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FINAL PROJECT REPORT

Project Title
Toward a rapid and reliable pathogen detection system in produce

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Objectives

1. *To develop and evaluate LAMP assays for STEC O157 and top non-O157 serogroups*
2. *To evaluate the robustness of the LAMP detection system using abusive temperature, pH, and the addition of soil, chlorophyll, and produce enrichment broth*
3. *To validate the system in complex produce matrices (cantaloupe, lettuce, pepper, spinach, sprout, and tomato) surface-inoculated with low levels of these pathogens*

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FINAL REPORT

Abstract

Salmonella, *Escherichia coli* O157:H7, and more recently non-O157 Shiga toxin-producing *E. coli* (STEC) are leading causes of produce-associated outbreaks in the United States. Rapid, reliable, and robust detection methods are needed to promptly identify pathogen contamination risks in the supply chain and better ensure produce safety. We recently developed a loop-mediated isothermal amplification (LAMP) assay for *Salmonella* detection as well as a LAMP suite for STEC detection, which were shown to be rapid, specific, sensitive, and robust. In this project, these LAMP assays were comprehensively evaluated against real-time quantitative PCR (qPCR) using a large panel of bacterial strains and in various produce items (cantaloupe, lettuce, pepper, spinach, sprouts, and tomato). To mimic real-world contamination events, produce samples were surface-inoculated with a low level (1 to 2 CFU/25 g) of individual pathogen cells representing ten *Salmonella* serovars (Braenderup, Enteritidis, Heidelberg, Javiana, Montevideo, Muenchen, Newport, Poona, Saintpaul, and Typhimurium) or belonging to seven STEC serogroups (O26, O45, O103, O111, O121, O145, and O157), and tested after aging at 4^{o}C for 48 h. Six DNA extraction methods were also compared using produce enrichment broths. The *Salmonella* LAMP assay was 100% specific among 168 strains examined. Similarly, all STEC targets and their subtypes were accurately detected by the STEC LAMP suite. The LAMP detection limits were 1 to 20 cells per reaction in pure culture and 10^4 to 10^6 CFU per 25 g (i.e., 10^2 to 10^4 CFU per g) in produce, except for STEC strains harboring *stx*$_{2c}$, *eae*-β, and *eae*-θ subtypes. After 6 to 8 h of enrichment, these LAMP assays achieved accurate detection of low-level *Salmonella* of diverse serovars or STEC strains with various *stx*$_2$ and *eae* subtypes and O serogroups in all produce varieties tested but not in sprouts. A similar trend of detection was observed for qPCR. PrepMan Ultra sample preparation reagent yielded the best results among the six DNA extraction methods. This project provided a rapid, reliable, and robust system for detecting *Salmonella* and STEC in produce during routine sampling and testing. The challenge with sprouts detection by both LAMP and qPCR calls for special attention to further analysis.

Background

Produce consumption in the U.S. has increased significantly in the past several decades, which unfortunately coincided with the surge of produce-related outbreaks. The U.S. Food and Drug Administration reported that between 1996 and 2010, there were approximately 131 produce-related outbreaks, resulting in 14,132 illnesses, 1,360 hospitalizations, and 27 deaths. Among approximately 20 produce commodities involved in these outbreaks, sprouts, leafy greens, tomatoes, and melons accounted for the majority of outbreaks. *Salmonella* and *E. coli* O157:H7 were the two prominent human pathogens involved in such outbreaks, many are large-scale, multistate. In September 2006, tainted pre-packaged spinach triggered an *E. coli* O157:H7 outbreak, resulting in 205 confirmed illnesses and 3 deaths in 26 states, as well as an estimated $37-74 million loss to the California produce industry. During 2005-2006, multistate *Salmonella* outbreaks associated with raw tomatoes caused 459 culture-confirmed cases of salmonellosis in 21 states. The 2008 outbreak of *Salmonella* Saintpaul infections associated with raw produce (jalapeño peppers, Serrano peppers, and raw tomatoes) led to a large outbreak in 43 U.S. states, the District of Columbia, and Canada, resulting in 1,500 cases and 2 deaths. Between 2009 and 2010, alfalfa sprouts were implicated in multiple *Salmonella* serovars outbreaks which

resulted in 419 illnesses in more than 26 states. In 2010, a multistate outbreak of non-O157 STEC serogroup O145 infections linked to shredded romaine lettuce from a single processing facility led to 26 confirmed cases in five states. This particular outbreak highlights the emerging importance of six non-O157 STEC serogroups (e.g., O26, O45, O103, O111, O121, and O145) in causing foodborne illnesses. Since June 2012, these non-O157 STEC serogroups have been regulated by the U.S. Department of Agriculture (USDA) as adulterants in raw, non-intact beef products. In May 2011, a massive outbreak of hemolytic-uremic syndrome (HUS; 852 cases) in Germany and several other countries was attributed to a rare STEC serotype O104:H4 in sprouts. Additionally, there are several recent multistate outbreaks caused by *E. coli* O157:H7 in romaine lettuce, organic spinach/spring mix blend, and ready-to-eat salads, and STEC O26 in clover sprouts, as well as several multistate salmonellosis outbreaks linked to cantaloupe, alfalfa sprouts, among others. The increasing implication of fresh produce in *Salmonella* and STEC outbreaks poses a significant threat to public health, and is detrimental to the produce industry.

