romaine crops (assayed as unwashed lettuce) were low, 7-15 CFU/25gm, irrespective of independent water source. No evidence for the presence of *E. coli* O157:H7 in the reservoir with highest generic *E. coli* populations, rolling geometric mean 85 to 824 MPN/100ml, was observed within the limits of the study methodology.
- Modified Moore Swabs were determined to facilitate filtration and bacterial capture of at least 10L of irrigation source water.
- These preliminary results indicated that presence/absence determinations of low numbers of viable *E. coli* O157:H7 may be detected in 10L of non-sterile irrigation source water, following an enrichment and concentration procedure.
- A Colony Blot Detection protocol for immunodetection of *E. coli* O157:H7 was successfully adopted to facilitate culture-confirmation from irrigation water source testing and related environmental or lettuce samples. This method for differentiation of false positives from bona fide colonies on recovery media will be combined with assessments of rapid test methods.

<u>Overall Conclusions</u>

Within the limits of methodology, access to representative irrigation reservoirs and lettuce fields, and combined with other data from 2003-2006 there does not appear to be a strong predictive value in irrigation source monitoring of generic *E. coli* populations in the Central Coast region. A correlation between generic *E. coli* and pathogens in reservoir sources appears nonexistent. No strong correlation between irrigation water quality and levels of generic *E. coli* on the irrigated lettuce is apparent. However, it is apparent from multiple years of surveying the same reservoirs that left untreated, populations of indicator bacteria do follow trends and the source/cause of the few reservoirs with consistently high levels of *E. coli* remains to be determined. Similarly, much larger volumes and frequencies of pathogen testing of these water sources should be done before dismissing the potential for surface water to be a focal point for contamination. These preliminary results indicated that presence/absence determinations of low numbers of viable *E. coli* O157:H7, at least 6 CFU, may be detected in 10L of non-sterile irrigation source water, following an enrichment and concentration procedure. The limits of detection with increasingly complex water sources and in-field validations studies remain to be conducted.

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Project Title: Assessment and Validation of Improved Methods for Irrigation Water Quality Compliance Monitoring and Detection of *Escherichia coli* O157:H7

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Background: A variety of indicator organisms (IO) have been used by the fresh produce industry to assess the suitability of water used in preharvest crop management prior to seasonal planting and concurrent with preharvest application, including up to the point of harvest (8, 9). Typically, total coliform, fecal coliform, and/or generic (commensal) *E. coli* have been used; however no clear and supportable standards have been available to establish microbial limits or criteria that define suitable versus unacceptable quality for the diverse sources and modes of application. No federal standards for irrigation water quality exist and international standards are considered too permissive to ensure adequate risk reduction. The potential for contaminated irrigation water to represent a significant risk to consumers due to external and, possible, internal contamination of leafy greens has been recently reviewed in some detail (3). Recent events, exemplified by the *E. coli* O157:H7 outbreak on spinach in Sept 2006, have accelerated the efforts of industry leadership to define practical and meaningful criteria, including irrigation water, which would be the preliminary benchmarks for monitoring and compliance assessments. Within these standards, various IO are monitored with the expectation that their presence is predictive of recent fecal contamination and the elevated potential for human enteric pathogens to be present. The adoption of meaningful and predictive standards or criteria, particularly for irrigation water quality, is significantly hampered by the apparent lack of correlation between indicator coliforms or generic *E. coli* levels and likely fecal contamination of public health significance or the potential for the detectable presence of pathogens such as *E. coli* O157:H7.

Microcosm and mesocosm studies (1, 7) have demonstrated the severe limitations of popular IO's, including commensal *E. coli*, in predicting pathogen presence or correlating to proportional survival following fecal contamination. Further, multi-year survey research conducted in the Salinas region has failed to establish a positive correlation between presumptive fecal IO and pathogen detection in well reservoirs or at the point of overhead irrigation emitters (10, 11, 12). Within these studies, it was demonstrated that the growth of IO and bacterial pathogens in stored irrigation water was possible at permissive temperatures; assessed from 12 to 25C (54 to 77F). Water temperatures within this range conducive for growth were observed, seasonally, in on-farm reservoirs and irrigation conveyances. Persistence of IO in the absence of detectable levels of pathogens and secondary growth strongly suggest that the use of current IO is compromised and renders decision-making based on presence/absence or numerical thresholds of IO an unnecessarily self-penalizing practice.

Purpose:

- To assess the seasonal variation in water quality indicators associated with selected irrigation reservoirs and the associated irrigated Romaine lettuce crop.
- To evaluate improved methods to re-assess irrigation water as a potential risk factor for *E. coli* O157:H7 or other enteric pathogen contamination in leafy greens production environments.

Objective 1: Assess the seasonal variation in presumptive fecal indicators in relation to the presence of *E. coli* O157:H7 and indicator populations on the associated irrigated crop

Three irrigation reservoirs within the Salinas region were selected for seasonal monitoring of thermotolerant coliforms (TTC), commensal *E. coli*, and *E. coli* O157:H7. These reservoirs were selected based on prior monitoring to represent typically low, medium, and high anticipated levels of generic *E. coli* in replicated 100 ml grab samples.

