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DEVELOPMENT AND EVALUATION OF MOLECULAR-BASED ASSAYS FOR DETECTING *SALMONELLA* SEROVARS IN VARIOUS FOOD COMMODITIES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in

The Department of Food Science

by
Qianru Yang
B.S., Southern Yangtze University, 2004
M.S., Oregon State University, 2009
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

As a leading cause of foodborne illnesses and outbreaks, *Salmonella* poses a major public health risk in the United States and worldwide. Various food commodities including meat and poultry, eggs, and fresh produce can serve as the transmission vehicles for *Salmonella* infections. To better ensure the safety of these products and protect public health, rapid, accurate, and reliable detection methods for *Salmonella* are needed. Molecular-based methods like loop-mediated isothermal amplification (LAMP), have gained wide applications in *Salmonella* detection, owning to their rapidity, specificity, and sensitivity. However, there is a paucity of data on the robustness of these assays. And very recently, bioluminescence assay in real-time (BART) was used as a new and effective platform to detect LAMP products, and this combination has not been evaluated before.

This dissertation research evaluated the robustness of two LAMP assays in comparison with PCR, examined the application of LAMP assays in detecting *Salmonella* specifically in food items, and developed a novel LAMP-BART assay for *Salmonella* detection. The LAMP assays achieved robust detection of *Salmonella* under abusive preparation and running temperatures, also demonstrated greater tolerance than PCR to various inhibitors. They achieved 100% accuracy among 185 strains. The limits of detection of LAMP for *Salmonella* strains belonging to ten serovars were 1 to 10 cells per reaction in pure culture, 100-fold more sensitive than PCR. In spiked egg homogenates, it could detect *Salmonella* serovars Enteritidis and Typhimurium down to 10^4 CFU/25 ml egg homogenates directly and 1 CFU/25 ml with 8 h enrichment. In spiked produce (cantaloupe, jalapeno pepper, tomatoes, sprouts, and lettuces), the detection limits ranged from 10^4 to 10^6 CFU/25 g produce, which were comparable to qPCR.

Coupled with 6 to 8 h of enrichment, LAMP consistently detected in produce samples spiked with very low levels of *Salmonella* cells, with the exception of sprouts.

Based on these evaluations and further development, LAMP demonstrated to be a rapid and robust alternative to PCR-based assays for *Salmonella* detection and could be adopted by food industries and regulatory agencies in routine product testing for *Salmonella* to improve product safety and protect public health.

CHAPTER 1: INTRODUCTION

The genus *Salmonella* is a Gram-negative, rod-shaped, non-spore-forming, and facultative anaerobe that causes typhoid fever, paratyphoid fever, and foodborne diseases in humans (48). Nontypoidal *Salmonella* is a collective name given to those strains that cause foodborne illnesses in human. *Salmonella* is widely distributed in nature with animals and humans being their primary reservoirs (22, 51). Food products may be contaminated with *Salmonella* at any step (production, harvest, processing, storage, distribution, retail, and consumption) through the farm to fork continuum, resulting in food safety problems (28, 49). Common vehicles implicated in *Salmonella* outbreaks include meat and poultry, eggs, dairy products, and fresh produce. In 2010, a nationwide *Salmonella* outbreak involving shell eggs resulted in 1,939 illnesses and a recall of over 500 million eggs (14). Among produce commodities implicated in *Salmonella* outbreaks, lettuce/leafy greens, tomatoes, and melons were the top three, accounting for 34.1%, 17.1%, and 15.9% of total outbreaks, respectively (95-97). Given the public health significance of *Salmonella*, it is critical for food industry and regulatory agencies to have access to rapid, reliable, and user-friendly detection techniques so that potential contamination problems can be identified promptly during the production, processing, and distribution of these high-risk food products.

In this dissertation research, loop-mediated isothermal amplification (LAMP), a novel molecular-based detection method, was evaluated for its robustness and application in *Salmonella* detection in shell eggs and various produce items. LAMP was also combined with bioluminescence assay in real-time (BART) to enable the rapid, reliable, and robust detection of *Salmonella*. Firstly, we tested the robustness of LAMP assays using abusive assay conditions and in the presence of potential inhibitors likely encountered in food applications, including culture

media, biological substances and food matrices. Secondly, we evaluated the performance of LAMP assays for detecting *Salmonella* Enteritidis and *Salmonella* Typhimurium in spiked egg homogenates. Thirdly, the capability of LAMP to detect *Salmonella* in spiked produce items was evaluated in comparison with real-time quantitative PCR (qPCR). Finally, we investigated the novel combination of LAMP and BART for the rapid, reliable, and robust detection of *Salmonella*, which may potentially be deployed in field applications.

This dissertation is organized as following:

Chapter 1 is the introduction.

Chapter 2 is a literature review where general information of *Salmonella*, food commodities involved in *Salmonella* illnesses and outbreaks and *Salmonella* detection methods are reviewed.

Chapter 3 presents the robustness of LAMP assays for *Salmonella* detection using abusive assay conditions and in the presence of inhibitors.

Chapter 4 shows the application of LAMP to detect *Salmonella* Enteritidis and *Salmonella* Typhimurium in shell eggs.

Chapter 5 demonstrates the comparative evaluation of LAMP and qPCR in detecting *Salmonella* in spiked produce.

Chapter 6 explores the novel combination of LAMP and BART in *Salmonella* detection.

Chapter 7 presents the conclusions for this dissertation research.

CHAPTER 2: LITERATURE REVIEW

General Information on *Salmonella*

Microbiology The genus *Salmonella* is a Gram-negative, rod-shaped (0.7- .5 × 2.0-5.0 μm), non-spore-forming, and facultative anaerobe, belonging to the family *Enterobacteriaceae* (4, 5). *Salmonella* is divided into two species, *Salmonella enterica* and *Salmonella bongori* (1). *S. enterica* is further divided into six subspecies, consisting of *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV) and *indica* (VI). Together, these subspecies are responsible for diseases in human and other warm-blooded animals and are of great public health concern (7, 34). In contrast, *S. bongori* (originally designated *S. enterica* subspecies V) is usually associated with illnesses in cold-blooded animals, although a few cases of human infections have been reported (34, 39). Within each species/subspecies, many serovars (or serotypes) are designated (7, 22). Currently > 2,500 *Salmonella* serovars are identified. Although most of them are motile with peritrichous flagella, nonflagellated variants, such as *Salmonella* Gallinarum, and nonmotile strains resulting from dysfunctional flagella do exist (61). *Salmonella* is mesophilic with an optimum growth temperature of 35-37^{o}C. They can grow at pH below 4 and above 9 with the optimal pH around 7 (49). Some *Salmonella* can grow under extreme environmental conditions, such as elevated temperature (54^{o}C) and refrigerator temperature (2 to 4^{o}C) (28). However, they are sensitive to high salt concentrations (4), though latest study show that they could be detected in sediment areas (9).

Salmonella mainly dwells in the intestinal tracts of animals, with poultry, eggs, livestock, pets, reptiles, and humans being as their primary reservoirs (22, 51). As a result, fecal materials loaded with *Salmonella* from these animals may lead to direct contamination of meat and poultry as well as secondary contamination of produce and environment, resulting in foodborne illnesses

and outbreaks (28, 49). In the winter of 2000-2001, an outbreak strain of *Salmonella* Enteritidis phage type 30 (PT30) was found in a farmer's orchards, suggesting secondary contamination from a large agriculture area (47).

Clinical Symptoms Foodborne diseases caused by *Salmonella* are termed salmonellosis, which is characterized by acute gastrointestinal infections with symptoms including diarrhea, nausea, vomiting, abdominal pains, headache, chills, and fever (63). These symptoms commonly develop within 12-14 h of exposure and last for 2-3 days. Most patients can recover without treatment. However, approximately 5% of patients, mainly immuno-compromised individuals, may become *Salmonella* carriers upon recovery (22). These patients are also more likely to develop other extra-intestinal focal infections, including meningitis, septic arthritis, osteomyelitis, cholangitis, and pneumonia (45).

The infective dose for *Salmonella* was reported to be more than 10^7-10^8 cells (60). However, very low infective dose such as 15 to 20 *Salmonella* cells has also been reported to cause quite a few outbreaks implicating high-fat food products (5). The minimum numbers for gastroenteritis should be determined based on the species of *Salmonella* and the health condition of different people groups.

Virulence Properties Fundamental to *Salmonella* virulence is its ability to invade and trespass the host cells (1). The virulence capability of *Salmonella* consists of many virulence determinants, such as prophages, integrons, pathogenicity islands and plasmids, and the evolution of *Salmonella* (53). The invasion gene (*invA*) on the *Salmonella* chromosome encodes an invasion protein InvA (35). This protein assists *Salmonella* to penetrate the gut lumen into the epithelium cells of host small intestine. And *Salmonella* can also actively invade both phagocytic

and non-phagocytic cells using two distinct type III secretion systems (T3SSs), encoded by pathogenicity island 1 (SPI-1 T3SS) and 2 (SPI-2 T3SS) (67).

Following internalization into host cells, *Salmonella* enters enterocytes, M cells, and dendritic cells (DCs) in the intestinal epithelium and subsequently reaches to the submucosa by resident macrophages. At this stage, the ability of *Salmonella* to survive in a variety of host cells is another important character to its success as a pathogen, which mainly associated with SPI-2 T3SS and factors involved in nutrient acquisition and avoiding induction of antibacterial mechanisms (46). Then *Salmonella* spreads through the blood stream and accumulates in mesenteric lymph nodes and spleen, causing inflammation which leads to salmonellosis (86). *Salmonella* can also produce enterotoxins and cytotoxins in the host intestinal tracts which only have minor effects on the infection (48). Therefore, *Salmonella* causes typical foodborne infection rather than intoxication.

Salmonella as a Leading Cause of Foodborne Illnesses and Outbreaks

Epidemiology *Salmonella* is one of the leading causes of foodborne illnesses worldwide. In the United States, the Centers for Disease Control and Prevention (CDC) estimated that approximately 1 million cases of foodborne illnesses were caused by *Salmonella* annually, accounting for 11% of total illnesses linked to bacteria-contaminated food (87). *Salmonella* is also estimated to result in 19,336 hospitalizations and 378 deaths annually (88). In 2011, the CDC's Foodborne Disease Active Surveillance Network (FoodNet) reported that *Salmonella* was responsible for 7,813 cases of laboratory-confirmed foodborne infections in 10 states, accounting for more than 41% of the total laboratory-confirmed infections caused by 10 pathogens under FoodNet surveillance (33). *Salmonella* also has the highest incidence rate of 16.45, the furthest from its healthy people 2010 target (6.8) (15). Compared to the start of FoodNet surveillance

during 1996–1998, the incidence of *Salmonella* infection in 2011 did not change significantly but increased 10% compared to that during 2006–2008 (Figure 2.1).

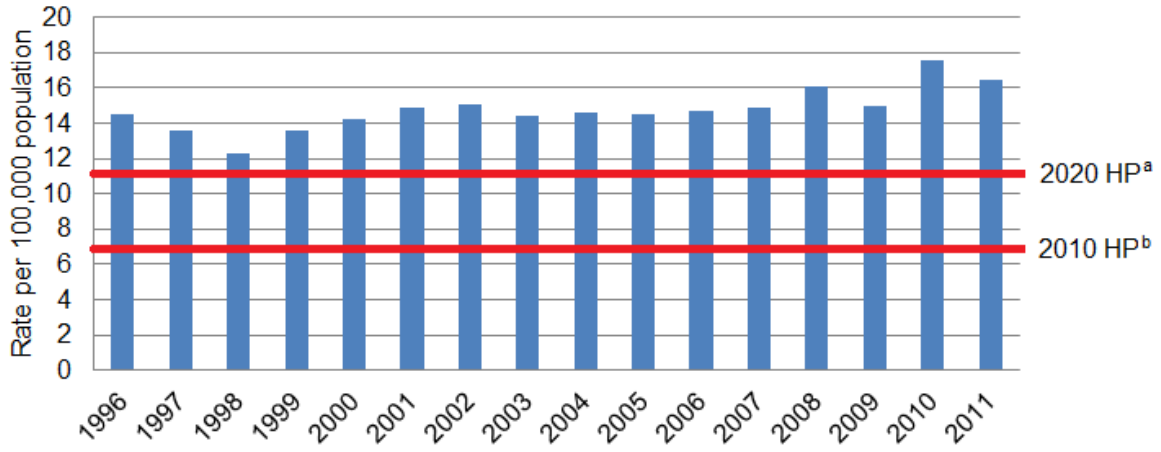

Figure 2.1 Incidence of *Salmonella* Infections in Foodnet Surveillance Area Reported by the CDC from 1996 to 2011.
[a] Healthy People 2020 objectives for incidence of *Salmonella* infections for year 2020 was 11.4 case per 100,000 persons.
[a] Healthy People 2010 objectives for incidence of *Salmonella* infections for year 2010 was 6.8 case per 100,000 persons.

Salmonella is also recognized as a leading cause of foodborne outbreaks in the United States. Between 2009 and 2010, the CDC's Foodborne Disease Outbreak Surveillance System indicated that *Salmonella* was responsible for 243 foodborne disease outbreaks, resulting in 7,089 cases of illnesses (17). And it was the most common cause of outbreak-related hospitalizations, causing 49% of total reported hospitalizations. And 5 out of the total 23 death were attributed to *Salmonella* infections.

Association between *Salmonella* Serovars and Food Commodities Not all *Salmonella* serovars are created equal in terms of their disease potential. Checking all case of *Salmonella* infection reported in FoodNet during 1996-2006, *Salmonella* Typhimurium caused the most of the hospitalizations when compared to 12 other common serovars (50). However, infections

linked to *Salmonella* Choleraesuis (57%) and Dublin (64%) had a significant higher proportion of invasive diseases than *S.* Typhimurium (6%).

Among over 1,000 *Salmonella* serovars that have caused illness in the U.S., *Salmonella* Enteritidis (SE), Newport, and Typhimurium consistently ranked among the top three (13, 81). In 2011, SE was most frequently reported by FoodNet (in 18% of laboratory-confirmed *Salmonella* infections), followed by *S.* Typhimurium (13%) and *S.* Newport (12%) (10). Compared with 1996–1998, a 58% increase in the incidence was observed for SE infection. Other common serovars involved in human illness include Javiana, Heidelberg, etc (50).

Food commodities commonly implicated in *Salmonella* outbreaks include meat and meat products, poultry and eggs, dairy products, and a variety of produce items, such as melons, tomatoes, sprouts, spinaches, and peppers (42). Historically, shell eggs, undercooked or raw, are the major culprit of *Salmonella*-implicated outbreaks, especially SE (14). The recent large-scale *Salmonella* outbreak associated with shell eggs was the 2010 *Salmonella* Enteritidis outbreak (16). From May 1 to November 30, 2010, approximately 1,939 illnesses were reported from multiple states that were likely to be associated with this outbreak. It is now generally accepted that eggs become contaminated with SE primarily through the transvarian route (52), although trans-shell penetration (enviromental contamination) also plays a role (31). An earlier risk assessment estimated that of the 47 billion eggs consumed annually as shell eggs, 2.3 million are contaminated with *Salmonella* Enteritidis (29). In response, a federal egg safety rule was published recently, requiring producers with more than 3,000 laying hens to implement measures by July 9, 2012 to prevent *Salmonella* from contaminating eggs on the farms (31). More vigilant environment monitoring and egg testing for SE is one measure included in this regulation. In recent years, in part due to the increasing consumption, fresh produce has emerged to be a major

category for *Salmonella* infections, as evidenced by an increasing number of *Salmonella* outbreaks caused by fresh produce (2, 44). From April 12 to July 5, 2011, a total of 25 persons from 5 state eating alfalfa sprouts and spicy sprouts were infected with an outbreak strain of *Salmonella* Enteritidis (11). During the same time period, another outbreak caused by *Salmonella* Panama linked to cantaloupes resulted in 20 illness identified in10 states (12). According to the Outbreak Alert! Database from the Center for Science in the Public Interest (CSPI) and CDC outbreak data, the *Salmonella* serovars most frequently associated with eggs, chicken and produce are compiled (Table 2.1) (24). Produce occupied 4 out of 9 foodborne outbreaks due to *Salmonella* contamination in 2011, which was a significant public health burden that might be largely preventable.

Table 2.1 *Salmonella* Serovars Commonly Associated with Egg, Chicken and Produce Outbreaks.

Outbreaks	No. of outbreaks							
Serovar	CDC OutbreakNet (1998 – 2010)				CSPI Outbreak Alert (1990 – 2010)			
	Produce	Eggs	Chicken	Total	Produce	Eggs	Chicken	Total
S. Enteritidis	23	76	25	124	36	302	26	364
S. Newport	31	-	7	38	29	-	8	27
S. Typhimurium	16	1	18	35	20	5	15	40
S. Heidelberg	3	5	12	20	4	13	14	31
S. Javiana	10	-	2	12	12	-	2	14
S. Braenderup	6	1	3	10	6	2	3	11
S. Saintpaul	8	-	1	9	8	-	2	10
S. Muenchen	5	-	3	8	5	-	1	6
S. Thompson	3	1	2	6	4	-	2	6
S. Litchfield	5	-	-	5	2	-	-	2
S. Infantis	4	-	-	4	6	2	-	8
S. Poona	4	-	-	4	5	-	-	5
S. Oranienburg	3	-	1	4	3	-	2	5
S. Anatum	3	-	-	3	2	-	1	3
S. Mbandaka	3	-	-	3	3	-	-	3
S. Baildon	3	-	-	3	2	-	-	2
S. Hartford	3	-	-	3	1	-	-	1
S. Senftenberg	2	-	-	2	4	-	-	4
S. Berta	2	-	-	2	3	-	-	3
S. Panama	2	-	-	2	1	-	-	1

The FDA Food Safety Modernization Act (FSMA), signed into law by President Obama on Jan. 4, 2011, enables FDA to focus more on preventing food safety problems rather than relying primarily on reacting to problems after they occur (32). As a key element of this preventive approach, the FSMA proposal rule for produce aimed to establish science-based, minimum standards for the safe growing, harvesting, packing, and holding of produce on farms to minimize contamination that could cause serious adverse health consequences or death. With these newly implemented and proposed regulations, it is imperative that the food industry and regulatory agencies have access to simple, rapid, accurate, and economic methods.

Detection Methods for *Salmonella*

Overview As with the detection of other foodborne pathogens in food, *Salmonella* detection needs to overcome many inherent challenges associated with food analysis (28). First of all, there are many forms of food products. They could be liquid or solid, homogenous or heterogeneous, raw or ready-to-eat, and so on. These distinctions make it difficult to develop a universal protocol for efficient food sampling, sample preparation, and analytical methods. And the complexity of the food matrices and compositions also limits the efficiency of microbiological analysis. Some compounds in the food matrices might interfere with the functional activity of key reagents in pathogen detection, leading to false positive or false negative results. For instance, PCR enzymes such as *Taq* polymerase are particularly vulnerable to inhibitors in the food samples, rendering the limited sensitivity and false negative results of PCR in many foods (101), whereas intrinsic peroxidase in fruits and vegetables might cause false positive reaction of ELISA as it uses peroxidase conjugates (28). Another concern is the high level of background flora naturally present in food samples, which makes the target microorganism accounting for a very small portion of the total microorganisms in foods and

undermines the efficiency of target pathogen detection. Moreover, low-dose presence of pathogen in real foods, heterogeneous distribution of target agents in foods, injuries of cells due to food processing further compromise the effective detection of pathogens in foods (38).

There have been great efforts in developing effective detection methods for *Salmonella* in food from sampling to results. Such methods are reviewed briefly in the following sections.

Sample Preparation Methods Due to the intrinsic factors mentioned above, sample preparation is essential to separate the target pathogen from the food samples or concentrate the DNA samples in the small volume for the molecular assays. To detect the low levels of *Salmonella* in food, a significant time for pre-enrichment is necessary in the sample preparation step (66). Gast and Holt's study (37) reported that approximately 10 *Salmonella* Enteritidis cells in egg samples could reach 10^5 CFU/ml in TSB with ferrous sulfate supplement within 12 h and 10^7 CFU/ml with 12 to 15 h of incubation. In addition, it has been reported that the enzymes in egg albumin inhibited *salmonella*'s growth (82), compromising the objective of pre-enrichment to bring the cell numbers to a sufficient level within a short period of time.

Concentration by membrane filters is another method used in sample preparation (6). A two-step filtration protocol has been developed to concentrate *Salmonella* cells in chicken carcass rinses and mung bean sprouts (102). Such method has also been applied to contaminated water and spinach samples (6). Although the recovery rate was high, the rate was significantly affected by the filter type and the conditions used to recover cells from the filters. Additionally, immunomagnetic separation (IMS) technique using magnetic beads coated with antibodies to specific pathogen have been used to concentrate *Salmonella* from food (90, 106).

