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Development and Application of Serogroup-independent and Serogroup-specific Loop-mediated Isothermal Amplification Assays for Detecting Shiga Toxin-Producing Escherichia coli

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DEVELOPMENT AND APPLICATION OF SEROGROUP-INDEPENDENT AND SEROGROUP-SPECIFIC LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAYS FOR DETECTING SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI*

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC), encompassing *E. coli* O157:H7 and non-O157 STEC, is a significant cause of foodborne illnesses and deaths in the United States and worldwide. Shiga toxins (encoded by *stx*) and intimin (encoded by *eae*) are important virulence factors for STEC strains causing infection. Although *E. coli* O157:H7 remains to be the single most common STEC causing disease, the clinical importance of non-O157 STEC is on the rise worldwide. And six major serogroups (O26, O45, O103, O111, O121, and O145) accounted for over 70% of non-O157 STEC infections in the United States.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification technology that has attracted great attention in recent years as a rapid, accurate, and cost-effective pathogen detection method in both food testing and clinical diagnostics. In this dissertation research, two sets of LAMP assays, one serogroup-independent and the other one serogroup-specific, were designed by targeting the *stx1*, *stx2*, and *eae* genes, and seven major STEC serogroup-specific genes (the *wzx* and *wzy* genes), respectively, for the rapid, specific, sensitive, and quantitative detection of STEC strains. The assay performances in pure culture, spiked ground beef, and human stools were evaluated and compared with qPCR. No false positive or false negative results were observed among 120 strains for assay specificity testing. The detection limits for all assays were approximately 1-20 CFU/reaction in pure culture and 10^3-10^4 CFU/g in spiked ground beef, which were comparable to qPCR. Standard curves generated suggested good linear relationships between STEC cell numbers and LAMP turbidity signals. When applied in ground beef samples spiked with two low levels (1-2 and 10-20 CFU/25 g) of STEC cultures, the LAMP assays achieved accurate detection after 6-8 h of

enrichment. The assays also consistently detected STEC in human stool specimens spiked with 10^3 or 10^4 CFU/0.5 g stool after 4 h enrichment, while qPCR required 4-6 h of enrichment.

Given the emerging and evolving nature of STEC serogroups involved in human illness, the LAMP assays developed in this research can serve as rapid and reliable methods for STEC detection in food so that proper control measures can be implemented promptly.

CHAPTER 1: INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC), encompassing *E. coli* O157:H7 and non-O157 STEC, is a leading cause of foodborne outbreaks and deaths worldwide. In the United States, STEC causes an estimated 176,000 illnesses, 2,400 hospitalizations, and 20 deaths annually through the food transmission route. Although non-O157 STEC strains are generally considered less pathogenic than *E. coli* O157:H7, some highly virulent ones (O26, O45, O103, O111, O121, and O145) have distinguished themselves from others by involving in outbreaks associated with the same severe human illnesses, like hemorrhagic colitis (HC) and uremic syndrome (HUS). Additionally, an unprecedented large outbreak of *E. coli* O104:H4 in Germany has resulted in a total of 4,075 cases (including 908 HUS) and 50 deaths as of July 21, 2011. Due to the severity of disease symptoms, STEC O157 was declared as an adulterant in raw ground beef and beef trim by the U.S. Department of Agriculture (USDA) in 1994. On September 13, 2011, USDA announced the intention to declare six additional serogroups of STEC (O26, O103, O45, O111, O121, and O145) as adulterants in non-intact raw beef and the regulation will be enforced beginning on March 5, 2012.

In contrast with the rising clinical importance of STEC strains, the effective detection, isolation, and characterization of this group of pathogens remain problematic, particularly for those strains belonging to various non-O157 serogroups, due to the lack of phenotypic characteristics distinguishable from generic *E. coli*. Immuno-based technology for Shiga toxins and a few STEC serogroups are commercially available, but with reported false positive results and long pre-enrichment treatment. Nucleic acid amplification tests (NAAT) such as PCR and qPCR are rapid, specific, and sensitive, and therefore have been applied for STEC detection by targeting genes coding for major STEC virulence factors and antigen. Nonetheless, the indispensable thermal cycling instrument limits their wide applicability.

In this dissertation research, a novel molecular-based detection method, loop-mediated isothermal amplification (LAMP), was adopted for the rapid, specific, sensitive, and quantitative detection of STEC strains. Two sets of LAMP assays consisting of 10 individual ones were developed and evaluated. In the first set, three LAMP assays were designed to identify all STEC strains with important virulence factors by targeting the *stx1*, *stx2*, and *eae* genes. The second set of seven LAMP assays targeted serogroups-specific genes (*wzx* or *wzy*) of seven major STEC O serogroups (O26, O45, O103, O111, O121, O145, and O157).

This dissertation consists of the following chapters:

1: Introduction about this dissertation research.

2: Literature review on general information and detection methods of STEC.

3: Describes a project on LAMP assays for detecting STEC in beef and human stools.

4: Describes a study on rapid and specific detection of STEC O26, O45, O103, O111, O121, O145 and O157 serogroups in ground beef by LAMP.

5: Conclusions of this study and future work.

Given the demonstrated rapidity, sensitivity, specificity, and robustness of the two sets of LAMP assays, they may effectively serve as serogroup-independent and serogroup-specific screening of STEC strains in ground beef and/or clinical samples, therefore facilitating the rapid and reliable identification of STEC contaminations in high-risk food commodities and prompt diagnosis of STEC infections in clinical laboratories.