Procedure

All methods and materials used in this research effort were previously detailed in reports to the CLRB (12) and, for brevity in this report, will not be repeated.

Results

Populations of presumptive IO in surface water grab samples from three on-farm reservoirs were variable among the individual locations and over time (Table 1-3). In general, location SVR 11 had the consistently highest levels of recoverable *E. coli*, up to log 3.16 CFU/100ml, equivalent to approximately 1445 MPN/100ml on a single date sample. In terms of establishing a rolling geometric mean for SVR 11, the first 5-point value would be 203 MPN/100ml and subsequent assay points were 824, 553, 376, 263, 151, and 85 MPN/100ml. SVR 10 and SVR 28 were consistently below the current target of 126 MPN/100ml with the exception of one single August date for SVR 28; 151 MPN/100ml.

As in previous years, no pattern or correlation between population densities of generic *E. coli* and thermotolerant coliforms (aka fecal coliforms) was observed. No clear pattern in IO populations and water temperature of the surface grab sample, measured at collection, was apparent. These temperatures, based on laboratory studies with well water blended with tailwater, are conducive for bacterial multiplication if sufficient nutrients are present. In earlier research surveys that included these same reservoirs, the average IO populations recovered from grab samples taken at sprinkler heads was 7 MPN/100ml (max 81) and 36 MPN/100ml (max 300) for *E. coli* and TTC, respectively.

No *E. coli* O157:H7 was detected in any 1L water sample within the limits of sample size and detection methodologies used for these studies. Due to circumstances beyond our control, permission to evaluate the lettuce crop for pathogenic *E. coli* was withdrawn.

Populations of generic *E. coli* on mature Romaine lettuce irrigated from these reservoirs were consistently low (Table 4). Plants irrigated with water from SVR 11 had a higher average population of TTC on leaves but other factors related to crop location, microclimate, and management may be responsible for these observations apart from the higher TTC levels in the source water, as measured by surface grab samples. An example of the variability in TTC levels on individual plants for SVR 11 is presented in Figure 1.

Conclusion: Within the limits of methodology, access to representative irrigation reservoirs and lettuce fields, and combined with other data from 2003-2006 there does not appear to be a strong predictive value in irrigation source monitoring of generic *E. coli* populations in the Central Coast region. A correlation between generic *E. coli* and pathogens in reservoir sources appears nonexistent. No strong correlation between irrigation water quality and levels of generic *E. coli* on the irrigated lettuce is apparent. However, it is apparent from multiple years of surveying the same reservoirs that left untreated, populations of indicator bacteria do follow trends and the source/cause of the few reservoirs with consistently high levels of *E. coli* remains to be determined. Similarly, much larger volumes and frequencies of pathogen testing of these water sources should be done before dismissing the potential for surface water to be a focal point for contamination.

Objective 2: Assess the performance of Modified Moore Swabs as a low-cost method of large volume irrigation source surveys

To improve the predictive value of irrigation source analysis in both research and commercial practice, we utilized a capture-swab, similar to the Moore Swab approach used by researchers monitoring sewer effluent, manure run-off, and pathogen-impacted irrigation water (5,6,13). More recently, Moore Swabs have increased the frequency of detection of *E. coli* O157:H7 in watershed run-off in the Salinas region (4). During this project period we preliminarily evaluated a modification of this sampling strategy with the intent to re-assess regional irrigation sources and modes of delivery (such as overhead irrigation) to trap the bacteria, either free or attached to suspended solids, by surveying larger volumes of source water than typical grab-samples used for IO evaluations. Modified Moore Swabs (MMS) were developed with the intent to analyze for the presence of *E. coli* O157:H7 and *Salmonella* using nonselective enrichment and immunomagnetic separation system (IMS) or membrane filtration coupled with PCR and confirmation plating on selective media. As suitable survey sites were not available during the project period, this report presents the outcome of laboratory assessments during the initial method development.

Preliminary Tests: Modified Moore Swabs have been used to capture and assess the presence of commensal *E. coli* (to facilitate safe handling during system development prior to introduction of pathogenic forms) from larger volumes of irrigation water than have previously been employed in typical grab samples. Early studies determined recovery efficiency in simulated tests with spiked irrigation water, of decreasing clarity, as briefly described below:

Procedure

Pre-autoclaved cheesecloth (90 cm by 30 cm) was used to create the MMS for filtering the inoculated water. The swab was placed inside a 14 cm long PVC piping (assembled using screwed-in ends that were glued on both sides; See Fig 2, 3, and 4) to create a filtration cassette. Inoculated water was filtered through the MMS unit by attaching the cassette to a peristaltic pump set at a flow rate of 1 liter per minute.

Three independent experimental setups evaluated capture sensitivity by inoculating 1.0 g of a clay-loam soil with different concentrations of commensal (generic) *E. coli* (TVS 356 transformed to express kanamycin resistance and GFP on a stable plasmid) and suspending in 10 L of nanopure water to achieve three different target concentrations. The water turbidity level after suspending the clay-loam soil with water was measured to be 14 FAU, typical of many irrigation reservoirs in the CA Central Coast.

