CPS 2014 RFP
FINAL PROJECT REPORT

Project Title
Rapid tests to specifically differentiate clinically significant from environmental STEC towards reducing unnecessary crop destruction

Project Period
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Objectives

1. *Test retained E. coli O157:H7 negative leafy greens samples from CPS Project 2011-136 for evidence of clinically significant STEC.*
2. *Test E. coli O157:H7 negative but STEC positive enrichments from commercial preharvest, raw material, and packaged leafy greens and culinary herb products for evidence of clinically significant STEC.*
3. *Characterize cultures identified as positive and negative by the ROKA Atlas EHEC screen for clinically relevant virulence markers by diagnostic multiplex PCR.*
4. *Characterize the clinical significance of EHEC/STEC isolates from nuisance bird populations exhibiting flocking and foraging behavior in leafy green vegetable fields. We will culture EHEC/STEC strains from bird cloacal samples following live-capture and release at two enrolled farms in proximity to confined and range beef cattle operations.*

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FINAL REPORT

1. Abstract

Although exceptionally rare events, relative to the scale of production and consumption, there is ample evidence that harvested produce intended for fresh market consumption sometimes contains pathogens of serious potential human health consequences. In the wake of the *E. coli* O157:H7 outbreak associated with spinach in 2006, the programmatic testing of leafy greens at preharvest, incoming raw material, and/or finished packaged product was essentially introduced as an expectation for the leafy greens category. Its application to preharvest qualification of fields greatly increased over subsequent years. A group of potentially serious bacterial pathogens, Shiga toxin–producing *E. coli* (STEC) from diverse fresh produce was recovered during multi-year sampling programs conducted by the USDA and FDA, largely at wholesale distribution centers and terminal markets. Leafy greens, herbs, and specifically spinach were singled out for concern due to a STEC prevalence rate exceeding 50% of the total isolates recovered. Many but not all producers use pre-screening of field lots, especially leafy greens and herbaceous culinary herbs, for bacterial pathogens, including STEC, and some include finished product testing in their program or for specific buyers. Unfortunately, not all testing platforms rapidly distinguish STEC likely to cause human illness from those that lack the genetic traits necessary for human infection. There has been a rapid expansion of platforms, kits, pathogen targets, and diversity of approaches to lot acceptance criteria. There has been an accelerating shift in product testing criteria within the group of target pathogens and STEC sub-groups, which includes the enterohemorrhagic *E. coli* (EHEC). Some commercial kit test systems screened for the top-seven EHEC (O157, O26, O45, O103, O111, O121, and O145) based on the premise that these sub-types are responsible for over 85% of clinical cases. However, due to the increasing recognition of diverse STEC in clinical cases, many commercial service labs had more recently been using detection and lot acceptance systems that employ the least diagnostic genetic markers for this group, namely the presence of *eae* (intimin; attaching and effacing) and *stx* (either of two key forms of Shiga toxin) in an enrichment culture without cultural confirmation. Presence of these two markers, alone or being contributed individually by independent cell lines, has resulted in frequent crop destruction involving many acres and substantial economic loss, at the individual grower level. Due to the high perishability of these commodities, testing can lead to destruction of a field due to false association with presumptively dangerous STEC. The project hypothesis was that the combined objectives of protecting consumers, reducing food loss, and improving sustainability could be enhanced by applying new advancements proposed in this research in specific detection of clinically relevant Shiga toxin–producing *E. coli* to risk management decisions and better defining the role of wildlife as vectors of preharvest contamination. This study was a starting point for a longer-term effort to consolidate and clarify the available information on risk associated with the diverse STEC group and to present this information in a guidance format that can help form industry-based standards of practice. An objective of this study was to explore the development of a set of recommendations relative to rapid virulence profiling and its application to routine compliance and lot acceptance testing for fresh produce.

2. Background

Shortly after a multi-state outbreak of *E. coli* O157:H7 on mesclun (spring mix) lettuce in May–June 1996 (originating from a small farm near the Central Coast of California, involving at least 61 victims, 21 hospitalizations, and 3 cases of hemolytic uremic syndrome), large salad processors began or increased their periodic or routine testing of agricultural inputs and/or product for this specific pathogen. At the time, and realistically up to late 2006, rapid commercial kit methods validated or performance tested on lettuce and leafy greens for *E. coli* O157:H7 were largely non-existent or not applied to fresh produce. Despite examples of sporadic human illness and regional outbreaks in the U.S., and globally, associated with fresh produce, and involving non-O157 STEC, testing was only for the EHEC O157:H7 sub-type.

After the *E. coli* O157:H7 outbreak on spinach in 2006, the focus of commercial lot acceptance testing was limited to the O157 group, although it was widely known that non-O157:H7 STEC also were isolated from the consumer bags of spinach associated with the 2006 outbreak lot code. At the time, the general industry attitude towards focused testing was dictated by several limiting methodological factors in the detection and confirmation of STEC as frequent human pathogens associated with farm-gate produce as well as the predominance of the O157:H7 type in U.S. outbreaks.

Since then there has been a rapid expansion of platforms, kits, pathogen targets, and diversity of approaches to lot acceptance criteria. In parallel with the experience and policy development at CDC, FSIS, and within the meat industry, there has been a gradual but accelerating shift in product testing criteria and policies for the group of pathogens that includes EHEC and STEC. Due to the increasing recognition of diverse STEC in clinical cases, many commercial service labs have more recently been using detection and lot acceptance systems that employ the least diagnostic genetic markers for this group, presence of *eae* (intimin; attaching and effacing) and *stx* (either of two key forms of Shiga toxin) in an enrichment culture. Presence of these two markers, alone or being contributed individually by independent cell lines, has resulted in frequent crop destruction involving many acres and substantial economic loss, at the individual grower level. First-hand knowledge from extension involvement in such decisions supports the reality of non-harvest or finished product destruction of 5 to >200 acres for multiple farm and handler operations in each year since 2007. Although the total acreage destroyed is a fraction of the total annual production, estimated at <2% of volume, these losses can be substantial for an individual. Food loss and food waste is a topic of keen focus as increased attention is being given to food security.

Some of these crop destruct decisions have arguably been made with only marginal risk-based data. The perishability of the crop and the reality that current methods for cultural confirmation among the non-O157 STEC may take as long as 8–18 days, or may never be successful in a presumptive positive enrichment, essentially preclude the use of culture techniques in lot acceptance decisions for this pathogen sub-group. In the absence of a clear, understandable, and consistent policy, there was disbelief and resentment among the grower-suppliers in the necessity for uniform crop destruction or recalls in every case of detection of *eae* and *stx*, alone, based on limited molecular evidence. These markers are known not to be unique to EHEC/STEC and may not be present in the same cell in a mixed enrichment culture or even in an *E. coli* cell line in the enrichment but carried in a different genus of related bacteria altogether. This fact, and the associated fact that not all environmental STEC are equally infectious or recognized as pathogenic to humans, provoked renewed and passionate discussion surrounding the need to develop and standardize criteria for rapid virulence profiling that would be necessary before a lot acceptance decision is finalized. A significant focus of this concern is around the belief that unnecessary crop and product destruction is diametrically opposed to sustainability initiatives; all inputs are wasted and re-plant intervals may be as long as one year in some non-regulatory schemes.

Most recently, the 2011 outbreak of *E. coli* O104:H4 centered in Germany and France has caused some service labs to include additional diagnostic markers to their test panel. The conventional EHEC or simplistic STEC tests would not identify this strain and related *E. coli* with alternative genetic mechanisms for attachment, invasion, and toxicity (Grad et al., 2013). Environmental isolates containing some but not the full complement of virulence traits is regularly being presumptively detected in the ag-environment and on product. Against this expanding palette of pathogen detection screens, prudent crop and finished product lot acceptance decisions must be made without certainty of the degree of risk.

There is ample evidence to support that produce samples do contain clinically relevant and serious non-O157 EHEC/STEC. Feng and Reddy (2013) published the results of analysis from the multi-year USDA

Microbiological Data Program. STEC isolates from diverse fresh produce were recovered from sampling programs, largely at wholesale distribution centers. Spinach was singled out for concern due to a STEC prevalence rate of 50% of the total isolates recovered. Less than 10% of the colonies had the *eae* gene but these belonged to known pathogenic EHEC, including O157:H7 and O26:H11. Other virulence markers, including *stx1, stx2, ehxA, saa*, and *subAB* genes, were detected at varying frequencies. The authors concluded that nearly half of the STEC strains from produce belonged to unremarkable serotypes and, in view of the uncertainties of some of putative virulence factors in causing human illness, it would be difficult to determine the health risk of frequently encountered STEC on produce.

An abundance of publications have evaluated prevalence, frequencies, and diversity of this diverse group of pathogens but few look beyond the major serotypes or differentiate general risk from human health risks. Research identified differential virulence between bovine vs. human clinical *E. coli* O157 but studies of this type do not appear to apply broadly to the complex class of STEC (Cooley et al., 2013; Feng et al., 2013). Perelle et al. (2007) found very low MPN values for STEC in positive foods tested with predicted contamination ranging from 1 to 2 MPN/kg of recognized human pathogenic serotypes. Based on these low but detectable numbers, with modern techniques of recovery and detection, they concluded that modeling the potential risk for consumers required much more data and epidemiological evidence.

The industry's current perspective on non-O157 EHEC and STEC seasonal and geographic prevalence on lettuce, leafy greens, and several other commonly tested fresh produce commodities emanates mainly from data generated by a few contract testing service laboratories. Most utilize AOAC-Research Institute certified test methods accessible for purchase by any lab and one lab—IEH Laboratories—provides pathogen testing services to the produce industry primarily using a proprietary system that has received AOAC Performance Tested certification. Public data disclosure of product testing for leafy greens is almost exclusively based on this IEH dataset and is held by many to reflect the reality of EHEC and STEC prevalence and consumer risk-exposure potential. While it is reasonable to draw preliminary conclusions in regards to contamination sources, seasonal patterns, or specific regional production from this data, it is important and prudent to develop additional science-based views of the validity of the testing outcomes. Confirming the prevalence of clinically-relevant STEC in environmental samples, product samples, and root cause investigative samples is critical to addressing risk–based questions. The ramifications of product testing design and implementation are being experienced across many commodities and are no longer restricted to lettuce and leafy greens.

The Roka Atlas EG2 test is known to detect pathotoxigenic *E. coli* O157 and STEC with a characterized determinative virulence factor (Livezey et al., 2015). This promising technology uses a novel single genetic marker as a surrogate for the detection of pathogenic STEC. This study evaluated the potential utility of single marker technology for the detection of non-O157 STEC in ag-environment samples.