To reduce the incidence of produce-associated outbreaks, a multifaceted approach from farm to table is required. In particular, the industry has drastically increased raw and finished product testing as a tool to better identify contamination risks. Nonetheless, pathogen detection in produce remains a challenging task. Due to the highly perishable nature of produce, a rapid test is critical. Produce items are also diverse and complex, many harboring assay inhibitors, therefore requiring effective sample preparation and commodity-specific method validation. Additionally, pathogens in produce are usually injured cells present at low levels in contrast to high-level normal flora, demanding a highly sensitive and specific assay. For *Salmonella*, the ability to detect broad serovars is necessary. In the case of STEC, the need to identify STEC as a group and certain STEC serogroups specifically adds yet another layer of complexity.

Owing to their rapidity, specificity, and sensitivity, molecular-based methods such as PCR and real-time quantitative PCR (qPCR) have gained wide applications in produce testing. Enrichment is commonly used to increase target cell numbers while simultaneously diluting assay inhibitors and normal flora in produce. However, false-positive and false-negative results are observed, and few PCR assays have been validated on a commodity-specific basis. Besides, a sophisticated thermal cycling instrument is indispensable to carry out these nucleic acid amplification tests (NAATs), limiting their wider applications.

Recently, a novel NAAT termed loop-mediated isothermal amplification (LAMP) has emerged as a promising alternative to PCR for pathogen detection. LAMP uses four to six specially designed primers and a strand-displacing *Bst* DNA polymerase to amplify up to 10^9 copies of target DNA under isothermal conditions (~65^{o}C) within an hour. Since it is isothermal, LAMP can be performed in much simpler instruments such as a heater or water bath. To date, multiple LAMP assays have been developed for *Salmonella* detection and successfully applied in food samples particularly eggs and produce, and shown to be rapid, specific, and sensitive. In the meantime, LAMP assays targeting STEC Shiga toxin genes (stx_1 and stx_2) have been developed and evaluated in food primarily beef, as have several others targeting the *E. coli* O157 *rfbE* gene (encoding perosamine synthetase). Very recently, we developed a suite of LAMP assays for STEC (targeting common virulence genes stx_1, stx_2, and *eae*) and the seven adulterant STEC serogroups (targeting *wzx* or *wzy* genes on respective O-antigen gene clusters). In another very recent paper, we also reported the robustness of our recently developed *Salmonella* LAMP assay (a CPS 2008 RFP funded project) for food applications. Despite these developments, the *Salmonella* LAMP assay and the STEC LAMP suite have not been evaluated using a large number of *Salmonella* strains representing broad *Salmonella* serovars or STEC strains harboring various *stx* and *eae* subtypes or tested in a variety of produce items using conditions

mimicking real-world contamination events (e.g., low-level surface inoculation, cold storage). In addition, despite the critical role sample preparation plays, there is a scarcity of data regarding the effectiveness of DNA extraction methods on *Salmonella* or STEC detection in produce.

The aims of this project were to comprehensively evaluate the *Salmonella* LAMP assay and STEC LAMP suite against qPCR using a large panel of bacterial strains and in various produce items (cantaloupe, lettuce, pepper, spinach, sprouts, and tomato) with conditions mimicking real-world contamination events, and to examine the effect of DNA extraction methods on assay performance. The qPCR assays tested were developed recently by scientists from the FDA for *Salmonella* and the USDA for STEC.

Research Methods and Results

Methods

Bacterial strains and target gene characterization

For *Salmonella* assay evaluation, a total of 141 *Salmonella* strains representing 54 serovars in *S. enterica* subsp. I, five other *S. enterica* subspecies, and *S. bongori*, and 27 non-*Salmonella* strains were used for specificity testing. Among them, ten *Salmonella* strains, one representing each of the ten serovars commonly involved in outbreaks of produce and other food: Braenderup, Enteritidis, Heidelberg, Javiana, Montevideo, Muenchen, Newport, Poona, Saintpaul, and Typhimurium were used for sensitivity testing and spiked-produce experiments. All strains were routinely cultured on Trypticase soy agar (BD Diagnostic Systems, Sparks, MD) or in broth for 37^{o}C overnight.