Recovery was accomplished by extracting the MMS from the cassette and placing the saturated swab (approximately 100ml of free water) in a sterile Whirlpak® Bag holding 100ml 2X TSB. The broth culture was incubated without shaking at 37ºC and duplicate15ml sample aliquots extracted at 4 and 7 hours. Each 15ml enrichment sub-culture was filtered with an IsoGrid Membrane system (Neogen Corp.; Lansing, MI) and the grid placed on TSA+Kan (50mg/L) + 0.1% Pyruvate at 37 ºC and CHROMAGAR™ ECC (DRG International Inc. Mountainside, NJ) + Kanamycin held at 22C for 2 h and transferred to 44.5 ºC. Grids were inspected for fluorescence typical of GFP at 18 and 36h.

Results
The enumerated levels of challenge *E. coli* inoculum were 638, 21, 2 CFU/10L. In all three enrichments, the generic *E. coli* kan:GFP used to inoculate the water was present on the grid. Fluorescent membrane grid-pores were absent in uninoculated water.

MMS Capture of *E. coli* O157:H7

Procedure
The experimental system for inoculated soil challenge in water was conducted as described above with a minor modification; 10 liters of irrigation reservoir water collected from a commercial farm in the Salinas region was inoculated with a marked, pathogenic *E. coli* O157:H7, (PTVS 81, GFP and KAN resistant). The target concentration was obtained by inoculating water with 100 µl of a 10^1 CFU/ml, adjusted from a standardized suspension in 0.1% BPW that had been held for 24h at 2.5C. Conditions for filtration capture, enrichment, and recovery were as described above. Duplicate aliquots of 15ml enrichment culture were filtered with IsoGrid Membrane and transferred to TSA+Kan+Pyruvate and ChromAgar O157+Kan, both held at 37 ºC for up to 36h.

A colony blotting procedure was adopted from published reports and operator proficiency verified in our lab to allow screening of agar cultures for the presence of *E. coli* O157:H7 from irrigation sources and lettuce (See Appendix 1).

Results
The measured inoculum delivered to the irrigation water source was 6 CFU/10L. Pathogenic *E. coli* kan:GFP used to inoculate reservoir water was present on filters, visualized by fluorescence under UV light (See Figure 5). Fluorescent membrane grid-pores were absent in uninoculated water.

No opportunities with grower cooperators to test this system and the Colony Blot Detection Method from regional irrigation sources were possible within this funding period.

Conclusion

These preliminary results indicated that presence/absence determinations of low numbers of viable *E. coli* O157:H7, at least 6 CFU, may be detected in 10L of non-sterile irrigation source water, following an enrichment and concentration procedure. The limits of detection with increasingly complex water sources and in-field validations studies remain to be conducted.

In actual practice, MMS capture-swabs are analyzed for the presence of *E. coli* O157:H7 and *Salmonella* using nonselective enrichment broth media and immunomagnetic separation system (IMS) or membrane filtration coupled with PCR detection. Preliminary detection is conducted using the BioControl Inc, Assurance GDS system for *E. coli* O157:H7 and *Salmonella* in comparison to similar systems provided by Qualicon BAX. All molecular virulence-marker positive samples for target pathogens are culture-confirmed by plating on selective and differential media followed by repeat molecular marker confirmation and immunodiagnostic tests.

Objective 2: Evaluation of a membrane based immunocapture technique for trapping *E. coli* O157:H7 cells from irrigation source water

Processing of large volumes of water from irrigation reservoirs by conventional filtration methods presents practical handling issues, depending on the source, due to suspended solids such as clay, silt, humus, algal particles, and other organic material. Concentration of water samples by vacuum filtering through membranes (<0.4 µ) or by ultrafitration across hollow-fiber membranes is a very tedious process and can be also be problematic if clay and suspended organic matter and colloids are present. Recovery efficiency problems may be reduced by tangential flow filtration devices but the unit cost is high. Within this project period we evaluated a recirculating immunocapture system to survey large volumes of irrigation quality water for the presence of *E. coli* O157:H7.

Procedure

Eight cm diameter circles cut out of PVDF membranes were coated on one side with 1µg of MAb diluted in 1 ml of Phosphate buffered saline (PBS) containing Tween-20 (T). The membranes were dried to render them hydrophobic and placed in a glass container fitted with closures containing extensions overfit with tubes for inlet and outlet. Water samples (3 L), spiked with *E.coli* O157:H7 bacterial cells (as described above) were circulated across the MAb coated PVDF membrane, at 50 to 200 ml/minute using a peristaltic pump. After four hours of circulation, which would allow at least 10 volumes (30 L) to be re-circulated, PVDF membranes are removed, rinsed briefly in sterile PBS-T, and transferred onto differential agar media CHROMagar plate or CT-SMAC plates such that the MAb coated surface comes in contact with agar directly. Plates were incubated overnight at 37C to allow bacteria to grow. Coating PVDF with 1 µg of MAb (ca. 4×10^{12} IgG molecules) would potentially allow more than a million bacterial cells per mm^2. Following 18h incubation, cells on the membrane-agar medium interface were bound to the MAb coated membrane, thus allowing examination of colonies using affinity-purified polyclonal O157:H7 specific IgG conjugated with horse radish peroxidase (Kirkegaard and Perry Laboratories Inc., Gaithersberg, MD). Residual colonies on CHROMagar or CT-SMAC allowed completion of confirmation tests, as described above. In addition, immunocapture membranes could be placed in liquid enrichment media for alternative presence/absence detection methods.