Commercial DNA extraction products have been developed for applying molecular-based detection assays in food. The Qiagen® DNeasy *mericon* Food Kit uses cetyltrimethylammonium

bromide (CTAB) to improve extraction rate of total cellular nucleic acids from food samples. As a nonionic detergent, CTAB is widely used for efficient extraction of total cellular nucleic acids from a wide range of tissue types based on the capability of complexion with cellular nucleic acids (in low-salt conditions) or cellular inhibitors (in high-salt conditions), such as polysaccharides, proteins, and plant metabolites (26, 27). With high quality product and efficiency (up to 30 samples can be processed in 2.5 hours), this Kit is the first universally applicable extraction method that generates optimal and reliable results even when using strongly inhibitory, highly processed, fatty, acidic, high, or low DNA content foods.

Culture-Based Methods Traditional culture-based methods are considered the "gold standard" for microbiological analysis in food and are used widely. The U.S. Food and Drug Administration's Bacteriological Analytical Manual (BAM) details the detection of various foodborne pathogens by traditional culture methods which generally include pre-enrichment, selective enrichment, selective plating, and identification (68). The BAM method for *Salmonella* detection include pre-enrichment in BPW, selective enrichment using Rappaport-Vassiliadis (RV) broth, or tetrathionate (TT) broth, selective isolation by streaking on Hektoen Enteric agar, Xylose Lysine Desoxycholate agar or Bismuth Sulfite agar and identification and confirmation via biochemical tests such as urease test, indole test, etc. (Figure 2.2) (3). However, this process is labor-intensive and time-consuming, taking up to 10-calendar days for a definitive result (30, 59). Therefore, rapid methods have been developed at a fast pace during the past several decades.

Immunological-Based Methods Immunological-based methods rely on the interaction between antibody and antigen for testing and have been used for many years to identify, serovar, and quantify bacteria. With recent decade, many quick detection kits have been developed based on this theory.

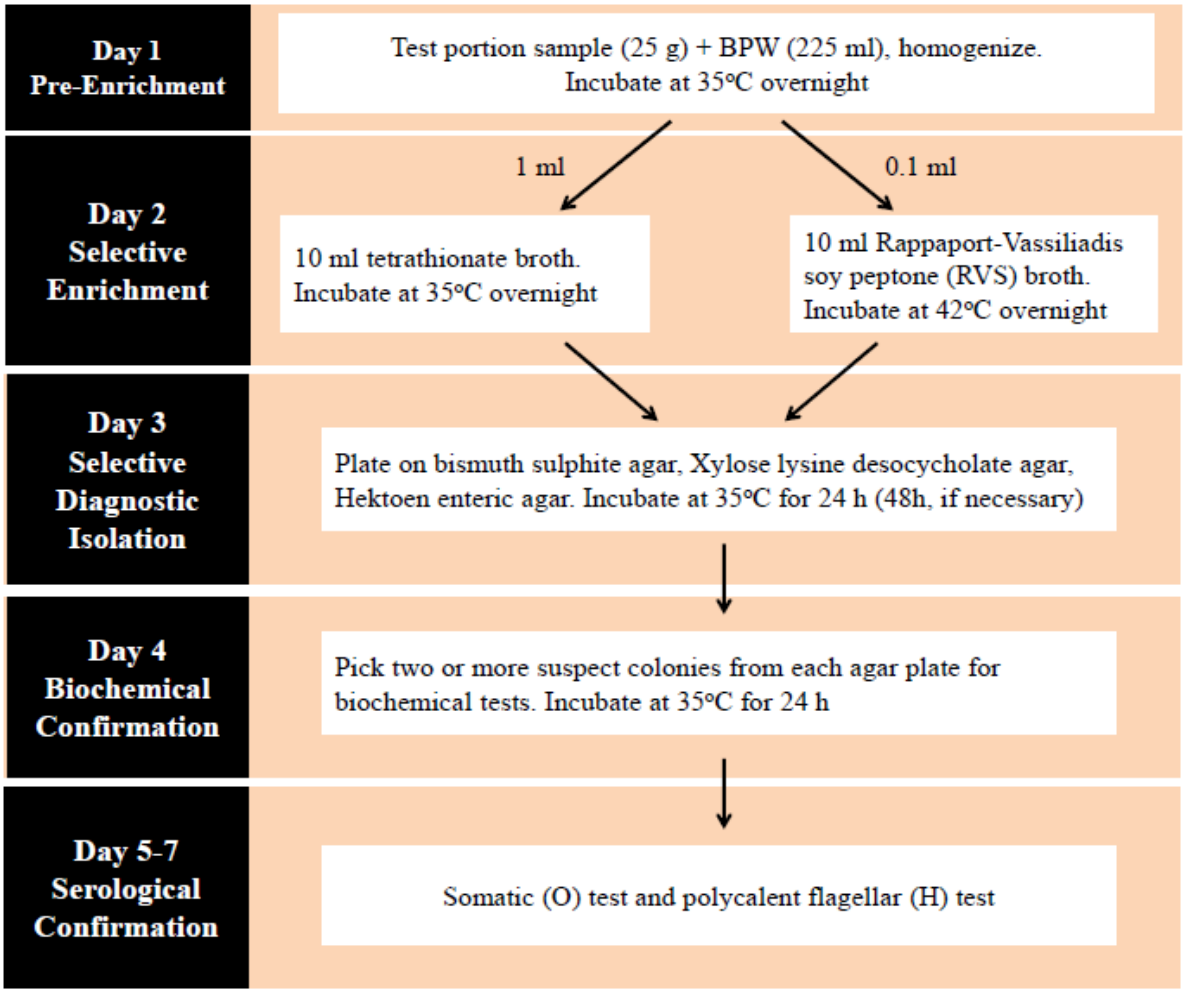

Figure 2.2 FDA BAM *Salmonella* Isolation Procedure.

Enzyme-linked immunosorbent assay (ELISA) is one of the various types of immunological-based assays that have been developed for the rapid microbiological detection in foods since 1970s (8). After being loaded into a 96-well microtiter plate, target pathogen binds to the specific antibody which has been pre-coated onto the wells of the microtiter plate. Then a secondary antibody linked to an enzyme is incubated together to again bind to the target pathogen, forming a sandwich structure. Following the washing step to get rid of non-specific bindings, a colorless substance for the enzyme is added that reacts with the bound enzyme and generates detectable color signals (23). Szabo et al. (92) compared different ELISA tests in

performance to detect *Salmonella* in various sample matrices, blood serum and meat juice of pig samples. The result showed that all tests used in this study could successfully detect the *Salmonella* in different samples. Although ELISA can significantly reduce the assay time, the drawbacks of poor sensitivity, low specificity, and lack of quantitative capability still greatly limit its application. The detection limit of ELISA is between 10^4-10^5 CFU/ml (28), which may be improved greatly with pre-enrichment. The poor binding affinity between antibody and antigen causes another major shortage of ELISA, low specificity. A study in Sweden (30) reported that ELISA performed worse in sensitivity and specificity compared with the standard culture methods and PCR assays on detection of *Salmonella*. The poor binding specificity of the antibodies lead to the poor ability of detecting *Salmonella* Livingstone and *Salmonella* Worthington.

Molecular-Based Methods In the 1980s, advances in basic DNA research stimulated the surge of molecular-based pathogen detection assays (48). As the representatives of molecular-based assays, PCR and real-time quantitative PCR (qPCR) feature rapid reaction time (requiring only several hours), high sensitivity, high specificity, and good reproducibility. In the last decade, a novel molecular-based assay, loop-mediated isothermal amplification (LAMP) has been developed and applied in pathogen detection. Here, we will review PCR, qPCR, and LAMP assays developed and applied for detecting *Salmonella* in food.

PCR is a powerful molecular-based nucleic acid amplification technique that has been widely used for foodborne pathogen detection. During PCR, a highly efficient DNA polymerase such as *Taq* polymerase is employed and within a few hours, the target DNA sequence can be exponentially amplified by 10^6 fold (71). A gel electrophoresis is then followed to examine the amplified PCR products under UV light. PCR assays are widely regarded to be rapid and

sensitive. A study for the detection of *Salmonella* in seafood samples by Kumar et al. (59) found that PCR assay which targeted at *Salmonella*-specific *invA* gene showed 31.6% positive results in a total of 214 seafood samples, while positive rates of 23.7% and 21.3% for ELISA and culture method, respectively. The greater sensitivity of PCR assay contributed to the higher detection rate of *Salmonella* in seafood samples. Koyuncu et al.(58) compared the performance between commercial PCR-based method and traditional culture-based method to detect *Salmonella* in six feed types. The result showed that two methods performed similarly to each other but PCR-based method have the advantage of sensitivity, specificity and rapid speed.

A multiplex PCR could detect several genes as targets at one run. It greatly improved the rapid identification and characterization of the microorganism by targeting several genes in one bacteria genus and detecting multiple target microorganisms in food samples simultaneously. Soumet et al. (89) developed a multiplex PCR assay to detect *Salmonella* and identify the two serovars Enteritidis and Typhimurium by amplifying a 429 bp fragment specific for the genus *Salmonella* within a randomly cloned sequence, a 559 bp target specific for *Salmonella* Typhimurium within the *fliC* gene and a 312 bp fragment specific for *Salmonella* Enteritidis within the *sefA* gene. With the enrichment on a Modified Semi-solid Rappaport Vassiliadis medium (MSRV) for 18-20 h, the m-PCR combined with MSRV had a better sensitivity (95%) than the bacteriological method (92.5%). And Li et al. (62) in 2004 established a multiplex PCR, in which three pairs of primers were used to identify *E. coli* O157:H7, *Salmonella*, and *Shigella*. This method could successfully detect the three bacteria in apple cider and detect down to 8×10^{-1} CFU/g after overnight enrichment. In 2009, with five specific primer pairs, Park et al. established a multiple PCR method to detect and distinguish *Salmonella* serovars Typhimurium and Enteritidis (80).

The drawback of this simple, convenient assay is that its sensitivity is dramatically reduced in food sample testing due to inhibitors from the complex composition of the food matrix. In some cases, the result from PCR-based detection cannot match the result of culture-based method, thus invalidating the application of PCR-based detection of *Salmonella.* Therefore, an enrichment step is generally required for the application in food samples which inevitably increases the complete analysis time (54, 56, 76).

The second generation of PCR assay, real-time PCR, also termed qPCR provides both detection and quantification of the target gene simultaneously. Quantification is achieved using fluorescent dyes or fluorescence-labeled DNA probes. A fluorescent dye bounds to double-stranded DNA (dsDNA) during PCR, and is used to track the amplification level of the target gene after each thermal cycle. The most economically used dye is SYBR Green I. The main drawback of using fluorescent dyes in real-time PCR is the potential inaccurate quantification due to nonspecific binding of PCR products. To improve the specificity, melting-curve analysis is usually conducted after amplification to get the melting temperature, which varies depending on sequence of the amplified product (83).Using the fluorescence-labeled DNA probes in real-time PCR is another way that can improve specificity since the probes are designed to be specifically targeting the target sequence. However, DNA probes are expensive and can be difficult to design.

Real-time PCR has been reported to be more rapid, sensitive, and specific than traditional PCR methods. The amplification cycle times of real-time PCR are usually shorter than that of conventional PCR, and it also eliminates the necessity of running gel, which is time-consuming and does not allow precise quantification. A specific probe was designed and used in a qPCR to

target *invA* gene of *Salmonella*, and the detection limit was 2 CFU per reaction, which was 100 fold more sensitive than conventional PCR reported previously (20, 21, 84).

As the third generation of PCR method, real-time reverse-transcriptase polymerase chain reaction (rt-RT-PCR) could detect the live pathogen cells in food samples. This technique further pushed the detection limit of *Salmonella* cells in culture as low as 10^2 CFU/ml (25). Compared with qPCR, the new assay has better sensitivity and lower false positive results by only detecting the live cells (40). However, the disadvantages of these mRNA-based techniques include low amplification efficiency when compared with DNA-based methods and limited sensitivity and specificity. Techathuvanan et al.(94) reported a method based on qPCR to detect *Salmonella* in the pork. This method can sensitively detect 10^6 CFU/ 25g in pork chop and sausage within less than 24hs, compared to more than one week with traditional methods. Van Blerk et al.(98) established a rapid and specific method to detect *Salmonella* in water samples based on real-time PCR, shorten the detection time from several days in standard culture-based method to less than 24hs. And in 2011, Real-time PCR was reported as a rapid screening method to detect *Salmonella* in liquid whole eggs (LWE) by Techathuvanan et al.(93). This method could detect as low as 10^7 CFU/25ml LWE, without enrichment, and 10^4 to 10^2 CFU/25ml LWE, with enrichment, within 24 h, which contribute to early detection and prevent transmission of egg-associated *Salmonella* in egg industry. However, these mRNA-based techniques depend on high quality of primers and high amplification efficiency of amplification cycles. When compared with DNA-based detection methods, Real-time PCR has much more strict requirements in sampling and operation, which limit its application to some extent.

LAMP was developed by a group of Japanese scientists in 2000 (74). This novel molecular based assay uses a set of four specific primers, two inner and two outer, targeting six

distinct regions of the target DNA sequence. To facilitate the auto-cycling amplification, one or two loop primers targeting the dumbbell-like region of the stem-loop structure are added into the reagents mix to a dumbbell-like structure (72) . The addition of loop primer(s) accelerates DNA amplification by increasing the number of starting points for DNA synthesis and the results can be detected within 30 min. It has high efficiency of specifically amplifying the target gene from a couple of cells to 10^9 copies within one hour under isothermal conditions (at 60 - 65^{o}C).

LAMP has been applied for the detection of many foodborne pathogens such as *Vibrio cholerae* (103), *Vibrio vulnificus* (41, 85), *Vibrio parahaemolyticus* (18, 73), *Escherichia coli* (57), *Campylobacter* (104, 105) and yielded promising results in food samples with less inhibition effect. It was reported that LAMP assay for detecting *V. vulnificus* in pure culture and raw oyster samples was found to be 10-fold and 1,000-fold more sensitive than the conventional PCR (41). It also showed that LAMP was able to successfully identified *Salmonella* in the total of 110 raw egg samples while PCR failed to detect 10% of them (77). Featured for its high specificity, LAMP has been developed to detect specific serovars (O4 and O9 group) of *Salmonella enterica* in food samples (78, 79). There are several studies developed LAMP methods for *Salmonella* detection by targeting the *invA* gene (19, 43, 65, 99). Hara-Kudo et al. (43) have pioneered the field of LAMP detection for *Salmonella* in 2005 with a sensitivity of 2.2 CFU/ test tube. And the LAMP assay designed by our group's previous work was able to identify as few as 1.3 *Salmonella* per reaction in pure culture (19).

LAMP is recognized as a simple, rapid, and cost-effective technique for DNA amplification and yielded highly specific and sensitive results. Since it is isothermal, simple equipment such as water bath or heating block that can maintain the temperature at around 60 - 65^{o}C is sufficient. Additionally, the large amount of DNA synthesized by LAMP can result in

turbidity change which can be observed by naked eyes (70). These advantages of LAMP make it easier to be adapted in a field application. Further improvement for field-adaption could be focus on development of lyophilized ready-to-use LAMP reaction mix. A recent study developed a disposable, low-cost, easy-to-use microfluidics-based diagnostic system that requires no instrument at all (100). Coupled with lyophilized LAMP master mix, the study presented examples of microfluidic functional elements—including mixers, separators, and detectors—as well as complete microfluidic devices that function entirely without any moving parts and external power sources. And another study developed a disposable, water-activated, self-heating, easy-to-use, polymeric cartridge for isothermal nucleic acid amplification (LAMP) and visual fluorescent detection of the amplification products (64). And Gene-Z, a device for point of care genetic testing was also based on LAMP assay, which consisted of (i) a disposable microfluidic chip with pre-dispensed and dehydrated primers, (ii) a compact and economic fluorescence detector, and (iii) a wirelessly-connected smart device (iPod Touch or iPhone) for control, data collection, display and analysis (91). In their study, the lowest copy number that could be detected was 13 copies *E. coli* DNA per reaction. All these studies gave a trend for molecular diagnostic test that indicated isothermal nucleic acid amplification, especially LAMP had a great potential to fit in the field test for food processing and handling.

Real-time LAMP can be conducted real-time which allows quantitative analysis of DNA amplification by correlating the amplification signals with the cell numbers (69). It was first designed running on a turbidimeter which monitors the increase in turbidity caused by the synthesis of magnesium pyrophosphate precipitates during amplification (69, 70). Recently, a Bioluminescent Assay in Real-Time (BART) for LAMP was developed by Gandelman et al. (36), which continuously reported the exponential increase of inorganic pyrophosphate (PPi) produced

during the isothermal amplification of a specific nucleic acid target through bioluminescent output. PPi produced stoichiometrically during nucleic acid synthesis was converted into ATP by the enzyme ATP sulfurylase, and then quantitatively detected by bioluminescence using thermostable firefly luciferase (75). The assay showed a unique kinetic signature for nucleic acid amplifications, an identifiable light output peak that reflected the concentration of original target nucleic acid. Since the quantification capability of BART is based on determination of peak time rather than absolute intensity of light emission, it can be run on simple light detectors, thus promotes the potential application of LAMP in field test with small and portable equipment. This novel real-time, closed-tube luminescent report system coupled with LAMP has been used for detecting genetically modified (GM) maize target DNA (55). The LAMP-BART was able to detect at low level of 0.1-5.0% GM and showed higher tolerance to plant sample-derived inhibitors than qPCR. The multiple choices of LAMP detecting platform enhance the possible application of LAMP assay for detecting *Salmonella* in fields, farms or food processing factories.

In summary, to reduce the frequency of *Salmonella*-associated foodborne illnesses and outbreaks, LAMP as a novel molecular-based method has shown great promise to be a rapid, reliable detection system that can be widely used in food detections. However, there is no research has been done to evaluate the possible obstacles when bringing LAMP from labs to fields especially related to food industry, such as assay robustness under unstable environmental temperature, various pH, and inhibitors in complex food matrices, and the development of more user-friendly result reading techniques, which are the knowledge gaps this dissertation research aimed to fill (detailed in Chapters 3-6) The validation of LAMP as a rapid, accurate, simple, and robust detection method to determining *Salmonella* contaminations will benefit farmers, harvesters, processors, distributors, retailers, and the ultimate consumers by better ensuring

Salmonella-free food, therefore reducing the infections and deaths associated with *Salmonella* contaminated food commodities.

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CHAPTER 3: ROBUSTNESS OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAYS FOR *SALMONELLA* DETECTION IN FOOD APPLICATIONS

Introduction

Salmonella is a leading cause of foodborne illnesses and outbreaks in the United States. The Centers for Disease Control and Prevention (CDC) estimated that *Salmonella* causes approximately 1 million cases of foodborne illnesses each year, , resulting in 19,336 hospitalizations and 378 deaths (27). In 2011, the CDC's FoodNet data showed that *Salmonella* was responsible for over 41% of the total laboratory-confirmed infections from 10 bacterial/parasitic enteric agents under FoodNet surveillance (9). Another recent CDC report on foodborne disease outbreaks in 2009 and 2010 also indicated *Salmonella* to be a leading pathogen causing most outbreak-related deaths (4). Various food commodities have been implicated in *Salmonella* outbreaks, including meat and poultry, eggs, dairy products, and fresh produce.

To identify potential contamination problems during the production, processing, and distribution of these high-risk food commodities, it is critical for the industry to have rapid, reliable, and user-friendly detection methods for *Salmonella* so that contamination events may be identified promptly. .There are three categories of methods commonly used for *Salmonella* detection, including culture-based, antibody-based, and nucleic acid-based assays (11). Traditional culture-based methods are reliable but limited by the lengthy time and labor consumption (2). Immunological assay, such as enzyme-linked immunosorbent assay (ELISA) has the drawback of low specificity (8). Molecular-based methods such as polymerase chain reaction (PCR) and real-time quantitative PCR (qPCR) have been widely applied in *Salmonella* detection, and demonstrated to be rapid, specific, and sensitive (3, 8, 16, 17). However, both

PCR and qPCR require a sophisticated thermal cycling instrument, limiting their wide application in food industry. Additionally, to detect PCR products, some toxic or mutagenic stains such as ethidium bromide are used (18). The *Taq* polymerase used in PCR amplification is reported to be rather susceptible to inhibitors (7).