From concept, the anticipated outcome was the development of a set of recommendations relative to rapid virulence profiling and its application to routine compliance and lot acceptance testing for fresh produce. This project sought to be the catalyst for a longer-term effort to consolidate and clarify the available information on risk associated with the diverse STEC group and present this information in a guidance format that could help form industry-based standards of practice. The potential utility of this approach for future root-cause investigations was anticipated to be demonstrated using the example of bird intrusions, the most common co-management challenge in pre-leafy green production. The outcomes were intended to lead to both immediate transferrable actions in pathogen testing protocols, serving as a first step towards fulfilling our collective responsibility to stewardship of the ag-environment, and associated regional landscape.
In response to the increased number of crop and product destruction incidents, a renewed and rising empirical root-cause association to flocking bird intrusion was debated among CA Central Coast growers.

Wild birds are a recognized source of EHEC/STEC in agricultural environments (Hancock et al., 1998; Langholz and Jay-Russell, 2013; LeJeune et al., 2008; Nielsen et al., 2004; Williams et al., 2011). Fresh produce may be contaminated with EHEC/STEC via direct contact with bird droppings, or indirectly through transfer of EHEC/STEC to agriculture water, soil amendments, equipment, or other potential vehicles in the pre-harvest production environment. Additionally, contact with contaminated beaks, feathers, or feet while foraging in produce fields is considered a potential microbial food safety hazard from bird intrusions. Likewise, interspecies transmission of EHEC/STEC between cattle reservoirs and wild bird populations may elevate the risk of pathogen transfer to nearby produce fields. Evidence of fresh produce contamination in preharvest pathogen testing following bird intrusions has resulted in rejected and destroyed products. In a prior CPS and CA Leafy Greens Research Board supported research study, the Suslow Lab associated numerous American crow intrusions into irrigation reservoirs supplying a Romaine lettuce block and numerous crow intrusions into baby spinach fields with the detection of presumptive EHEC/STEC, which triggered full crop destruction decisions for both. While molecular and culture-confirmed *E. coli* O157:H7 and non-O157 EHEC were identified in both cases, multiple enrichments yielded only STEC isolates lacking a detectable attachment factor or hemolysin. Taken together, these experiences contributed to the development of associated objectives to further assess the single surrogate test for clinically significant STEC.

3. Research Methods

3.1. Sampling and STEC testing of crops and environmental samples

3.1.1. Special study-site sampling

As part of a grower participatory study, 145 samples were collected from two sites in five different sampling events, between August 2015 to October 2016. All samples from 2015 (n=32) were collected in duplicate to test two enrichment methods; 177 samples were tested in total. Samples of basil, bok choy, leeks, kale, chard, collard, parsley, soil and water were collected using clean latex gloves and scissors, and individual samples were placed in separated bags. Basil and parsley plots were located 110 m downwind from a concentrated animal feeding operation (CAFO), site one. Leek and bok choy plots were located between 10–30 m from a CAFO, site one. Kale, chard, and collard plots were 20 m upwind from a CAFO, site two. Plant samples were gathered as a composite of multiple plants within 3 m in the same bed. Soil samples (furrow) were collected right across from CAFO down to 5–10 cm from the surface in an area 20 x 20 cm^2. Water was collected from ditches nearby the plants (cattle and crop run-off); approximately 1-L of sample was placed into a sterile bottle. Between all individual samples, a new pair of gloves was donned and scissors were sanitized with 70% isopropanol. Samples were stored and transported on ice. Samples were processed within 12–18 h after collection.

3.1.2. Sample enrichment

All samples were processed for STEC detection. Nevertheless, to increase the robustness of the process, in the two sampling events of 2015, direct enrichment of the plant material and capture by filtration using modified Moore swabs (MMS) were tested in parallel (**Figure 1**).

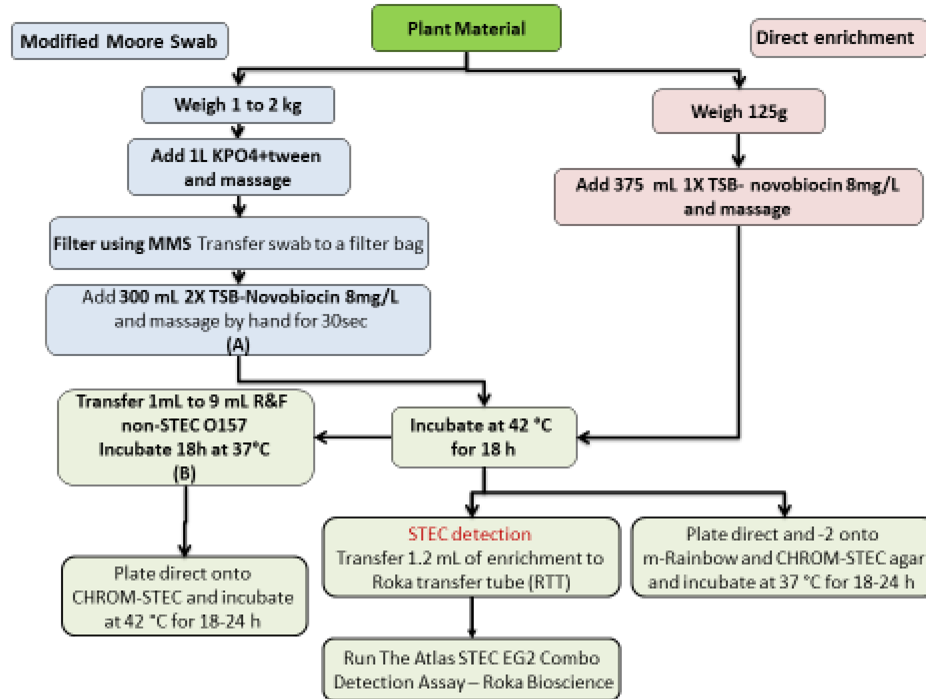


Figure 1. *Direct and modified Moore swab enrichments*

The MMS method was used for STEC cells recovered in 2016, since more samples exhibited positive outcome with this method (**Table 1**). The protocol is illustrated in Figure 1. Briefly, for the two-step enrichment (A and B), plant samples were weighed and placed into a sterile bag holding 1 L of sterile potassium phosphate buffer supplemented with 0.05% Tween 20, and then massaged for 1 min to detach bacteria. Buffer was carefully collected into a sterile bag and filtered following the standardized MMS method. After filtration, the saturated swab was placed in a sterile Whirl-Pak bag (Nasco, Modesto, CA) with 300 ml of pre-warmed double strength (2X) tryptic soy broth (TSB; Becton, Dickinson) containing 8 mg L^{-1} of novobiocin (2X-nTSB). Swabs were massaged for 1 min and incubated for 24 h at 42°C. One mL of enrichment was transferred to a 15-mL tube containing R&F non-O157 STEC (R&F) enrichment broth (R&F Laboratories, Downers Grove, IL) and incubated for 18–24 h at 42°C. Also, a small number of samples were transferred to Actero STEC Enrichment media (Food Check) enrichments after initial incubation in 2X-nTSB.

For soil enrichment, 100 g of homogenized soil was placed into a sterile Whirl-Pak bag holding 200 mL of 2X-nTSB. Samples were massaged for 1 min and processed as described below. Water samples were filtered using the MMS method. Filtration was performed with a flow rate of 0.5 L/min for 10 to 30 min depending upon the water volume and turbidity. MMS were placed into a sterile bag containing 300 mL of 2X-nTSB and processed as described below.

3.1.3. Molecular STEC testing by single genetic marker

After enrichment, 1.2 mL of 2X-nTSB (Roka STEC EG2 test was validated with this media broth) were transferred to Roka Transfer Tubes (RTT) and samples were analyzed using the Atlas® STEC EG2 Combo Detection Assay (Roka Bioscience) in the Atlas® System, a fully automated molecular pathogen detection system. This system uses a single genetic marker (*ecf1*) to detect pathogenic STEC (Livezey et al., 2015). A percentage of R&F non-O157 STEC and Actero STEC enrichments were tested in the Atlas® System for comparison.

3.2. Isolation and molecular screening of isolates from crops and environmental samples

3.2.1. Cultural isolation and presumptive isolate screening

Non-O157 STEC and incidental *E. coli* O157:H7 presence was determined by plating 100 µL of 1:10, 1:100 and direct enrichments onto CHROMagar STEC (CHROMagar Microbiology, Paris, France). Rainbow agar O157 (Biolog, Hayward, CA) supplemented with novobiocin (20 µg/mL; Sigma Aldrich) and tellurite (0.8 µg/mL; Invitrogen) (RBA) and STEC heart infusion washed blood agar with mitomycin-C (SHIBAM) were tested in a subset of samples. Plates were incubated at 37°C for 24 hours. At least three presumptive mauve colonies were transfered to CHROMagar STEC at least two times more for purification. Pure isolates were storage in 25% glycerol frozen at −80°C until use.

3.2.2. Detection of *stx1*, *stx2* and *eae* by multiplex PCR

Presumptive positive (mauve) colonies were eluted in 200 µL in Butterfield's phosphate buffer (BPB). Colony suspensions were incubated at 95°C for 10 min and the supernatant was used as DNA template. Multiplex polymerase chain reaction (PCR) assays for detection of *E. coli 16s rDNA*, *stx1*, *stx2,* and *eaeA* gene were performed with the Gen Amp PCR system 2700 (Applied Biosystems). Primers and predicted lengths of amplication are listed in **Table 2**. The optimized protocol was carried out with a 25-µl mixture containing 5X Green GoTaq® Flexi Buffer (Promega); 1.25 U of Hot start *Taq* DNA polymerase (Promega); 0.2 mM deoxynucleoside triphosphates (Thermo); 3 mM $MgCl_2$ (Promega); 10% DMSO (Sigma-Aldrich); a 0.5 µM concentration of primers stx1, stx2 and eaeA; 0.2 µM of *16s rDNA* primers; and 1 µl of the DNA template. The PCR program was initial 95°C for 5 min followed by 40 cycles of 95°C for 30 seconds, 60°C for 50 seconds, and 72°C for 1.5 min. The final cycle was followed by 72°C incubation for 5 min. PCR products were then electrophoresed on a 1.5% agarose gel and stained with SYBR Safe DNA gel stain (Invitrogen). Gels were visualized with UV illumination, and imaged with a Gel Logic 100 imaging system (Kodak).

3.2.3. Detection of STEC virulence-associated markers by PCR

The template DNA was prepared as described below. The primers and expected sizes from the PCR assays are included in **Table 3**. The assay was set up as follows: each 25-µl mixture contained 5X Green GoTaq® Flexi Buffer (Promega), 0.25 U of *Taq* DNA polymerase (Promega), 0.2 mM deoxynucleoside triphosphates (Thermo), 2 mM $MgCl_2$ (Promega), 10% DMSO (Sigma-Aldrich), a 0.2 µM concentration of primers, and 1 µl of the DNA template. The PCR program was initial 95°C for 5 min followed by 40 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 min. The final cycle was followed by 72°C incubation for 5 min. PCR products were then electrophoresed on a 1.2% agarose gel and stained with SYBR Safe DNA gel stain. Gels were visualized with UV illumination, and imaged with a Gel Logic 100 imaging system.