For STEC assay evaluation, a total of 123 *E. coli* strains belonging to 41 serogroups and 33 non-*E. coli* strains were used for specificity testing. Among them, seven STEC strains, one from each of the seven adulterant serogroups, were used for sensitivity testing and spiked-produce experiments. *E. coli* strains were examined for the presence of STEC virulence genes (*stx*$_1$, *stx*$_2$, and *eae*) by PCR, followed by restriction fragment length polymorphism (RFLP) to determine their respective gene subtypes.

LAMP assays

The *Salmonella* LAMP assay (Chen et al., 2011, Applied and Environmental Microbiology, 77:4008-4016) and the STEC LAMP suite of 10 assays (Wang et al., 2012, Journal of Clinical Microbiology, 50:91-97; Wang et al., 2012, Applied and Environmental Microbiology, 78:2727-2736) developed recently by our research group were evaluated. The *Salmonella* LAMP assay targeted the *invA* gene. In the STEC LAMP suite, the first three assays targeted common STEC virulence genes (*stx*$_1$, *stx*$_2$, and *eae*), while the remaining seven targeted genes (*wzx* or *wzy*) on the O-antigen gene clusters of the seven adulterant STEC serogroups. Each LAMP assay employed five to six specially designed LAMP primers, two outer, two inner, and one or two loop that recognized specific regions of the target DNA sequences.

The assays were performed as described previously. Briefly, the LAMP reagent mix (25 µl) contained 1× ThermoPol reaction buffer (New England Biolabs, Ipswich, MA), 6 mM $MgSO_4$, 1.2 mM each deoxynucleoside triphosphate (dNTP), 0.1 µM each outer primer (Integrated DNA Technologies, Coralville, IA), 1.8 µM each inner primer, 1 µM each loop primer, 10 U of *Bst* DNA polymerase (New England Biolabs), and 2 µl of template DNA. All LAMP reactions were

carried out at 65°C (63°C for *Salmonella*- and O157-LAMP) for 1 h and terminated at 80°C for 5 min in an LA-320C real-time turbidimeter (Eiken Chemical Co., Ltd., Tokyo, Japan). Turbidity readings at 650 nm were obtained every 6 s, and time threshold values (T_t; in min) were determined when the turbidity increase measurements (differential values of moving average of turbidity) exceeded 0.1.

qPCR assays

As a comparison, a *Salmonella* qPCR assay (Cheng et al., 2008, Journal of Food Protection, 71:2436-2441) and 10 STEC qPCR assays (Fratamico et al., 2010, Food Analytical Methods, 3:330-337; Fratamico et al., 2011, Foodborne Pathogens and Disease, 8:601-607) developed by FDA and USDA scientists, respectively, were performed. Similarly, the *Salmonella* qPCR assay targeted the *invA* gene. For STEC qPCRs, ten sets of primers/probes were used, three targeting common STEC virulence genes (stx_1, stx_2, and *eae*) and seven targeting *wzx* or *wzy* genes on the O-antigen gene clusters of the seven adulterant STEC serogroups.

The qPCR assays were carried out as described previously. The reagent mix (25 µl) consisted of 1× PCR buffer, 4 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 µM each primer (supplemental table), 0.1875 µM probe, 1.5 U of GoTaq Hot Start polymerase (Promega, Madison, WI), and 2 µl of template DNA. The qPCR reactions were conducted using 40 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 50 s in a SmartCycler II system (Cepheid, Sunnyvale, CA). Fluorescence readings were acquired using the 6-carboxyfluorescein (FAM) channel, and cycle threshold values (C_T; in number of cycles, approximately 2 min per cycle) were recorded when the fluorescence readings reached 30 units.

Specificity and sensitivity tests

For assay specificity, DNA templates of the bacterial strains (168 for *Salmonella* assay evaluation and 156 for STEC assay evaluation) were prepared by heating at 95°C for 10 min. Aliquots (2 µl) were subjected to LAMP and qPCR, and repeated twice.

Assay sensitivity (limit of detection) was determined by using 10-fold serial dilutions (10^8 CFU/ml to extinction) of *Salmonella* (n = 10) and STEC (n = 7) cultures. DNA templates were prepared from stationary-phase cultures as described previously. Aliquots (2 µl) were tested by LAMP and qPCR, and repeated three times (five times for *Salmonella* assays).