Loop-mediated isothermal amplification (LAMP) was developed as a novel DNA amplification technique in 2000 (20). This assay is technically simple and doesn't require sophisticated thermal cycling equipment, making it easy to be implemented. Since 2000, LAMP assays have been adopted to detect multiple bacterial and viral agents including foodborne pathogens such as *Salmonella* and shown to be rapid, specific, and sensitive (12, 20-22, 25, 33, 34). LAMP assays were also found to be more robust than PCR against inhibitors in clinical samples, such as monovalent salt, serum, blood, urine and stools (10, 14). However, there is a lack of studies on its performance in food samples.

During food sample testing, one major obstacle associated with molecular-based detection assays is effect derived from the food matrix. Previous studies reported that humic acid and plant polysaccharides were the major factors in plants that inhibited PCR reactions (1, 7, 30). To evaluate the potential application of LAMP assay in testing plant-based food and other foods, this study compared robustness of two LAMP assays and one PCR and one qPCR assay for their performance in detecting *Salmonella* under abusive assay conditions and in the presence of inhibitors likely encountered during food testing.

Materials and Methods

Bacterial strains and DNA template preparation. *Salmonella enterica* serovar Typhimurium LT2 (ATCC 700720) was used for LAMP sensitivity testing. The *Salmonella* strain was cultured using trypticase soy agar (TSA; BD Diagnostic Systems, Sparks, MD) at

37°C overnight. Three to four single colonies were transferred to 5 ml of fresh trypticase soy broth (TSB; BD Diagnostic Systems) and incubate at 37°C for 16 h with shaking to reach ~10^9 CFU/ml (A_{600} = 1). Ten-fold serial dilutions were made with 0.1% peptone (from 1 ml to 9 ml). The initial cell count was determined by direct plating of 100 µl aliquot on TSA in duplicate and enumerating colonies after overnight incubation. To prepare templates, 500 µl of the each dilution was transferred into a microcentrifuge tube, boiled at 95°C for 10 min, and centrifuged again at 12,000 ×g for 2 min. The supernatant was stored at -20°C till use.

LAMP assays. Two sets of LAMP primers (Table 3.1) targeting the *Salmonella* invasion gene (*invA*, Genbank accession number M90846) were used in this study. Primer sets for LAMP1 and LAMP2 were designed by Hara-Kudo et al. (12) and our research group (5), respectively.

LAMP assays were performed using conditions described previously (5). The LAMP reaction mix in a total volume of 25 µl consisted of 1 × thermal buffer, 6 mM $MgSO_4$, 1.2 mM deoxynucleotide triphosphate (dNTP), 0.1 µM each outer primer, 1.8 µM each inner primer, 1.0 µM loop primer, 10 unit of *Bst* DNA polymerase (New England Biolabs, Ipswich, MA) and 5 µl of DNA template (for assay condition tests) or 1 µl of DNA template with inhibitor solutions. A negative control was included in each LAMP run. The LAMP assay was carried out at 63°C for 40 min and terminated at 80°C for 5 min in a real-time turbidimeter (LA-320C; Eiken Chemical Co., Ltd., Tokyo, Japan), which acquired the turbidity readings at 650 nm every 6 s. The time threshold values (*Tt*; min) were collected for all samples when the turbidity increase measurements (the differential value of the moving average of turbidity) exceeded a threshold of 0.1.

Table 3.1 LAMP, PCR and qPCR Primers Used in the Study.

Assay	Primer name	Sequence (5′-3′)	Gene location	Size (bp)	Ref.
LAMP 1	Sal-F3	GGCGATATTGGTGTTTATGGGG	225-246	Ladder-like bands for LAMP; 244 bp for F3/B3 PCR	(12)
	Sal-B3	AACGATAAACTGGACCACGG	449-468		
	Sal-FIP	GACGACTGGTACTGATCGAT-AGTTTTTCAACGTTTCCTGCGG	327-346(F1c) 271-292(F2)		
	Sal-BIP	CCGGTGAAATTATCGCCAC-ACAAAACCCACCGCCAGG	368-386(B1c) 414-431(F2)		
	Sal-Loop-F	GACGAAAGAGCGTGGTAATTAAC	297-319		
	Sal-Loop-B	GGGCAATTCGTTATTGGCGATAG	391-413		
LAMP 2	F3	CGGCCCGATTTTCTCTGG	503-520	Ladder-like bands for LAMP; 180 bp for F3/B3 PCR	(5)
	B3	CGGCAATACGCGTCACCTT	665-682		
	FIP	GCGCGGCATCCGCATCAATA-TGCCCGGTAAACAGATGAGT	573-592 (F1c) 527-546 (F2)		
	BIP	GCGAACGGCGAAGCGTACTG-TCGCACCGTCAAAGGAAC	593-612 (B1c) 635-652 (B2)		
	Loop-F	GGCCTTCAAATCGGCATCAAT	547-567		
	Loop-B	GAAAGGGAAAGCCAGCTTTACG	613-634		
PCR	invA-139	GTGAAATTATCGCCACGTTCGGGCAA	371-396	285	(24)
	invA-141	TCATCGCACCGTCAAAGGAACC	634-655		
qPCR	invA3F	AACGTGTTTCCGTGCGTAAT	1598-1610	263	(6)
	invA3R	TCCATCAAATTAGCGGAGGC	1840-1859		
	invA3-Probe1	FAM-TGGAAGCGCTCGCATTGTGG-BHQ-1	1631-1650		

PCR and qPCR assays. As a comparison, the same DNA templates used for LAMP assays were also used for PCR and qPCR reactions. The PCR mix with a total volume of 25 µl contained 1 × PCR buffer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.4 µM each forward or reverse primer from Rahn et al. (24) (Table 3.1), 0.625 U of GoTaq Hot Start Polymerase (Promega, Madison, WI), and 5 µl of DNA template (for assay condition tests) or 1 µl of DNA template with inhibitor solutions. The PCR reactions were conducted using initial denaturation at 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 64°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 7 min in a Bio-Rad C1000 Thermal Cycler (Hercules, CA). Aliquots (10 µl) of PCR products were analyzed by electrophoresis on

1.5% agarose gel containing ethidium bromide, and visualized under UV light. Gel images werer documented by a Gel Doc XR system (Bio-Rad).

The qPCR reagent mix (25 µl) consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 10 µM each forward or reverse primer, 2.5 µM probe (Integrated DNA Technologies, San Jose, CA), 1.5 U of Taq DNA polymerase, and 5 µl of DNA template (for assay condition tests) or 1 µl of DNA template with inhibitor solutions. Reactions were performed in a iQ^{TM}5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) at 95°C for 3 min, which was followed by 50 cycles of 15 s at 95°C and 30 s at 60°C. The cycle threshold (*Ct*) value was obtained when the fluorescence reading exceeded a threshold value of 30 units.

Abusive assay condition tests. Various abusive assay preparation temperatures, assay running temperatures, and pH (Table 3.2) were tested. Five µl of DNA templates prepared above at 10^6 CFU/ml were used in LAMP, PCR, and qPCR assays described above. The experiments were repeated three times.

Inhibitor tests. Inhibitors including culture media used for enrichment and dilution, plant polysaccharide, and humic acid (Table 3.3), actual food juices (chicken, eggs, meat products, and produce) and soil solution (Table 3.4) were tested. These potential inhibitors were added in certain proportions (0, 1%, 2%, 5%, 10%, 20%, and 30%) to the final reaction mix. One µl of DNA templates prepared above at 10^7 CFU/ml were used in LAMP, PCR, and qPCR assays described above. ,). The experiments were repeated three times.

To prepare the food juices, whole chicken, shell eggs, ground beef, ground pork, peanut butter, and produce (alfalfa sprout, cantaloupe, tomato, and jalapano pepper) were purchased from a local grocery store and test immediately. The whole chicken was rinsed with 400 ml

buffer peptone water (BPW) with vancomycin (8 mg/liter) and mixed manually for 5 min. For all the rest food, 25 g sample was mixed with 225 ml BPW with vancomycin (8 mg/liter) and manually mix for 1 min. Soil sample was obtained in the campus and mixed with 225 ml BPW at the ratio of 1:10. All the samples were checked to be *Salmonella*-free following the U.S. Department of Agriculture procedure (31).

Data analysis. Means and standard deviations of *Tt* for LAMP and *Ct* for qPCR were calculated by Microsoft Excel (Microsoft, Seattle, WA). These values were sorted by condition parameter and inhibitor and compared by using the analysis of variance (SAS for Windows, version 9.2; SAS Institute Inc., Cary, NC). Differences between the mean values were significant when the *P* value was <0.05.

Results

Assay performance affected by abusive assay conditions. Under abusive assay preparation temperatures/holding times, the *Tt* values of LAMP1 increased significantly ($P < 0.05$) when the holding time increased from 10 min to 30 min (Table 3.2). In contrast, the *Tt* values for LAMP2 did not change significantly ($P > 0.05$) under various abusive preparation conditions. Similarly, PCR consistently gave positive results under all of these preparation conditions. For qPCR, significant increase in *Ct* values was observed when the assay was not prepared at 4^{o}C and held for various time periods.

Under different assay running temperatures, both LAMP assays had the lowest *Tt* values at 65°C, which increased significantly as the running temperature deviated from that optimum temperature (Table 3.2). For qPCR, the Ct values were lowest at an annealing temperature of 60°C. When the temperature was raised above 70°C, none of the assays could generate amplification signals. In comparison, qPCR actually stopped amplification at 68^{o}C (Table 3.2).

The pH values obviously affected the performance of these molecular assays. The functional pH ranges for LAMP1 and LAMP2 were 7.8-8.8 and 8.3-8.8, respectively (Table 3.2). The qPCR obtained a slow performance by adding the Tris buffer at pH 7.3. Negative results were observed for all PCR possibly due to insufficiency of copy number that was amplified. Additional test showed PCR required higher concentration level of template to generate band (data not shown).

Table 3.2 Performances of LAMP, qPCR and PCR Assays under Abusive Assay Conditions.

Condition	LAMP1 *Tt* (min)	LAMP2 *Tt* (min)	qPCR *Ct* (cycles)	PCR
Preparation temperature/holding time				
4°C	12.32±0.40(A)	16.85±0.74(A)	21.53±0.13(A)	+
25°C10min	12.70±0.14(A)	17.39±1.01(A)	22.33±0.14(B)	+
25°C30min	13.68±0.35(B)	17.75±1.10(A)	23.55±0.21(C)	+
37°C10min	12.98±0.07(AB)	18.30±0.75(A)	23.30±0.19(C)	+
37°C30min	14.97±0.62(C)	18.45±1.02(A)	23.44±0.13(C)	+
Assay running temperature				
57°C	30.30±0.75(D)	27.60±2.08(D)	30.83±0.02(C)	+
60°C	20.13±1.70(B)	20.28±0.21(C)	26.42±0.18(A)	+
63°C	12.40±0.52(A)	17.02±0.49(AB)	28.02±0.82(B)	+
65°C	12.00±0.04(A)	15.32±0.40(A)	28.28±0.06(B)	+
68°C	22.40±0.00(C)	18.37±0.05(BC)	-	+
70°C	-	-	-	-
pH				
6.8	-	-	-	-
7.3	-	-	29.41±6.12[a]	-
7.8	20.53±1.46(A)	-	-	-
8.3	19.48±0.60(A)	22.14±1.57(A)	-	-
8.8	24.85±6.29(A)	23.80±0.00(A)	-	-
9.3	-	-	-	-
9.6	-	-	-	-

The data was based on three independent repeats. In each column each different conditions, mean values followed by different upper case letters in parenthesis are significantly different ($P < 0.05$).

[a] There was only 2 round of qPCR worked of out 3 repeats at this pH level.

Inhibitory effects of enrichment media and biological substances As shown in Table 3.3, with the exception of humic acid, adding other inhibitors (up to 30%) into the reaction mix still resulted in positive LAMP amplification by both LAMP1 and LAMP2. Most of the LAMP reactions were completed within 25 min except for plant polysaccharide added at 20 or 30%. In contrast, the performance of PCR or qPCR was obviously affected by the addition of all of the inhibitors added, especially at concentrations above 20%. It is also notable that with the increase in the concentration of inhibitors added, increasing trends with both *Tt* and *Ct* values were observed; some were statistically significant ($P < 0.05$).

When added into 30% of the final reaction mix, plant polysaccharides (1.25% w/v) slowed down both LAMP reactions. The *Tt* value of LAMP1 and LAMP2 increased from 11.00 min to 25.98 min and 15.90 min to 40.84 min, respectively, which was more than doubled. qPCR and PCR failed to detect *Salmonella* at this concentration. The strongest inhibition effect came from humic acid(0.005% w/v), which completely inhibited the amplification by both LAMP assays, qPCR and PCR at the addition portion of 20%, 5% and 2% of the final reaction mix, respectively.

Inhibitory effects of food juices. The food juices involved in the tests were meat products, whole chicken, shell egg, and fresh produce. LAMP1 showed good tolerance to various food juices (Table 3.4). Except for egg homogenate (20% and 30%) and jalapeno pepper juice (30%), LAMP1 generated positive reactions. LAMP2 demonstrated comparable results as LAMP1 in chicken rinse, cantaloupe, tomato, alfalfa sprouts, and peanut butter tests, but it was not as robust as LAMP1 in other tests. When less than 20% produce juices were added, qPCR performance was slowed down but still within 25 min. The amplification was noticeably inhibited by the juice from meat or poultry, and completely stopped with 2% chicken rinse or

ground beef juice, or 10% ground pork juice. Egg homogenate inhibited all the assays at 20% or higher level and dramatically impacted the performance of LAMP2.

Table 3.3 Comparison of Robustness of LAMP, PCR and qPCR Assays with Addition of Potential Inhibitory Compound Solutions.

Substance	Portion in reaction (%)	LAMP1 *Tt* (min)	LAMP2 *Tt* (min)	qPCR *Ct* (cycles)	PCR
TSB	0	11.84±0.47(A)[a]	16.53±0.25(A)	20.11±1.93(A)	+
	1	11.80±0.38(A)	16.84±0.66(A)	21.15±1.05(A)	+
	2	11.97±0.33(A)	16.75±0.49(A)	22.23±0.49(AB)	+
	5	12.30±0.24(A)	16.42±0.12(A)	22.52±0.39(AB)	+
	10	12.79±0.02(A)	16.84±0.37(A)	22.85±0.51(AB)	+
	20	15.07±0.42(B)	18.32±0.54(B)	24.40±1.46(B)	-
	30	19.37±0.99(C)	21.27±0.47(C)	-	-
0.1% peptone	0	11.04±0.05(A)	16.77±0.42(A)	21.29±1.61(A)	+
	1	11.12±0.16(AB)	17.02±0.49(AB)	22.61±1.10(A)	+
	2	11.30±0.18(AB)	17.50±0.42(ABC)	22.77±1.15(A)	+
	5	11.90±0.47(ABC)	17.67±0.23(BC)	22.78±1.15(A)	+
	10	12.07±0.33(BC)	18.04±0.37(C)	22.90±1.26(A)	+
	20	12.37±0.47(C)	18.10±0.24(C)	23.04±1.10(A)	+
	30	12.88±0.78(C)	18.31±0.30(C)	23.08±1.08(A)	+
BPW	0	11.00±0.00(A)	16.32±0.02(A)	21.30±0.31(A)	+
	1	11.05±0.07(A)	16.70±0.33(AB)	21.80±0.12(AB)	+
	2	11.24±0.33(AB)	17.10±0.61(ABC)	22.58±0.88(AB)	+
	5	11.58±0.21(B)	17.38±0.49(BC)	22.95±1.07(AB)	+
	10	12.17±0.23(C)	17.87±0.37(C)	23.18±0.79(B)	+
	20	13.77±0.09(D)	19.47±0.14(D)	25.03±0.52(C)	-
	30	17.25±0.25(E)	23.34±0.09(E)	-	-
Plant Polysaccharides (1.25% w/v)	0	11.00±0.00(A)	15.90±0.81(A)	22.15±0.04(A)	+
	1	11.00±0.00(A)	17.30±0.24(B)	23.07±1.17(A)	+
	2	11.59±0.12(AB)	18.37±0.09(C)	23.19±1.01(A)	+
	5	12.45±0.03(AB)	19.35±0.07(D)	23.52±0.76(A)	+
	10	13.72±0.02(B)	20.72±0.35(E)	23.32±1.34(A)	+
	20	16.85±0.25(C)	25.75±0.03(F)	23.83±1.02(A)	+
	30	25.98±2.90(D)	40.84±0.09(G)	-	-
Humic acid (0.005% w/v)	0	11.00±0.00(A)	17.24±0.47(A)	22.74±0.33(A)	+
	1	12.29±0.02(B)	18.07±0.33(AB)	24.28±0.45(B)	+
	2	12.90±0.18(B)	19.02±0.02(B)	24.49±0.51(B)	-
	5	14.54±0.05(C)	21.52±0.12(C)	-	-
	10	19.34±0.80(D)	27.28±0.92(D)	-	-
	20	-	-	-	-
	30	-	-	-	-

[a] Data labeled with the same character has no significant difference ($p > 0.05$).

Table 3.4 Comparison of Robustness of LAMP, PCR and qPCR Assays with Addition of Food Juices and Soil Solution.

Substance (%)	LAMP1 (*Tt*)	LAMP2(*Tt*)	qPCR(*Ct*)	PCR
Ground beef juice				
0	11.85±0.17(A)	15.57±0.42(A)	21.60±2.21(A)	+
1	12.09±0.16(A)	15.82±0.35(A)	26.58±6.18(A)	+
2	12.32±0.30(A)	16.40±0.42(A)	-	+
5	12.87±0.33(A)	17.33±0.14(AB)	-	+
10	13.40±0.24(A)	18.90±0.14(B)	-	+
20	17.82±0.64(B)	23.64±1.65(C)	-	-
30	25.17±2.12(C)	-	-	-
Ground pork juice				
0	11.01±0.01(A)	16.19±0.87(A)	21.66±2.09(A)	+
1	11.17±0.23(A)	16.30±0.89(A)	25.47±1.04(A)	+
2	11.28±0.35(A)	16.80±0.81(AB)	33.48±0.54(B)	+
5	12.20±0.24(A)	17.54±0.62(AB)	36.61±1.81(B)	+
10	13.02±0.21(A)	18.68±0.64(B)	-	+
20	16.60±1.23(B)	-	-	-
30	25.14±3.73(C)	-	-	-
Chicken rinse				
0	11.00±0.00(A)	16.19±1.11(A)	21.13±0.99(A)	+
1	11.18±0.07(A)	17.02±1.68(A)	24.51±0.79(A)	+
2	11.55±0.07(A)	17.90±1.60(A)	-	+
5	13.03±0.00(B)	20.80±3.63(A)	-	+
10	16.25±0.31(C)	24.40±5.09(AB)	-	+
20	24.02±0.54(D)	31.47±8.10(B)	-	-
30	35.45±0.11(E)	42.45±2.47(C)	-	-
Egg homogenate				
0	11.40±0.04(A)	18.62±0.12(A)	22.24±1.41(A)	+
1	25.69±0.45(B)	45.95±4.45(B)	27.44±2.42(AB)	+
2	34.62±0.40(C)	-	28.55±3.87(AB)	+
5	44.37±4.24(D)	-	30.69±4.17(B)	+
10	48.29±0.54(D)	-	39.52±2.20(C)	+
20	-	-	-	-
30	-	-	-	-
Cantaloupe juice				
0	11.45±0.21(A)	15.40±0.00(A)	21.62±1.32(A)	+
1	11.90±0.24(AB)	15.88±0.02(AB)	22.94±1.65(AB)	+
2	12.04±0.23(B)	15.95±0.03(B)	23.28±1.87(AB)	+
5	12.32±0.26(B)	16.34±0.23(B)	23.62±1.80(AB)	+
10	12.95±0.31(C)	16.95±0.17(C)	23.53±1.62(AB)	+
20	15.79±0.26(D)	19.97±0.37(D)	26.95±2.15(B)	-
30	21.59±0.12(E)	25.55±0.31(E)	-	-

(Table 3.4 continued)

Substance (%)	LAMP1 (*Tt*)	LAMP2(*Tt*)	qPCR(*Ct*)	PCR
Jalapeno pepper juice				
0	12.05±0.17(A)	15.25±0.78(A)	21.91±0.93(A)	+
1	12.72±0.26(AB)	16.34±0.66(AB)	23.55±1.06(AB)	+
2	12.95±0.11(BC)	16.52±0.49(AB)	23.88±1.41(AB)	+
5	13.75±0.03(C)	17.85±0.96(BC)	24.79±0.81(B)	+
10	15.47±0.00(D)	19.63±0.71(C)	25.36±0.62(B)	+
20	21.77±0.80(E)	-	29.25±0.24(C)	-
30	-	-	-	-
Tomato juice				
0	11.52±0.59(A)	16.12±0.97(A)	22.78±0.39(A)	+
1	11.64±0.52(A)	16.79±0.87(A)	23.94±0.11(A)	+
2	11.97±0.80(AB)	16.80±0.75(AB)	24.21±0.48(A)	+
5	12.27±0.76(AB)	16.98±0.92(AB)	24.67±0.46(A)	+
10	12.68±0.40(AB)	16.75±1.10(AB)	25.28±0.42(A)	+
20	14.24±0.76(B)	17.05±0.82(AB)	33.08±5.56(B)	-
30	17.62±2.10(C)	19.27±1.65(B)	-	-
Alfalfa sprouts juice				
0	11.25±0.16(A)	16.18±0.49(A)	22.61±0.21(A)	+
1	11.59±0.02(A)	16.49±0.54(A)	23.92±0.71(AB)	+
2	11.77±0.05(AB)	16.55±0.64(A)	23.92±0.78(AB)	+
5	12.15±0.07(AB)	17.02±0.54(AB)	24.15±0.56(B)	+
10	13.22±0.16(B)	17.95±0.59(B)	24.31±0.44(B)	-
20	16.40±0.24(C)	21.07±0.05(C)	26.24±0.35(C)	-
30	25.58±1.62(D)	27.62±0.69(D)	-	-
Peanut butter juice				
0	11.24±0.33(A)	15.50±0.66(A)	21.20±1.05(A)	+
1	11.59±0.12(AB)	15.87±0.28(AB)	22.55±2.17(A)	+
2	11.67±0.09(B)	16.10±0.10(AB)	24.51±1.20(AB)	+
5	11.72±0.07(B)	16.95±0.03(BC)	33.47±5.53(B)	+
10	12.29±0.02(C)	17.07±0.05(BC)	45.02[a](C)	+
20	14.87±0.00(D)	18.20±0.18(C)	-	-
30	20.29±0.16(E)	24.34±1.36(D)	-	-
Soil solution				
0	11.47±0.42(A)	15.45±0.31(A)	21.46±1.08(A)	+
1	12.02±0.30(AB)	15.69±0.40(A)	22.17±1.34(A)	+
2	12.45±0.40(AB)	15.92±0.30(AB)	22.63±1.15(A)	+
5	13.39±0.54(B)	16.60±0.28(B)	22.93±1.27(A)	+
10	16.29±0.97(C)	18.69±0.12(C)	24.27±1.96(A)	-
20	23.17±0.09(D)	25.15±0.39(D)	-	-
30	34.65±1.539(E)	-	-	-

[a] There was only one positive result in one of the triplicate qPCR test rounds.