3.3. Real time PCR screening for *stx1*, *stx2* and *eae* in enrichment broth

3.3.1. DNA extraction for screening by real-time PCR

One milliliter of 2X-nTSB was transferred to a microcentrifuge tube, and cells were harvested by centrifugation at 16,000 g for 3 min. The supernatant was removed, and the pellet was resuspended in 100 µL of the PrepMan Ultra reagent (Applied Biosystems). Samples were heated in a boiling water bath for 10 min, cooled to room temperature, and then centrifuged for 3 min at 16,000 g. The supernatant was transferred to a sterile tube and stored at −20°C until use as a template for the real-time PCR assays.

3.3.2. *stx1*, *stx2* and *eae* detection by real-time qPCR assay in enrichment broth

Additionally, for the detection of STEC single genetic marker in the enrichments, the presence of the STEC main screening markers (*stx1*, *stx2*, and *eaeA*) was determined by TaqMan™ real-time PCR. Oligonucleotides are listed in **Table 4**. All real-time PCR amplifications were performed in a final volume of 20 µL consisting of 10 µl Sso Advanced Universal Probes Supermix (BioRad, Hercules, California), 0.45 µM of

each primer, 0.125 µM of each probe, and 2.45 µL of molecular grade water (Invitrogen). A 5-µL sample of the supernatant of the enrichment broth lysate was used as source of template DNA. Samples were amplified using a CFX96 Touch™ Real-Time PCR Detection System (BioRad). The following thermocycling conditions for the amplifications were used: one cycle of denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s and annealing at 60°C for 1 min. Samples were considered positive if the cycle of quantification (Cq) values were ≤32.

3.4. STEC testing from *E. coli* O157:H7–negative leafy greens samples retained enrichments

3.4.1. Secondary recovery enrichment and cultural isolation

Retained enrichments (n=120) from Dr. Elaine Berry's lab (USDA-MARC) from CPS project 2011-136 were received. Samples were stored in glycerol at −80°C. Each homogenized sample (500 µL) was transferred into a tube containing 4.5 mL of pre-warmed TSB containing 8 mg 1^{-1} of novobiocin (nTSB) and also to modified enterohemorrhagic *E. coli* (mEHEC) broth (Hardy Diagnostics) for comparison. Samples were incubated at 42°C for 18 h. After the incubation, 1 mL of enrichment was transferred to R&F broth and incubated for 18–24 h at 42°C. Enrichments were plate onto CHROMagar STEC and RBA and incubated at 37°C for 24 h. Presumptive mauve colonies were transfer to CHROMagar STEC at least two times more for purification. Purified isolates were eluted in 200 µL in BPB. Colony suspensions were incubated at 95°C for 10 min and the supernatant was used as DNA template. Multiplex PCR was performed for detection of *E. coli 16s rDNA*, *stx1*, *stx2,* and *eaeA* gene, as previously described.

3.4.2. Molecular detection of STEC by single genetic marker

1.2 mL of Each enrichment (1.2 mL) was transferred to Roka Transfer Tube (RTT) and the Atlas® STEC EG2 Combo Detection Assay was carried out, using the Atlas® EG2 system as described above.

3.4.3. DNA extraction and Real time qPCR assay for detection of *stx1*, *stx2* and eae

After incubation, 1 mL of nTSB was centrifuged at 16,000 g for 3 min. The pellet was washed three times with BPB, and resuspended in 300 µL of molecular grade water (Invitrogen). Samples were placed in a heating block for 10 min at 95°C. The presence of the STEC main screening markers (*stx1, stx2*, and *eaeA*) was determined by TaqMan™ real-time PCR, as described above.

3.5. Characterization of *E. coli non-O157*, and presumptive STEC retained cultures by Roka Atlas® screening and virulence markers

A total of 165 retained *E. coli* isolates from different sources (130 retained presumptive STEC isolates from previous CPS Rapid Response project, and 35 from TVS Lab culture collection) were grown on tryptic soy agar (TSA) at 37°C for 18–24 h. One colony was transferred into 5 ml of TSB and incubated for 4 h. After incubation, 1.2 mL of the broth was transferred to RTT and the Atlas® STEC EG2 Combo Detection kit was used for STEC testing. Each strain was also screened for STEC markers (*stx1, stx2, eae*) and associated virulence marker, as described above.

3.6. Characterization of presumptive isolates from STEC-positive enrichments in commercial labs

Twenty-six cultures recovered from STEC-positive enrichments in commercial labs were screened: 16 from leafy greens and 10 from irrigation water. Bacterial isolates were frozen at −80°C in TSB containing 28% glycerol and were subcultured on CHROM STEC and TSA agar plates prior to testing. Strains were screened for STEC markers (*stx1, stx2, eae*) and associated virulence marker, as previously described.

3.7. Characterization of the clinical significance of EHEC/STEC isolates from nuisance bird populations exhibiting flocking and foraging behavior in leafy green vegetable fields

Study sites: Birds were captured and sampled at two study sites in California in 2016. (Efforts to initiate this objective in 2015 were not possible due to a change of ownership and one-year delay in re-establishing site access permissions, and no acceptable alternative site could be found during 2015.) One of the study sites is a commercial produce ranch in San Benito County, on the Central Coast foothills. The other site is a National Wildlife Refuge in Yolo County on the delta of the Central Valley, where land use is predominantly agriculture. In both sites, mist nets and traps were placed in proximity to a water body (reservoir or river). Forty-four sampling events took place over a period of a year (e.g., two sampling days per site and month). Nine to ten nets were used year-round, and mist netting was supplemented with ground traps at each site. Both sites were adjacent to cow-calf beef cattle herds on rangeland. The team was able to collect cattle fecal samples from the Yolo County site (Elk Grove), but did not have access to the cattle herd in San Benito County. Cattle feces were collected monthly throughout 2016. "Convenience" fecal samples were collected from wildlife (e.g., deer, feral pigs) observed during visits. Samples were stored on ice and taken to the lab for processing within 24 h of sample collection.

Detection of EHEC/STEC in bird and cattle samples: Fecal samples from all tagged birds were pre-enriched by placing cloacal swabs collected in the field into TSB. Samples were then incubated for 2 h at 25°C with agitation at 100 rpm, followed by 8 h at 42°C with agitation, and held overnight at 6°C, using a Multitron programmable shaking incubator (Eppendorf, Hauppauge, NY).

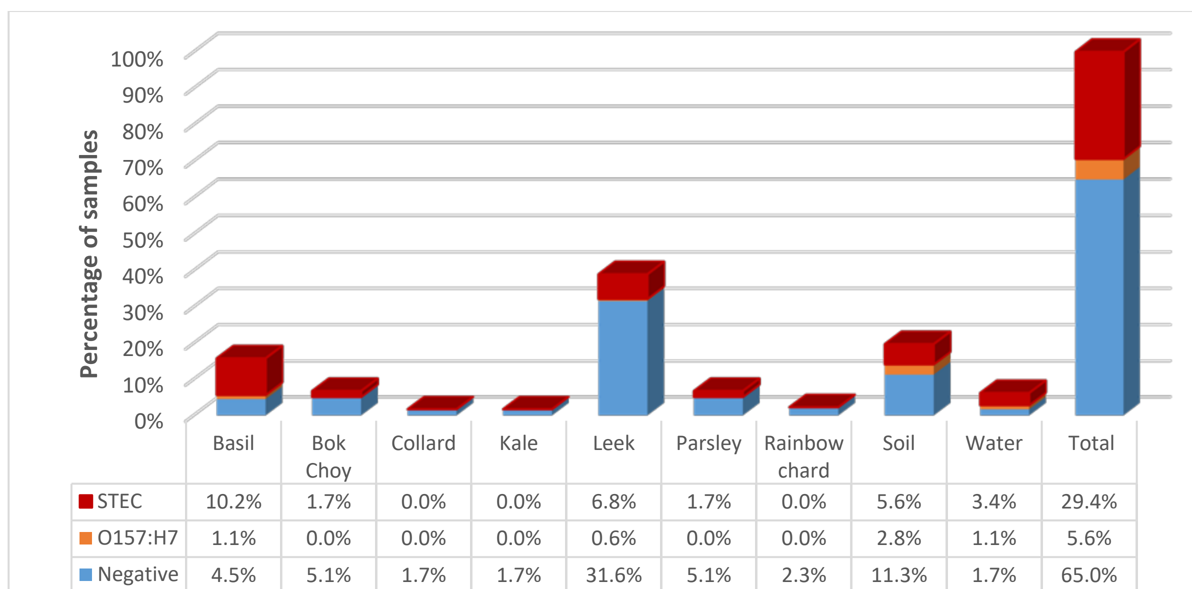
For detection of *E. coli* O157, immunomagnetic separation (IMS) using Dynal anti-*E. coli* O157 beads (Invitrogen/Dynal, Carlsbad, CV) was performed on TSB enrichment broths with the automated Dynal BeadRetriever (Invitrogen) per the manufacturer's instructions (Cooley, Jay-Russell et al., 2013). After incubation and washing, 50 µL of the resuspended beads were plated onto Rainbow agar (Biolog) with novobiocin (20 mg/L) and tellurite (0.8 mg/L) (MP Biomedicals, Solon, OH). Fifty µL of the resuspended beads were also plated onto MacConkey II agar with sorbitol supplemented with 500 µl of potassium tellurite solution and 100 µl Cefixime (CT-SMAC); plates were streaked for isolation and incubated for 24 h at 37°C.

To detect non-O157 STEC, the pre-enrichment broth was incubated in mEHEC selective media (Biocontrol, Bellevue, WA) for 12 h at 42°C, followed by plating on CHROM STEC (DRG International Inc., Springfield, NJ) and incubating. Up to 6 presumptive STEC positive colonies were confirmed for the presence of *stx1* and/or *stx2* genes by real-time PCR (Eppendorf, Hauppauge, NY). Confirmed STEC isolates were then characterized for virulence genes (*stx1*, *stx2*, *eaeA*, *hlyA*, *fliC* and *rfbE*) using conventional PCR. Selected isolates were submitted to the Pennsylvania State *E. coli* reference laboratory for O-serogrouping.