Assay evaluation in produce with high-level inoculation (assay sensitivity in produce**)**

For *Salmonella* assay evaluation, nine produce items including cantaloupe, pepper (jalapeño), and varieties of lettuce (iceberg, romaine), sprouts (alfalfa, clover, mung bean), and tomato (red round, roma) were tested. For STEC assay evaluation, eight produce items including varieties of lettuce (iceberg, romaine), spinach (baby, savoy, semi-savoy), and sprouts (alfalfa, clover, mung bean) were tested. The produce samples were obtained from a local grocery store and analyzed within 2 h of collection. Briefly, cantaloupe, pepper, and tomatoes were sliced into wedges (2.5 cm^3 cubes or 1/8 fruit wedges) using a sterile knife, and divided into 25-g sample portion. Lettuce and spinach leaves were cut into 4-cm^2 pieces using sterile scissors and 25-g samples were weighed out. Sprouts were also divided into 25-g analysis portions.

To determine assay sensitivity in produce, for *Salmonella* assay evaluation, 60 samples per produce variety (one sample per strain [n = 10] per inoculation level [n = 6]) were inoculated and

three samples were included as uninoculated controls. For STEC assay evaluation, 35 samples per produce variety (one sample per strain [*n* = 7] per inoculation level [*n* = 5]) were inoculated and three samples were included as uninoculated controls. Spot inoculation on the produce surface was performed as described previously. Briefly, 1.5 ml of 10-fold serially diluted overnight *Salmonella* or STEC cultures ranging between 10^4 (10^5 for STEC) and 10^9 CFU/ml was added to each 25-g test sample. For lettuce and spinach, the inocula were divided equally among the number of leaf pieces; whereas for other produce items, the samples were grouped into three equal portions and 500 µl of the inocula were added onto the surface of each portion. Aerobic plate counts were determined for the uninoculated controls (n = 3) by standard pour plate method. All samples were air-dried in a laminar flow biosafety cabinet for 2 h followed by storage at 4°C for 48 h.

After cold storage, each sample was homogenized with 225 ml of buffered peptone water (BD Diagnostic Systems) for 1 min in a food stomacher (model 400; Seward, Cincinnati, OH). Aliquots (1 ml) of each homogenate were centrifuged at 16,000 × *g* for 3 min, and pellets were suspended in 100 µl of PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA). The mixtures were heated at 95°C for 10 min and centrifuged again at 12,000 × *g* for 2 min. The supernatants (2 µl) were tested by both LAMP and qPCR, and repeated three times.

Assay evaluation in produce with low-level inoculation

For this application, the same produce items described above for each assay evaluation were used following similar inoculation, processing, and testing procedures but with three major exceptions: inoculation level, enrichment, and replication scheme. The inoculation level applied was 10^0 CFU/25 g, with two additional ones (10^2 and 10^3 CFU/25 g) for sprouts only. After surface inoculation and cold storage, the samples were first incubated at 37°C (42°C for STEC assay evaluation) for up to 24 h, and aliquots (1 ml) were removed at 6, 8, and 10 h (also 24 h for STEC) for processing by PrepMan Ultra and testing by LAMP and qPCR as described above. The experiments were independently repeated three times with different produce samples (on separate sampling trips). In total, for *Salmonella* assay evaluation, there were 30 inoculated samples (one sample per strain [*n* = 10] in three repeats [*n* = 3]) and three uninoculated controls tested per produce item; while for STEC assay evaluation, there were 21 inoculated samples (one sample per strain [*n* = 7] in three repeats [*n* = 3]) and three uninoculated controls. The number of inoculated samples was tripled for sprouts varieties (90 and 63 for *Salmonella* and STEC assay evaluations, respectively) due to two additional inoculation levels tested.

Comparison of DNA extraction methods

For STEC, six DNA extraction methods were evaluated using the same eight produce items above spiked with 1.2-1.8 CFU/25 g (or 1.2-1.8 × 10^2 CFU/25 g for sprouts varieties) of *E. coli* O157:H7 strain MDL 3562 and enriched for various periods (6, 8, 10, 24 h). Each produce item was independently repeated three times with different samples (on separate sampling trips). These methods were: 1) raw enrichment broth, i.e., direct testing without any DNA preparation step; 2) boiling preparation at 95°C for 10 min; 3) two-step centrifugation, first centrifuging at 500 × *g* for 1 min to remove produce tissues, followed by centrifuging again at 16,000 × *g* for 5 min to pellet bacterial cells, and resuspending the pellet in 100 µl of TE buffer; 4) two-step centrifugation followed by boiling, i.e., heating the bacterial suspension in TE buffer prepared in method 3) at 95°C for 10 min; 5) PrepMan Ultra as describe above; and 6) FTA Elute, briefly, adding 65 µl of enrichment broths onto an FTA Card (Whatman Inc., Florham Park, NJ), punching out two 2-mm disks after absorption and dry, washing the disks with sterile water and

then heating with water at 95°C for 30 min. Aliquots (2 µl) of DNA templates prepared by all six methods were tested by *eae*-LAMP and *eae*-qPCR.