Discussion

LAMP assay is a rapid, accurate, and cost-effective diagnostic method for bacterial and viral detection (19). To bring LAMP close to application in food testing, the robustness of the assay need to be evaluated under abusive assay conditions and in the presence of potential inhibitors in the food matrix and during food processing (11). A study conducted by Francois group reported that in clinical applications, LAMP was particularly robust across 2 pH units (7.3-9.3) and over the temperature values between 57-67°C (10). Our results showed that LAMPs were robust to abusive preparation temperatures and assay running temperatures ranging from 57 to 68°C, which agreed with their findings. Compared to LAMPs, both qPCR and PCR were robust to the abusive preparation conditions as well due to the usage of GoTaq® Hot Star polymerase (Promega, Madison, Wisc.). This polymerase needs to be activated during the initial denaturation step when amplification reactions are heated at 94–95°C for at least two minutes which avoid its activity below 70°C.

The pH ranges for the successfully amplification of target *Salmonella* DNA by LAMP1 and LAMP2 were 7.8-8.8 and 8.3-8.8, respectively. These ranges were relatively narrower than the robust pH range (7.3-9.3) for LAMP in Francois' study. This might be caused by the different concentration of the DNA templates. Francois et al. used the positive control DNA from the commercial kit which might have higher concentration and purer DNA than our direct-boiled template. In this study, we also did qPCR and PCR tests parallel to LAMP tests and gained some evidences to support this assumption. When applied the same template used in LAMP to PCR, a concentration of 10^4 CFU/reaction was too low for PCR to work. But it could conduct the amplification at pH 9.3 (data not shown) when the template concentration increased to 10^5 CFU/reaction. Therefore, LAMPs are more robust than PCRs against the lower template

concentration, with the pH ranges around 9.0. Two reasons may account for the difference. In the earlier study, 4 µl of positive control *Salmonella* DNA in the LAMP kit (purified DNA with unspecified concentration) was used while 1 µl of directly-boiled DNA template (8.5×104 CFU/reaction) was used in the present study. The amount of Tris buffers added was 4 µl in the present study but 1 µl in the earlier study, which would result in different pH in the final reaction mix. Nonetheless, PCR and qPCR tested in parallel performed markedly poorer than the two LAMP assays. Comparison between LAMP and PCR or qPCR was not performed in the earlier study (10).

Based on the *Salmonella* procedure in the laboratory guidebook of USDA Food Safety and Inspection Service (FSIS), BPW is one of the common media used in the enrichment step for food sample testing. And TSB and 0.1% peptone water are usually involved in the *Salmonella* culturing and making 10-fold series dilution. Kaneko et al. reported that some media, such as saline, phosphate-buffered saline (PBS), and Minimum Essential Medium (MEM) could inhibit PCR reaction at 30% (v/v), while showed no impact on LAMP performance at the same level (14). The investigation of the influence brought by media during sample preparation would help to improve the procedure to achieve better quality templates for molecular tests. With 20% or more TSB or BPW presented in the reaction, PCR was completely stopped; qPCR was noticeably slowed down; but both LAMP assays gave positive results within 20 min (Table 3.3). However, 0.1% peptone water almost had no effects on inhibiting any assay's performance. This outcome was similar to Rossen group's finding which reported there was no inhibitory effects by 10% (v/v) regular peptone water against PCR performance (26). These results suggest that resuspend the sample liquid in 0.1% peptone water instead of other media broth can reduce the inhibition on DNA amplification methods. Acidic polysaccharides are well-known compounds in

plant tissue that can be inhibitory to nucleic acids amplification assays (7). When the plant polysaccharide stock was made at a very low concentration as 1.25% (w/v), all of the detection methods worked efficiently (Table 3.3). Compared to the mild effects by plant polysaccharides stock, another well-documented PCR inhibitory compound, humic acid displayed strong impact against the performances of these assays even at the concentration as low as 0.005% (Table 3.3) (30). The amplifications of LAMP1, LMAP2, qPCR and PCR were abolished by humic acid in the reactions at the concentration of 20%, 20%, 5%, and 2%, respectively.

Due to complex texture and compounds of different food commodities, although food compounds have been accepted as one category of inhibitors for molecular amplifications, the mechanisms are still unknown. Rossen et al. found that some inhibitory effects were assigned to salami, chicken salad and cheese (26). In Witham et al.'s paper, the food compounds in ground beef caused the failure of PCR amplification (32). Comparable results were observed in our study. Meat products or chicken caused more serious loss of functions of PCR, especially qPCR, than produce samples (Table 3.4). Even as the same technique, LAMP1 had a slightly better robustness than LAMP2 because of the different designs of primer sets. Thus, a well-designed primer set could improve the tolerant ability of the assay against the inhibition from food matrix.

Another interesting finding is the effects of egg homogenate on these assays. Eggs are high in lipid and protein content, and contain lysozyme in the egg white. The former factors set up obstacles for detection methods and the later one may catalyze the artificially inoculated bacteria and affect the result outputs. Price et al.'s study has reported that egg albumin had inhibition effect on ELISA (23). In their experiment, the albumin-only samples did not yield any positive result with the initial 10 organism inoculation through all the incubation period (24 – 96 h). Another study on detection of castor toxin in milk and eggs found that the egg matrix

inhibited PCR amplification and interfered with two of the three methods tested for DNA extraction (13). Comparing with the two main parts in eggs, egg yolk had a greater negative effect on PCR amplification than the egg white. It was also reported that PCR gave false-negative results in dried egg samples (28). In this study, LAMP1, qPCR, and PCR could detect *Salmonella* cells with 10% egg homogenate in the reaction. But LAMP2 could only succeed with up to 1% level. These phenomena also proved that the different designs of primer sets also could affect the assay robustness to the inhibitions.

The produce juice, peanut butter juice and soil solution gave the same trend: noticeable inhibitory effects on PCR, less on qPCR, and least on LAMP assays. Overall, LAMP assays, especially LAMP1 is more robust than PCR-based assays. Considering its other features, LAMP has more potential for quick screening of foodborne pathogens in field.

The two *Salmonella invA*-based LAMP assays, LAMP1 and LAMP2 were rapid, specific, sensitive, and only require 40 min to 1h to obtain the results (5, 12). And for initial testing foods, it took less than 3 hours to complete LAMP assay from sample preparation to result output. It has same accuracy as the FDA-BAM culture-based method, uses significantly less time than the culture method, and has 100-fold better sensitivity than PCR method. The isothermal feature gives this assay the capability of becoming an approach for field testing. Several portable detection systems have been developed based on LAMP technology, such as Gene-Z device (Gene Z, Columbus, Ohio) and BART-LAMP system (Lumora, UK) (15, 29). The approval of the assay robustness would promote the application of these LAMP-based quick detection systems for food testing.

In conclusion, this study demonstrated the advantages of LAMP assays in tolerance to abusive operation temperatures, extra pH factors, and inhibitors that would possibly occur in

food matrix and during sample preparation. In the assay condition tests, qPCR and PCR were as tough as LAMP assays against the instability potentially exists in test preparation conditions and assay running temperatures, but they were extremely sensitive to pH changes. Humic acid strongly inhibited all the assays even at very low concentrations, which also partially revealed the mechanism of inhibition caused by soil solution. LAMPs showed its high tolerance to about 20% of inhibitor liquids in one reaction which assigned to media used for sample preparation, food compounds and food matrix including meat products, poultry, eggs, fresh produce, peanut butter, and potential compounds that might be contained in produce—soil. Additionally, LAMP assays are technically simpler and more cost effective than PCR because they do not require an expensive thermal cycler. This rapid, accurate, easy-operating, and cost-effective method is very robust to be operated in the field and used as a reliable tool to detect *Salmonella* in various food commodities for better controlling potential microbial hazards in foods and defending the public health.

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CHAPTER 4: DETECTING *SALMONLLA* SEROVARS IN SHELL EGGS BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

Introduction

Nontyphoidal *Salmonella* is a leading cause of foodborne illness in the United States, responsible for an estimated 1 million cases, 19,336 hospitalizations, and 378 deaths each year (31). Among over 1,000 *Salmonella* serovars that have caused illness in the U.S., *Salmonella* Enteritidis (SE), Newport, and Typhimurium (ST) consistently ranked among the top three (4, 29). In 2011, SE was most frequently reported by FoodNet (in 18% of laboratory-confirmed *Salmonella* infections), followed by ST (13%) and *Salmonella* Newport (12%) (3). In addition, a 58% increase in the incidence of SE infection was observed compared with 1996-1998 (3), underscoring the continued significance of SE in causing foodborne illnesses in the U.S.

Shell eggs, undercooked and raw, have been identified as the predominant sources of SE infection (5). Of particular note is the 2010 nationwide SE outbreak involving shell eggs, which resulted in 1,939 illnesses and a recall of over 500 million eggs (11). It is now generally accepted that eggs become contaminated with *Salmonella* primarily through the transovarian route (18), although trans-shell penetration (environmental contamination) also plays a role (10). An earlier risk assessment estimated that 2.3 million SE-contaminated shell eggs are produced annually, posing significant food safety and public health concerns (1). In response, a federal egg safety rule was published recently, requiring producers with more than 3,000 laying hens to implement measures by July 9, 2012 to prevent SE from contaminating eggs on the farm. More vigilant environmental monitoring and egg testing for SE is one measure included in this regulation (10).

To detect SE in egg samples, traditional culture-based methods specified in the U.S. Food and Drug Administration (FDA)'s Bacteriological Analytical Manual (BAM) (9) are used, although rapid methods equivalent in accuracy, precision, and sensitivity are acceptable (10).

The BAM method is reliable but labor-intensive and time-consuming, demanding up to 10-calendar days for a definitive result (9). Rapid methods, though capable of reducing the time to results significantly, still require days rather than hours for a complete analysis (13). Among the four methods currently achieved FDA's equivalency status (12), the PCR-based BAX system requires 55 hours (8) and the real-time PCR-based TaqMan system needs 27 hours (21). Besides, both PCR and real-time PCR require a sophisticated thermal cycler to carry out the nucleic acid amplification.

Recently, multiple *Salmonella* assays using a novel nucleic acid amplification technology termed loop-mediated isothermal amplification (LAMP) (25) have been developed (7, 16, 20, 23, 27, 28, 32, 38-40). Salient features of LAMP include isothermal (i.e., no need for the thermal cycling instrument), rapid, specific, sensitive, easy result-reading, and tolerance to biological substances (17). Several of these LAMP assays have been applied to detect *Salmonella* in liquid eggs or on egg shells (26, 33, 40). However, none of the studies have evaluated two LAMP assays simultaneously and applied them to detect and quantify *Salmonella* in shell eggs.

This study aimed to evaluate two LAMP assays side by side to detect *Salmonella* serovars in shell eggs, using PCR as the comparison method. One assay chosen was the first *Salmonella* LAMP assay reported (16) and the other one was developed most recently by our research group (7). Both LAMP assays target the *Salmonella* invasion gene (*invA*), which possesses a broad specificity for *Salmonella* serovars (30).

Materials and Methods

Bacterial strains and culture conditions Thirty-three *Salmonella enterica* subsp. *enterica* strains of 18 serovars (Table 4.1) were used for specificity testing. Among them, SE strain S50, previously recovered from a retail organic chicken in Louisiana (19), and ST strain

LT2 (BEI Resources, Manassas, VA), originally isolated from a chicken in Indian in the 1940s (22), were used for sensitivity testing and spiked-egg-homogenate experiments. The cultures were stored at -80^{o}C in trypticase soy broth (BD Diagnostic Systems, Sparks, MD) supplemented with 20% glycerol. All strains were routinely cultured on trypticase soy agar and incubated at 35^{o}C for 24 h.

Table 4.1 Thirty-three *Salmonella enterica* Strains (18 Serovars) Used in this Study

Salmonella serovar	No. of strains	Strain ID [a]	Other designations/Source/References
Anatum	1	NR-4291	Roma tomato (2004 Pennsylvania outbreak)
Anatum var. 15+	1	1637 H	Meat meal
Braenderup	1	10 N	Raw chicken
Enteritidis	5	20 N	Lasagna
		SE 5	Unknown
		SE 9	Unknown
		SE 22	Unknown
		S50 [a]	Raw chicken (19)
Hartford	1	2807 H	Raw oysters
Heidelberg	1	1364 H	Raw oysters
Infantis	1	1102 H	Meat meal
Javiana	3	2080 H	Frog legs
		7 N	Unknown
		NR-4296	Human stool (2004 Pennsylvania outbreak)
Mbandaka	1	37 N	Halva candy
Montevideo	2	1 H	Whole eggs
		NR-172	ATCC BAA-710, G4639; Human clinical
Muenchen	2	1501 H	Feather meal
		NR-4311	Human stool (2004 Pennsylvania outbreak)
Newport	1	1240 H	Dried yeast
Oranienburg	2	1410 H	Feather meal
		NR-171	ATCC 9239; Human (foodborne outbreak)
Poona	1	2861 H	Pet turtles
Saintpaul	1	1358 H	Mixed vegetable macaroni
Stanley	1	1243 H	Bone meal
Thompson	1	NR-4319	Human stool (2004 Pennsylvania outbreak)
Typhimurium	7	NR-169	ATCC 43971, CIP 60.62; derived from LT2
		NR-170	ATCC 6994
		NR-173	ATCC 13311, NCTC 74; human stool
		LT2 [a]	NR-174; Chicken (22)
		NR-4333	Human stool (2004 Pennsylvania outbreak)
		NR-4341	ATCC 14028, CDC 6516-60; Chicken
		NR-13555	A36, ATCC BAA-1834

[a] The two strains were also used for sensitivity testing and spiked-egg-homogenate experiments.

LAMP assays. Two LAMP assays developed by Hara-Kudo et al. (16) and Chen et al. (7) were evaluated and designated LAMP1 and LAMP2, respectively. Both sets of LAMP primers (Figure 4.1) targeted the *Salmonella invA* gene (GenBank accession number M90846) and consisted of two outer (F3 and B3), two inner (FIP and BIP), and two loop (Loop-F and Loop-R) primers. The LAMP assays were performed as previously described (7). Briefly, the reagent mix in a total volume of 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 DNA template. The LAMP reactions were carried out at 63^{o}C for 40 min and terminated at 80^{o}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 the time threshold (*Tt*; in min) was determined when the turbidity increase measurement (differential value of moving average of turbidity) exceeded 0.1.

PCR assay. In comparison, a *Salmonella* PCR assay described previously (30) targeting the *invA* gene was carried out. The PCR mix (25 µl total) consisted of 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 µM each primer (Fig. 1), 0.625 U of GoTaq Hot Start polymerase (Promega, Madison, WI), and 2 µl of DNA template. The PCR reaction was conducted using 95°C for 2 min to activate the polymerase followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 64°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 7 min in a Bio-Rad C1000 thermal cycler (Hercules, CA). PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide and visualized under UV light. Gel images were documented by a Gel Doc XR system (Bio-Rad).

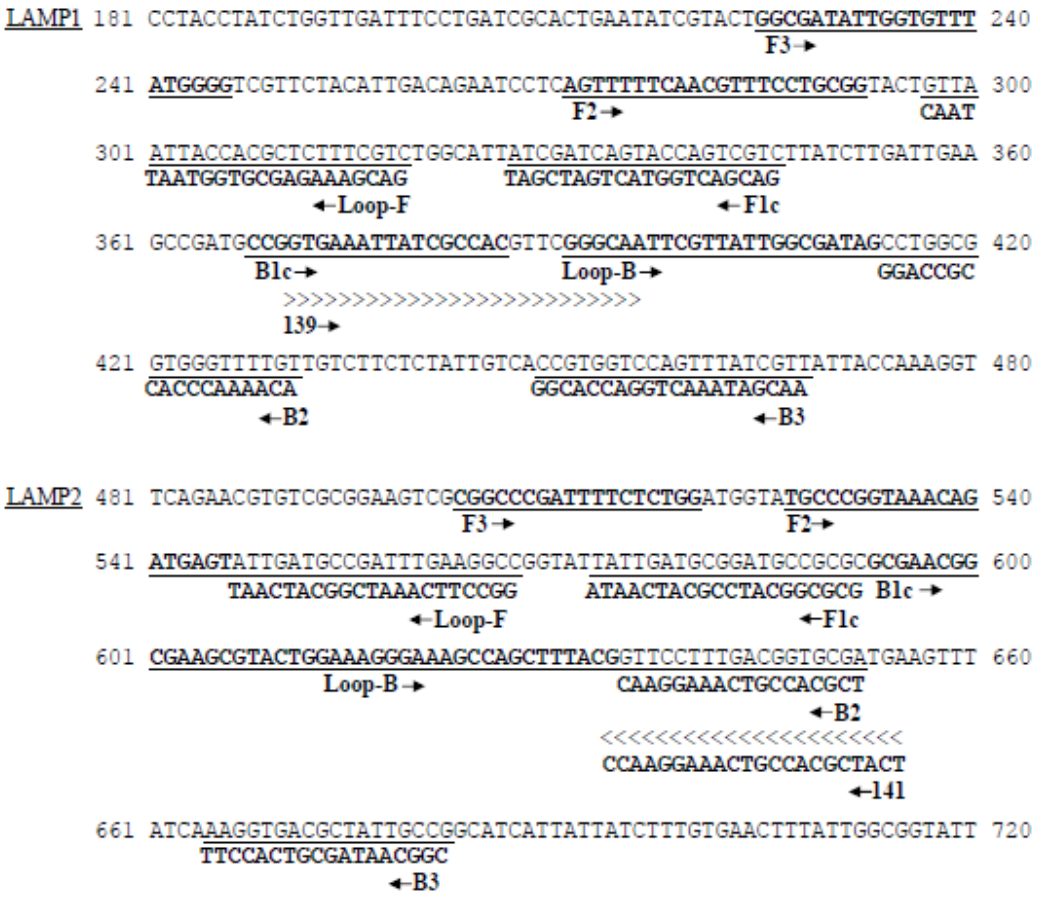

Figure 4.1 Partial Nucleotide Sequence of Tthe *Salmonella enterica* Serovar Typhimurium Invasion Gene (invA; Genbank Accession Number M90846), Target Regions, and Primers Used for the Two LAMP Assays and One PCR Assay. Underlined sequences are the target regions. Oligonucleotide sequences in bold were used as LAMP primers. F3 and B3 are the forward and backward outer primers, respectively. FIP and BIP are the forward and backward inner primers, respectively. FIP is a combination F1c and F2, whereas BIP is a combination of B1c and B2. Loop-F and Loop-B (underlined and bolded) are the forward and backward loop primers, respectively. PCR primers 139 and 141 are shown in forward and backward arrows, respectively.