4. Results

4.1. Crop and environmental samples: STEC testing

Of 177 samples collected in this special grower-site study, 29.4% (n=52) of enrichments were positive for STEC marker, 5.6% (n=10) were positive for *E. coli* O157:H7, and 65% (n=115) were negative using Atlas® STEC EG2 Combo Detection Assay (**Figure 2**, **Table 5**). Of the 52 STEC-positive samples, basil samples were most often positive (10.2%), followed by leek (6.8%), soil (5.6%), water (3.4%), and parsley (1.7%).



	Basil	Bok Choy	Collard	Kale	Leek	Parsley	Rainbow chard	Soil	Water	Total
STEC	10.2%	1.7%	0.0%	0.0%	6.8%	1.7%	0.0%	5.6%	3.4%	29.4%
O157:H7	1.1%	0.0%	0.0%	0.0%	0.6%	0.0%	0.0%	2.8%	1.1%	5.6%
Negative	4.5%	5.1%	1.7%	1.7%	31.6%	5.1%	2.3%	11.3%	1.7%	65.0%

Figure 2. *Detection of STEC and O157:H7 in 177 samples using Atlas® STEC EG2 Combo Detection Assay*

A total of 393 presumptive colonies were recovered from enrichments, of which 373 were confirmed as *E. coli*; 7.8% (n=29), 1.3% (n=5), and 11.8% (n=44) were positive for *stx1*, *stx2*, and *eae* markers, respectively. Only 1.9% (n=7) isolates were positive for both *stx* and *eae* genes (*stx*/*eae*). Basil samples had the highest number of isolates positive for *stx1* (3.5%). Soil and leek samples yielded most of the isolates carrying only *eae* gene (4.6% and 4%, respectively). The crop and environmental samples from which strains carrying STEC marker were isolated are summarized in **Table 6**. Recovery of STEC isolates from all enrichments was conducted as described above. Of the 52 STEC-positive enrichments, recovery of isolates with the individual STEC markers was observed from fifteen (28.8%) samples (**Table 7**). However, only four enrichments (7.7%) yielded isolates with both *stx*/*eae* markers. Additionally, strains carrying STEC genes were isolated from 15 (13.9%) STEC-negative enrichments.

4.1.1. Molecular screening of isolates from crops and environmental samples

PCR was used to screen for the presence of virulence markers described above. Isolates carrying either *stx* and/or *eae* genes were included. In this study, 32 different marker profiles were observed from all isolates characterized (including culture collection and retained strains). Fifteen of these marker arrays were detected in strains recovered from the four samplings. One strain (Profile 2) was positive for *stx1, stx2* and *eae* genes, and also for α-hemolysin (*hlyA*), putative intestinal colonization factor (*adfO)* (Imamovic et al., 2010) and open reading frame (ORF) associated with *eae*-positive STEC (Delannoy et al., 2013). Eight strains containing *stx1* and *eae* genes were observed; these were distributed into two different profiles (10 and 13), which showed very similar virulence-associated pattern to profile 2 (*hlyA+, adfO+,* and *Z2098+*). Profile 10 showed the same virulence marker pattern as profile 2, and profile 13 did not carry the *adfO* gene.

Additionally, eighteen strains carried only *stx1* as STEC marker; these were distributed in three different marker profiles (15, 18 and 19). Among *stx1* positive strains, profile 15 showed a similar virulence-associated pattern as profile 2, and also was positive for the plasmid and phage-carried entero-hemolysin (*ehxA*). Profile 18 showed the same pattern as 2, and 19 was only positive for STEC autoagglutinating adhesion *saa* gene (Paton et al., 2001). Four isolates with very similar virulence-associated profile (23 and 24) were positive only for *stx2.* The only difference observed between these marker arrays was that 24 was positive for *Z2098* gene.

Forty-one isolates were positive only for the *eae* gene; they were grouped in eight different marker profiles (25, 26, 27, 28, 29, 30, 31, and 32). Profile 25 showed the same virulence-associated pattern as profile 15; profile 26 the same as 2 and 15; profile 27 was positive only for hemolysin genes (*hlyA+, ehxA+*), profile 28 for *hlyA and adfO* genes, 29 carried only *saa* and subtilase cytotoxin (*subAB*) (Paton et al., 2004) genes. Profile 31 and 32 were only positive for *subAB* and *adfO* genes, respectively. Isolates carrying STEC markers were recovered from enrichments negative for STEC/O157:H7 on the Atlas® system; however, the majority of the isolates (23 of 31) were only carrying *eae*, and had no *stx* genes.

4.1.2. Real-time qPCR assay detection of *stx1*, *stx2* and eae in enrichments from samplings

Enrichments were screened for STEC markers genes by TaqMan real-time PCR. Outcomes were compared with results from the Atlas® Roka System, which uses a single genetic marker (*ecf1*) to detect pathogenic STEC. Overall, eight real-time PCR profiles (A to H) of genetic markers were observed (**Table 9**). Thirty-four samples (19.2%) (Profile A), showed a positive outcome for all three markers (*stx1, stx2* and *eae*); however, five (2.8%) of those enrichments were negative in Atlas®. A total of 19 enrichments (10.8%) were positive for both *stx1* and *stx2* genetic markers (Profile B); six (3.4%) showed a negative outcome in Atlas®. Nine samples (5.1%) were s*tx2* and *eae* positive (Profile C); seven of these enrichments were considered negative in Atlas®. Only seven samples (4%) showed a positive outcome for both *stx1* and *eae* markers (Profile D); four were negative in Atlas®. Eleven enrichments (6.2%) showed a positive result only for *stx1* marker (Profile E), seven of these samples were negative in Atlas®. Eighteen samples (10.2%) were positive for *stx2* gene (Profile F); all eighteen had a negative outcome in Atlas®. Eighteen samples (10.2%) corresponded to Profile G (only *eae* gene positive); thirteen of these samples had a negative outcome in Atlas® and five (2.8%) were positive for STEC. Sixty-one enrichments (34.5%) were negative for all three STEC markers when tested with TaqMan real-time PCR; 55 of the samples were negative in Atlas®. From a regulatory action perspective (Feng and Reddy, 2013), enrichments only *eae* positive (Profile G) are consider STEC negative. Thus, the total negative samples by real-time PCR would be 79 (44.7%).

A set (n=82) of the second step enrichments—R&F (n=38) and Actero STEC (n=48)—were tested in the Atlas® System in parallel with the first step nTSB enrichment (**Table 10** and **Table 11**, respectively). Regarding samples with a negative outcome in nTSB, four enrichments (two from R&F and two from Actero) showed a positive outcome for STEC. Out of four STEC positive samples in nTSB, only two showed a positive outcome when R&F was tested. However, the positive enrichments on R&F corresponded to different samples in nTSB. A similar outcome was observed when Actero STEC was tested: eight nTSB enrichments STEC positive were negative in Actero. One of the two enrichment O157:H7 positive in nTSB was negative in R&F, two of the four positive in nTSB showed a negative outcome in Actero, and two samples were considered STEC positive instead of O157:H7.

4.2. STEC testing from retained enrichments

120 retained enrichments (storage at −80°C) from bioaerosol deposit plots, from a study conducted by Dr. Elaine Berry at the USDA-MARC facility in Nebraska during CPS project 2011-136 (Berry et al., 2015), were processed for screening using the Atlas® System. Two broth media were tested: nTSB and mEHEC. The percentage of STEC positive samples was low (1.7% nTSB and 3.3% mEHEC) (**Table 12**). Only five and seven enrichments showed a positive result for *E. coli* O157:H7 in nTSB and mEHEC, respectively. In the previous study (Berry et al., 2015), O157:H7 results were higher (n=30); however, three samples considered negative for O157:H7 before, showed a positive outcome when tested in Atlas® (**Table 13**). Twelve samples showed a different outcome when different enrichment broths were tested (nTSB vs mEHEC).

Recovery of non-O157 STEC from enrichments was carried out according to the protocol described above. A total of 146 colonies (20 from CHROM STEC and 126 from RBA) were isolated and purified from the

secondary enrichments. All isolates were negative for STEC markers by multiplex PCR. Most the strains corresponded to generic E. *coli*. Fifteen samples did not show any type of bacterial growth on both agars.

4.2.1. Real-time qPCR detection of *stx1*, *stx2* and *eae* in retained enrichments

TaqMan real-time PCR was also used to screen enrichments for STEC genes and results were compared with outcomes from the Atlas® System. Three simplex TaqMan real-time PCR assays targeting *stx1, stx2* and *eae* genes were performed. Only six of the previously described eight real-time PCR profiles were observed (**Table 14**). Based on real-time PCR analysis, only one enrichment was positive for the three markers (Profile A), however, this sample showed a negative outcome in Atlas®. Eleven samples (9.2%) were positive for both s*tx2* and *eae* (Profile C); of these samples in Atlas®, seven (5.8%) from nTSB and four (3.3%) from mEHEC were negative. Only three samples (2.5 %) yielded a positive outcome for both *stx1* and *eae* markers (Profile D), but they were negative in Atlas®. Only one sample (0.8%) showed positive outcome for the s*tx2* marker (Profile F), but was negative in Atlas®.

Twenty-two samples (18.3 %) showed a positive outcome only for the *eae* gene by real-time PCR (Profile G); of these samples, 20 (16.7%) from nTSB and 19 (15.8%) from mEHEC exhibited a negative outcome in Atlas®; two (1.7%) from nTSB and one (0.8%) from mEHEC were positive for STEC, while two (1.7%) from the mEHEC enrichment were positive for O157:H7. Eighty-two samples (68.3%) were negative for all markers, but one of these samples was O157:H7/STEC positive in the Atlas® System.

Since samples only *eae* positive (Profile G) are technically considered STEC negative, the total negative samples by real-time PCR would be 110 (88.6%), which was very close 113 (94.2%) and 109 (90.8%) in the Atlas® system when nTSB and mEHEC, respectively, were used as the enrichment broth.

STEC marker screening results using TaqMan real-time PCR were compared with the outcome for *E. coli* O157:H7 obtained in the initial study (Berry et al., 2015) (**Table 15**). Out of the 30 enrichments that were O157:H7 positive in the Berry study, eight (6.7%) were positive for both s*tx2* and *eae* (Profile C), one (0.8%) for *stx1* and *eae* (Profile D), ten (8.3%) s*tx2* positive (Profile F), one (0.8%) for *eae* only (Profile G), and ten (8.3%) showed negative outcome for three STEC markers (Profile H).

4.3. Characterization of presumptive STEC retained cultures and *E. coli* non-O157 isolates, by Roka Atlas® screening and virulence markers

EG2 Combo Detection Assay, multiplex PCR for STEC markers and virulence-associated markers were carried out as described above. A total of 164 retained presumptive STEC and *E. coli* non-O157 isolates (stored at −80°C) from different sources were investigated: 130 from the CPS-supported project (including leafy greens, irrigation water, soil and feces samples) and 34 from the TVS Lab culture collection (including strains from animal and human sources). All 130 presumptive isolates from the previous CPS funded project, and seven strains from the culture collection showed a negative outcome for STEC/O157 in the Atlas® system. However, ten retained cultures from the CPS project and all strain isolates from the culture collection were positive for STEC markers in multiplex PCR. Thus, 44 strains were suited for characterization.