For *Salmonella*, four DNA extraction methods (methods 2, 4, 5, 6 described above) were evaluated using the same nine produce items above spiked with 1-2 CFU/25 g (or 1-2 × 10^2 CFU/25 g for sprouts varieties) of *Salmonella* Typhimurium strain NR-4333 and enriched for various periods (6, 8, 10 h).

Data analysis

Means and standard deviations of T_t for LAMP and C_T for qPCR were calculated by Microsoft Excel (Seattle, WA). The T_t and C_T values sorted by assay target, gene subtype, produce type, enrichment time, and DNA extraction method were compared by using the analysis of variance (SAS for Windows, version 9.2; Cary, NC). Differences between the mean values were considered significant when P was < 0.05.

Results

Assay specificity

Among 168 and 156 bacterial strains tested by the *Salmonella* LAMP assay and the STEC LAMP suite, respectively, false-positive or false-negative results were not observed; i.e., LAMP results matched 100% with known strain characteristics for all of the target genes. The overall mean T_t values for the *Salmonella* LAMP assay ranged from 13.5 to 24 min, and for the STEC LAMP suite, the range was from 16.1 min for *eae*-LAMP to 24.3 min for stx_2-LAMP.

The *Salmonella* qPCR assay had no false-positive or false-negative results, with overall mean C_T values ranging from 14 to 19.7 cycles. For STEC qPCR assays, the vast majority of specificity tests detected the 156 strains accurately with the overall mean C_T values ranging from 12.8 cycles for *eae*-qPCR to 20 cycles for O103-qPCR. However, false-negative results were consistently generated by stx_2-qPCR for two strains (ESC0603 and N5789) carrying the stx_{2g} gene.

Assay sensitivity in pure culture

In pure-culture sensitivity testing, the *Salmonella* LAMP assay and the STEC LAMP suite consistently detected 10-20 CFU/reaction of target *Salmonella* or STEC strains. In two to five out of five repeats, the *Salmonella* LAMP assay was capable of detecting all of the ten *Salmonella* strains representing ten serovars at an even lower concentration, i.e., 10^0 CFU/reaction. Among the STEC LAMP assay, stx_2- and O45-LAMP detected 1 CFU per reaction of respective STEC strains in one or two out of three repeats. However, the stx_2- and *eae*-LAMP assays were less sensitive (up to 100-fold) for strains carrying certain target gene subtypes, e.g., stx_{2c}, *eae*-β, and *eae*-θ.

The detection limits for *Salmonella*-qPCR consistently fell between 1 and 20 CFU per reaction, however, the assay failed to detect Enteritidis 20 N, Heidelberg 1364 H, Montevideo 1 H, and Poona 2861 H at the 10^0 CFU/reaction level. While the STEC-qPCR assays had detection limits of 1 to 20 CFU per reaction for all assay targets and their subtypes.

Assay sensitivity in produce

For the uninoculated controls, aerobic plate counts averaged 10^6-10^8 CFU/g in sprouts varieties and 10^4-10^5 CFU/g in all other produce types, and all target genes tested negative by LAMP and qPCR.

In the majority of produce items tested, the detection limits of the *Salmonella* LAMP assay was between 10^4 and 10^5 CFU per 25 g produce (approximately 10^2 -10^3 CFU/g, equivalent to 0.2-2 CFU/reaction). In several strain/produce combinations (Braenderup 10 N, Poona 2861 H, and Javiana NR-4296 in mung bean sprouts, Enteritidis 20 N and Montevideo 1 H in jalapeño peppers, and Muenchen NR-4311 in all three sprouts varieties), 10^6 CFU/25 g was required. By qPCR, although the majority of strain/produce combinations were detected at between 10^4 and 10^5 CFU per 25 g levels, Braenderup 10 N required up to 10^7 CFU/25 g for detection in all three sprouts varieties. Small variations in detection limit (10-fold) among varieties of the same produce type were observed by either LAMP or qPCR.

In lettuce and spinach varieties, the STEC LAMP suite detected down to 10^5 CFU per 25 g produce (approximately 10^3 CFU/g, equivalent to 2 CFU/reaction). In alfalfa (but not clover and mung bean) sprouts, 10-fold higher cell concentrations (10^6 CFU/25 g) were needed for accurate detection by *stx*$_1$-, O111-, and O145-LAMP. Regardless of produce type or variety, *stx*$_2$- and *eae*-LAMP were less sensitive (up to 1,000-fold) for strains containing *stx*$_{2c}$, *eae*-β, and *eae*-θ subtypes. In comparison, all of the qPCR assays detected down to 10^5 CFU/25 g in lettuce, spinach, and sprouts varieties. Small variations in detection limit (10-fold) among varieties of the same produce type were observed by either LAMP or qPCR.