CHAPTER 2: LITERATURE REVIEW

General Information on *Escherichia coli* O157:H7 and Related STEC

Microbiology. *Escherichia coli* is a common component of intestinal microflora of human and warm-blooded animals. As a member of the Enterobacteriaceae family, *E. coli* is Gram-negative, rod-shaped, and facultative, possessing both respiratory and fermentative metabolism pathways (Doyle et al., 2001a). Most *E. coli* strains are motile with peritrichous flagella; meanwhile, a number of non-motile variants also exist. On solid culture media, *E. coli* appears as colorless, translucent round colony with entire margin and smooth surface (Fig. 2.1).

FIGURE 2.1. A typical *Escherichia coli* strain grown on trypticase soy agar (TSA). Photo courtesy of Eddy Perez, 2011.

E. coli strains are generally considered harmless in healthy people and animals. Further, it is a good indicator organism reflecting the possible fecal contamination in food and water, due to its similar characteristics as other major foodborne pathogens in terms of reservoir, transmission route, and growth speed (Doyle et al., 2001a). However, some *E. coli* strains are pathogenic and capable of causing diseases ranging from mild diarrhea to lethal complications (Brooks et al., 2005). Currently, diarrheagenic *E. coli* is grouped into six major groups: diffuse-adhering *E. coli*

(DAEC), enteroaggregative *E. coli* (EAEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli*, (EPEC), and enterotoxigenic *E. coli* (ETEC) (Kaper et al., 2004). EHEC can be distinguished from other diarrheagenic *E. coli* by its highest incidence in foodborne outbreaks of hemorrhagic colitis (HC) and life-threatening hemolytic uremic syndrome (HUS) in the United States and worldwide (Brooks et al., 2005; Pennington, 2010). Since nearly all EHEC strains produce Shiga toxins (encoded by *stx1* or *stx2*), they are classified into another group termed Shiga toxin-producing *E. coli* (STEC). The main difference between EHEC and STEC is that some STEC strains are only found in animal host and have not been associated with human illness in the past (Gyles, 2007; Johnson et al., 2006). Among STEC, *E. coli* O157:H7 is the most widely recognized serotype to date, causing significant food safety and public health concerns (Pennington, 2010).

Physiologically, most *E. coli* strains including *E. coli* O157:H7 can survive under a broad range of temperatures between 4^{o}C and 46^{o}C, with the optimum temperature usually around 37^{o}C. Studies on heat resistance of *E. coli* O157:H7 in ground beef revealed that it is thermal sensitive under high temperature conditions, with D values of 270 s, 45 s, 24 s, and 9.6 s at 57.2^{o}C, 60^{o}C, 62.8^{o}C and 64.3^{o}C, respectively (Doyle and Schoeni, 1984). As a result, heating is commonly adopted in food industry and clinical setting as an easy and effective treatment for *E. coli* elimination. *E. coli* O157:H7 can propagate over a wide range of pH values as well, though its acid-resistant ability varies according to many intrinsic and extrinsic factors, including strain serotype, genetic profiles, acid type, food type, environmental conditions, and others. For example, *E. coli* O157:H7 at high inoculation level can live in fermented sausage (pH 4.5) for up to 2 month at 4^{o}C (Glass et al., 1992), and in apple cider (pH 3.6-4.0) for 10 to 31 days at 8^{o}C (Zhao et al., 1993).

Clinical Symptoms. Symptoms of *E. coli* O157:H7 infection range from asymptomatic to lethal. Once a person is infected, initial symptoms may include abdominal cramps, a short-lived fever, and watery diarrhea. Nearly half of the patients may also experience vomiting in this phase. After one or two days, much more severe bloody diarrhea may occur, usually accompanied with increased abdominal pain, which may last up to 10 days (Pennington, 2010). Although most people infected with *E. coli* O157:H7 will recover without seqealae if timely diagnosis and proper treatment were initiated, there are still approximately 10% of patients who will develop hemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS), particularly in children younger than 10 years old and senior people (Ethelberg et al., 2009; Gyles, 2007; Nataro and Kaper, 1998). HC is characterized by severe abdominal cramps and grossly bloody diarrhea with little to no fever. HUS was initially described in 1955 in *Shigella dysenteriae* infection, characterized by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia (Mead and Griffin, 1998; Rowe et al., 1998; Slutsker et al., 1997). In adult infected with O157 STEC, thrombotic thrombocytopenic purpura (TTP) may develop similar signs and symptoms as HUS plus neurological symptoms (Griffin and Tauxe, 1991). HUS can cause high percentage of permanent renal injury and up to 5% of deaths. One 6-year study on 180 cases of HUS in Scotland showed typical outcomes: 48% recovered and were released home; 13% had renal impairment; 7% became dependent on dialysis; 4% had neurological impairment; and 4% died (Pennington, 2010). The infectious dose for *E. coli* O157:H7 is low, as few as 10-100 cells may occasionally cause illnesses for immuno-compromised person and child under 4 or 5 years old (Rangel et al., 2005). The three to four days of incubation period is normal for STEC, but in some cases it can be either as long as 5 to 8 days, or as short as 1 to 2 days. Unlike *Salmonella*

typhi infection, long-term carriage of *E. coli* O157:H7 in infected patients has not been recorded (Mathusa et al., 2010).