Four replicate spiking experiments with populations, 0, 1, 5, 10, 100, and 1000 cells L^{-1} in samples were tested against MAb coating amounts of 0.1, 0.25, 0.5 and 1 µg IgG, per 8 cm diameter PVDF circle.

Results

The immunocapture antibodies were demonstrated to recognize most, but not all, *E. coli* O157:H7 lettuce-associated and other isolates commonly used in our research (Table 5, Fig. 6). Generic *E. coli* and non-O157 *E. coli* gave negative outcomes. The benchtop system was observed to have very good specificity but very low capture efficiency as water quality declined. For this reason studies were not completed and data from preliminary tests are not included in this report.

Immunoblotting procedures were successfully introduced into the detection and recovery program used for environmental testing associated with lettuce and leafy greens. The technique was determined to be very useful in screening presumptive colonies in conjunction with PCR-based colony screens.

Objective 3: Determine the degree of correlation between levels of fecal coliform, generic *E.coli*, and presence of F^+RNA phage in irrigation source water

While the persistence of these IO bacteria in water distribution systems is comparable to that of some bacterial pathogens, the relationship between bacterial indicators and the presence of true pathogens is poor and often absent. Due to the limitations of developing practical and predictive irrigation water quality standards based on bacterial IO indicators, although easier and less costly than pathogen testing, it is clear that there remains an acute need to develop improved indicators of fecal contamination. Ideally these methods would be rapid and simultaneously provide immediate directional information as to the most likely source/host of that pollution so corrective investigations and actions could be initiated. The overall goal of this project was the field assessment, based on preliminary lab results, of an integrated detection method, which was reported to simultaneously determine the presence of fecal pollution and also the source, animal or human, of that pollution, in one hour. The method is based on rapid concentration and identification of F^+RNA phages, and simultaneous characterization of the phages to determine their source (which allows for an indication of the source of fecal pollution). F^+RNA phages have attracted interest as useful alternatives to bacterial indicators because their survival characteristics closely resemble broad groups of pathogens of concern and have been used to reliably detect the presence and source of fecal pollution in water.

Procedures

During this project period, irrigation water collection sites from reservoirs receiving pumped ground water that were not treated with an antimicrobial agent were not available. To allow some assessment to proceed and to develop a research interaction with Dr. Larry Goodridge of CSU, composite site irrigation runoff water that passes through a municipality was collected on four separate dates. Replicated, concentrated 1 L samples of this water was shipped with gel-ice chilled, insulated containers to Goodridge for analysis for the presence of F^+RNA phage using conventional coliphage techniques and a prototype lateral flow device Assessment of fecal coliform and generic *E.coli* from split samples of the same source was conducted in the Suslow lab. Samples were obtained from July to August 2007 from a site in the Salinas Valley. Samples were circulated through a Pathatrix system and captured onto cationically charged beads based on the following method. Briefly, 250 ml of sample was placed into a Whirlpak bag and inserted into the Pathatrix system. The system was allowed to recirculate for 120 min at 25^{o}C, and after the capture step, the cationic beads were removed and placed into a 2 ml microcentrifuge tube. The phages were eluted off the beads with the use of 2 ml of elution buffer (1.5% beef extract, 0.25 M glycine, 0.2% tween 80, pH 9.5) with mixing at room temperature for 120 min. At the end of the elution step, the tube was placed into a Dynal portable magnet for 10 minutes, during which time the cationic beads were attracted to the magnet. The eluate was removed and placed into a sterile tube.

Isolation and Purification of Phages

Approximately 500 µl of the eluate was spot plated onto a lawn of *E. coli* C3000 (using a double layer technique), which is a suitable bacterial host for the FRNA phages. The plate was incubated overnight at 37$_o$C. The next day, the entire top layer (containing phage plaques) was scraped off the plate into 5 mls of lambda diluent (NaCl 5.8g, MgSO4.7H2O 2.0g, 1M Tris.HCl (pH 7.5) 50ml, Gelatin (Difco) 0.01%), and the phages were purified by mixing with an equal volume of chloroform, followed by

centrifugation at 10,000 RPM for 10 minutes. The aqueous layer was removed and filtered through a 0.2 µm syringe filter.

Detection and Characterization of FRNA Phages

Two methods were used to detect the presence of FRNA phages. The first method consisted of a FRNA Real Time Polymerase Chain Reaction (RT-PCR) assay, carried out using a multiplex primer set specific for the two genera of the FRNA (i.e. the *Leviviridae* and *Allolevivirida*e). Alternatively, we enriched the eluate overnight (shaking at 37^{o}C) in the presence of *E. coli* C3000. After the enrichment, an aliquot of the phage lysate was removed, syringe filtered as above, and 200 µl was placed on a newly developed lateral flow device (LFD), that was developed to detect group I FRNA phages.