Effect of DNA extraction methods

The effect of DNA extraction methods on *Salmonella*-LAMP assay performance were in the following order (increasing T_t values): PrepMan Ultra = two-step centrifugation > FTA Elute > boiling preparation. The same trend was obtained for qPCR. However, qPCR was not able to generate consistent and meaningful results for samples prepared by the FTA Elute procedure.

For the STEC LAMP suite, positive LAMP results were obtained using all six methods but at different enrichment hours. For samples enriched for 6 h, PrepMan Ultra sample preparation reagent was the only one consistently gave positive LAMP results. FTA Elute and raw enrichment broth required 8 and 10 h of enrichment, respectively, to generate positive LAMP results. For qPCR, 8 h of enrichment was needed for the majority of methods and 10 h was needed for two-step centrifugation. Boiling facilitated LAMP and qPCR detection as evidenced by lower T_t and C_T values in boiled samples and/or shorter enrichment time needed for detection. Among all six methods, FTA Elute tended to generate the highest T_t or C_T values at each enrichment period in baby spinach.

In other spinach varieties, minimum enrichment time required for LAMP detection was 6 h (8 h for qPCR) when two-step centrifugation followed by boiling or PrepMan Ultra sample preparation reagent was used. However, at least 10-h enrichment was required for both LAMP and qPCR when using FTA Elute and at least 24-h enrichment for the other three methods (raw enrichment broth, boiling preparation, and two-step centrifugation). In two lettuce varieties, regardless of DNA extraction method, samples enriched for 6 to 8 h were accurately detected by LAMP and qPCR. In three sprouts varieties spiked with 1.2-1.8 × 10^2 CFU/25 g of STEC cells, 6 to 8 h enrichment was sufficient for LAMP and qPCR detection, except for alfalfa and clover sprouts by FTA Elute which needed 10 h.

Rapid detection of low-level pathogens in produce

The *Salmonella* LAMP assay consistently detected the ten *Salmonella* strains representing ten serovars in all of the produce items tested with 6-8 h enrichment, except for sprouts varieties, where there was no detection in several strain/produce combinations even after 10 h enrichment. A similar trend of detection was observed for *Salmonella*-qPCR.

For the STEC LAMP suite, after 6-h enrichment, all assays in the suite successfully detected such low-level STEC in baby spinach, except for stx_1- and O157-LAMP, and stx_2- and *eae*-LAMP in strains carrying stx_{2c} and *eae*-β, respectively. Significantly higher T_t values were observed for samples enriched for 6 or 8 h than those for longer periods ($P < 0.05$). A similar trend of detection was observed for qPCR, although O45- rather than stx_1-qPCR failed to detect at 6-h enrichment period.

In other spinach and lettuce varieties, both LAMP and qPCR achieved accurate detection after 6 to 8 h of enrichment, except for stx_2-LAMP in savoy spinach spiked with CVM 9790 (stx_{2c}) which required 10-h enrichment (data not shown). In contrast, neither LAMP nor qPCR detected such low-level (1.2-1.8 CFU/25 g) STEC strains in sprouts varieties with up to 24 h enrichment (data not shown). The 10^2-CFU/25 g inoculum resulted in positive LAMP and qPCR results in sprouts after 6 to 8 h enrichment for 10 target gene and subtype combinations (stx_{1a}, stx_{2a}, stx_{2c}, stx_{2d}, *eae*-β, *eae*-γ1, O26, O45, O103, and O145). All 15 targets/subtypes were detected in sprouts samples spiked with 10^3 CFU/25 g after 6 to 8 h of enrichment.

Outcomes and Accomplishments

Objective 1: To develop and evaluate LAMP assays for STEC O157 and top non-O157 serogroups

STEC assay development and initial evaluation

We developed two sets of LAMP assays for detecting STEC strains. The first set consisted of three LAMP assays, each targeting one of the three common STEC virulence genes (stx_1, stx_2, and *eae*). The second set consisted of seven LAMP assays, each targeting one of the leading STEC serogroups (O157, O26, O45, O103, O111, O121, and O145).

Upon initial development, we evaluated both LAMP assay sets for their sensitivity, specificity, and quantitative capability. The assays were able to detect 1-20 STEC cells per reaction. The assays were specific, with 100% inclusivity for target strains and 100% exclusivity for non-target strains. Good linear relationships were observed between STEC cell numbers and LAMP turbidity signals, suggesting quantitative assays.

Two refereed papers have been published based on findings from these studies (Wang et al., 2012, Journal of Clinical Microbiology, 50:91-97; Wang et al., 2012, Applied and Environmental Microbiology, 78:2727-2736).