Similar to *E. coli* O157:H7, clinical manifestations of non-O157 STEC infections range from watery diarrhea to HC, HUS, and even death (Brooks et al., 2005; Johnson et al., 2006). However, in general, non-O157 STEC is considered to be less virulent than *E. coli* O157:H7, since it tends to be associated with mild symptoms shown in the early stage of infection, but seldom induces severe complications at later stage of diseases (Brooks et al., 2005). This statement is backed up by the data collected and analyzed by U.S. Centers for Disease Control and Prevention (CDC), which found a lower incidence of non-O157 STEC in HUS (1.7% for non-O157 STEC *v.s.* 6.3% for O157 STEC) and death (0.1% for non-O157 STEC *v.s.* 0.6% for O157 STEC) (Gould, 2009). In another smaller scale study conducted between 2000 and 2006, a similar finding was reported (Hedican et al., 2009) by testing stool cultures of STEC-infected patients that non-O157 strains were less likely to result in bloody diarrhea (54% *v.s.*78%), hospitalization (8% *v.s.*34%), and HUS (0 *v.s.*7%) than O157 STEC strains. Among over 100 different STEC O serogroups historically involved in sporadic HUS cases, O111 is the second most frequent one right after STEC O157 (Brooks et al., 2005). Other important ones included O26, O103 and O145 (Johnson et al., 2006).

Virulence Determinants. By definition, all STEC strains have the potential to produce Shiga toxins, the major virulence factors contributing to STEC pathogenicity. There are two types of Shiga toxins secreted by *E. coli*, Shiga toxin 1 and 2, with different typical genetic and antigenic characteristics (Gyles, 2007). Molecular sequence analysis of their coding genes revealed that the *stx*1 gene was more conservative than the *stx*2 gene, with the same sequence or only three bases difference from that of *Shigella dysenteriae* (Jackson et al., 1987). The *stx2* gene has at least 11

variants identified to date, including *stx2*, *stx2c*, *stx2d*, *stx2e*, *stx2f, stx2g* and others (Brett et al., 2003; Russmann et al., 1995; Schmidt et al., 2000). Pairwise sequence alignments among randomly picked two *stx2* gene variants revealed that the homology score ranged from 91% to 98%, demonstrating close relationship in evolution.

The molecular weight of purified Shiga toxin was estimated to be 62,000 Dalton (Yutsudo et al., 1986), consisting of two different functional subunits, A and B, when recognizing the host cell and triggering disease progress. Generally, the B subunit binds to neutral glycolipids on host cells and mediates cellular uptake of the Shiga toxin with trafficking to the endoplasmic reticulum. Thereafter, the A subunit is translocated across endoplasmic reticulum membrane to the cytoplasm, and achieve the access to its final target ribosome, where it can bind to the 28S RNA, cleave off a specific adenine, and prevent aminoacyl t-RNA binding, resulting in the inhibition of protein synthesis and the initiation of proinflammatory cytokine expression (Johnson et al., 2006).

A given STEC strain may produce either one or both Shiga toxins, and Stx2 have been identified to be closely associated with highly pathogenic STEC strains, especially when co-existing with the *eae* gene (Boerlin et al., 1999; Brooks et al., 2005). Some studies found frequent appearance of *stx2*-positive *E. coli* O157:H7 isolates in HUS cases, whereas the detection of strains carrying only *stx1* gene in HUS cases was not reported (Ostroff et al., 1989). Besides, purified Stx2 presented 1,000 times more toxic for human renal microvascular endothelial cells than Stx1, probably due to their major differences in crystal structure (Gyles, 2007).

Since STEC strains are not invasive, it is critical for them to attach and colonize the intestinal epithelial cell first and then release the Shiga toxins. After intensive research on the mechanisms of adherence and colonization presented by highly pathogenic STEC, including *E. coli* O157:H7, one characteristic histopathological feature, attaching and effacing lesion (A/E), was elucidated (Kaper et al., 2004). It enables the intimate attachment of the bacteria to the plasma membranes of the host epithelial cells, localized destruction of the brush border microvilli, and assembly of highly organized pedestal-like actin structures. Only the expression of A/E lesion is believed to be sufficient to cause nonbloody diarrhea, while the production of Shiga toxin is responsible for the development of bloody diarrhea and HUS.

The A/E lesion is associated with a group of functional proteins, which are encoded by a gene cluster located on a chromosomal pathogenicity island, referred as the locus of enterocyte effacement (LEE). These proteins include components of a type III secretion system (TTSS), intimin, translocated intimin receptor (Tir), and others (Garmendia et al., 2005). Among these proteins, intimin has been widely used as the target in developing method for identification of LEE. The intimin is a 94-kDa outer membrane protein encoded by the *eae* gene, and it determines the unique pattern of attachment and interaction of STEC with epithelial cells: Tir is provided by the pathogen itself and translocated into the host cell to serve as the receptor for intimin, so that intimate attachment could be established (Johnson et al., 2006).