Twenty-one of the 32 marker profiles were observed (**Table 16**). The first three profiles (1, 2 and 3) were positive for *stx1*, *stx2* and *eae* markers, and for *adfO* and *Z2098* genes. Profile 1 contained both hemolysin genes (*hlyA* and *ehxA*), Profile 2 contained only *hlyA,* and Profile 3 was negative for both. One strain from the culture collection (Profile 3) showed a negative outcome for STEC/O157 in the Atlas® system.

Twenty-three isolates were positive for *stx1* and *eae* markers, and were distributed in nine different profiles (4–12). Profile 4 contained strains carrying *hlyA, ehxA, saa, adfO* and *Z2098* genes. Profiles 5–9 showed a very similar pattern to Profile 4, but these profiles were missing one or two genes from Profile 4 (*saa, adfO*,

or *Z2098*). All isolates from these profiles were positive for STEC in the Atlas® system, although one strain (Profile 7) showed a positive outcome for O157:H7 instead of STEC. Profile 10 was positive only for *hlyA*, *adfO* and *Z2098* genes, Profile 11 for *adfO* and *Z2098*, and Profile 12 only for *Z2098*. Strains with Profile 11 and 12 (n=4) were negative for STEC/O157 in the Atlas system®.

One strain, Profile 14, was positive for *stx1* and *stx2* genes but negative for the *eae* gene. This isolate was also positive for *hlyA*, *ehxA*, and *saa* genes, and had a negative outcome for STEC/O157 in the Atlas system®.

Ten isolates distributed in three marker array profiles (15, 16 and 17) were positive only for the *stx1* gene. All were negative for STEC/O157 in the Atlas system®. Profile 15 showed the same associated virulence markers as Profile 1. Strains belonging to Profile 16 were positive for *hlyA*, *ehxA*, *saa*, and *subAB*, whereas Profile 17 was positive for three of these genes (*hlyA*, *ehxA*, and *subAB*).

Seven strains were positive for *stx2*, distributed in three profiles (20, 21 and 22). Profile 20 contained three isolates that were positive for the *eae* gene as well, and showed a positive outcome for STEC in Atlas®. Profile 20 exhibited the same associated virulence markers as Profile 1. Profiles 21 and 22 were positive only for *stx2*; Profile 21 had the same marker array as Profile 1, and Profile 22 the same as Profile 16. Both profiles (21 and 22) were negative in the Atlas system®.

Finally, one isolate was only positive for *eae* gene, Profile 32, which only carried *ehxA*.

4.4. Associated virulence markers profile of presumptive STEC cultures isolated in commercial labs from positive enrichments

Additionally, 25 presumptive STEC strains (16 from leafy greens and 9 from irrigation water) isolated in commercial labs were screened for associated virulence markers (**Table 17**). These isolates came from STEC-positive enrichments in the Atlas® system. Five of the isolates showed positive outcome for *stx1* and *eae* genes; they grouped in three profiles previously described (6, 9 and 10). One strain was positive for *stx2* and *eae* genes, showing virulence marker Profile 20. Five strains were positive only for stx2, which corresponded to Profile 22. Thirteen strains carried only the *eae* gene, and grouped in three profiles previously described (25, 30 and 31).

4.5. Overall review of sample testing

In the present study, 177 purified samples from produce/environment, and 120 retained enrichments were tested for STEC single marker (Atlas® System), STEC marker profiles (*stx*1/2 and *eae*) by real time PCR, and cultural assessment. In addition, 164 retained isolates were examined for STEC single marker and three-target STEC marker profiles; also, 25 strains recovered from commercial labs from positive enrichments were characterized by PCR. Overall, 29.4% (52) of the produce/environmental samples, and 3.3% (4) of retained enrichments, were positive for STEC by single marker analysis, while outcomes for testing by real-time PCR three-marker screening were 39.1% (69) and 12.5% (15), respectively (**Table 18**). The prevalence of STEC culture positive samples in this study was 10.7% (19), which was lower than molecular assessment, as expected. It has been reported that close to 1% of produce in the U.S. was positive for STEC (Feng and Reddy, 2013), which is considerably lower than the prevalence found in the current study. Is important to consider that 9% (16) of the molecular single marker positive samples in this study were water or soil, which have shown high prevalence of STEC (12 to 18%) (Cooley et al., 2013, 2014).

Assessment of the virulence genes have been used to predict whether STEC could present significant risk to human health (Delannoy et al., 2013). In the present study, >140 strains were suited for associated virulence marker characterization: 72 strains recovered from samplings, 11 from retained isolates from CPS supported

projects, 34 strains from the lab culture collection, and 25 recovered in commercial labs (**Table 19**). Isolates from Profiles 1 to 10, 13 and 20 were positive for single marker *efc1* Atlas®. All single marker positive profiles contained isolates carrying *stx* and *eae* genes. Most of the single marker positive profiles were also carrying *hlyA*, *ehxA*, *adfO* and *Z2098* genes.

4.6. Field capture of birds and site-convenience samples and characterization of STEC Isolates

In San Benito, a total of 234 birds were captured. From these birds, 234 fecal samples, 156 oral swabs and 18 feet/feather swabs were collected (**Table 20**). Samples were tested for *E. coli* O157 and non-O157 STEC and two birds were positive (**Table 21**): one male red-winged blackbird and one golden-crowned sparrow. The former was positive in feces and feet/feathers, whereas the latter was positive in feet/feathers only. Both birds were sampled on the same day, by the end of September. This month coincided with a peak in bird abundance (second most abundant month of sampling, after March). All isolates belonged to serogroup O26 (**Table 22**).

In the delta of the Central Valley (Elk Grove, Yolo County), 349 individual bird fecal samples were collected (**Table 20**), along with 32 pooled samples opportunistically collected from flocks of Canada geese, greater white-fronted geese and sandhill cranes. Additionally, 197 oral swabs and 213 feet/feather swabs were collected from the mist-netted/trapped individuals. Of the pooled fecal samples, 12.5% were positive for non-O157 STEC (**Table 21**); these belonged to greater white-fronted geese sampled in March (serogroups O26 and O84) and Canada geese sampled in July (serogroup O163) (**Table 22**). There were no other positive findings until late fall, by the end of November. The feces of one Bewick's wren were positive for non-O157 STEC (serogroup pending). On the same day and site, the feces of a ruby-crowned kinglet were positive for *E. coli* O157:H7. One week later the feces of another ruby-crowned kinglet was positive for *E. coli* O157:H7 also. The first event coincided with the day of the highest abundance of the year, but the abundance one week later was lower than the mean. A comparison of the number of bird samples collected in the two sites is provided in **Table 23**.

Among 265 cattle fecal samples collected, *E. coli* O157 and non-O157 STEC were detected in 4 (1.5%) and 97 (37%), respectively. Non-O157 STEC prevalence was over 50% in September to December. *E. coli* O157 was isolated from both cattle feces (February, August, September, and October) and ruby-crowned kinglets (November, December). Birds and cattle at the Yolo Country site shared non-O157 STEC serogroups including O26 (great white-fronted goose, red-winged blackbird, golden-crowned sparrow) and O163 (Canada goose). Data on other non-bird convenience sampling is provided in **Table 24**.

5. Outcomes & Accomplishments

This study evaluated the potential utility of single marker technology for the detection of non-O157 STEC in ag-environment samples. The Roka Atlas EG2 test is known to detect pathotoxigenic *E. coli* O157 and STEC with a characterized determinative virulence factor. This promising technology uses a novel single genetic marker as a surrogate for the detection of pathogenic STEC. The baseline data collected and the genetic virulence profiling the team completed within this effort, resulted in the adoption and ongoing use of the Roka Atlas EG2 system with secondary Shiga-toxin gene target screening as one of the primary testing platforms for fresh produce in California and across the U.S.

- Calibration of standardized protocols and operator proficiency assessments: Several rounds of verification, standardization, and proficiency training were conducted to ensure technical execution of the multiple STEC detection platforms and genetic markers. Two lab assistants and one postdoctoral researcher were trained on the Roka system and real-time polymerase chain reaction (PCR) assay.

- Analyzed retained STEC cultures from Lab Rapid Response collections: The team screened all of the 120 feedlot surface and spinach samples (received from Dr. Elaine Berry, U.S. Meat Animal Research Center (MARC), Nebraska) and more than 600 isolates from the principal investigator's lab collection. These were characterized by Roka reaction and 12 Marker Profile Group patterns. This confirmed the basic functionality of the Roka EG2 platform and identified the need for secondary rapid confirmation tests to minimize the potential for false positive results leading to crop destruction.

- Conducted deferred pathogen detection tests on Roka Transfer Tubes (TT) and retained enrichments from industry submissions: Due to limited actual vs. projected submissions, the team only had access to 25 samples not pre-screened with a Roka system but reported as presumptive positive for EHEC/STEC. The test showed that 13 of the 25 were false positive by genetic marker screening.

- Recovered isolate characterizations: More than 600 potential STEC isolates from diverse farm, animal, and crop sources were characterized for virulence-associated markers. A total of 32 different Marker Profile Groups were identified. Long term, a micro-array test platform, direct from the enrichment culture, may be the preferred detection method to ensure elimination of both false positive and false negative results, which are equally troubling. Overall, 29.4% (n=52) of the produce/environmental samples were positive for STEC by single marker analysis, in contrast with 39.1% (n=69) by real-time PCR three-marker screening, which is very likely to be a consequence of signal from target genes carried by different cells/strains.

- Conducted initial study site evaluations and coordination/informational meetings with enrolled cooperators: For targeted pre-trial environmental sampling, a total of 177 samples were tested. Samples of basil, bok choy, leeks, kale, chard, collard, parsley, soil, and water were collected, which provided over 350 isolates for molecular and cultural confirmation. Both known EHEC and STEC isolates were correctly identified by the Roka system.