Note: This portion of research was completed by Dr. Beilei Ge's research group while she was an Associate Professor in the Department of Food Science at Louisiana State University using internal funds.

Further evaluation of STEC and *Salmonella* assays

We further evaluated the specificity and sensitivity of LAMP assays developed by our research group for *Salmonella* and STEC (Chen et al., 2011, Applied and Environmental Microbiology, 77:4008-4016; Wang et al., 2012, Journal of Clinical Microbiology 50:91-97; Wang et al., 2012, Applied and Environmental Microbiology, 78:2727-2736) by using a large collection of bacterial strains (168 for *Salmonella* and 156 for STEC) maintained in the PI's laboratory and at FDA.

All of the LAMP assays were confirmed to be highly specific, with 100% inclusivity for target strains and 100% exclusivity for non-target strains. Additionally, the LAMP assays targeting STEC virulence genes (*stx*$_1$, *stx*$_2$, and *eae*) also successfully detected strains possessing major variants/subtypes of these genes associated with human illnesses.

For sensitivity, the *Salmonella* LAMP assay was able to detect 1-20 cells per reaction for ten leading *Salmonella* serovars implicated in outbreaks of produce and other food. Similarly, the overall detection limit for STEC LAMP was 1-20 cells per reaction, but the *stx*$_2$-LAMP and *eae*-LAMP were 100-fold less sensitive when applied to strains containing *stx*$_{2c}$, *eae*-β, and *eae*-θ subtypes.

Objective 2: To evaluate the robustness of the LAMP detection system using abusive temperature, pH, and the addition of soil, chlorophyll, and produce enrichment broth

Salmonella LAMP assay robustness

We evaluated the robustness of our published *Salmonella* LAMP assay (Chen et al., 2011, Applied and Environmental Microbiology, 77:4008-4016) in comparison with PCR. Parameters evaluated included assay preparation temperature, assay running temperature, pH, the addition of culture media, humic acid, plant polysaccharide, soil and produce rinses. The data suggested that humic acid had a strong inhibitory effect against LAMP assays, while the LAMP assays were rather robust with other conditions. In comparison, PCR was found to be less robust than LAMP.

One refereed paper based on findings from this study has been published recently (Yang et al., 2014, Journal of Applied Microbiology, 116:81-88).

Note: This portion of research was completed by Dr. Beilei Ge's research group while she was an Associate Professor in the Department of Food Science at Louisiana State University using internal funds. We did not evaluate the robustness of the STEC LAMP suite since representative data on LAMP robustness have already been obtained using the *Salmonella* LAMP assay.

Objective 3: To validate the system in complex produce matrices (cantaloupe, lettuce, pepper, spinach, sprout, and tomato) surface-inoculated with low levels of these pathogens

Assay evaluation in produce with high-level inoculation (assay sensitivity in produce)

We evaluated the sensitivity of the *Salmonella* LAMP assay and the STEC LAMP suite using a large variety of produce items (cantaloupe, lettuce, pepper, spinach, sprouts, and tomato) surface-inoculated with *Salmonella* strains representing ten serovars or STEC strains of seven adulterant serogroups. The assays were performed after aging at 4^{o}C for 48 h. The assay

sensitivity was 10^4 to 10^5 CFU per 25 g produce, comparable to that in pure culture testing, i.e., 1-20 cells per reaction, suggesting no significant inhibition of LAMP assays from the produce matrix. However, lower sensitivity was observed in the three types of sprouts (alfalfa, clover, mung bean). The abundant natural flora present in sprouts, 2-3 logs higher than those in leafy greens, may account for the discrepancies. Similarly for STEC, LAMP assays could detect most targets in spiked produce down to levels of 10^5 to 10^6 CFU per 25 g, except for strains harboring strains containing *stx*$_{2c}$, *eae*-β, and *eae*-θ subtypes, for which, 10- to 100-fold less sensitivity was observed.

Comparison of different DNA extraction methods from produce enrichment broths

We compared six methods for DNA extraction from spiked produce samples and evaluated their effects on the assay outcomes. The spiked produce samples were consistently detected after 6 h enrichment when prepared with PrepMan Ultra or two-step centrifugation. Longer enrichment periods (e.g., 8-24 h) were required to detect the targets processed with four other methods. Overall, PrepMan Ultra sample preparation yielded the best results, while the five other methods also generated satisfactory results with samples enriched up to 24 h. FTA Elute has the advantage of preserving sample DNAs up to two years and without centrifugation steps; however, the final DNA amount extracted was approximately 100-fold less concentrated than other methods, requiring more cells in the enrichment broth or prolonged enrichment time.