Except for Shiga toxin and A/E lesion, many other putative virulent genes are found on the conserved plasmids (pO157, pO113, and others) in some STEC strains (Brunder et al., 2006; Newton et al., 2009). For instance, the F-like plasmid pO157 has approximately 100 open reading frames, of which 19 may be potentially involved in disease, including the one encoding EHEC hemolysin. This hemolysin toxin is secreted across both the cytoplasmic and outer

membranes of pathogenic *E. coli* to form the membrane pores in the host immune system cells and cause dysfunction and death. Recently, non-Lee enfectors (*nle*) have been identified to be strongly associated with STEC pathogenicity (Coombes et al., 2008). Taken together, the STEC virulence is due to a combination of various factors, and the attempt to describe it with single trait is difficult (Grant et al., 2011). More researches are still needed to clarify the relationship between these diverse virulence factors associated with STEC infection in human.

O Serogroups. Serotyping *E. coli* isolates is normally based on three major surface antigens, the O, H, and K antigens, among which O antigen identifies the serogroup, and H antigen identifies the serotype (Doyle et al., 2001c). The application of serotyping to isolates associated with diarrheal disease has shown that particular serogroups often fall into one category of pathogenic *E. coli*, while others (i.e. O55, O111) will appear in more than one (Doyle et al., 2001c). Since *E. coli* O157:H7 was firstly identified as a new foodborne zoonosis in 1982 (Riley et al., 1983), about 250 different O serogroups of *E. coli* have been identified to produce Shiga toxin (Johnson et al., 2006).

FIGURE 2.2. The open reading frame of O antigen gene cluster for *E. coli* O157

Genes involved in the synthesis of O-antigen are located in the O antigen gene cluster (10-15 kb) between the *galF* and *gnd* genes on the *E. coli* chromosome (Samuel and Reeves, 2003). O antigen gene clusters generally contain 8 to 20 genes (Fig. 2.2), some of which encode the protein carrying out specific assembly or processing steps to convert the O unit to the O

antigen as part of the complete lipopolysaccharide, therefore, they are O serogroup specific and can be selected as target to develop novel serotyping method (Wang and Reeves, 1998).

Emerging Clinical Importance of Non-O157 STEC Serogroups

Epidemiology of *E. coli* O157:H7. Ruminants particularly cattle are the major reservoirs for *E. coli* O157:H7 strains (Pennington, 2010). The prevalence rates of STEC was as high as 60% in bovine herds in some countries; however, the rates of 10-25% were mostly reported (Michael P. Doyle et al., 2001c). Generally, the isolation rates of *E. coli* O157:H7 are much lower than those of non-O157 STEC. For example, two major surveys conducted in the U. S. revealed that 31 (3.2%) of 965 dairy calves and 191 (1.6%) of 11,881 feedlot cattle were positive for *E. coli* O157:H7. Additionally, 0.4% of feedlot cattle were positive for *E. coli* O157:NM (Zhao et al., 1995). The number of *E. coli* O157:H7 in calf feces ranged from less than 100 CFU/g to 10^5 CFU/g. Some studies found a robust pattern that up to 80% of the *E. coli* O157:H7 transmissions on the farm originated from 20% most infectious cattle, which were defined as Super-Shedders by their ability to release higher numbers of this organism for a longer period (Menrath et al., 2010).

E. coli O157:H7 has been the reason of many major outbreaks of severe illnesses worldwide since 1982. In the United States, STEC O157 causes an estimated 63,153 illnesses, 2,138 hospitalizations, and 20 deaths annually through food transmission route (Scallan et al., 2011). Based on CDC's surveillance data, STEC O157 outbreaks in the United States has increased from an average of 2 cases per year between 1982 and 1992 to 29 cases annually between 1993 and 1998 (Doyle et al., 2001b), and it fluctuates around 22 to 40 outbreaks each year (CDC, 2006, 2010a) in recent 10 years (1998-2007). However, if the increase of population

is counted, the incidence of *E. coli* O157:H7 has actually dropped by 44% to 0.9 per 100,000 people in 2010 (Fig. 2.3) (CDC, 2010b).