Specificity and sensitivity test. For assay specificity, DNA templates of the 33 *Salmonella* strains (Table 4.1) were prepared by heating at 95^{o}C for 10 min as described previously (7). Aliquots (2 µl) of each template were subjected to LAMP1, LAMP2, and PCR, and repeated twice each.

Assay sensitivity (limit of detection) was determined by using 10-fold serial dilutions of SE S50 and ST LT2 cultures. DNA templates were prepared from stationary-phase cultures as

described previously (36). Aliquots (2 µl) of each template were tested by LAMP1, LAMP2, and PCR, and repeated five times each.

Assay evaluation in spiked shell eggs. Shell eggs (grade A) were obtained from a local grocery store and processed within 2 h of collection following procedures described in the BAM (9). Briefly, the egg shells were disinfected, and twenty eggs (ca. 1 liter) were cracked aseptically into one bag and mixed thoroughly by gloved hands. Two 25-ml portions of the pooled eggs were removed and analyzed for the presence/absence of *Salmonella* by culture using the BAM method (9). Confirmed *Salmonella*-negative egg samples were used for the following spiking experiments, which were independently repeated twice.

To determine assay sensitivity in shell eggs, a previous method (36) was used with slight modifications. Briefly, each test portion (25 ml) was inoculated with 2 ml of 10-fold serially diluted *Salmonella* S50 or LT2 overnight cultures, resulting in spiking levels between 10^9 and 10^3 CFU/25 ml. Another sample was included as the uninoculated control, for which aerobic plate counts were performed by standard pour plate method. All samples were homogenized with 225 ml of buffered peptone water (BPW; BD Diagnostic Systems) for 1 min in a food stomacher (model 400; Tekmar Company, Cincinnati, OH). Aliquots (1 ml) of the homogenate were subjected to DNA extraction by PrepMan Ultra sample preparation reagents (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Two microliters of the sample DNA extracts were used for LAMP1, LAMP2, and PCR, and repeated three times each.

The assay's capability to detect low levels of *Salmonella* cells in shell eggs was also evaluated. For this application, egg samples were spiked with *Salmonella* S50 or LT2 overnight cultures at two levels: 10^0 and 10^1 CFU/25 ml. After homogenization in 225 ml of pre-warmed BPW supplemented with vancomycin (8 mg/liter), the samples were incubated at 35°C for up to

24 h. Aliquots (1 ml) of the enrichment culture were removed at 6, 8, 10, 12, and 24 h, and processed similarly by PrepMan Ultra sample preparation reagents. Two microliters of the sample DNA extracts were subjected to LAMP1, LAMP2, and PCR, and repeated three times.

Data analysis. The *Tt* values obtained by LAMP1 and LAMP2 were compared by using analysis of variance (SAS for Windows, version 9; Cary, NC). Differences between the means were significant when $P < 0.05$. The detection limits (CFU/reaction in pure culture and CFU/25 ml in shell eggs) were presented as the lowest numbers of *Salmonella* cells that could be detected by the assays. In spiked-egg-homogenate experiments, CFU/reaction was calculated by using CFU/25 ml ÷ 250 × 10 × 2 × 10^{-3}, i.e., CFU/25 ml × 8 × 10^{-5}. Standard curves to quantify *Salmonella* in pure culture and spiked egg homogenates were generated by plotting *Tt* values against log CFU/reaction or log CFU/25 ml, respectively, and the quantification capabilities of the assays were derived based on the correlation coefficients (R^2) from the standard curves.

Results

LAMP specificity. All of the 33 *Salmonella* strains belonging to 18 serovars (Table 4.1) were accurately detected by the two *invA*-based LAMP assays, indicating 100% inclusivity. By LAMP1, the mean *Tt* values ranged from 11 to 14.6 min with an average of 11.5 ± 1.0 min. By LAMP2, the mean *Tt* values fell between 14.8 and 25.2 min with an average of 18.5 ± 3.3 min. The difference in *Tt* values obtained by LAMP1 and LAMP2 was statistically significant ($P < 0.05$). Similarly, the PCR assay included for comparison achieved accurate detection of all of the *Salmonella* strains tested.

LAMP sensitivity and quantification capability. Table 4.2 summarizes the sensitivity and quantification capability of LAMP1, LAMP2, and PCR when testing pure cultures of 10-fold serially diluted SE S50 and ST LT2 strains. In five repeats, both LAMP assays consistently

detected down to 1 CFU per reaction of either *Salmonella* strain, while PCR had a detection limit of approximately 100 cells for both strains. The mean *Tt* values obtained for the SE S50 template series (between 10^6 and 10^0 CFU/reaction) ranged from 15.1 to 27.2 min by LAMP1 and from 16.3 to 28.8 min by LAMP2 (data not shown). While for ST LT2, the mean *Tt* values for the same concentration series (10^6-10^0 CFU/reaction) ranged from 13.9 to 25.2 min by LAMP1 and from 17 to 27.9 min by LAMP2 (data not shown). For both strains, the *Tt* values obtained by LAMP1 were significantly smaller (by 1 to 5 min) than those by LAMP2 at every cell level tested ($P < 0.05$), except for the 10^0 CFU/reaction level, where no significant difference was observed (data not shown). However, inconsistent false positive results were also observed for LAMP1 at cell levels less than 1 CFU/reaction and in negative control samples (data not shown).

Based on the standard curves generated (data not shown), the correlation coefficients (R^2) of LAMP1 and LAMP2 when testing SE S50 and ST LT2 dilution series ranged from 0.87 to 0.94 (Table 4.2), suggesting a good linear relationship between *Salmonella* cell numbers (log CFU/reaction) and LAMP turbidity signals (*Tt*). PCR based on the end-point product analysis, on the other hand, is not quantitative.

Table 4.2 Sensitivity and Quantification Capability of the Two LAMP Assays and One PCR Assay when Testing 10-Fold Serial Dilutions of *Salmonella enterica* Serovar Enteritidis S50 and *S. enterica* Serovar Typhimurium LT2 Cells in Pure Cultures.

Strain ID	Serovar	Assay	Detection limit (CFU/reaction)	Quantification equation[a]	Linear R^{2a}
S50	Enteritidis	LAMP1	1	y = -1.052x + 19.313	0.88
		LAMP2	1	y = -1.324x + 22.363	0.87
		PCR	102	N/A[b]	N/A
LT2	Typhimurium	LAMP1	1.1	y = -1.094x + 18.551	0.89
		LAMP2	1.1	y = -1.324x + 23.596	0.94
		PCR	108	N/A	N/A

[a] Quantification equations and correlation coefficients (R^2) were calculated based on the linear relationship of mean *Tt* values and log CFU/reaction for *Salmonella* cell levels ranging from 10^1 to 10^5 CFU/reaction.
[b] N/A, not applicable.

Rapid and sensitive detection of *Salmonella* in spiked shell eggs. All of the uninoculated control samples tested negative for *Salmonella* by LAMP or PCR (data not shown). No visible colonies were identified on the standard plate count agar after 48 h incubation. Table 4-3 shows the sensitivity of LAMP1, LAMP2, and PCR when testing egg homogenates spiked with 10-fold serially diluted SE S50 or ST LT2 cells. In direct testing (i.e., no enrichment), regardless of the *Salmonella* strain used for inoculation, both LAMP assays consistently detected down to 10^4 CFU per 25 ml egg homogenate (equivalent to approximately 1 cell in the reaction mixture). In contrast, PCR was unable to detect either *Salmonella* strain in egg homogenate samples spiked with less than 10^6 CFU/25 ml. In one repeat for SE S50, 10^7 CFU/25 ml was needed for positive PCR results (Table 4.3).

Table 4.3 Sensitivity of the Two LAMP Assays and One PCR Assay when Testing Egg Homogenates Spiked with 10-Fold Serial Dilutions Of *Salmonella enterica* S50 or LT2 Cells via Direct Testing or after Enrichment.

Strain ID	Serovar	Assay	Detection limit of direct testing (CFU per 25 ml egg homogenate)	Minimum enrichment time needed for positive detection in egg homogenate samples spiked at levels	
				10^1 CFU/25 ml	10^0 CFU/25 ml
S50	Enteritidis	LAMP1	1×10^4	8 h	8 h
		LAMP2	1×10^4	8 h	8 h
		PCR	1×10^6 - 1×10^{7a}	12-24 h[b]	24 h
LT2	Typhimurium	LAMP1	1.1×10^4	8 h	8 h
		LAMP2	1.1×10^4	8 h	8 h
		PCR	1.1×10^6	12-24 h[b]	24 h

[a] One out of two repeats was positive for the lower detection limit.
[b] One out of two repeats required longer enrichment time for detection.

Table 4.3 also summarizes LAMP1, LAMP2, and PCR results in egg homogenates spiked with two low levels (10^0 and 10^1 CFU/25 ml) of SE S50 or ST LT2 and tested after various enrichment periods. Regardless of the *Salmonella* strain used for inoculation or the spiking level, none of the 6-h enrichment samples tested positive by LAMP1, LAMP2, or PCR (data not shown). Positive LAMP results appeared at 8-h enrichment by either LAMP1 or

LAMP2 for samples spiked with either *Salmonella* strain at both levels. In contrast, PCR required at least 12 h of enrichment for samples spiked with either *Salmonella* strain at 10^1 CFU per 25 ml egg homogenate and 24 h for those with 10^0 CFU/25 ml (Table 4.3).

Discussion

Both *invA*-based LAMP assays (7, 16) evaluated in the present study were rapid (11 to 40 min), specific (100% inclusivity for 33 *Salmonella* strains tested), sensitive (1 CFU/reaction in pure culture and 10^4 CFU/25 ml in spiked egg homogenate), and quantitative (R^2 = 0.87 to 0.94). With 8 h of enrichment, the assays accurately detected two low levels (10^0 and 10^1 CFU/25 ml) of SE S50 and ST LT2 strains in shell eggs. This is the first study applying two LAMP assays simultaneously for the detection of *Salmonella* serovars in shell eggs.

We chose to evaluate LAMP assays that detect *Salmonella* serovars in general not SE specifically since serovars other than Enteritidis (e.g., Braenderup, Heidelberg, and Typhimurium) have been implicated in egg-related outbreaks (2). This approach is therefore more proactive than that focusing on SE. For detecting *Salmonella* serovars, assays targeting the *invA* gene were chosen as this gene has been shown previously to possess a broad specificity for *Salmonella* serovars. For example, the *invA*-PCR assay had 99.4% inclusivity for 630 *Salmonella* strains comprising over 100 serovars (30). The LAMP1 assay developed by Hara-Kudo et al. (16) accurately detected 220 *S. enterica* subsp. *enterica* strains of 39 serovars and 7 *S. enterica* subsp. *arizonae* strains. LAMP2 developed by Chen et al. (7) demonstrated the specific detection of 28 *Salmonella* strains of 10 serovars. Similarly, 100% inclusivity was shown by both LAMP1 and LAMP2 when evaluated in the present study using additional *Salmonella* strains; some belonging to serovars (e.g. Anatum var. 15+ and Hartford) not tested before (7, 16, 30).

No obvious difference was observed between LAMP1 and LAMP2 in terms of specificity, sensitivity, and quantification ability. LAMP1 was notably faster than LAMP2 with significantly smaller (by 1 to 5 min) *Tt* values ($P < 0.05$), although false positive results were noted for LAMP1 at cell levels less than 1 CFU/reaction and in negative control samples (data not shown). As illustrated in Figure 4.1, primers for LAMP1 and LAMP2 were located in adjacent regions of the *invA* gene sequence, with PCR forward and reverse primers falling within the regions targeted by LAMP1 and LAMP2, respectively. Additionally, each LAMP primer set included two loop primers to accelerate the reactions (24). Besides LAMP1 and LAMP2, four other *Salmonella invA*-based LAMP assays have been reported to date (23, 32, 38, 40). Three of them (23, 38, 40) were developed by the same research group, using essentially the same primer set (one nucleotide addition/deletion at the 3' end of FIP and BIP primers) with no loop primers. LAMP primers in all four assays were located downstream of PCR primers.

The detection limits reported for LAMP1 and LAMP2 at the time of assay development were 2.2 and 1.3 CFU/reaction, respectively (7, 16). In the present study, both LAMP assays were capable of detecting approximately 1 cell of SE strain S50 or ST strain LT2 per reaction in pure cultures, corroborating previous findings. This level of sensitivity fell within the range reported for other *Salmonella* LAMP assays, ranging from 10^0 to 35 CFU/reaction (20, 23, 27, 28, 38-40). Both LAMP1 and LAMP2 were 100-fold more sensitive than PCR run in parallel. The improved sensitivity (by at least 10-fold) of LAMP over PCR has been reported previously (6, 7, 15, 16, 27, 28, 38) but qPCR was as sensitive as LAMP (7, 36, 37, 39). It is noteworthy that with a running time of 40 min, LAMP is markedly faster than either PCR or qPCR.

In the majority of *Salmonella* LAMP studies, amplicons were detected by end-point product analysis using gel electrophoresis or naked eye observation of white turbidity or color

change (20, 23, 38-40). Intercalating DNA dyes such as YO-PRO-1 have been used to facilitate fluorescence-based detection of *Salmonella* by LAMP (16). Close monitoring of turbidity changes occurring during LAMP amplification via a real-time turbidimeter have been used for quantitatively detecting some foodborne pathogens (6, 14, 15, 36, 37), but rarely for *Salmonella* (7). In this study, the R^2 values of LAMP1 and LAMP2 ranged from 0.87 to 0.94 for *Salmonella* cells between 10^1 and 10^5 CFU/reaction, suggesting a good quantification capability.

LAMP assays have been applied previously to detect *Salmonella* serovars in various food samples, including eggs (26, 33, 40), milk (20, 32), pork (35, 39), and produce (7, 41), usually with overnight enrichment. An earlier survey in Japan showed that LAMP was as sensitive as culture, but clearly more sensitive than PCR, in detecting *Salmonella* serovars from 110 naturally contaminated liquid eggs (26). The number of contaminated *Salmonella* cells ranged from < 1 to 2.4×10^2 CFU/g and overnight pre-enrichment in BPW was necessary (26). Using an *in situ* LAMP method, Ye et al. (40) demonstrated the detection of 1 CFU of *Salmonella* per cm^2 egg shells after 4 h enrichment in Luria Bertani medium. However, the *in situ* procedure was rather lengthy, including fixation for 8 h, permeabilization with lysozyme for 10 min, followed by fluorescence microscopic examination (40). Recently, Techathuvanan et al. (33, 35) coupled reverse transcription with LAMP1 (RT-LAMP) to detect viable *Salmonella* in pork and eggs. In spiked liquid whole eggs, the detection limit was 10^8 CFU/25 ml without enrichment and 16 h of enrichment was required for samples spiked at 10^0 CFU/25 ml (33). In the present study, LAMP could detect 10^4 CFU/25 ml in spiked egg homogenate and required 8 h of enrichment for samples spiked at two low levels, suggesting much improved assay sensitivity and speed. The *invA*-PCR, on the other hand, was 100-fold less sensitive and required 12-24 h of enrichment.

Several recent studies have compared the performance of LAMP and qPCR with culture in detecting *Salmonella* from food samples (34, 41). Zhang et al. (41) reported similar sensitivity and accuracy of three molecular methods (qPCR, RT-qPCR, and LAMP1) to the BAM culture method for detecting *Salmonella* in produce, with a detection limit of 2 CFU/25 g produce after 24 h enrichment. Another study also found comparable performance of RT-LAMP and RT-PCR with culture for *Salmonella* detection from pork processing environments, with detection limits of 1 log CFU/ml after 10 h enrichment (34). Although the culture method was not directly evaluated in this study, both LAMP assays achieved excellent sensitivity in detecting *Salmonella* serovars in shell eggs (10^0 CFU/25 ml after 8 h enrichment).

With the newly implemented egg safety final rule, it is imperative that rapid and accurate detection methods are available to facilitate the prompt identification of *Salmonella* contamination problems in poultry farms and shell eggs. The two *invA*-based LAMP assays were demonstrated to be rapid, specific, sensitive, and potentially quantitative (for direct testing, i.e., without enrichment) for detecting *Salmonella* serovars. The complete assay took about 10 h to complete when testing shell eggs, which included 8 h of enrichment. Therefore, the assays may be adopted in routine egg testing for *Salmonella* to improve egg safety and protect public health.

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CHAPTER 5: APPLICATION OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION FOR THE RAPID, RELIABLE AND ROBUST DETECTION OF *SALMONELLA* IN PRODUCE

Introduction

Nontyphoidal *Salmonella* is the leading bacterial cause of foodborne illness and a major public health concern in the United States and worldwide. Between 2009 and 2010, the CDC's Foodborne Disease Outbreak Surveillance System indicated that *Salmonella* was responsible for 243 foodborne disease outbreaks, resulting in 7,089 cases of illnesses (9). And it was the most common cause of outbreak-related hospitalizations, causing 49% of total reported hospitalizations. Symptoms of salmonellosis include gastroenteritis, bacteremia, and other extra-intestinal infections (32). Among more than 2,500 *Salmonella* serovars identified to date, Enteritidis, Typhimurium, Newport, Javiana, and Heidelberg rank among the top serovars associated with human illness. Various food products including meat and poultry, eggs, dairy products, and fresh produce could serve as vehicles of *Salmonella* infection (8). Among produce items implicated in *Salmonella* outbreaks, FDA reported that lettuce/leafy greens, tomatoes, and melons were the top three, accounting for 34.1%, 17.1%, and 15.9% of total outbreaks, respectively (38-40).

The consumption of fresh produce has increased sharply in the United States for the past several decades (41, 42). According to recent U.S. Department of Agriculture's reports, the amount of produce consumption per capita rose from 497.9 pounds in 1981 up to 616.4 pounds in 2010 (42). Unfortunately, the increase in consumption of produce coincides with the surge of produce-related outbreaks. Data from the Center for Science in the Public Interest (CSPI) demonstrated that produce-linked foodborne illnesses increased about 10% from 1999 to 2008 (12). To reduce the incidence of produce-associated foodborne outbreaks, multifaceted

approaches along the farm-to-table continuum are needed. Among them, it is important for the produce industry to have an access to rapid, reliable, and robust methods to test the raw and finished products for *Salmonella*. . However, there are many inherent challenges associated with pathogen detection in produce, including complex produce matrices, assay inhibitors, high background flora, injured target cells, and heterogeneous distribution of pathogens in produce (17). To overcome these challenges, sample preparation steps including enrichment and DNA extraction are commonly used.

Methods to detect *Salmonella* can be divided into three categories: culture-based, immunological-based, and molecular-based. The reference culture-based method is specified in the U.S. Food and Drug Administration (FDA)'s Bacteriological Analytical Manual (BAM), which involves pre-enrichment, selective enrichment, plating on selective media, and subsequent biochemical and serological identification of suspected colonies (11). Major drawbacks of these methods are time consuming and labor intensive. Immunological assays such as enzyme-linked immunosorbent assay (ELISA) and the AOAC International–approved VIDAS method have been used to detect the presence of *Salmonella* (33). These methods are much faster than the conventional culture methods but low in specificity (16). Molecular-based methods, PCR and real-time quantitative PCR (qPCR) have been commonly used for the detection of *Salmonella* (21, 30, 34). These assays are rapid and sensitive, but require an expensive, sophisticated, large instrument to run the thermal cycles resulting in low potential application as portable field screening tools. As a promising alternative to PCR, loop-mediated isothermal amplification (LAMP) assays have been widely used for the rapid, sensitive, and accurate detection of many foodborne pathogens, inclding *Campylobacter* (45), Shiga toxin producing *Escherichia coli* (43), norovirus (46), *Staphylococcus aureus* (26), and *Vibrio vulnificus* (19), as well as *Salmonella* (10,

20, 25, 29, 44). LAMP operates under isothermal conditions (60 to 65°C) and uses four specially designed primers and a strand-displacing *Bst* DNA polymerase to produce target-specific stem-loop DNA structure, which is followed by quasiexponential amplification of this structure. LAMP can generate 10^9 copies of the target DNA within an hour (28). The loop primers accelerate the LAMP reaction by hybridizing to stem-loop DNAs and facilitating strand displacement and amplification (27). The isothermal condition enables LAMP to be conducted in much simpler instruments such as a heater or water bath. To date, several portable instruments have been developed based on the LAMP assay (36).