Assay evaluation in produce with low-level inoculation

We evaluated the LAMP assays for detecting *Salmonella* and STEC in various produce items surface-inoculated with a low level of target bacteria (1-2 CFU/25 g). The spiked samples were subjected to 48-h aging and various enrichment periods. Regardless of target bacteria and produce type, all of the assays could detect the targets consistently and accurately after 6-8 h enrichment, except for those spiked in sprouts, which required up to 1,000-fold higher bacterial inoculum and 8-10 h of enrichment. Again, the normal flora or natural compounds in sprouts released during processing may affect Salmonella and STEC survival during enrichment. For samples enriched longer than 8 h, more stable and shorter T_t values were observed. A similar trend of detection was observed for qPCR.

Summary of Findings and Recommendations

Upon comprehensive evaluation using a large panel of bacterial strains and a variety of produce items, the *Salmonella* LAMP assay and the STEC LAMP suite of 10 assays were demonstrated to be rapid (10 to 45 min), reliable (no false-positive or false-negative results), and robust (under conditions mimicking real-world contamination events, e.g., surface contamination, cold storage). Coupled with an effective DNA extraction method, the assays accurately detected a low level (1-2 CFU/25 g) of these pathogens in all produce items tested (but not sprouts) after 6 to 8 h of enrichment. A similar trend of detection was observed for qPCR. The challenge with sprouts detection by both LAMP and qPCR calls for special attention to further analysis.

The availability of such a detection system for routine pathogen testing in produce provides a valuable tool for the produce industry and regulatory agencies to better identify contamination risks and ensure produce safety, therefore reducing produce-related *Salmonella* or STEC outbreaks and illnesses in the long run.

APPENDICES

Publications and Presentations (required)

Publications

1. Wang, F., Q. Yang, Y. Qu, J. Meng, and B. Ge. 2014. Evaluation of a loop-mediated isothermal amplification suite for the rapid, reliable, and robust detection of Shiga toxin-producing *Escherichia coli* in produce. Applied and Environmental Microbiology (revision submitted)
2. Yang, Q., F. Wang, K. L. Jones, J. Meng, W. Prinyawiwatkul, and B. Ge. 2014. Evaluation of loop-mediated isothermal amplification for the rapid, reliable, and robust detection of *Salmonella* in produce. Food Microbiology (under preparation)

Presentations

1. Wang, F., Q. Yang, J. Meng, and B. Ge. 2013. Evaluation of a suite of loop-mediated isothermal amplification assays for the rapid, reliable, and robust detection of Shiga toxin-producing *Escherichia coli* in produce. Abstr. 4489. International Association for Food Protection 2013 Annual Meeting, Charlotte, NC, July 28-31, 2013.
2. Yang, Q., F. Wang, K. L. Jones, J. Meng, W. Prinyawiwatkul, and B. Ge. 2013. Evaluation of loop-mediated isothermal amplification for the rapid, reliable, and robust detection of *Salmonella* in produce. Abstr. 4596. International Association for Food Protection 2013 Annual Meeting, Charlotte, NC, July 28-31, 2013.
3. Wang, F., Q. Yang, J. Meng, and B. Ge. 2013. Toward a rapid and reliable pathogen detection system in produce. Abstr. The 4th Annual Center for Produce Safety Produce Research Symposium, Rochester, NY, June 25-26, 2013.
4. Wang, F., Q. Yang, J. Meng, and B. Ge. 2013. Toward a rapid and reliable pathogen detection system in produce. Abstr. The Western Food Safety Summit, Salinas, CA, May 9-10, 2013.
5. Wang, F., Q. Yang, J. Meng, and B. Ge. 2012. Toward a rapid, reliable, and robust pathogen detection system in produce. Abstr. The 2nd Annual FDA Foods Program Science and Research Conference, Silver Spring, MD, August 1-2, 2012.
6. Wang, F., Q. Yang, J. Meng, and B. Ge. 2012. Loop-mediated isothermal amplification assays for detecting seven major Shiga toxin-producing *Escherichia coli* serogroups in produce. Abstr. 2236. International Association for Food Protection 2012 Annual Meeting, Providence, RI, July 22-25, 2012.
7. Wang, F., Q. Yang, J. Meng, and B. Ge. 2012. Toward a rapid and reliable pathogen detection system in produce. Abstr. The 3rd Annual Center for Produce Safety Produce Research Symposium, Davis, CA, June 27, 2012.

Budget Summary (required)

Nearly 70% ($103,000) of funds were used for personnel (salary and benefits) support. Approximately 20% ($31,000) for materials and supplies, 3% for travels, and 5% for indirect cost. The funds ($152,000) were sufficient for completing the project.

Tables and Figures (optional)

Suggestions to CPS (optional)