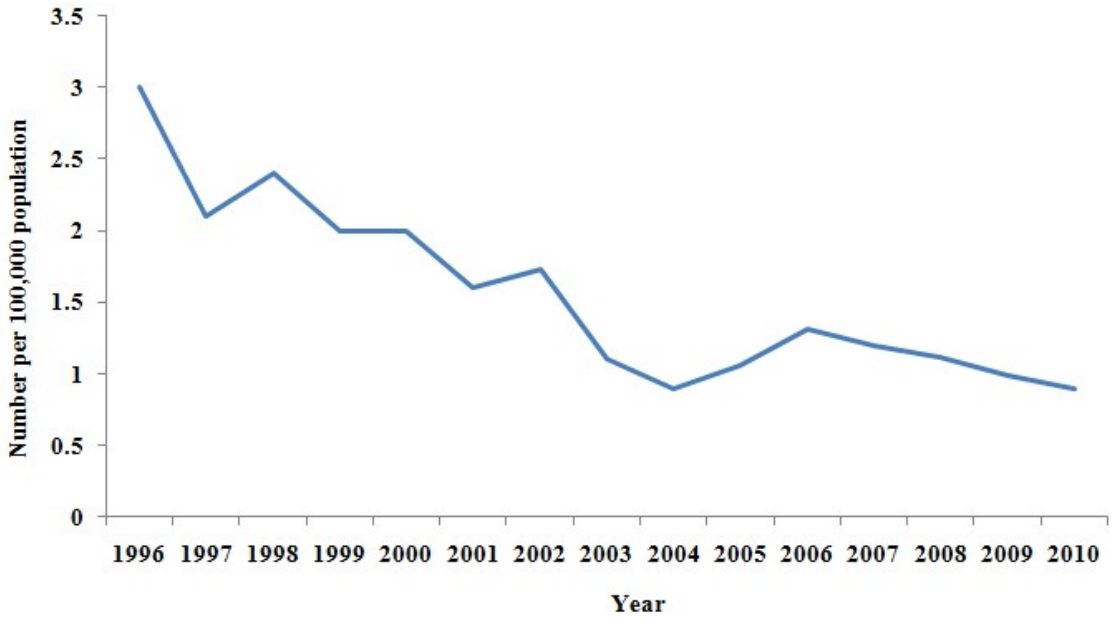

FIGURE 2.3. Incidence of STEC O157-Foodborne Diseases Active Surveillance Network, United States, 1996-2010 (CDC, 2010b)

A variety of foods have been identified as vehicles of *E. coli* O157:H7 infections, including ground beef, produce, milk, and juice (Pennington, 2010). Among the 196 *E. coli* O157:H7 outbreaks (by 1998) reported in the United States for which a vehicle has been identified, 48 (33.1%) were associated with ground beef, 4 (2.8%) with raw milk, and 3 (2.1%) with roast beef (Doyle et al., 2001b). Another review analyzed the source of 24 multistate outbreaks recorded between 1992 and 2002, of which 16 were related to ground beef and 6 to produce (Pennington, 2010). If the food/outbreaks combination was examined based on the country, geographical distribution may be clearly unveiled, indicating the difference in local food preference and culinary customs. For example, the butcher-associated *E. coli* O157:H7 outbreaks

have occurred more often in the United Kingdom other than ground beef (Rangel et al., 2005). Secondary spread is another primary cause for *E. coli* O157:H7 infections, mainly through person to person transmission (Rangel et al., 2005).

Epidemiology of Non-STEC Serogroups. Non-O157 STEC has the same reservoirs and transmission route as *E. coli* O157:H7, but with relatively higher prevalence. Regarding the original source, one six-month study found 63.2% of cattle in one herd carrying STEC by testing feces sample for the *stx* gene, and no isolates belonged to O157 serogroup (Beutin et al., 1997). Similarly, the high non-O157 STEC prevalence was also estimated in dairy cattle (74%), or cattle at slaughter house (70.1%) in the United States (Hussein and Bollinger, 2005a, b; Hussein and Sakuma, 2005). Talking about the food items frequently associated with STEC contamination and infection, data from numerous countries indicates that while *E. coli* O157:H7 is rarely present in more than 1% of raw beef products, the prevalence of non-O157 STEC ranges from 2.4% to 49.6% (Grant et al., 2011). One recent published study (Bosilevac and Koohmaraie, 2011) examined the prevalence of non-O157 STEC isolated from 4,133 commercial ground beef samples, representing the main regions of the United States. The overall suggested prevalence of STEC in ground beef was 24.3%, and it varied among different regions (13.1% to 39.4%). Nine serogroups (O113, O8, O22, O117, O163, O174, O171, O116, and O20) accounted for 53% of all isolates that were identified (Bosilevac and Koohmaraie, 2011). Such prevalence variation may depend on environmental factors and management practices.

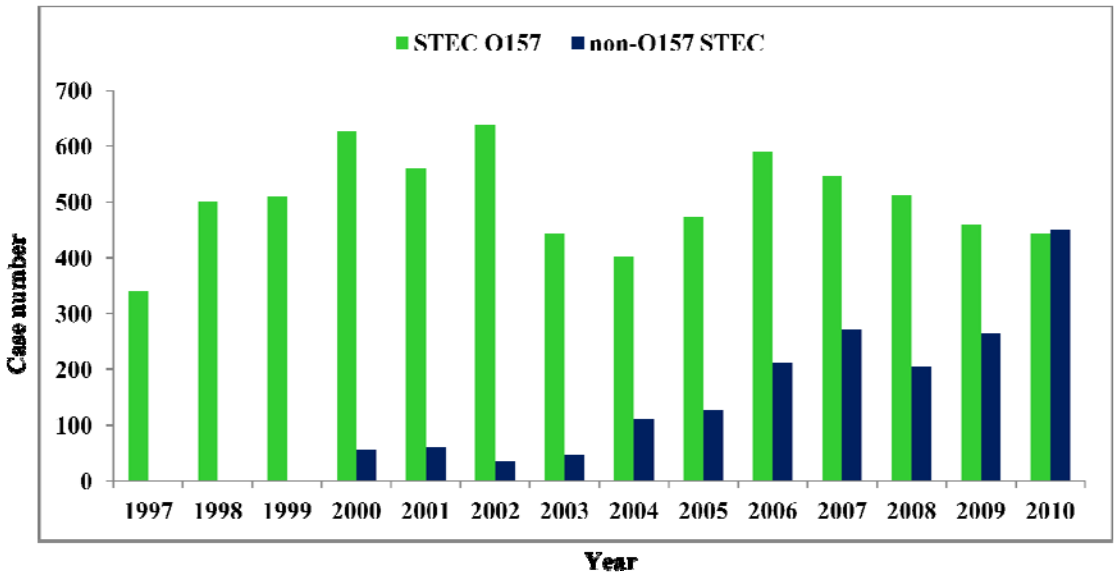

FIGURE 2.4. The reported STEC O157 and non-O157 STEC infections by CDC's FoodNet, 1997-2010 (CDC, 2010b)