Results

High densities of thermotolerant coliforms and moderate levels of generic *E. coli* were present in the run-off water (data not shown as the samples collected were not used for irrigation). FRNA phages were detected but low overall water quality may have interfered with capture. In Figure 7, the presence of two amplicons in the PCR gel indicate that both genera of FRNA phages were present in the sample. We are currently testing the sample with different primer sets to determine the FRNA groups (I – IV) that the phages belong to. This will provide information about a possible host source of fecal contamination in the sample. Figure 8 shows the LFD detection result. The LFD (capable of detecting group I FRNA phages) was designed as a proof of concept device to determine whether these devices could be used in the field to detect FRNA phages. The results indicate that, after an enrichment, a positive test result was observed. The low intensity of the band is due to the low sensitivity of the LFD (which we are currently trying to improve), and not a low concentration of FRNA phages in the sample. Also, since group I FRNA phages are known to be found in animal waste, this result provides preliminary information that suggest that this irrigation run off sample contained animal

Taken collectively, these results indicate the presence of FRNA phages in irrigation water runoff, and also indicate that these phages can be detected in a lab setting with RT-PCR or a field based setting with a LFD. The detection of F^{+}RNA phage was demonstrated in two of four sets of samples at very low titre.

Conclusion: Improvements in phage capture and detection are required before this technique will be field-ready.

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Table 1. Summary of water observations from irrigation reservoir SVR 10

						E. coli	TTC
Lab code	Date	Time	Temp ˚C	pH	EC dS	log cfu/100ml	log cfu/100ml
SVR 10	7/12	08:40	20.8	7.1	0.74	-0.05	-0.05
SVR 10	7/18	10:15	23.2	7.3	0.75	1.48	1.85
SVR 10	7/24	09:35	24.2	6.2	0.76	1.70	3.27
SVR 10	8/1	10:00	22.8	7.6	0.81	1.48	2.85
SVR 10	8/7	13:10	24.6	8.5	0.76	1.78	2.93
SVR 10	8/14	-	-	-	-	-	-
SVR 10	8/21	10:50	22.4	7.6	0.91	0.78	4.67
SVR 10	8/28	12:20	21.9	8.0	0.58	1.45	3.78
SVR 10	9/5	10:40	23.2	7.6	0.70	-0.05	4.22
SVR 10	9/11	12:15	26.1	4.6	0.72	0.85	4.02
SVR 10	9/18	14:20	25.7	5.4	1.39	1.00	2.41

Table 2. Summary of water observations from irrigation reservoir SVR 11

						E. coli	TTC
Lab code	Date	Time	Temp ˚C	pH	EC dS	log cfu/100ml	log cfu/100ml
SVR 11	7/12	09:10	21.1	7.4	1.30	2.26	1.08
SVR 11	7/18	10:40	22.1	7.7	1.67	2.15	2.11
SVR 11	7/24	08:40	22.2	7.2	1.25	2.32	2.36
SVR 11	8/1	10:30	22.1	8.2	1.21	2.60	2.87
SVR 11	8/7	14:05	23.1	8.1	1.17	1.95	4.20
SVR 11	8/14	09:30	22.4	8.1	1.23	3.16	2.68
SVR 11	8/21	10:30	20.9	8.2	0.99	2.45	4.56
SVR 11	8/28	11:45	22.2	7.7	0.71	2.30	4.20
SVR 11	9/5	11:10	22.3	6.6	0.83	2.18	2.23
SVR 11	9/11	11:50	24.3	6.7	1.16	1.60	2.83
SVR 11	9/18	13:30	24.4	7.0	2.00	1.30	2.70

Table 3. Summary of water observations from irrigation reservoir SVR 28

						E. coli	TTC
Lab code	Date	Time	Temp ˚C	pH	EC dS	log cfu/100ml	log cfu/100ml
SVR 28	7/12	09:30	20.7	7.2	1.77	-0.05	-0.05
SVR 28	7/18	11:00	23.4	7.4	1.19	-0.05	1.71
SVR 28	7/24	09:10	21.0	6.8	1.75	-0.05	2.10
SVR 28	8/1	11:00	20.7	8.2	1.71	-0.05	1.89
SVR 28	8/7	13:40	21.2	7.4	1.72	0.48	2.15
SVR 28	8/14	10:00	21.0	7.4	1.75	-0.05	-0.05
SVR 28	8/21	11:15	21.6	7.8	1.43	2.18	2.40
SVR 28	8/28	12:00	22.5	7.7	1.43	1.60	2.08
SVR 28	9/5	11:30	21.6	7.2	1.55	-0.05	-0.05
SVR 28	9/11	11:30	22.8	7.5	0.66	0.00	1.00
SVR 28	9/18	14:00	22.4	6.1	1.94	-0.05	-0.05

Table 4. Summary of 'indicator' populations on Romaine lettuce at harvest

Reservoir Code	Field Code	Avg. *E. coli* Log CFU/25g	std. dev.	Avg. TTC Log CFU/25g	std. dev.
SVR 10	SVL 50	0.91	0.01	3.59	0.80
SVR 11	SVL 53	1.18	0.28	4.34	0.67
SVR 28	SVL 54	1.00	0.16	2.99	0.23

Forty plants were harvested per field and assayed individually in duplicate by sectioning the entire head, topped and tailed, into 4 in^2 pieces, randomizing all outer and inner sections, and removing 25g sub-samples for bacterial enumeration.