To enhance its application in the food testing, our study aimed at validating this method in produce using complex produce matrices mimicking real-world contamination events with surface contamination and addition of aging time under refrigerated storage condition. Additionally, comparison of four quick DNA extraction methods was also included in this study.

Materials and Methods

Bacterial strains and DNA template preparation. Ten *Salmonella* strains (Table 5.3), belonging to ten serovars and isolated from various sources were used in this study. An additional 141 *Salmonella* strains and 27 non-*Salmonella* strains (Table 5.1) were used to evaluate the assay specificity. *Salmonella* strains were cultured using trypticase soy agar (TSA; BD Diagnostic Systems, Sparks, MD) at 37°C overnight. Non-*Salmonella* strains were grown on TSA or blood agar (BD Diagnostic Systems) and *Campylobacter* strains were grown under microaerophilic conditions (85% N_2, 10% CO_2, and 5% O_2).

To make DNA templates for specificity testing, several colonies were suspended in 500 µl of TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA; Sigma-Aldrich, St. Louis, MO) and heated at 95°C for 10 min in a dry heating block. After centrifuging at 12,000 *g* for 2 min, the

supernatants were stored at -20°C until use. For sensitivity testing, 3-4 *Salmonella* colonies from the overnight plates were inoculated to 8 ml of trypticase soy broth (TSB; BD Diagnostic Systems, Sparks, MD) and incubated at 37°C for 16 h to achieve the mid-log phase (OD_{600} = 1; approximately 10^9 CFU/ml). Each *Salmonella* culture was 10-fold serially diluted in 0.1% peptone water and the exact cell number was determined by standard plate counting. Aliquots (500 µl) of each dilution were used to prepare DNA templates for sensitivity testing similarly by the boiling method.

Table 5.1 Strains Used in the Specificity Test.

Bacteria group	Genus/Species/Serovars (no. of strains tested)	Total No. of strains
Salmonella	4,5,12:b:- (1), 4,5,12, i- (7), Agona (1), Alachua (1), Albany (1), Anatum (1), Anatum var 15+ (1), Braenderup (2), Brisbane (1), Cerro (2), Chester (1), Choleraesuis (1), Cubana (1), Dublin (1), Enteritidis (5), Fresno (1), Gaminara (1), Gera (1), Give (1), Hadar (1), Hartford (1), Heidelberg (1), Indiana (1), Infantis (2), Inverness (1), Javiana (2), Johannesburg (1), Kentucky (1), Kunzendorf (1), Mbandaka (2), Meleagridis (1), Michigan (1), Minnesota (1), Muenchen (2), Muenster (1), Newport (2), Oranienburg (2), Paratyphi B (1), Poona (1), Pullorum (1), Rubislaw (1), Saintpaul (1), Saphra (1), Schwarzengrund (1), Senftenberg (2), Sloterdijk (1), Stanley (1), Thompson (1), Tornow (1), Typhi (1), Typhimurium (19), Urbana (2), Vietnam (1), Virchow (2), Worthington (1), *S. enterica* subsp. (II) (9), *S. enterica* subsp.(IIIa) (8), *S. enterica* subsp.(IIIb) (8), *S. enterica* subsp. (IV) (10), *S. bongori* (V) (6), *S. enterica* subsp. (VI) (5)	158 (61 serovars)
Non-*Salmonella* strains	*Campylobacter* (3), *Citrobacter* (n=1), *Escherichia coli* (n =8), *Enterobacter* (n=1), *Hafnia* (n=1), *Listeria* (n=6), *Shigella* (n=3), *Vibrio* (n=4)	27

LAMP and qPCR conditions. The LAMP primers (Table 5.2) described previously (10) were used in this study, which targeted the *Salmonella* invasion gene . LAMP conditions were as those described (10) Briefly, the LAMP reagent mix in a total volume of 25 µl contained 1× ThermoPol reaction buffer (New England Biolabs, Ipswich, MA), 6 mM $MgSO_4$, 1.2 mM dNTP,

0.1 µM F3 and B3, 1.8 µM FIP and BIP, 1 µM Loop-F and Loop-B, 10 U of *Bst* DNA polymerase (New England Biolabs), and 2 µl of DNA template. The reaction was carried out at 63°C for 60 min and terminated at 80°C for 5 min in a real-time turbidimeter (LA-320C; Eiken Chemical Co., Ltd) which obtained the turbidity readings at 650 nm every 6 s. The time threshold (*Tt*; min) value was determined when the turbidity increase measurement (the differential value of the moving average of turbidity) exceeded a threshold value of 0.1.

Table 5.2 LAMP and qPCR Primers Used in this Study for *Salmonella* Detection.

Primer name	Sequence (5'-3')	Reference
LAMP primers		
F3	CGGCCCGATTTTCTCTGG	(10)
B3	CGGCAATAGCGTCACCTT	
FIP	GCGCGGCATCCGCATCAATA-TGCCCGGTAAACAGATGAGT	
BIP	GCGAACGGCGAAGCGTACTG-TCGCACCGTCAAAGGAAC	
Loop-F	GGCCTTCAAATCGGCATCAAT	
Loop-B	GAAAGGGAAAGCCAGCTTTACG	
qPCR primers		
invA3F	AACGTGTTTCCGTGCGTAAT	(11)
invA3R	TCCATCAAATTAGCGGAGGC	
invAProbe1	FAM-TGGAAGCGCTCGCATTGTGG-BHQ-1	

As a comparison, a qPCR assay targeting the *Salmonella invA* gene was performed using primers (Table 5.2) and conditions described previously (11). The qPCR reagent mix (25 µl) consisted of 1× Colorless GoTaq Flexi Buffer (Promega, Madison, WI), 10 µM each primer, 2.5 µM probe, 1.5 U of Taq DNA polymerase, and 2 µl of DNA template. The qPCR reaction was conducted at 95°C for 3 min, followed by 50 cycles of 15 s at 95°C and 30 s at 60°C in an iQ5 System (Bio-Rad, Hercules, CA). The cycle threshold (*Ct*) value was obtained when the fluorescence reading exceeded a threshold value of 30 units.

Specificity and sensitivity. A total of 168 bacterial strains (Table 5.1) were used to determine LAMP specificity. Aliquots (2 µl) of each DNA template as prepared above were subjected to both LAMP and qPCR amplifications. Specificity tests were repeated twice.

To determine LAMP sensitivity, aliquots (2 µl) of the 10-fold serially diluted *Salmonella* strains templates prepared above were subjected to both LAMP and qPCR amplifications. Sensitivity tests were repeated five times.

Detection of *Salmonella* in spiked produce. Nine produce samples [cantaloupe, lettuce (romaine), lettuce (iceberg), pepper (jalapeno), sprouts (alfalfa), sprouts (mung bean), sprouts (clover), tomato (red round), tomato (roma)] were purchased from local supermarket. To facilitate homogenization, leafy greens were cut into 4 cm^2 square using sterile scissors, and cantaloupe, pepper and tomatoes were sliced into fresh-cut size pieces (2.5 cm^3 cubes and 1/8 fruit wedge, respectively) using a sterile knife. Each produce was weighed 25 g out as one sample. After checking for the presence/absence of endogenous *Salmonella* following BAM method (1), confirmed *Salmonella*-negative produce was inoculated with 1.5 ml of bacterial suspensions (Table 5.1) to obtain the target inoculum level (10^9-10^4 CFU/25g for high level and 1-20 CFU/25 g for low level). The spiked samples were air-dried in a laminar flow biosafety hood for 2 h and stored at 4°C for 48 h.

After refrigerator storage, direct testing was applied to the high-level (specify the levels) inoculated samples. Each sample was mixed with 225 ml of pre-warmed buffered peptone water (BPW; BD Diagnostic Systems) and homogenized for 2 min in a food stomacher (Model 400; Tekmar Company, Cincinnati, OH) to produce 1:10 produce-BPW homogenate. Then, 1 ml of food homogenate was centrifuged at 900 *g* for 3 min to remove large produce tissues. The DNA was extracted using the PrepMan® Ultra sample preparation reagent (Life Technologies, Grand

Island, NY). Aliquots (2 µl) of the extracted DNA were used for both LAMP and qPCR amplifications. For the low-inoculated sample, enrichment was performed by incubating the *Salmonella*-spiked produce homogenate at 37^{o}C for 6, 8, and 10 h. After enrichment, the homogenate was processed similarly as described above for direct testing. The produce tests were repeated three times.

Comparison of DNA extraction methods. Four quick DNA extraction methods (direct boiled, TE extraction, PrepMan extraction, and FTA card extraction) were used on produce inoculated with low level (1-20 CFU/25 g) of *Salmonella* Typhimurium strain NR4333. One ml of enriched produce homogenates were used to prepare templates using the four different methods (Table 5.3).

Table 5.3 DNA Extraction Methods Used Ffor Template Preparation.

Method	DNA type	Processing method
Direct boiled	Crude DNA	Directly boiled at 95°C for 10 min
TE extraction	Crude DNA	Centrifuge at 500 *g* for 1 min, then 16,000 *g* for 5 min, resuspend in 100 µl TE
PrepMan extraction	Purified DNA	Use FTA Cards, follow instruction
FTA card extraction	Purified DNA	Use PrepMan reagents, follow instruction

The PrepMan extraction was followed the manufacture protocol of PrepMan® Ultra sample preparation reagent (Life Technologies, Grand Island, NY). And the Whatman® FTA Cards (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) procedure was as following: adding 0.65 ml produce enrichment broth onto the FTA Cards, punch a 2-mm disk from the absorbed material, and stored at room temperate. For each LAMP/qPCR reaction, 2 µl of liquid template or one disc was used. The comparison tests were repeated three times

Data analysis. For data from sensitivity test, produce test, and the extraction method comparison test, means and standard deviations of *Tt* or *Ct* values for detecting 10-fold serially diluted *Salmonella* in pure culture and spiked produce samples were calculated using Microsoft

Excel (Microsoft, Seattle, WA). The detection limits (CFU/reaction in pure culture or CFU/25 g in spiked produce) were presented as the lowest number of cells that could be detected by the assays.

Results

Specificity and sensitivity in pure cultures Both LAMP and qPCR demonstrated 100% inclusivity and 100% exclusivity when testing the 168 bacterial strains. For the sensitivity test, the average *Tt* values of LAMP ranged from 15.8 to 27.0 min on the designated 10 strains with the template concentration ranging from 10^6 to 10^1 CFU/ reaction (Figure 5.1). The sensitivity of qPCR achieved similar results in the same template concentrations, with the *Ct* value ranged from 21.2 to 37.5 cycles. When the cell number of *Salmonella* went down to 10^0 levels, qPCR was not able to detect the strains of Enteritidis 20N, Heidelberg 1364H, Newport 1240H, and Javiana NR-4296, while LAMP could detect them in some repeats (Table 5.4).

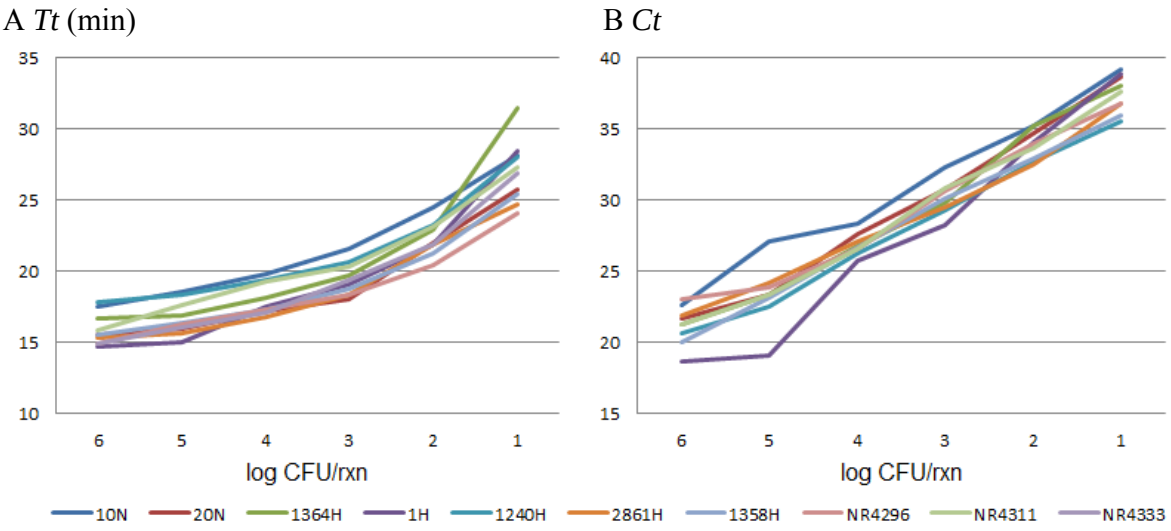

Figure 5.1 Comparison of Sensitivity of LAMP and qPCR Assays when Test 10-Fold Serial Dilution of *Salmonella* Cultures Ranged from 10^6 to 10^1 CFU/reaction. (A) LAMP sensitivity in pure cultures. (B) qPCR sensitivity in pure cultures.

Table 5.4 Detection Capability of LAMP and qPCR at the Level of 10^0CFU/reaction in the Pure Culture of 10 Designated *Salmonella* Strains.

Serovar	Strain ID	Detection at the 10^0CFU/reaction[a]	
		LAMP	qPCR
Braenderup	10N	2/5	1/5
Enteritidis	20N	5/5	N/A
Heidelberg	1364H	2/5	N/A
Javiana	NR4296	3/5	1/5
Montevideo	1H	2/5	N/A
Muenchen	NR4311	3/5	5/5
Newport	1240H	3/5	1/5
Poona	2861H	5/5	N/A
Saintpaul	1358H	4/5	3/5
Typhimurium	NR4333	3/5	1/5

[a] The number in the table indicated the positive result out of five repeats.

Evaluation LAMP and qPCR in produce samples Nine produce samples were artificially inoculated with 10 different *Salmonella* strains on surface individually. There was no obvious difference on detection limit of LAMP and qPCR in direct test of each produce category, with the average detection limit from 10^4 to 10^5 CFU/ 25 g sample, which equals to 10^1 to 10^2 CFU/ reaction (Table 5.5). Compared with the results from tomatoes, lettuces, pepper and cantaloupe, both assays showed lower sensitivity in the three types of sprouts.

The differences caused by produce type turned more noticeable when low-level inoculation (1-20 CFU/ 25 g sample) was applied. All the other artificially inoculated produce samples could give positive signals after 6-8 h of enrichment (Table 5.6). No positive result was found in three sprouts sample even with up to 10 h incubation. Additional tests were applied for 1 log higher inoculation in sprouts. And only part of them could be detected when the enrichment time went up to 10 h.

Table 5.5 Comparison of Sensitivity of LAMP and qPCR Assays in Detecting *Salmonella* in Spiked Produce Samples.

Strain	Assay	Detection level (log CFU/25g)								
		Cantaloupe	Pepper (Jalapeno)	Tomato		Lettuce		Sprouts		
				Red round	Roma	Romaine	Iceberg	Alfalfa	Clover	Mung bean
Braenderup 10N	LAMP	5[a]	4	5	5	5(2/3)[b]	5(2/3)	4(1/3)	5	6
	qPCR	5	4	5	5(2/3)	5	5	7	7	7
Enteritidis 20N	LAMP	4	6	4(2/3)	4(1/3)	4(2/3)	4	4	5	4
	qPCR	4	6	5	6	4	4(1/3)	4	5	4
Montevideo 1H	LAMP	4(1/3)	6	4(1/3)	4(1/3)	4(2/3)	4(2/3)	5	5(2/3)	4(2/3)
	qPCR	4(1/3)	5(1/3)	4(1/3)	5	4(1/3)	5	5	5	4(1/3)
Newport 1240H	LAMP	5	5	4(1/3)	4(1/3)	5	5(1/3)	5	5	6(2/3)
	qPCR	5	4(1/3)	5	5	5	5	5	5	5
Saintpaul 1358H	LAMP	4(2/3)	4(2/3)	4	4(1/3)	5	5	5(2/3)	5	5
	qPCR	4	4	4(1/3)	5	4(1/3)	5	5	4(1/3)	5
Heidelberg 1364H	LAMP	4(1/3)	5	4	4(1/3)	5(1/3)	5	5(2/3)	5(1/3)	5(1/3)
	qPCR	4(1/3)	5	4(1/3)	5	5	5	5	5	5(2/3)
Poona 2861H	LAMP	4	4(1/3)	4(1/3)	5	5	5	5(2/3)	5	6
	qPCR	4(1/3)	4(2/3)	4(1/3)	4(2/3)	4(2/3)	5	5	5	5
Javiana NR4296	LAMP	4(2/3)	4(2/3)	5	4	5	5	5	5	6
	qPCR	4	4	4(1/3)	4	4(1/3)	5	5	4(1/3)	5(2/3)
Muenchen	LAMP	5	4(1/3)	4	5	6	5(2/3)	6	5	6
NR4311	qPCR	5	5	4	4(1/3)	5	5	5	5	5
Typhimurium	LAMP	4	4(2/3)	4	4	5	4(2/3)	4(2/3)	5(2/3)	5(1/3)
NR4333	qPCR	4	4(2/3)	4	4	4	5	4(2/3)	5	6

[a] The lowest concentration that can be detected by assays.

[b] Time of positive results got out of three repeats.

Table 5.6 Comparison of LAMP and qPCR Assays in Detecting Low-Level Inoculated *Salmonella* in Spiked Produce Samples Coupled with 6, 8, or 10 h of Enrichment.

Strain	Assay	Detection level (hrs of enrichment)								
		Cantaloupe	Pepper (Jalapeno)	Tomato		Lettuce		Sprouts[b]		
				Red round	Roma	Romaine	Iceberg	Alfalfa	Clover	Mung bean
Braenderup 10N	LAMP	6(1/3)[a]	6(2/3)	6(1/3)	6(1/3)	8(2/3)	8(3/3)	-	-	10(1/3)
	qPCR	6(2/3)	6(1/3)	6(1/3)	6(1/3)	6(1/3)	8(2/3)	10(1/3)	-	10(1/3)
Enteritidis 20N	LAMP	6(2/3)	6(2/3)	6(1/3)	6(1/3)	8(2/3)	6(1/3)	6(1/3)	6(1/3)	6(1/3)
	qPCR	10(2/3)	6(1/3)	8(3/3)	8(3/3)	8(3/3)	8(3/3)	10(2/3)	8(1/3)	10(2/3)
Montevideo 1H[c]	LAMP	8(1/4)	6(1/4)	6(1/4)	6(1/4)	8(2/4)	8(3/4)	10(1/4)	10(1/4)	10(1/4)
	qPCR	8(1/4)	6(1/4)	6(1/4)	6(1/4)	8(3/4)	8(3/4)	-	10(1/4)	-
Newport 1240H	LAMP	6(3/3)	6(1/3)	6(1/3)	6(1/3)	8(3/3)	8(3/3)	10(2/3)	8(1/3)	8(1/3)
	qPCR	6(3/3)	6(1/3)	6(1/3)	8(3/3)	8(3/3)	8(3/3)	10(1/3)	-	8(1/3)
Saintpaul 1358H	LAMP	6(1/3)	6(1/3)	8(3/3)	6(1/3)	8(3/3)	8(3/3)	10(1/3)	-	-
	qPCR	8(3/3)	6(1/3)	8(3/3)	6(1/3)	8(3/3)	8(3/3)	-	-	-
Heidelberg 1364H	LAMP	8(2/3)	8(1/3)	6(1/3)	8(3/3)	8(3/3)	8(3/3)	-	-	-
	qPCR	8(2/3)	8(3/3)	8(3/3)	6(1/3)	8(3/3)	8(3/3)	10(1/3)	10(1/3)	10(1/3)
Poona 2861H	LAMP	6(1/3)	6(1/3)	6(2/3)	6(1/3)	6(1/3)	8(3/3)	-	-	8(1/3)
	qPCR	6(2/3)	6(2/3)	8(3/3)	10(3/3)	8(3/3)	8(2/3)	-	8(1/3)	10(2/3)
Javiana NR4296	LAMP	6(1/3)	6(2/3)	6(2/3)	6(2/3)	8(3/3)	6(2/3)	6(1/3)	-	8(2/3)
	qPCR	8(2/3)	6(1/3)	6(3/3)	6(2/3)	6(1/3)	6(2/3)	8(1/3)	8(1/3)	10(3/3)
Muenchen	LAMP	6(1/3)	6(2/3)	8(3/3)	8(3/3)	6(1/3)	6(1/3)	10(2/3)	6(1/3)	6(1/3)
NR4311	qPCR	6(1/3)	6(1/3)	6(1/3)	6(1/3)	6(1/3)	8(1/3)	10(1/3)	8(1/3)	6(1/3)
Typhimurium	LAMP	8(3/3)	6(2/3)	6(2/3)	8(3/3)	8(3/3)	8(3/3)	-	-	6(1/3)
NR4333	qPCR	6(2/3)	6(2/3)	6(2/3)	6(1/3)	8(2/3)	8(3/3)	8(1/3)	10(3/3)	6(1/3)

[a] The shortest enrichment hours that can be detected by assays. The number in the parentheses indicated the time of positive results got out of three repeats.