Although non-O157 STEC strains are generally thought to be less virulent than *E. coli* O157:H7, some O serogroups are still frequently associated with sporadic foodborne outbreaks of HC and HUS. Non-O157 STEC may annually cause 112,752 illnesses and 271 hospitalizations in the United States (Elaine Scallan et al., 2011), accounting for 20% to 50% of all STEC infections (Grant et al., 2011). In contrast to the decreasing trend of STEC O157 infection, non-O157 STEC infection has been on the rise since it became nationally notifiable in 2000 (Fig. 2.4), and a ten-fold increase in incidence was recorded in CDC FoodNet Report between 2000 and 2010 (0.12 cases per 100,000 to 1 case per 100,000 people) (CDC, 2010b). Even non-O157 serogroups were not equally pathogenic either; epidemiology data showed that several serogroups (O26, O45, O103, O111, O121, and O145) were repeatedly isolated from 70% of confirmed non-O157 STEC infections in the United States (Brooks et al., 2005). And very recently, an unprecedented large outbreak of *E. coli* O104:H4 in Germany has resulted in a total of 4,075 cases (including 908 HUS) and 50 deaths as of July 21, 2011(WHO, 2011). Due to

the severity of disease symptoms, STEC O157 was declared as an adulterant in raw ground beef and beef trim by the U.S. Department of Agriculture (USDA) in 1994. On September 13, 2011, USDA announced the intention to declare six additional serogroups of STEC (O26, O103, O45, O111, O121, and O145) as adulterants in non-intact raw beef and the regulation will be enforced beginning on March 5, 2012.

Detection Methods for *E. coli* O157:H7 and Related STEC

Importance of Pathogen Detection. Microorganism with the ability to cause food spoilage and foodborne illness has always raised concerns of food quality and safety. Effective detection methods with high sensitivity and speed, although not a solution, may serve as the powerful tool in identifying the problem source and outlining solutions. Recently, numerous technologies have been developed to enumerate the total and groups of microorganisms and to detect specific pathogens and toxins in foods (Ge and Meng, 2009). Traditional methods mainly rely on appropriate selective and differential agar to detect specific microorganism in food. Although it has the highest sensitivity (several cells) among all the detection methods, and progress has been made to improve the formulation of the enrichment agar and differential medium, this method is still time-consuming and labor intensive. Advanced techniques, including convenience-based, antibody-based, and molecular-based assays, have successfully reduced the process to several hours with high specificity. In addition, such assays may also serve as the effective tool to provide the comprehensive genetic or metabolic profiles of the organism.

Culture-based Methods. Culture-based method is regarded as the "gold standard" in food detection, due to its high sensitivity and ability to detect live cells. Additionally, it is the only method so far to provide the access to purified isolates from the background flora. Multiple steps

must be included in one complete test round, consisting of sample preparation, pre-enrichment, selective-enrichment, plating on differential agars and subsequent confirmation via biochemical, serological, and molecular test (Feng, 2007). As a result, the total analysis needs several days. The hardcore part of such traditional detection method for pathogens is the suitable selective and differential media. Selective media enhances the growth of target pathogens to detectable level, and simultaneously suppresses the growth of the rest. Differential agar, on the other hand, relies on the specific biochemical traits possessed by the target organisms, which are usually reflected on the color changes of the growth media. Numerous types of selective and differential agar have been commercialized for decades, targeting the main foodborne pathogens, including *E. coli* O157:H7, *Campylobacter*, *Salmonella*, *Listeria monocytogenes*, *Vibrio* and others. Continuous effects have also been made on the selection of specific physiological metabolism traits in microorganisms, and the improvement on the formulation of selective and differential media. For instance, the enrichment broth mEC + n used for *E. coli* O157:H7 detection is replaced by an improved one, mTSB + n (modified tryptone soy broth with Novobiocin and casamino acids) in the revised USDA protocol (U.S. Department of Agriculture, 2008).

E. coli O157:H7 can be easily distinguished from other pathogenic and generic *E. coli* by their inability to ferment sorbitol within 24 hours (March and Ratnam, 1986). Recently, three types of differential agar are recommended by regulatory agencies and widely used in scientific and clinical community, including sorbitol-MacConkey agar (SMAC), cefixime tellurite-sorbitol MacConkey agar (CT-SMAC), and CHROMagar O157 (Fig. 2.5). Typical *E. coli* O157:H7 colony will present mauve or pink color on CHROMagar O157, but no color on the other two medium after 16-24 h incubation at 37^{o}C. With regards to sensitivity, CHROMagar O157 and CT-SMAC are better than SAMC (Church et al., 2007; Zadik et al., 1993). Presumptive colony

needs to be confirmed by O157 specific antiserum or O157 latex reagent before it is documented and reported as *E. coli* O157:H7 (March and Ratnam, 1989). Usually the culture-based method can detect as low as one *E. coli* cell or even fewer in 65 gram tested sample after enrichment (Bosilevac et al., 2010), but the confirmation of this preliminary data needs several days or even weeks.