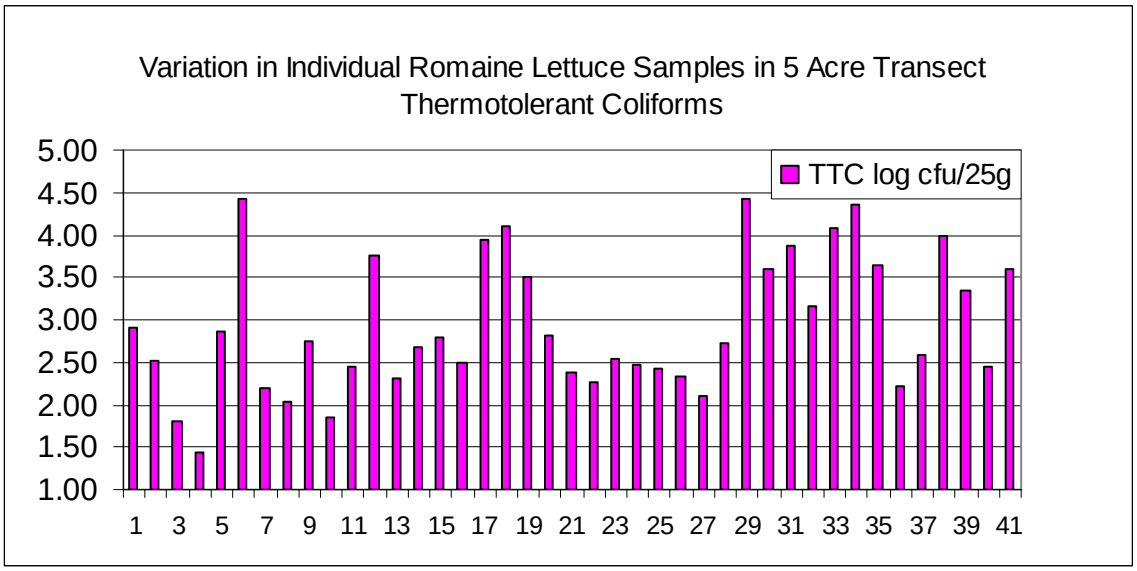


Figure 1. - Example of TTC populations on freshly harvested Romaine lettuce irrigated from reservoir source SVR 11. Forty heads were harvested along a transect that evenly divided a 5 acre section on a tangent to the direction of the seedbeds and sprinkler lines.

Figure 2: Modified Moore Swab cassette

Figure 3. Laboratory setup to evaluate capture efficiency in single-pass filtration with Modified Moore Swab

Figure 4 – Sediments captured from 10L of irrigation source water.

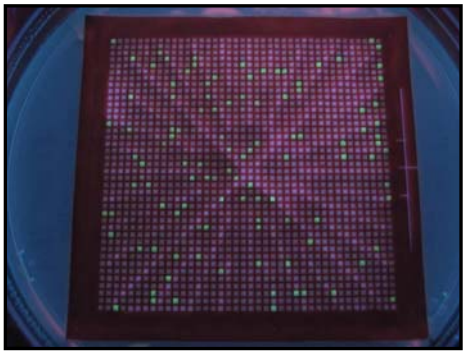


Figure 5 - Membrane capture filter (IsoGrid) with fluorescing pores on kanamycin containing media. Fifteen ml capture from initial 6 CFU/10L following 7h enrichment.

Table 5. Assessment of recognition of polyclonal and monoclonal antibodies towards E. coli O157:H7 isolates originating from lettuce outbreaks and non-toxigenic forms intended for use in controlled field inoculation studies. A nonpathogenic *E. coli* and some laboratory manipulated isolates (LJH 539, PTVS 17 and 18) retain virulence markers but lack antigens recognized by the Mab used in these tests.