- Bird trapping, STEC detection and recovery assessments / environmental sampling and detection activities: More than 1,300 samples from birds were tested in two study sites. The majority of those trapped were passerine (songbird) species. The results of the bird trapping suggest that only a small proportion of birds carry EHEC/STEC, and some species may be more prone than others. For example, golden-crowned sparrows forage on the ground, where they spend most of their time. Red-winged blackbirds are also ground foragers, and they breed in wet places like marshes and are known for their association with cattle and other livestock. However, the STEC finding for ruby-crowned kinglets was surprising as they feed on insects pecked from the surface of leaves and branches. The results also indicated that there is a strong seasonality of carriage of EHEC/STEC by birds, but this differs between taxonomic orders (spring and summer in Anseriformes; fall in Passeriformes). To the team's knowledge, this is the first report of EHEC/STEC isolated from the feet/feathers of birds, which raises further questions on the potential of transmission and contamination by birds. The conclusion was that the general frequency of internal or external carriage of STEC is likely to be low. Industry standards and food safety policy may benefit from deemphasizing knee-jerk reactions to farm specific bird intrusion and develop grower guidance and decision trees for an assessment of risk, which would include both quantitative assessments, flocking behavior, general bird species assessments, and other relevant factors.

6. Summary of Findings & Recommendations

Over the course of the project period, more than 600 samples from diverse agricultural production and farmscape environments were collected and analyzed by several test methods for the presence of STEC contamination. In addition, 120 retained samples from pre-project commercial and research lab isolations were screened. All confirmed *E. coli* were further genetically characterized with 12 virulence-associated genetic markers. The activities required multiple trips to commercial production farms for collection of crop samples, irrigation water, and soil samples. During the project period, 177 purified samples from produce/environment, and 120 retained enrichments were tested for STEC single marker (Atlas® System, Roka Biosciences), STEC marker profiles (*stx1*/*2* and *eae*) by real-time PCR, and cultural assessment. In addition, 164 retained isolates were examined for STEC single marker and three-target STEC marker profiles; also, 25 strains recovered from commercial labs from positive enrichments were characterized by PCR.

Of the 400 purified STEC colonies recovered from all environmental project sources, 32 variants of the combined twelve identity-confirming or virulence-associated genetic markers were observed (Marker Profile Groups). Of these, the Roka system positively identified 97% of those that met U.S. FDA criteria as STEC of presumptive clinical significance for human illness.

Early in the project performance period, only 25 presumptive positive enrichment samples were obtained from commercial testing labs. Of these, eight Marker Profile Groups were observed, including those aligning with environmental isolates, as above. Twelve samples yielded colonies confirmed as presumptive clinical STEC and were positive by Roka, while 13 samples were determined to be false positive in the original platform detection screening, based on our 12 Marker Profile patterns and a negative reaction by Roka.

In a separate, related objective to generate additional isolates for genetic characterization, birds were captured and sampled at two study sites in 2015 and 2016. One of the study sites is a commercial produce ranch in San Benito County, on the Central Coast foothills. The other site is a National Wildlife Refuge in Yolo County on the delta of the California Central Valley, where land use is predominantly agriculture. In both sites, mist nets and traps were placed in proximity to a water body (reservoir or river). Of the 1,369 bird-associated samples, including 54 bird species from the study areas, 0.7% were positive for a presumptive clinically significant STEC. In addition, 12.5% of composited samples of Canada goose fecal mass were positive for a presumptive clinically significant STEC.

Since the end of the first performance period in 2015, commercial producers in CA alone have tested tens of thousands of samples of diverse leafy greens, culinary herbs, and other fresh and fresh-cut produce. The concerns for economic losses due to false-positive reports have essentially ceased to be raised to our extension program or at industry meetings. The research team holds this to be the major success of the project. In addition, the project outcomes further supported prior CPS research that showed, while birds can be a source of clinically significant STEC contamination, the prevalence is very low. Current data do not support implementing environmentally damaging measures or immediate crop destruct or broad-scale buffering in response to minor bird presence or intrusion to leafy greens fields. Science-based Best Practices regarding bird intrusion is a major successful outcome.

In summary, without comment to the need or advisability of pathogen testing of fresh produce in food safety programs, the team achieved its performance goal to expand and confirm the scientific basis for a rapid two-step detection protocol for routine compliance and lot acceptance testing of the presence or absence of clinically significant EHEC/STEC for fresh produce. Major service labs that are utilized by the fresh and fresh-cut industries rapidly adopted the study outcomes, in collaboration with and complimentary studies conducted by Roka Biosciences, early in the project performance period. The application of this two-step protocol remains in use today, and the original issue of potential false-positive test results has been managed to a minimal occurrence across the industry (personal communication with several large handlers).

APPENDICES

Presentations

2017
Single marker detection and virulence gene profiling of non-O157 STEC in produce and associated farmscape samples. Poster: July 9, 2017. IAFP Annual Meeting, Tampa, FL.

Rapid tests to specifically differentiate clinically significant from environmental STEC towards reducing unnecessary crop destruction. Presentation: June 21, 2017. CPS Research Symposium, Denver, CO.

Produce Testing: Current state, benefits and challenges. January 29, 2017. Western Growers Association Lunch & Learn Webinar.

2016
Developing a Comfort Level with Myths, Mysteries, and Befuddlements in Pathogen Testing on Fresh Produce. September 23, 2016. Bonduelle Foods – Management Group, UC Davis Mann Lab.

Micro Testing and Rapid Pathogen Test and Hold Challenges. September 13–15, 2016. Fresh-Cut Products: Maintaining Quality and Safety, Buehler Alumni Center, UC Davis.

STEC Testing in Soil, Manure and Compost. August 24–25, 2016. CDPH/FDB and CalFERT Commodity Specific Training, Soil Systems and Management with Soil Amendments of Biological Origin, California Department of Food and Agriculture, Sacramento, CA.

Rapid tests to specifically differentiate clinically significant from environmental STEC towards reducing unnecessary crop destruction. Poster: June 28–29, 2016. CPS Research Symposium, Seattle, WA.

Preharvest to Postharvest Produce Safety: Preparing for FSMA. May 17, 2016. HRT 200B, Horticulture and Agronomy Graduate Group, UC Davis.

Basics of Microbiological Testing for Key Compliance Expectations of FSMA. May 4, 2016. Western Food Safety Summit, Hartnell College, Salinas, CA.

Developing a Comfort Level with Myths, Mysteries, and Befuddlements in Pathogen Testing on Fresh Produce. February 25, 2016. What Microbial Testing Can and Can't Do For You – A PMA Webinar.

Practical Issues and Current Systems in STEC Detection during Crop Input and Produce Lot Acceptance Testing. February 23, 2016. National Advisory Committee on Microbiological Criteria for Foods STEC Subcommittee, Washington, DC.

New Developments in Rapid Pathogen Testing. January 6, 2016. CA Melon Research Board Annual Meeting, San Diego, CA.

2015
Clarifying the issues surrounding product testing for EHEC/STEC. November 2, 2015. Advanced Produce Safety Workshop: A Science-based Framework, UCD Postharvest Technology Center.

Clarifying the Issues Surrounding Product Testing for EHEC/STEC. September 6, 2015. CA Leafy Green Research Board and Central Coast Grower Shipper Association Special Meeting, Salinas, CA.

Applied Research on Microbial Safety of Perishable Horticultural Food Crops. June 26, 2015. CDC DFWED Seminar Series, Atlanta, GA.

Applied Research on Microbial Safety of Perishable Horticultural Food Crops. May 12, 2015. Walmart Food Safety & Health Emerging Issues Series, Bentonville, AK.

Overview of Microbial Food Safety Challenges for the US Fresh Produce Industry. April 23, 2015. FSMA Implementation and Food Safety Concerns, BABCOCK Laboratories Inc. Team Event, Riverside, CA.

Key Safety Challenges for the Fresh Produce Industry. April 17, 2015. California Chapter of the American Society of Farm Managers and Rural Appraisers, Inc., Agribusiness Conference, Paso Robles, CA.

Microbial Testing Considerations. January 22, 2015. Quality and Safety of Fresh Cut Products, Cardiff, Wales.

Publications
(none)

Budget Summary

All grant funds awarded ($329,481) will be utilized by the end of the project period.

Tables and Figures

Tables 1–23

Table 1. *Direct enrichment vs MMS capture: STEC detection*

Sampling event	Method	STEC outcome - Roka	
		Negative	STEC
Sampling 1-2015	Direct	11	3
	MMS	8	6
	Total S-1	19	9
Sampling 2-2015	Direct	15	3
	MMS	14	4
	Total S-2	29	7
	Total	48	16

Table 2. *Multiplex STEC PCR: Primer sequences and lengths of PCR products*

Primer	Direction	Primer sequence (5'-3')	Fragment size (bases)	Reference
E. coli 16 rDNA	Forward	GGAAGAAGCTTGCTTCTTTGCTGAC	544	(Sabat et al. 2000)
	Reverse	AGCCCGGGGATTTCACATCTGACTTA		
stx1	Forward	ACACTGGATGATCTCAGTGG	614	(Fagan et al. 1999)
	Reverse	CTGAATCCCCCTCCATTATG		
stx2	Forward	CCATGACAACGGACAGCAGTT	779	(Fagan et al. 1999)
	Reverse	CCTGTCAACTGAGCAGCACTTTG		
eaeA	Forward	GTGGCGAATACTGGCGAGACT	890	(Gannon et al. 1997)
	Reverse	CCCCATTCTTTTTCACCGTCG		

Table 3. *Virulence-associated factors: Primer sequences and PCR sizes products*

Primer	Direction	Primer sequence (5'-3')	Fragment size (bases)	Reference
saa	Forward	CGTGATGAACAGGCTATTGC	119 bp	(Feng and Reddy 2013)
	Reverse	ATGGACATGCCTGTGGCAAC		
adfO	Forward	ATTGACGACCTGACCAGACC	339 bp	This study
	Reverse	ACGGGTTTCATCATTTGCAT		
aggR	Forward	CTAATTGTACAATCGATGTA	457 bp	(Feng and Reddy 2013)
	Reverse	AGAGTCCATCTCTTTGATAAG		
hlyA	Forward	GCATCATCAAGCGTACGTTCC	534 bp	(Paton and Paton 1998)
	Reverse	AATGAGCCAAGCTGGTTAAGCT		
Z2098	Forward	CGCGCGGAGATTAAATCATA	560 bp	This study
	Reverse	TCTGGTAGCGACATCCAGTG		
subAB	Forward	GTGTACAGGACTCATGG	783 bp	(Feng and Reddy 2013)
	Reverse	ATCACCAGTCCACTCAG		
ehxA	Forward	CCCAGGAGAAGAAGTCA	1108 bp	(Feng and Reddy 2013)
	Reverse	CTTCACCTGAGGCATCTT		