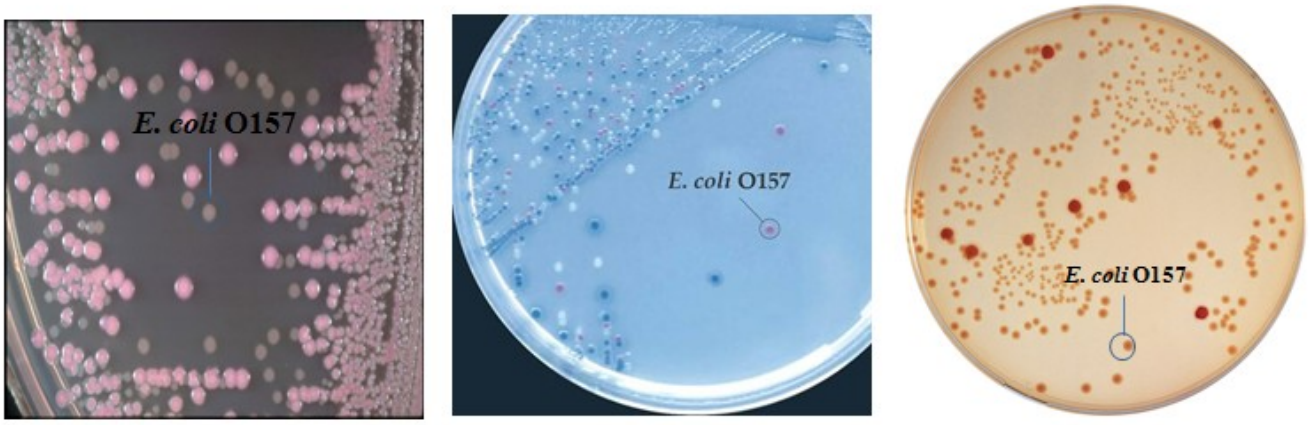

FIGURE 2.5. A typical *Escherichia coli* O157 strain grown on SMAC (left), CHROMagar O157 (middle) and CT-SMAC (right).

Standard methodology of non-O157 STEC detection is still challenging, since no characteristic physiological trait in this group of bacteria can be utilized to develop a differential agar. Recently several differential agars designed for some non-O157 STEC serotypes have been reported, however, the resulted color change is not consistent and sometimes hard to interpret, due to many unpredictable interfering factors like the incubation time, how crowded the colony is, and what food matrix they are isolated from (Catarame et al., 2003; Hiramatsu et al., 2002; Posse et al., 2008a, b). In most public health laboratory, the intention to isolate non-O157 STEC starts with the screening test for Stx presence in enrichment samples via enzyme immunoassay (EIA) or PCR method, then followed by plating the Stx-positive sample on relatively less

selective agar (MacConkey agar). Those well-isolated colonies are randomly picked out for serotype test. Hence it is possible that many potential STEC strains are omitted.

Immunology-based Methods. Immunology-based methods use antibodies to directly identify the presence of foodborne pathogens in food or assist in the detection by separating microbial cells from food matrix. Enzyme-linked immunosorbent assay (ELISA) and Immunomagnetic separation (IMS) are the typical examples in each category. In ELISA, the 96-well microtiter plate is pre-coated with specific antibody, which will recognize and capture the target organism in food sample. After the initial binding, the secondary antibody linked with an enzyme will bind with the target organism again to form the vivid "sandwich" structure. Then the indicator substance is added to react with the enzyme and generate color, fluorescence, or electrochemical signal, indicating the presence and amount of target organism. Similar as the ELISA method, one special designed magnetic bead coated with specific antibody is employed in IMS to capture the target organism in enrichment culture first, and then separated from the food matrix for further detection by other technology. The IMS method can result in the concentrated target organism and inhibitor-free solution, both factors served to improve the sensitivity of molecular-based tests. Recently, the antibody and bead for major foodborne pathogens have already been commercialized and widely used in various places other than food industry.

Shiga-toxin production as the only universally shared trait in STEC strains is used to design enzyme immunoassay, defined as Stx-EIA. This method was introduced into the United States in the early 1990s, and has successfully improved the low detection rate of STEC in food and clinical samples. Four commercial EIA kits have been approved by FDA, including The Premier EHEC, the ProSpecT Shiga Toxin *E. coli* Microplate Assay, the Immunocard STAT! EHEC, and the Duopath Verotoxins Gold Labeled Immunosorbent Assay. Most assays are

conducted after the enrichment, allowing the detection limit to reach a few cells in examined samples. The directly application of EIA test in stool samples may generate the result from 20 minutes to 4 hours, but with the compromised sensitivity and specificity. Other studies point out that EIA may fail to detect a subset of O157 STEC with unknown mechanism (Klein et al., 2002; Manning et al., 2007), and false-positive results cannot be avoided when other pathogens are presented (CDC, 2001). STEC serotyping mainly uses agglutination reactions between antisera and specific O-antigen; however, the process can only be conducted in specialized laboratories (CDC, 2009), and the cross-reaction of antisera with multiple O serogroups often occurs (Fratamico et al., 2005).

IMS coupled with culture-based or PCR method may increase the isolate rate of STEC from complicated food stuff. The key component, immunocapture bead, has been developed for those major STEC serogroups (O26, O45, O103, O111, O121, O145 and O157) associated with high prevalence and severe manifestations (Mathusa et al., 2010); however, due to the huge diversity in STEC serogroups, it is impossible to design the specific bead for all serogroups.