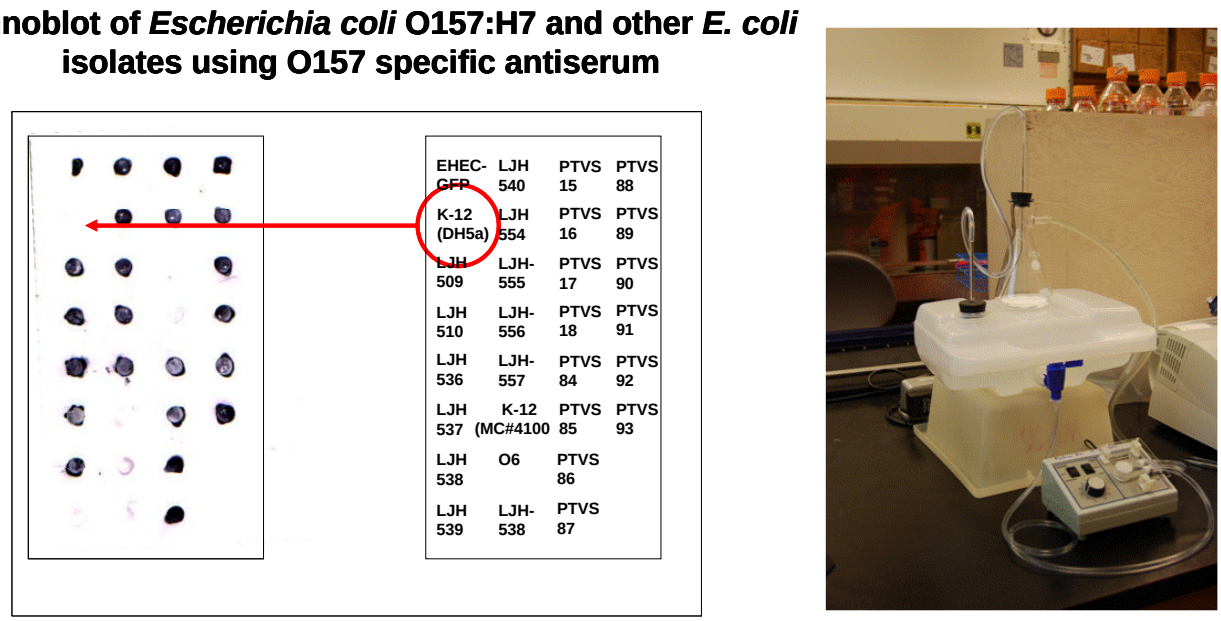
	Polyclonal (BD Bioscience)	C65310M (Biodesign Intl)	C65160M (Biodesign Intl)	13B3[2]	Stx1	Stx2	UidA
EHEC GFP	+	+	+	+	+	+	+
K-12[1]	-	-	-	-	-	-	-
LJH- 509	+	+	+	+	+	+	+
LJH- 510	+	+	+	+	+	+	+
LJH-536	+	+	+	+	+	+	+
LJH-537	+	+	+	+	+	+	+
LJH-538	+	+	+	+	+	+	+
LJH-539	-	-	-	-	+	+	+
LJH-540	+	+	+	+	+	+	+
LJH-554	+	+	+	+	+	-	+
LJH-555	+	+	+	+	+	+	+
LJH-556	+	+	+	+	+	-	+
LJH-557	+	+	+	+	+	+	+
LJH-569	-	-	-	-	-	-	-
LJH-570	-	-	-	-	-	-	-
LJH-538	-	-	-	-	-	-	-
PTVS-15	+	+	+	+	+	+	+
PTVS-16	+	+	+	+	+	+	+
PTVS-17	-	-	-	-	+	+	+
PTVS-18	-	-	-	-	+	+	+
PTVS-19	+	+	+	+	+	+	+
PTVS-84	+	+	+	+	+	+	+
PTVS-85	+	+	+	+	+	+	+
PTVS-86	+	+	+	+	+	+	+
PTVS-87	+	+	+	+	+	+	+
PTVS-88	+	+	+	+	+	+	+
PTVS-90	+	+	+	+	-	-	+
PTVS-91	+	+	+	+	+	+	+
PTVS-92	+	+	+	+	+	+	+
PTVS-93	+	+	+	+	-	-	+

[1] LJH-569 is a nonpathogenic *E. coli* K12 strain and should not be recognized by O157 antibodies
LJH-570 belongs to O6 serogroup and should not be recognized by O157 antibodies
PTVS-90 is same as ATCC 43888; PTVS-93 is same as ATCC 700728. Both were obtained from the American Type Culture Collection and are listed as non-toxigenic strains, lacking both stx1 and stx 2, but should be recognized by O157 antibodies.

[2] 13B3 is a monoclonal antibody provided by the USDA-ARS, Clay Center, NE. and is reported to be inclusive of O157

Stx 1 – shiga-like toxin 1; a virulence marker
Stx 2 – shiga-like toxin 2; a virulence marker
uidA – a variant a single nucleotide alteration in the uidA gene (T93G), which is specific to *E. coli* O157: H7.

Figure 6 – Example of binding and immunoblot detection of colonies to be used in model irrigation water capture tests. A benchtop recirculating immunocapture system was evaluated and determined to have very good specificity but low recovery efficiency relative to Modified Moore Swabs.



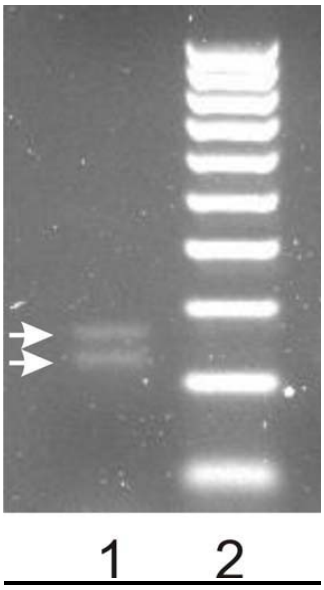


Figure 7 - Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) of the irrigation water runoff eluate. Lane 1 shows the presence of two amplicons (see the arrows) representing the two FRNA genera (*Leviviridae* and *Allolevivirdae*). Lane 2 contains a 100 base pair DNA ladder.