Table 4. *TaqMan real-time PCR primers and probes*

Primers and Probes	Sequences (5' -3')	Amplicon	Reference
stx1-F	GTGGCATTAATACTGAATTGTCATCA	109 bp	(Yoshitomi, Jinneman et al. 2006)
stx1-R	GAAGAGTCCGTGGGATTACGC		
stx1-P	VIC-TGATGAGTTTCCTTCTATGTGTC- MGBNFQ		
Stx2-F	TGGAAAACTCAATTTTACCTTTAGCA	83 bp	
Stx2-R	GCAAATAAAACCGCCATAAACATC		
Stx2-P	FAM-CCGCCATTGCATTAACAGA- MGBNFQ		
eae-F	CTTTGACGGTAGTTCACTGGAC	170 bp	(Fratamico, Bagi et al. 2011)
eae-R	CAATGAAGACGTTATAGCCCAAC		
eae-P	FAM-CTGGCATTTGGTCAGGTCGGGGCG-BHQ1		

Table 5. *STEC testing results from crops and environmental samples*

	Atlas STEC EG2 combo outcome® N (%)			
Crop/sample	**Negative**	**O157:H7**	**STEC**	**Total**
Basil	8 (4.5)	2 (1.1)	18 (10.2)	28 (15.8)
Bok choy	9 (5.1)	0	3 (1.7)	12 (6.8)
Collard	3 (1.7)	0	0	3 (1.7)
Kale	3 (1.7)	0	0	3 (1.7)
Leek	56 (31.6)	1 (0.6)	12 (6.8)	69 (39)
Parsley	9 (5.1)	0	3 (1.7)	12 (6.8)
Rainbow chard	4 (2.3)	0	0	4 (2.3)
Soil	20 (11.3)	5 (2.8)	10 (5.6)	35 (19.8)
Water	3 (1.7)	2 (1.1)	6 (3.4)	11 (6.2)
Total	115 (65)	10 (5.6)	52 (29.4)	177

Table 6. *Strains isolated and incidence of STEC markers by sample*

Number of isolates recovered (% of total)					
	E. coli	***stx1***	***stx2***	***eae***	***stx/eae*[a]**
Basil	65 (17.4)	13 (3.5)	2 (0.5)	6 (1.6)	2 (0.5)
Bok choy	3 (0.8)	0	1 (0.3)	2 (0.5)	0
Collard	11 (2.9)	0	0	0	0
Kale	0	0	0	0	0
Leek	130 (34.9)	3 (0.8)	2 (0.5)	15 (4)	1 (0.3)
Parsley	31 (8.3)	3 (0.8)	0	0	0
Rainbow chard	8 (2.1)	0	0	0	0
Soil	87 (23.3)	9 (2.4)	0	17 (4.6)	3 (0.8)
Water	38 (10.2)	1 (0.3)	0	4 (1.1)	1 (0.3)
Total	373	29 (7.8)	5 (1.3)	44 (11.8)	7 (1.9)

[a]*Positive if eae and stx1 and/or stx2 positive*

Table 7. *Molecular STEC outcome and isolate recovery carrying STEC markers*

Roka Outcome (N)		**Number of enrichments culture positive (% of Outcome)**				
		stx1	***stx2***	***eae***	***stx/eae***	**Total**
Negative	115	1 (0.9)	3 (2.6)	11 (9.6)	0	15 (13.9)
O157:H7	10	1 (10)	0	1 (10)	2 (20)	4 (40)
STEC	52	6 (11.5)	1 (2.3)	4 (7.7)	4 (7.7)	15 (28.8)
Total	177	8	4	16	6	34

Table 9. *Screening of STEC marker of crops and environmental enrichments: TaqMan real-time PCR vs* Atlas® *System (Roka)*

	STEC marker TaqMan real-time PCR			Roka Outcome N (% of Outcome)			
Profile	***stx1***	***stx2***	***eae***	**STEC**	**O157:H7**	**Negative**	**Total**
A	+	+	+	25 (14.1)	4 (2.3)	5 (2.8)	34 (19.2)
B	+	+	-	9 (5.1)	4 (2.3)	6 (3.4)	19 (10.8)
C	-	+	+	2 (1.1)	0	7 (4)	9 (5.1)
D	+	-	+	2 (1.1)	1 (0.6)	4 (2.3)	7 (4)
E	+	-	-	4 (2.3)	0	7 (4)	11 (6.2)
F	-	+	-	0	0	18 (10.2)	18 (10.2)
G	-	-	+	5 (2.8)	0	13 (7.3)	18 (10.2)
H	-	-	-	5 (2.8)	1 (0.6)	55 (31.1)	61 (34.5)
				52	10	115	177

Table 10. *Detection of STEC in Atlas® System (Roka): nTSB vs R&F non-O157 broth*

Atlas system outcome nTSB	Outcome R&F non-O157 broth		
	STEC	**0157:H7**	**Negative**
Negative (n=32)	2	0	30
O157:H7 (n=2)	0	1	1
STEC (n=4)	2	0	2
Total (n=38)	4	1	33

Table 11. *Detection of STEC in Atlas® System (Roka): nTSB vs Actero STEC broth*

Atlas system outcome nTSB	Outcome Actero STEC broth		
	STEC	**0157:H7**	**Negative**
Negative (n=21)	2	0	19
O157:H7 (n=4)	2	1	1
STEC (n=23)	15	0	8
Total (n=48)	19	1	28

Table 12. *Atlas STEC EG2 Combo Detection Assay® results from retained enrichments of bioaerosol deposition plots*

Previous result	Outcome from nTSB (% of total)			Outcome from mEHEC (% of total)		
	Negative 113 (94.2)	**O157:H7** 5 (4.2)	**STEC** 2 (1.7)	**Negative** 109 (90.8)	**O157:H7** 7 (5.8)	**STEC** 4 (3.3)
Generic *E. coli* negative (n=66)	64 (53.32)	2 (1.7)	0	60 (50)	3 (2.5)	3 (2.5)
Generic *E. coli* positive (n=54)	49 (40.8)	3 (2.5)	2 (1.7)	49 (40.8)	4 (3.3)	1 (0.8)
E. coli O157:H7 negative (n=90)	88 (73.3)	2 (1.7)	0	87 (72.5)	3 (2.5)	0
E. coli O157:H7 positive (n=30)	25 (20.8)	3 (2.5)	2 (1.7)	22 (18.3)	4 (3.3)	4 (3.3)

Table 13. *Atlas STEC EG2 Combo Detection Assay® results vs previous results from retained samples*

Previous study outcome		Atlas outcome	
***E. coli* O157:H7**	**Generic *E.coli***	**nTSB**	**mEHEC**
Positive	Positive	Negative	**O157:H7**
Negative	Positive	**O157:H7**	**O157:H7**
Negative	Positive	**O157:H7**	**O157:H7**
Positive	Negative	Negative	**O157:H7**
Positive	Negative	Negative	**STEC**
Positive	Positive	Negative	**O157:H7**
Positive	Positive	**O157:H7**	Negative
Positive	Negative	Negative	**O157:H7**
Negative	Negative	Negative	**O157:H7**
Positive	Negative	**O157:H7**	**STEC**
Positive	Negative	**O157:H7**	**STEC**
Positive	Positive	**STEC**	Negative

Table 14. *STEC gene markers screening (TaqMan real-time PCR) of 120 retained enrichments*

Marker profile	STEC markers TaqMan real-time PCR			Roka Outcome nTSB N (% of Outcome)			Roka Outcome mEHEC N (% of Outcome)			Total
	stx1	*stx2*	*eae*	STEC	O157:H7	Negative	STEC	O157:H7	Negative	
A	+	+	+	0	0	1 (0.8)	0	0	1 (0.8)	1 (0.8)
B	+	+	-	0	0	0	0	0	0	0
C	-	+	+	0	4 (3.3)	7 (5.8)	2 (1.7)	5 (4.2)	4 (3.3)	11 (9.2)
D	+	-	+	0	0	3 (2.5)	0	0	3 (2.5)	3 (2.5)
E	+	-	-	0	0	0	0	0	0	0
F	-	+	-	0	0	1 (0.8)	0	0	1 (0.8)	1 (0.8)
G	-	-	+	2 (1.7)	0	20 (16.7)	1 (0.8)	2 (1.7)	19 (15.8)	22 (18.3)
H	-	-	-	0	1 (0.8)	81 (67.5)	1 (0.8)	0	81 (67.5)	82 (68.3)
				2 (1.7)	5 (4.2)	113 (94.2)	4 (3.3)	7 (5.8)	109 (90.8)	120

Table 15. *STEC markers previous study outcome from retained crop enrichment samples*

Real-time PCR STEC marker profile	Previous study outcome (% of total)		Total
	E. coli O157:H7 positive (n=30)	*E. coli* O157:H7 negative (n=90)	
A	0	1 (0.8)	1
B	0	0	0
C	8 (6.7)	3 (2.5)	11
D	1 (0.8)	2 (1.7)	3
E	0	0	0
F	10 (8.3)	12 (10)	22
G	1 (0.8)	0	1
H	10 (8.3)	72 (60)	82
Total	30	90	120

Table 16. *Marker profiles and Atlas® outcome from retained isolates (culture collection–CPS project)*

Marker profile	STEC marker			Virulence associated genes							Roka Outcome of retained isolates			Total isolates
	stx1	*stx2*	*eae*	*hlyA*	*ehxA*	*saa*	*subAB*	*adfO*	*Z2098*	*aggR*	STEC	O157:H7	Negative	
1	+	+	+	+	+	-	-	+	+	-	2	0	0	2
2	+	+	+	+	-	-	-	+	+	-	1	0	0	1
3	+	+	+	-	-	-	-	+	+	-	1	0	1	2
4	+	-	+	+	+	+	-	+	+	-	5	0	0	5
5	+	-	+	+	+	+	-	-	+	-	1	0	0	1
6	+	-	+	+	+	-	-	+	+	-	8	0	0	8
7	+	-	+	+	+	-	-	+	-	-	1	1	0	2
8	+	-	+	+	-	+	-	+	+	-	1	0	0	1
9	+	-	+	+	+	-	-	-	+	-	1	0	0	1
10	+	-	+	+	-	-	-	+	+	-	1	0	0	1
11	+	-	+	-	-	-	-	+	+	-	0	0	3	3
12	+	-	+	-	-	-	-	-	+	-	0	0	1	1
14	+	+	-	+	+	+	-	-	-	-	0	0	1	1
15	+	-	-	+	+	-	-	+	+	-	0	0	1	1
16	+	-	-	+	+	+	+	-	-	-	0	0	5	5
17	+	-	-	+	+	-	+	-	-	-	0	0	4	4
20	-	+	+	+	+	-	-	+	+	-	3	0	0	3
21	-	+	-	+	+	-	-	+	+	-	0	0	1	1
22	-	+	-	+	+	+	+	-	-	-	0	0	3	3
32	-	-	+	-	+	-	-	-	-		0	0	1	1
											25	1	18	44