[b] All the sprouts samples were inoculated with *Salmonella* culture targeting at 10^2 CFU/ 25g sample.

[c] *Salmonella* Montevideo 1H test was repeated 4 times due to its low survival rate in produce.

Comparison of DNA extraction methods With the same sample, the LAMP results showed that the extraction efficiency ranked in the following order: PrepMan > TE > FTA card > direct boiled. The LAMP results in cantaloupe shown in Figure 5.2. All the other produce except sprouts gave similar results (data not shown). The same trend was obtained from the qPCR test (data not showed). However, qPCR did not get constant and meaningful results from the FTA card templates.

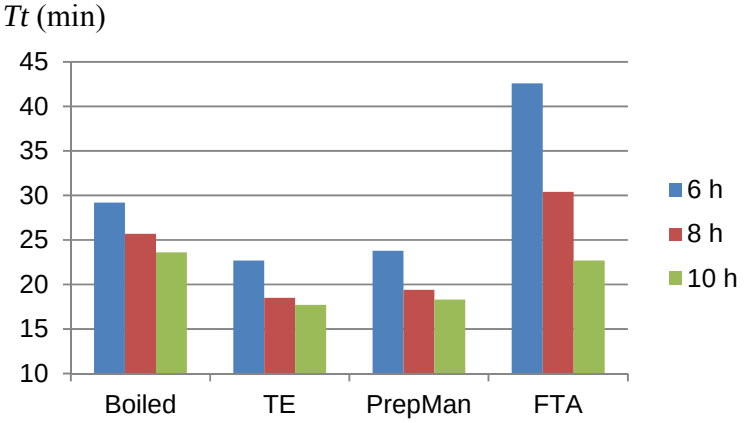

Figure 5.2 Effects of Four DNA Extraction Methods on *Salmonella* Detection in Produce by LAMP.

Discussion

It is well known that salmonellosis was commonly originated from meat and poultry products (2, 14, 15, 18). However, recent documents indicated that the foodborne outbreaks associated with fresh produce have been on the rise (5-7). The 10 *Salmonella* serovars used in this study was selected according to their high relevance associated with *Salmonella*-linked foodborne outbreaks (13). There was no significant difference in detection rates between these strains. Therefore, for further study of LAMP in food application, one or two serovars, such as *Salmonella* Enteritidis and Typhimurium, would be sufficient to present the trend as representatives for *Salmonella*.

The nine produce were picked based on their frequency of being involved in *Salmonella* outbreaks (13). The contamination of produce could be from multiple sources including contaminated manure, irrigation water, fertilizers, wildlife, wash water, processing equipment, or packaging (22). It has been reported the genetic basis of *Salmonella* attachment to plants was that *Salmonella* regulated production of cellulose using the *bcsA* gene and the O-antigen capsule gene *yihO* to attach and colonize plants (3). Thus, our study used surface inoculation for artificial contamination in produce. And 48 h of ageing time under refrigerated temperature was involved in the procedure to mimic the real-world events. Previous studies concluded that low level of bacteria recovered from produce may be resulted from the binding of bacteria cells to plant tissue, death of bacteria due to exposure to plant antimicrobial compounds, or drying after inoculation (4, 37). The attachment strength of pathogen cells varies among types of produce. In Patel and Sharma's study, the overall attachment strength of *Salmonella* to three produce types was in the order of romaine lettuce > iceberg lettuce > cabbage (31). Additionally, it has been reported that the dehydration stress imposed on bacteria was different from produce types as well (4). Overall, surface inoculation and ageing time increase the stress on bacteria survival in produce. When compared to previous study done in our lab (10), the direct test limit changed from 10^3 CFU/g in previous study to 10^4 –10^5 CFU/ 25g in this experiment for cantaloupe.

To closely monitor the pathogen existence in produce, a rapid, high precision, simple, as well as economic method is required. Based on our data, both LAMP and qPCR could serve as the quick screening tool for *Salmonella* detection due to their comparable sensitivity and specificity. With the isothermal feature, LAMP has better potential to fit on small, simple, portable machines.

Sample preparation is one of the key steps in a complete analysis for pathogens and toxins in foods (17). As pathogens are usually represented in low numbers in food products, the main purpose of sample preparation is to concentrate or increase the target bacteria, separate the target from the large background flora, or reduce the inhibition effects in the samples. In this study, two commercial quick DNA extraction products were evaluated. In TE and PrepMan procedure, 100 µl templates were made from 1ml of produce homogenates. It is reasonable that their templates gave out positive signal faster than the direct boiled method since there was a 10-fold concentration in these templates than in the direct boiled ones. The FTA card was featured for its room temperature storage for DNA sample. However, it was not use-friendly, not quantitative, and very labor-intensive and time consuming if a large quantity of samples were applied. Moreover, the paper disc had noticeable impact when it was used in qPCR detections, possibly due to the block of fluorescence reception. Overall, liquid templates would be more welcome to molecular assays.

Coupled with enrichment, the effects of different food commodities on detection limit became more obvious. In red round tomato and roma tomato samples, LAMP could detect as low as 1.4 CFU/ 25g tomato with 6 h enrichment, while qPCR required 8 h to generate positive results. Kim et al. reported that the acidic pH in tomatoes would cause reduction in viable *Salmonella* cells (24). Shearer et al.'s study also showed that PCR only could detect *Salmonella* in 3 out of 6 repeats with the inoculation level of 1 CFU/ 25g tomato after 24 h enrichment (35). Thus, LAMP may be more tolerant to organic acids in tomatoes than qPCR. LAMP and qPCR demonstrated similar performance in cantaloupe, jalapeno pepper, romaine lettuce and iceberg lettuce homogenates with constant detection at around 8 h. The three sprouts samples, mung bean sprouts, alfalfa sprouts, and clover sprouts, showed strong inhibition on the two molecular

assays. The study by Shearer group demonstrated that BAX PCR generated faint DNA bands in the alfalfa sprouts inoculated samples, while clear bands were displayed in cantaloupe and tomato samples (35). Besides the possible plant compounds that may bring in some inhibition, the main inhibitory factor in spouts should be its large volume of background flora. The sample test showed that the aerobic plate counts (APCs) for spouts were 10^6 to 10^7 CFU/g, while the APCs for other produce ranged 0 to 100 CFU/g. It agreed with a previous report that showed the APCs increased from 10^3 to 10^4 CFU/g in seeds to 10^7 CFU/g in sprouts during sprouting (23).

In conclusion, the LAMP assay was demonstrated as a rapid, reliable, and robust method for the detection of *Salmonella* in produce. Nonetheless, the low efficiency associated with sprout detection warrant further investigation.

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CHAPTER 6: DEVELOPMENT OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) COUPLED WITH BIOLUMINESCENT ASSAY IN REAL-TIME (BART) FOR *SALMONELLA* DETECTION

Introduction

Salmonella is an important cause of human illness in the United States and worldwide. According to the U.S. Centers for Disease Control and Prevention, nontyphoidal *Salmonella* is estimated to cause one million illnesses each year(31). The diseases caused by *Salmonella* infection are termed salmonellosis, which is gastroenteritis in most cases. However, severe or fatal symptoms may occur when the bacteria spread from the intestine to the blood stream and other sites of the body (28). Many foods could serve as vehicles for *Salmonella* infection by either harboring the pathogens by themselves or obtaining from other foods through cross-contamination during handling (2). A recent outbreak caused by cantaloupes contaminated with *Salmonella enterica* serovar Typhimurium and Newport resulted in a total of 261 illnesses from 24 states. Among them, 94 persons were hospitalized and three deaths were reported in Kentucky (5). And the U.S. Food and Drug Administration (FDA) announced a nationwide recall of cantaloupe by Chamberlain Farms of Owensville, Indiana. To better ensure food safety and reduce foodborne illnesses and outbreaks resulted from the consumption of *Salmonella*-contaminated foods, rapid, reliable, and robust methods are required to conduct more vigilant monitoring by the food industry.

Many nucleic acid amplification methods have been developed for *Salmonella* detection (14, 27, 29). Polymerase chain reaction (PCR) and real-time PCR (qPCR) assays are featured as fast and accurate screening tool. . But the required instruments for thermal cycling as well as lights detection are usually big, sophisticated and expensive, which eliminates their wide applications. Loop-mediated isothermal amplification (LAMP) assay, a novel nucleic

amplification technique, was first developed in 2000 (24). LAMP has been widely used in detecting foodborne pathogens, such as *Campylobacter* (36), Shiga toxin producing *Escherichia coli* (34), norovirus (37), *Staphylococcus aureus* (19), and *Vibrio vulnificus* (11), as well as *Salmonella* (6, 12, 17, 35). Under isothermal conditions (60 to 65°C), LAMP used six specially designed primers to recognize eight target regions, and a strand-displacing *Bst* DNA polymerase to produce a dumbbell shape stem-loop DNA structure, followed by quasiexponential amplification of this structure. Several copies of target DNA can be amplified to 10^9 copies within an hour (23, 24).

LAMP products may be detected through the the production of inorganic pyrophosphate (PPi), a low-molecular weight byproduct from all polynucleotide amplification (30, 33). Due to the large quantity of DNA generated by LAMP, there is visible white precipitation due to the formation of magnesium pyrophosphate, resulting in turbidity change in the reaction tube. (26) This turbidity change may be observed either through end-point detection or real-time monitoring using a turbidimeter.(20, 21). Recently, a Bioluminescent Assay in Real-Time (BART) was developed by Gandelman et al. (10), which reported the exponential increase of inorganic pyrophosphate (PPi) produced during the isothermal amplification of a specific nucleic acid target through bioluminescent output. PPi produced stoichiometrically during nucleic acid synthesis was converted into ATP by the enzyme ATP sulfurylase, and then quantitatively detected by bioluminescence using thermostable firefly luciferase (25). The assay showed a unique kinetic signature for nucleic acid amplifications, an identifiable light output peak that reflected the concentration of original target nucleic acid. Since the quantification capability of BART is based on determination of peak time rather than absolute intensity of light emission, it can be run on simple light detectors, thus promotes the potential application of LAMP in field

test with small and portable equipment. This novel real-time, closed-tube luminescent report system coupled with LAMP has been used for detecting genetically modified (GM) maize target DNA which detected at low level of 0.1-5.0% GM and showed higher tolerance to plant sample-derived inhibitors than qPCR (15).

Given the promising results shown in LAMP-BART detection of GM products, we hypothesized that this platform may be adopted for the detection of foodborne pathogens such as *Salmonella*. The aim of this study was to develop a LAMP reagent mix suitable for the BART platform and examine this novel LAMP-BART combination in detecting *Salmonella* in spiked produce samples.

Materials and Methods

Bacterial strains and DNA template preparation. *Salmonella enterica* serovar Typhimurium LT2 was cultured on trypticase soy agar (TSA; BD Diagnostic Systems, Sparks, MD) at 37^{o}C overnight. Several well-isolated *Salmonella* colonies were picked from the overnight TSA plates and transferred to 8 ml of trypticase soy broth (TSB; BD Diagnostic Systems) and incubated at 37^{o}C for 16 h to achieve the mid-log phase (OD_{600} = 1; approximately 10^9 CFU/ml). The *Salmonella* culture was 10-fold serially diluted in 0.1% peptone water and the exact cell number was determined by standard plate counting. To test assay sensitivity, aliquots (500 µl) of each dilution were used to prepare DNA templates by the boiling method (95^{o}C, 10 min). Among them, the solution with the bacteria level of 10^6 CFU/ ml was used as the template for positive control.

LAMP-BART and LAMP conditions. The *Salmonella* invasion gene (*invA*; GenBank accession number M90846) was used as the target for designing LAMP primers. A set of six

primers (Table 6.1), two outer (F3 and B3), two inner (FIP and BIP), and two loop (Loop-F and Loop-B) were designed by the PrimerExplorer 4 software (Fujitsu Limited, Tokyo, Japan).

Table 6.1 Primers Used in Two LAMP Platforms in this Study.

Primer name	Sequence (5'-3')	Position[a]	Reference
Sal4-F3	GAACGTGTCGCGGAAGTC	484-501	This study
Sal4-B3	CGGCAATAGCGTCACCTT	665-682	
Sal4-FIP	GCGCGGCATCCGCATCAATA-	573-592 (F1c)	
	TCTGGATGGTATGCCCGG	516-533 (F2)	
Sal4-BIP	GCGAACGGCGAAGCGTACTG-	593-612 (B1c)	
	TCGCACCGTCAAAGGAAC	635-652 (B2)	
Sal4-Loop-F	TCAAATCGGCATCAATACTCATCTG	538-562	
Sal4-Loop-B	GAAAGGGAAAGCCAGCTTTACG	613-634	
Sal8-F3	CGGCCCGATTTTCTCTGG	503-520	(6)
Sal8-B3	CGGCAATAGCGTCACCTT	665-682	
Sal8-FIP	GCGCGGCATCCGCATCAATA-	573-592 (F1c)	
	TGCCCGGTAAACAGATGAGT	527-546 (F2)	
Sal8-BIP	GCGAACGGCGAAGCGTACTG-	593-612 (B1c)	
	TCGCACCGTCAAAGGAAC	635-652 (B2)	
Sal8-Loop-F	GGCCTTCAAATCGGCATCAAT	547-567	
Sal8-Loop-B	GAAAGGGAAAGCCAGCTTTACG	613-634	

[a] The positions are numbered based on the coding sequence of *Salmonella invA* gene (GenBank accession number M90846).

The LAMP-BART reagent mixes were formulated according to two previous published studies (10, 15). Two formulas were used, one termed half panel and the other one full panel. The LAMP-BART half panel mix in a total volume of 25 µl contained 1× ThermoPol reaction buffer (New England Biolabs, Ipswich, MA), 6 mM $MgSO_4$, 1.2 mM dNTP, 0.1 µM F3 and B3, 1.8 µM FIP and BIP, 1 µM Loop-F and Loop-B, 8 U of *Bst* DNA polymerase (New England Biolabs), 100 µg/ml luciferin potassium salt (LH_2; Sigma-Aldrich, St. Louis, MO), 250 µM adenosine 5' phosphosulfate (APS; Sigma-Aldrich), 0.5 U/ml ATP sulfurylase (New England Biolabs), 5.6 µg/ml Ultra-Glo firefly luciferase (UGrLuc; Promega, WI), and 2 µl of DNA template. The LAMP-BART full panel mix included all the reagents in the half panel as well as 60 mM KCl, 0.4 mg/ml PVP, 10 mM DTT, and 87 mM trehalose (Sigma). The reaction was carried out at 60°C for 75 min in a 3M™ Molecular Detection Instrument (3M, St. Paul, MN).

The peak time (T_{max}; min) was determined when the bioluminescence signal reached the peak value in the amplification graph.

As a comparison, LAMP was performed under the condition described previously (6); the same reagent mix and templates were carried out at 63°C for 60 min and terminated at 80°C for 5 min in a real-time turbidimeter (LA-500; Eiken Chemical Co., Ltd) which obtained the turbidity readings at 650 nm every 6 s. The time threshold (*Tt*; min) value was determined when the turbidity increase measurement (the differential value of the moving average of turbidity) exceeded a threshold value of 0.15.

Sensitivity and quantitative capability Half panel and full panel were tested to determine the detection limit of assays. The aliquots (2 µl) of the 10-fold serially diluted *Salmonella* LT2 templates prepared above were subjected to both LAMP-BART and LAMP amplifications. Sensitivity tests were repeated three times.

Effects of facilitators on assay performance Four facilitators (KCl, PVP, DTT, and trehalose) at the concentration that used in full panel were added in the reaction individually and then combined in two or three. Half panel and full panel were tested as well. Each test included two positive samples and three negative controls, and was repeated three times each.

Detection of *Salmonella* in spiked produce Five produce samples (cantaloupe, jalapeno pepper, iceberg lettuce, round red tomato, and alfalfa sprouts) were purchased from local supermarket. To facilitate homogenization, leafy greens were cut into 4 cm^2 square using sterile scissors, and cantaloupe, pepper and tomatoes were sliced into fresh-cut size pieces (2.5 cm^3 cubes and 1/8 fruit wedge, respectively) using a sterile knife. Each produce was weighed 25 g out as one sample. After *Salmonella* check following BAM method (1), confirmed *Salmonella*-negative produce was inoculate 1.5 ml of *Salmonella* Typhimurium NR4333 to obtain the target

inoculum level (10^9-10^4 CFU/25g for high level and 1-20 CFU/25 g for low level). The spiked samples were air-dried in a laminar flow biosafety hood for 2 h and stored at 4°C for 48 h.

After refrigerated storage, direct testing was applied to the high-level inoculated samples. Each sample was mixed with 225 ml of pre-warmed buffered peptone water (BPW; BD Diagnostic Systems) and homogenized for 2 min in a food stomacher (Model 400; Tekmar Company, Cincinnati, OH) to produce 1:10 produce-BPW homogenate. The homogenate was analyzed for the presence/absence of endogenous *Salmonella* following methods. Briefly, 1 ml of food homogenate was centrifuged at 900 *g* for 3 min to remove large produce tissues. The DNA was extracted using the PrepMan® Ultra sample preparation reagent (Life Technologies, Grand Island, NY). Aliquots (2 µl) of the extracted DNA were used for both LAMP and qPCR amplifications. For the low-inoculated sample, enrichment was performed by incubating the *Salmonella*-spiked produce homogenate at 37°C for 6, 8, and 10 h. After enrichment, the homogenate was processed similarly as described above for direct testing. The produce tests were repeated three times.

Data analysis For sensitivity data, means and standard deviations of *Tt* or *Ct* values for detecting 10-fold serially diluted *S.* Typhimurium LT2 in pure culture were calculated by using the Microsoft Excel software (Microsoft, Seattle, WA). The detection limits (CFU/reaction in pure culture) were presented as the lowest number of cells that could be detected by the assays. Standard curves to quantify *Salmonella* in pure culture were generated by plotting *Tmax* or *Tt* values against log CFU/reaction, and linear regression was calculated using Microsoft Excel. Quantitative capabilities of the assays were derived based on the correlation coefficient (R^2) values from the standard curves. For data obtained from LAMP-BART and LAMP, means and

standard deviations of *Tmax* or *Tt* values for detecting 10^6 CFU/ml *Salmonella* in pure culture were calculated using Microsoft Excel as well.

Results

Sensitivity and quantitative capability of assays in pure cultures Table 6.2 summarizes the sensitivities and quantitative capability of the LAMP-BART and LAMP assays when testing 10-fold serial dilutions of *Salmonella* Typhimurium LT2. In pure-culture testing, half and full panel of LAMP-BART assay consistently detected down to 1.8×10^4 CFU/ ml, which equals to 36 cells per reaction (representative result generated by LAMP-BART was shown in Figure 6.1).

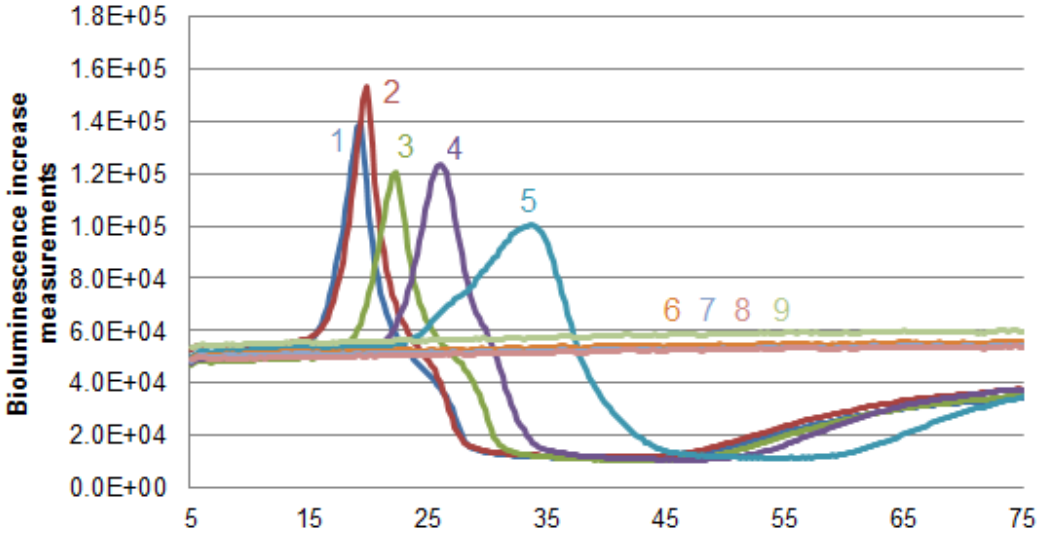

Figure 6.1 A Standard Curve Generated by LAMP-BART Coupled with Sal8 Primer Set when Testing 10-Fold Serially Diluted *Salmonella* Typhimurium LT2 in Pure Culture. Symbols 1 to 7 correspond to 10-fold serial dilutions of LT2 ranging from 3.6×10^5 CFU/reaction to 36 CFU/reaction; symbol 8 and 9 are water as negative controls.