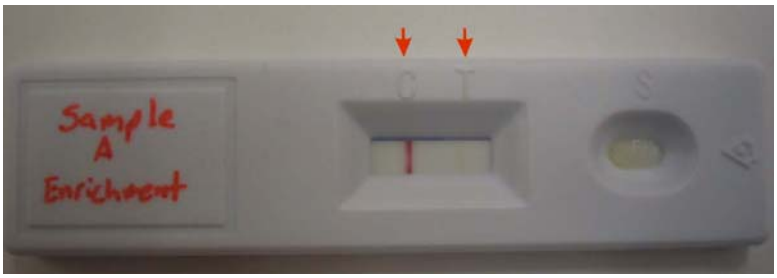


Figure 8 - Lateral flow detection of group I FRNA phages in the irrigation runoff eluate.

The arrows indicate the quality control (C) and test (T) lines.

Appendix 1: Colony Blotting Procedure for the Detection of *Escherichia coli* O157:H7

Materials:

PVDF-P membrane (Millipore),
Polyclonal rabbit anti *E. coli* O157:H7 antibody (Difco)
Polyclonal goat anti-rabbit IgG conjugated with alkaline phosphatase)
Bovine serum albumin
Methanol
Tween-20
PBS (NaCl 8.0 g, KCl 0.2g, Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g; pH 7.4)
NBT/BCIP substrate (Roche)
Tris-Cl substrate buffer for NBT/BCIP stock solution from Roche (0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl, 0.05 M $MgCl_2$)
10X PBS-T: NaCl 80 g, KCl 2g, Na_2HPO_4 11.5 g, KH_2PO_4 2 g, Tween-20 2 ml)

Protocol:

1. Obtain colonies on agar medium in a circular or square Petri dish.
2. Cut Circular (83-mm-diameter) Immobilon-P PVDF membranes (Millipore), saturate with 100% methanol for 30 seconds. Immerse the membrane in sterile milli-Q water for 2 min.
3. While still damp, lay the membrane on the plate containing bacterial colonies. Tap the membrane lightly with the wide end of a 200-µl pipette tip to ensure antigen transfer to the membrane.
4. Place a single loopful of broth-grown *E. coli* O157:H7 cells to serve as positive control.
5. Dry the PVDF membrane on a paper towel for 10 to 15 min. Once dried, blocking is not required for PVDF membranes. Wash the membrane three times with PBS-T for 5 minutes with gentle shaking.
6. Incubate the blot in 20 ml of primary antibody solution (rabbit anti O157:H7 serum diluted 1:1000 in PBS-T containing 0.1 % BSA). Check manufacturer's recommended dilution from the brochure.
7. Rinse the membrane two times with BPS-T, and wash the membranes three times with PBS-T on a shaker for 5 min.
8. Incubate the membrane in a 20 ml antirabbit polyclonal goat IgG labeled with alkaline phosphatase, diluted 1:10,000 in PBS-T containing 0.1% BSA for 1 hour with gentle shaking.
9. Wash as in step 9.
10. Mix 200 µl NBT/BCIP reagent in 10 ml substrate buffer, develop the blot by rocking the dish with hands gently for 2 to 10 min. Stop the reaction by rinsing the membranes in milli-Q water.

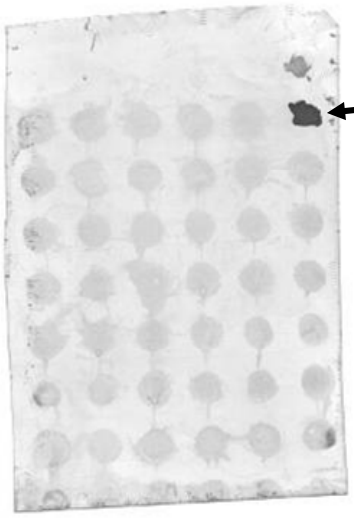
Notes:

1. For smaller blots 10 ml of reagents would be adequate.

2. For overcrowded plates, it is best to inoculate the colonies into 96 well plates, incubate overnight and transfer the growth onto membranes using 8x6 or 8x12 replicators. If this is a problem use nitrocellulose membrane which does not need prewetting with methanol and water. If using nitrocellulose paper, block the membrane by incubating the blot in 10-20 ml blocking buffer (3% bovine serum albumin in PBS-T) for 1 hour with gentle shaking (30-50 rpm).

3. Free water on PVDF membranes may allow bacterial cells to disperse. Shake off excess water and blot transfer the culture quickly before the membrane starts drying out.

4. Instead of polyclonal O157 specific IgG, monoclonal antibodies (expensive) can also be use. If monoclonal antibody is used, use appropriate secondary antibody-alkaline phosphatase conjugate.



Example from lettuce 2007
- **88 presumptive colony positives from enrichments**
- **all negative PAB**
- **all negative multiplex PCR (eae, hylA, stx1, stx2, uidA)**