Table 17. *Virulence marker profiles of isolates recovered in commercial labs*

Marker profile	No of isolates	STEC marker			Virulence associated genes					
		stx1	*stx2*	*eae*	*hlyA*	*ehxA*	*saa*	*subAB*	*adfO*	*Z2098*	*aggR*
6	2	+	-	+	+	+	-	-	+	+	-
9	1	+	-	+	+	+	-	-	-	+	-
10	3	+	-	+	+	-	-	-	+	+	
20	1	-	+	+	+	+	-	-	+	+	-
22	5	-	+	-	+	+	+	+	-	-	-
25	10	-	-	+	+	+	-	-	+	+	-
30	2	-	-	+	-	-	-	+	-	-	-
31	1	-	-	+	-	-	-	-	+	-	-

Table 18. *Overall sampling results*

Real-time PCR profile enrichments				Single marker outcome (Atlas)	STEC marker profile of purified colonies						Total	
	stx1	stx2	eae		stx1 + stx2 + eae +	stx1 + stx2 - eae +	stx1 + stx2 - eae -	stx1 - stx2 + eae -	stx1 - stx - eae +	Culture negative		
A	+	+	+	Negative					1	4	5	34
				O157:H7		2	1			1	4	
				STEC		1	4	1	1	18	25	
B	+	+	-	Negative				1	2	3	6	19
				O157:H7					1	3	4	
				STEC		1			3	5	9	
C	-	+	+	Negative					2	5	7	9
				STEC						2	2	
D	+	-	+	Negative			1			3	4	7
				O157:H7						1	1	
				STEC			1			1	2	
E*	+	-	-	Negative						7	7	11
				STEC		1				3	4	
F*	-	+	-	Negative				1		17	18	18
G*	-	-	+	Negative					5	8	13	18
				STEC	1		1			3	5	
H	-	-	-	Negative				1	1	53	55	61
				O157:H7						1	1	
				STEC						5	5	
				Total	1	5	8	4	16	143	177	177

*Samples positive for *eae* alone or only *stx* positive are not considered clinically significant STEC

Table 19. *Comparison among isolates identified by single marker ecf1 and broader virulence markers*

Isolates Serotype* - Source (n)	Single marker	Markers Profile	STEC marker			Virulence associated genes						
			stx1	*stx2*	*eae*	*hlyA*	*ehxA*	*saa*	*subAB*	*adfO*	*Z2098*	*aggR*
O111 Human (2)	+	1	+	+	+	+	+	-	-	+	+	-
O111 Unknown (1), Leek (1)	+	2	+	+	+	+	-	-	-	+	+	-
O145 Unknown (1)	-	3	+	+	+	-	-	-	+	+	+	-
O111 Unknown (1)	+	4	+	+	+	-	-	-	-	+	+	-
O26 Human (2), O26 Unknown (3),O45 Human (1), O45 Unknown (1), O103 Human (3), O145 Beef (1), O145 Unknown (2), Water (2)	+	5	+	-	+	+	+	-	-	+	+	-
O103 Unknown (2), Water (1)	+	6	+	-	+	+	+	-	-	-	+	-
O145 Human (2)	+	7	+	-	+	+	+	-	-	+	-	-
O26 Human (1), Soil (4), Water (1)	+	8	+	-	+	+	-	-	-	+	+	-
O45 Unknown (1)	+	9	+	-	+	+	-	+	-	+	+	-
Soil (1)	+	10	+	-	+	+	-	+	+	-	-	-
O26 Beef (1), O103 Human (1), O103 Unknown (1)	-	11	+	-	+	-	-	-	-	+	+	-
O103 Beef (1)	-	12	+	-	+	-	-	-	-	-	+	-
O121 Human (1), O121 Unknown (1), Leafy greens (1)	+	13	-	+	+	+	+	-	-	+	+	-
Unknown (1)	-	14	+	+	-	+	+	+	-	-	-	-
Cattle (1), Basil (1)	-	15	+	-	-	+	+	-	-	+	+	-
Basil (4), Leek (4), Parseley (4)	+	16	+	-	-	+	-	-	-	+	+	-
Water (1)	+	17	+	-	-	+	-	-	-	-	+	-
Leafy greens (1)	-	18	-	+	-	+	+	-	-	+	+	-
Leafy greens (5)	-	19	-	+	-	+	+	+	+	-	-	-
Bok Choy (1), Leeks (1)	+	20	-	+	-	-	+	-	-	+	+	-
Basil (1)	-	21	-	+	-	-	+	-	-	+	-	-
Leafy greens (11), Water (3)	+	22	-	-	+	+	+	-	-	+	+	-
Basil (2), Leek (1), Soil (4)	+	23	-	-	+	+	-	-	-	+	+	-
Bok Choy (1), Soil (1), Water (2)	+	24	-	-	+	+	-	-	-	+	-	-

* Basil isolates were recovered from STEC positive enrichment, but cattle strains tested negative
** Isolates from STEC-positive enrichments; strains were not tested directly in the Atlas
*** Mix of isolates from STEC-negative and STEC-positive enrichments

Table 20. *Amount and type of bird samples collected*

Location	Type of collection	Fecal samples	Oral swabs	Feet/ feather swabs	Total
San Benito	Individual	234	156	188	578
Elk Grove	Individual	349	197	213	759
	Pooled	32	-	-	32
Total		**615**	**353**	**401**	**1369**

Recaptures: 15 in San Benito, 16 in Elk Grove

Table 21. *Number of positive bird samples and prevalence*

Sample type	*E. coli* O157	Non-O157 STEC
Individual fecal samples	2/583 (0.34%)	2/583 (0.34%)
Pooled fecal samples	0	4/32 (12.5%)
Oral swabs	0	0
Feet/feathers swabs	0	2/401 (0.5%)

Table 22. *Serogroup and virulence factors of bird isolates*

Location	Date	Bird species	Sample type	*hlyA*	*eaeA*	*stx 2*	*stx 1*	*ehxA*	Serogroup/ serotype
Elk Grove	3/15/2016	Greater white-fronted geese	Pooled feces	0	0	0	1	1	O26
Elk Grove	3/15/2016	Greater white-fronted geese	Pooled feces	1	1	0	1	1	O84
Elk Grove	7/19/2016	Canada geese	Pooled feces	1	0	1	1	1	O163
Elk Grove	7/19/2016	Canada geese	Pooled feces	1	0	1	1	1	O163
San Benito	9/24/2016	Red-winged blackbird	Feces	1	1	0	1	1	O26
San Benito	9/24/2016	Red-winged blackbird	Feet & feathers	1	1	0	1	1	O26
San Benito	9/24/2016	Golden-crowned sparrow	Feet & feathers	1	1	0	1	1	O26
Elk Grove	11/30/2016	Bewick's wren	Feces	1	1	0	1	1	Pending
Elk Grove	11/30/2016	Ruby-crowned kinglet	Feces	1	1	1	1	1	O157:H7
Elk Grove	12/07/2016	Ruby-crowned kinglet	Feces	1	1	1	1	1	O157:H7

Table 23. *Comparison of bird group and species collection from sampling sites*

Group	Species	Number of samples collected in San Benito/Elk Grove
Sparrows and their allies	**White-crowned sparrow***	**75**/ 15
	Song sparrow	**25**/ 16
	Lincoln sparrow	13/ 1
	California towhee	5/ 1
	Golden crowned sparrow	10/ **47**
	Lark sparrow	1/ 0
	Fox sparrow	1/ 11
	Spotted towhee	1/ 16
	Savannah sparrow	14/1
	Oregon junco	0/ 20
Icterids	Great tailed grackle	3 / 0
	Brown-headed cowbird	0/ **24**
	Brewer's blackbird	0/ 5
	Red-winged blackbird	**26**/ 4
	Bullock's oriole	0/ 2
Game birds	California quail	9/ 2
Finches	House finch	3/ 5
	Lawrence's goldfinch	2/ 0
	Purple finch	1/ 0
	American goldfinch	0/ 6
Corvids	Western scrub jay	0/ 4
Grosbeaks	Black-headed grosbeak	0/ 10
Buntings	Lazuli bunting	0/ 1
Tyrant flycatchers	Black phoebe	9/ 5
	Pacific slope flycatcher	1/ 15
	Ash-throated flycatcher	1/ 7
	Willow flycatcher	1/ 2
	Western kingbird	0/ 1
	Gray flycatcher	0/ 1

Group	Species	Number of samples collected in San Benito/Elk Grove
Swallows	Northern rough-winged swallow	3/ 0
	Tree swallows	0/ 3
Shrikes and vireos	Warbling vireo	1/ 1
Pigeons and doves	Mourning dove	1/ 2
Wading birds	Green heron	1/ 0
Woodpeckers	Downy woodpecker	1/ 0
	Northern flicker	0/ 2
	Nuttall's woodpecker	0/ 3
Wrens	Bewick's wren	0/ 4
	House wren	0/ 2
Mimids	California thrasher	1/ 0
	Northern mockingbird	0/ 13
Chickadees and their allies	Oak titmouse	2/ 1
	White-breasted nuthatch	0/ 1
	Bushtit	0/ 10
Wood warblers	Orange crowned warbler	1/ 4
	Common yellowthroat	2/ 1
	Townsend's warbler	0/ 1
	Yellow-rumped warbler	18/ **29**
	Wilson's warbler	0/ 1
	Yellow warbler	0/ 1
Kinglets	Ruby-crowned kinglet	1/ 23
Kingfishers	Belted kingfisher	1/ 0
Wrentits	Wrentit	0/ 6
Geese	Canada goose – pooled samples	0/ 16
	Greater white-fronted geese – pooled samples	0/ 10
Hawks	Sharp-shinned hawk	0/ 1
Cranes	Sandhill crane – pooled samples	0/ 6

*In bold are the three most frequently captured species in each location

Table 24. *Non-bird convenience samples*

Species	Location	Date	***E. coli*** **O157**	**Non-O157 STEC**
Lizard	San Benito	3/28/2016	Negative	Negative
Rabbit	San Benito	3/28/2016	Negative	Negative
Deer	San Benito	4/25/2016	Negative	Negative
Deer	San Benito	4/25/2016	Negative	Negative
Lizard	San Benito	4/25/2016	Negative	Negative
Coyote	Elk Grove	5/03/2016	Negative	Negative
Otter	Elk Grove	7/19/2016	Negative	Negative
Feral pig	San Benito	8/15/2016	Negative	Negative
Feral pig	San Benito	8/15/2016	Negative	Negative
Feral pig	San Benito	8/15/2016	Negative	Negative
Feral pig	San Benito	8/15/2016	Negative	Negative
Feral pig	San Benito	8/15/2016	***E. coli*** **O157:H7**	Negative
Feral pig	San Benito	8/15/2016	Negative	Negative
Otter	Elk Grove	10/21/2016	Negative	**Positive – serogroup pending**

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