In one out of three repeats, the LAMP-BART assay using Sal8 primer set was capable of detecting at a one log lower concentration (i.e., 3.6 cells per reaction). For *Salmonella* cells between 3.6×10^4 and 36 CFU/reaction, the average *Tmax* values of half panel LAMP-BART assays ranged from 11.7 to 22.3 min for the Sal4 set, and 10.3 to 19.4 min for the Sal8 set (data

not shown). There was a striking difference of LAMP-BART performance between the half panel and the full panel. Using the same templates, the *Tmax* values of full panel assays were in the range of 29.3 to 43.4 min for the Sal4 set and 18.8 to 33.1 min for the Sal8 set, respectively. However, false positive was an obvious problem occurred in half panels using either primer set (Table 6.2).

Table 6.2 Comparison of Sensitivity and Quantification Capabilities of Half and Full Panel LAMP-BART and Conventional LAMP Assays when Testing Serially Diluted *Salmonella enterica* serovar Typhimurium LT2 in Pure Culture.

Primer set	Panel	Assay	Detection limit (CFU/reaction)	Quantification equation[b]	Linear R^2	False positive[c]
Sal4	Half	LAMP-BART	36	y = 2.58x + 8.02	0.9238	5/6[d]
		LAMP	3.6	y = 2.52x + 16.20	0.9109	0/6
	Full	LAMP-BART	36	y = 3.76x + 24.20	0.9613	0/6
		LAMP	36 (2/3)[a]	y = 3.71x + 30.35	0.8522	0/6
Sal8	Half	LAMP-BART	3.6 (1/3)	y = 2.20x + 7.36	0.9440	6/6[e]
		LAMP	36	y = 2.21x + 16.55	0.9245	0/6
	Full	LAMP-BART	36	y = 3.52x + 13.36	0.8987	0/6
		LAMP	3.6 (1/3)	y = 2.65x + 26.41	0.8671	0/6

[a] The number in the parentheses indicates the positive results out of the three repeats at that level.
[b] Quantitative equation and R^2 were calculated based on the linear relationship of average *Tmax* or *Tt* values and log CFU/reaction between the cell level ranging from 10^1 to 10^5 CFU/reaction for pure culture.
[c] The odds of false positive shows in the total of six repeats.
[d] The *Tmax* range for Sal4 half panel was from 36-44 min.
[e] The *Tmax* range for Sal8 half panel was from 22-33 min.

Effects of facilitators for polynucleotide amplification on LAMP-BART. The effects of the four facilitators on the amplification efficiency of LAMP-BART and conventional LAMP assays were tested by the addition of each facilitator individually or by two or three combinations (Figure 6.2).

It showed that KCl obviously slowed down both LAMP-BART and LAMP assays by as much as 30 min in Sal4 primer set. For further analysis, LAMP-BART panels with Tmax or Tt less than 25 min were picked out. Comparing their false positive rates, all the combinations containing trehalose demonstrated better performances than others, which had short response time for

Salmonella template at the level of 1.8 $\times 10^6$ CFU/ml while remaining relatively low false positive rates. With 100% false positive rate, half panels were not desirable for further evaluation. With single compound addition, the four compounds showed their inhibition effects on LAMP-BART in the order of PVP < DTT < Trehalose < KCl. Among them, KCl strongly inhibited the amplification in Sal4 LAMP-BART.

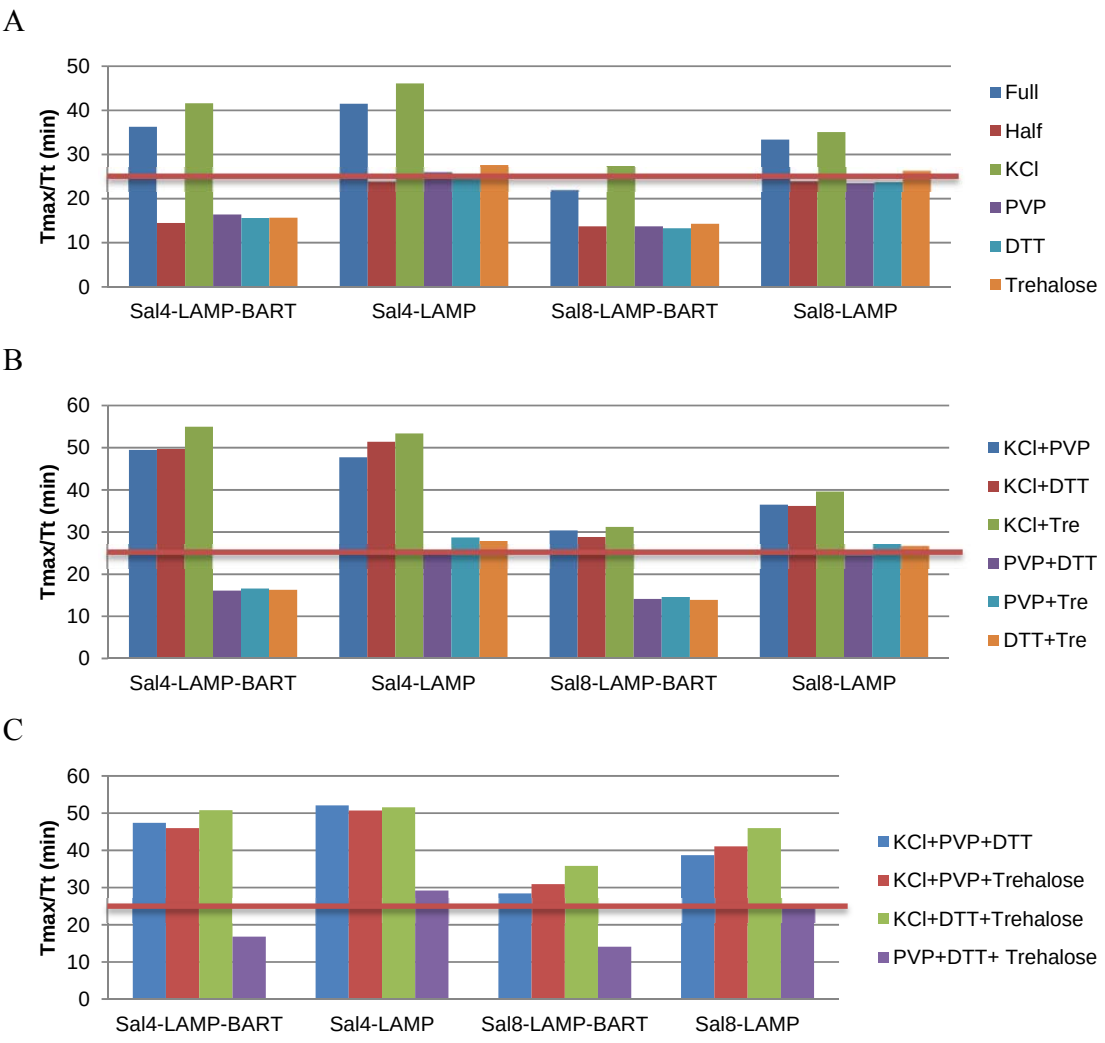

Figure 6.2 Effects of Four Facilitators on LAMP-BART and LAMP Performance Individually and by Combination. (A) Comparison of performances of LAMP-BART and LAMP assays with full, half and half panel combined with single compound. (B) Comparison of performances of LAMP-BART and LAMP assays with half panel formula combined with two compounds. (C) Comparison of performances of LAMP-BART and LAMP assays with half panel formula combined with three compounds. And the red line in each chart stands for the *Tmax* level of 25 min.

The addition of two or three compounds combination tests confirmed that KCl would postpone the *Tmax* value for LT2 template significantly. As well as detection efficiency, less false positive rate is another parameter that needs to be concerned. Among all the LAMP-BART with the capability of detecting *Salmonella* at 1.8×10^6 CFU/reaction level within 25 min, all the non-KCl combinations and the Sal8 full panel demonstrated acceptable outcomes (Table 6.3).

Table 6.3 False Positive Rate of the LAMP-BART Assays with Relatively Better Performance on Detecting *Salmonella* LT2 culture at the Level of 10^6 CFU/ ml.

Panel		Sal4		Sal8	
		False positive rate (%)[a]	*Tmax* range (min)	False positive rate (%)	*Tmax* range (min)
Half		82	36-44	100	22-33
Half + 1 compound	PVP	100	30-51	100	23-27
	DTT	100	37-50	100	26-32
	Trehalose	56	50-65	89	34-41
Half + 2 compounds	PVP+DTT	100	30-40	78	28-33
	PVP+Trehalose	33	50-75	44	36-42
	DTT+Trehalose	44	46-63	78	32-52
Half + 3 compounds	PVP+DTT+Tre	40	52-69	67	31-42
Full		NA[b]	NA	16	40-66

[a] The rate is calculated by the time of false positive results out of the total repeats, for example, full panel was tested in the effects test as well as the sensitivity pre-test and sensitivity test and repeated 23 times in total.

[b] The full panel LAMP-BART with Sal4 set was not selected since it gave slow response on positive templates (Tmax > 25 min).

Detection of *Salmonella* in spiked produce After comparison, Sal8 full panel was used for testing spiked produce samples. In the direct testing, the average *Tmax* valves were increased from 22.5-23.2 to 33.3-34.7 min when the cell levels decreased from 10^9 CFU/25g produce to 10^4 CFU/25g (data not shown), which were comparable to the detection limit of LAMP applied in produce testing (as described in Chapter 5). There was no obvious difference among the produce types tested except for Jalapeno pepper (Table 6.4).

Table 6.4 Detecting *Salmonella* in Spiked Produce Samples by LAMP-BART.

Produce	Detection limit (CFU/25g)	Detection in Enriched samples (h)		
		6	8	10
Cantaloupe	10^4	-	+ (2/3)	+
Jalapeno pepper	10^5	+ (1/3)	+ (2/3)	+
Red round tomato	10^4	+ (2/3)	+	+
Alfalfa sprouts	10^4	-	-	-
Iceberg lettuce	10^4	-	+ (2/3)	+

In the produce spiked with low level of *Salmonella* cells (1-10 CFU/25g), LAMP-BART could detect the target gene after 8 h enrichment (Table 6.4). Agreeable with the data from LAMP testing, the *Salmonella* cells in sprouts samples were not detectable by LAMP-BART.

Discussion

BART is a novel detection platform for isothermal nucleic acid amplification techniques such as LAMP (10). The bioluminescent signal was converted from ATP and originally from PPi produced during the nucleic acid amplification (25). The half panels only included all the essential compounds for LAMP-BART reaction which were LAMP reagents, substrates and enzymes for Enzymatic Luminometric Detection of Inorganic pyrophosphate Assay (ELIDA). In both Sal4 and Sal8 primer sets, half panel LAMP-BART was about 8 to 10 min faster than LAMP. Previously, the lowest reported detection limits for LAMP-BART were 5.5 copies DNA /reaction (10). The lowest detectable number by our LAMP-BART assay was 36 *Salmonella* cells per reaction, agreeable with the results obtained in a previous study (40 copies) (15). Instead of pure DNA, the present study only used crudely boiled to prepare the templates. The purity of templates might be not enough to get to higher sensitivity. One unexpected result was the detection limit of half panel LAMP with Sal4 primer set (3.6 CFU /reaction) that was one log lower than half panel Sal4 LAMP-BART. This actually was the same level as the limit of conventional LAMP targeting *Salmonella invA* gene that reported before (6, 12). In a positive LAMP-BART assay, the concentration of all four dNTPs, APS, PPi and ATP change

continuously (10). ATP and dATP are luciferase substrates, other dNTPs are competitive inhibitors of luciferase and PPi would stimulate/inhibit the reaction depends on its concentration (8, 9). Therefore, changes of their levels have a significant impact on BART light output. Half panel LAMP-BART achieved the linear correlation (R^2) of 0.92 and 0.94 for Sal4 and Sal8 set, respectively (Table 6.2), which were higher than corresponding LAMP assays. The same trend was observed in the full panel. The quantitative correlation reported in Gandelman et al.'s study was 1, which was fitted well in the template with the levels between 3 to 9 log CFU/reaction. The linear relationship between the positive signals and the template concentrations poorly fitted the linear regression when the cell number or DNA copy number was lower than 10^2 CFU (copies) per reaction. The results we obtained was comparable to the linear correlation (R^2 = 0.97) for quantitating viable *Salmonella* cells in pure culture ranging from 10^2 to 10^5 CFU/reaction in the study by Chen et al (6).

Among the four facilitators added into the LAMP-BART reagent mix, KCl exhibited a significant effect on the assay performance. The potassium ions bind to the charged phosphate ions of the DNA backbone and stabilize double strand formation. Increasing the KCl concentration of a PCR buffer will cause longer DNA to denature slower than shorter DNA, allowing for the preferential amplification of shorter molecules (100 bp – 1000 bp) (13). The optimal range of KCl was 50 to 100 mM in a standard PCR reaction (3). The effect of KCl concentration on LAMP based has not been studied yet. The present study found that KCl strongly inhibited the LAMP-BART and increased *Tmax* by approximately 13 min for Sal8 set and 25 min for Sal4 set, respectively (Figure 6.2). PVP has been reported as an enhancer for PCR using silicon-glass chips due to the significant surface passivation of silicon matrics (18). But it also might inhibit PCR depends on its concentration (16). DTT is known as an enzyme stabilizer

because of the protection of the sulfhydryl groups of cysteine residues (7). And the previous study showed that DTT enhanced PCR efficiency by stabilizing the *Taq* DNA polymerase (22). Besides PVP and DTT, trehalose is also an enhancer that greatly facilitates the yield of the PCR amplification products by reducing the DNA melting temperature and thermostabilizing the *Taq* polymerase (4, 32). In this study, the results in the effect comparison part did not show noticeable enhance from PVP, DTT, or trehalose. To act as a desirable facilitator, the compound needs to display good impact on promotion or maintenance of the assay efficiency as well as reduction of false positive or false negative (Table 6.3). Trehalose might be a good facilitator for LAMP-BART assays, especially coupled with the Sal4 set. This might be the reason that it was included in the recent LAMP-BART application rather than the assay just developed (15). For a quick evaluation, Sal8 full panel was selected for produce sample tests due to its acceptable response time and low false positive rate.

In the produce testing, LAMP-BART showed comparable results to LAMP assay (reported in Chapter 5). The use of the BART platform is simple and robust, and may potentially be applied in the field or resource-limited area using small portable instrument. Further extensive evaluation on the application of LAMP-BART in various food commodities and environmental samples is needed to bring this assay close to application.

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CHAPTER 7: CONCLUSIONS

In this dissertation research, an isothermal nucleic acid amplification technique termed loop-mediated isothermal amplification (LAMP) was evaluated for its robustness and application in the detection of *Salmonella* serovars from various food commodities, in particular, shell eggs and various produce items. LAMP was further developed by coupling with bioluminescent assay in real-time (BART) which promises an easier platform and potentially wider application. LAMP assays achieved robust detection of *Salmonella* cells under abusive assay preparation conditions (25 and 37°C with holding up to 30 min), running temperatures (60-68°C), and pH values, while PCR performed markedly poorly under abusive pH values. In the presence of inhibitors including culture media and biological substances, LAMP assays also demonstrated greater tolerance than PCR and real-time quantitative PCR (qPCR). When 20% or more food juices, including chicken rinse, egg homogenate, and ground beef juice, ground pork juice, produce homogenate and soil sample were added into the reaction mix, PCR amplifications were inhibited completely, but not LAMP. This study revealed the potential of LAMP serving as a rapid and robust alternative to PCR-type assays for the routine testing of *Salmonella* in food.

Extensive evaluation of LAMP assays also confirmed the highly specific and sensitive nature of LAMP. The detection limits were approximately 1 cell per reaction for both *Salmonella* Enteritidis and *Salmonella* Typhimurium, which was 100-fold more sensitive than PCR. In artificially contaminated egg homogenates, LAMP assays could detect as low as 10^4 CFU/ 25 ml egg homogenate without enrichment and about 1 cell per 25 ml egg homogenate with 8 h enrichment for both *Salmonella* serovars. Standard

curves generated in direct testing suggested a good linear relationship between cell numbers and LAMP turbidity signals. In contrast, PCR was not able to detect either *Salmonella* serovar in egg homogenates with inoculum less than 10^7 CFU/ 25 ml egg homogenate with or without enrichment. It indicated that LAMP assay could be used a rapid and reliable tool to detect *Salmonella* in shell eggs for controlling potential microbial hazards in eggs.

Another food application study evaluated LAMP performance in comparison with real-time quantitative PCR (qPCR) using a large panel of strains, and validated the method for the rapid, reliable, and robust detection of *Salmonella* in various produce items. The spiked produce samples (cantaloupe, lettuces, pepper, sprouts, and tomatoes). were surface-inoculated with low levels (1-20 cells per 25 g of produce) of *Salmonella* and detected after aging at 4°C for 48 h to mimic the real-world events. No false-positive or false-negative results were observed among the 168 strains by either LAMP or qPCR assays. The limits of detection of various *Salmonella* strains belonging to various serovars were 1 to 10 cells/ reaction in pure culture and 10^4 to 10^6 CFU/25 g in spiked produce samples, which were comparable to qPCR. In produce samples (25g) spiked with 1-10 cells of respective *Salmonella* strains, LAMP consistently achieved accurate detection after 6 to 8 h of enrichment, with the exception of sprouts. Thus, the LAMP assay was also a rapid, reliable, and robust method for *Salmonella* screening in produce.

Finally, we developed a LAMP-BART assay for *Salmonella* detection. The assay was capable of detecting approximately 3.6-36 CFU per reaction of *Salmonella* in pure culture and 1.5×10^4 CFU/g in spiked food samples. When applied real-time LAMP for the quantitative detection of *Salmonella* by targeting the *invA* gene, standard curves

generated in both Sal4 and Sal8 set showed good linear relationship between *Salmonella* cell counts and the light or turbidity signals. Given that BART uses light detection rather than turbidity detection, this new platform can potentially achieve more simple and robust *Salmonella* detection in various food commodities.

In conclusion, this dissertation research provided comprehensive development and evaluation on LAMP performance against abusive conditions, assay inhibitors and food matrices. The speed, specificity, sensitivity, and robustness of LAMP assays demonstrated their potential to be used as an invaluable tool for the food industry and regulatory agencies to facilitate the prompt identification of *Salmonella* contamination problems in various high-risk food commodities, thereby reducing the incidence of foodborne illnesses and deaths resulted from the consumption of *Salmonella*-contaminated food. With the newly implemented egg final rule and the produce preventive measures, such assays would prove beneficial to better ensure the safety of our food supply and protect public health.

VITA

Qianru Yang was born in Changshu, Jiangsu Province, People's Republic of China. After completing high school in her hometown, she attended Southern Yangtze University, Wuxi, China, and graduated with a Bachelor of Science in Food Science and Technology in June 2004. She obtained her Master of Science in Food Science and Technology from Oregon State University in January 2009. After her technician work in the quality assurance department of Fisher Nuts John B. Sanfilippo & Son, Inc. in Chicago, she joined Louisiana State University for her doctoral program in Food Science in August 2009. Currently, she is a candidate for the degree of Doctor of Philosophy in Food Science in the College of Agriculture. She will receive her doctoral degree in spring 2013.

During her graduate career, Qianru Yang has been actively involved in multiple research projects and an active member in the Food Science Club. She is a student member of the Institute of Food Technologists (IFT), the American Society for Microbiology (ASM), and the International Association for Food Protection (IAFP). She received Economic Development Assistantship (EDA) Award by the Louisiana Board of Regents and Graduate School Supplement Award by the LSU Graduate School from 2009 to 2013. She was awarded Gamma Sigma Delta Graduate Student Merit Honor Roll in 2011.