Molecular-based Methods. The breakthrough on DNA amplification theory and subsequent discovery of highly efficient and thermal-tolerant DNA polymerase enable the emergence and evolvement of molecular-based pathogen detection assay. Methods such as PCR and real-time PCR are widely used in detection, diagnosis, genetic characterization, and other biological research area, because of their high speed, sensitivity, specificity and reproducibility.

PCR can exponentially generate thousands to millions of copies of a particular DNA sequence in vitro across several orders of magnitude (Mullis et al., 1986). This method relies on two key components, primer and DNA polymerase, along with the repeated cycles of heating and

cooling of the reaction to enable the selective and repeated DNA amplification. Normally, a gel electrophoresis is coupled to examine the PCR products under the UV light; however, this drawback has been overcome by the development of real-time PCR. PCR can detect foodborne pathogens by the amplification of specific DNA genes and region, hence reducing the massive time previously spent on the agar preparation, and various biochemical tests. Meanwhile, it is easier to interpret the PCR result than the traditional culture-based method, where the non-differentiable color change often happens. The other desirable feature of PCR is that more than one pair of primers can be incorporated in the single test; therefore it is possible to simultaneously detect two or three targets in one run (Claustres et al., 1989). It is noteworthy that some inhibitors in food matrix may interfere with the PCR by affecting the DNA polymerase activity, so it is critical to design the internal control and separate the target organism from enrichment culture as complete as possible (Hoorfar et al., 2003).

Real-time PCR, also named as quantitative PCR (Q-PCR or qPCR), is an advanced PCR assay, providing near instantaneous amplification and detection at the same time. Different from conventional PCR, which distinguish the end point product for analysis, this fluorescence-based method is using a DNA-binding dye or hybridization probes to quantify input nucleic acid by measuring the number of thermal cycles required to reach a certain level of product. The identity of tested organism is reflected by sequence-dependent melting temperature or target-specific probe. It is generally agreed that real-time PCR is more sensitive than traditional one, along with the less running time. Both PCR assays now can detect the live cell after the incorporation of EMA or PMA as a dead DNA eliminating agent (Chen et al., 2011; Nocker and Camper, 2006; Nocker et al., 2006; Wagner et al., 2008). However, neither one can supply the purified isolate as

culture-based method. Meanwhile, the expensive equipment for accurate thermal control and data reading makes it unaffordable in small business.

TABLE 2.1. Comparison of detection limits reported using molecular methods

#	Target	Assay type	Detection limit	Reference
1	*stx 1* and *2*	PCR	20-200 CFU/R	(Pollard et al., 1990)
2	*stx 1* and *2*	PCR	1 CFU/g [a]	(Gannon et al., 1992)
3	*stx 1* and *2*	PCR	100 CFU/R	(Read et al., 1992)
4	*stx 1* and *2*	PCR-ELISA	10 CFU/R	(Ge et al., 2002)
5	*stx 1* and *2*, *eae*	qPCR	10 CFU/R	(Ibekwe and Grieve, 2003)
6	*stx 1* and *2*, *eae*	qPCR	10 CFU/R	(Sharma and Dean-Nystrom, 2003)
7	*stx 1* and *2*, *eae*	qPCR	50 CFU/R	(Fratamico et al., 2011)
8	*stx 1* and *2*	LAMP	0.7-2.2 CFU/R	(Hara-Kudo et al., 2007)
9	*stx 1* and *2*, *rfbE*	LAMP	2-20 CFU/R	(Zhao et al., 2010)
10	*stx 2*, *rfbE* and *fliC*	LAMP	26 CFU/R	(Zhu et al., 2009)

[a] This sensitivity was achieved with pre-enrichment procedure.

PCR and real-time PCR methods targeting the *stx1* and *stx2* genes are mainly used for STEC screening, strain confirmation and genetic characterization. According to the primer nature and detection strategy, some assays may differentiate the *stx1* from *stx2* gene (Gannon et al., 1997; Meng et al., 1997). Additionally, the assay has also been developed for the detection of specific O-serogroup and other virulence factors, such as intimin, and enterohemolysin (DebRoy et al., 2005; DebRoy et al., 2004; Fratamico et al., 2009; Paton and Paton, 1998, 1999). The reported detection limit of PCR and real-time PCR assay ranges from 10 to 10^2 CFU per reaction in pure culture (as shown in Table 2.1), which equals to 10^4-10^5 CFU per gram or milliliter sample. The result can be accessed within several hours after the 8-24 hours enrichment (Ibekwe and Grieve, 2003; O'Hanlon et al., 2004; Sharma and Dean-Nystrom, 2003). Although PCR method is with the high speed, sensitivity, and specificity, it can only be used in public health laboratories for confirmatory testing, but not approved by FDA for clinical diagnosis, because

the virulent genes somehow may not be translated into final pathogenic products. Another interesting finding is that no PCR-based method is used to detect STEC in those laboratories serving Foodborne Disease Active Surveillance Network site, based on one survey in 2007 (Hoefer et al., 2010). One latest strategy for non-O157 STEC strains detection and isolation proposed an additional serotyping step by PCR before immuno magnetic separation (IMS), therefore, the target bacteria can be selected and concentrated before plating on selective agar and the recovery rate is increased (Fratamico et al., 2011; Perelle et al., 2007). The flow chart is presented in Fig. 2.6.

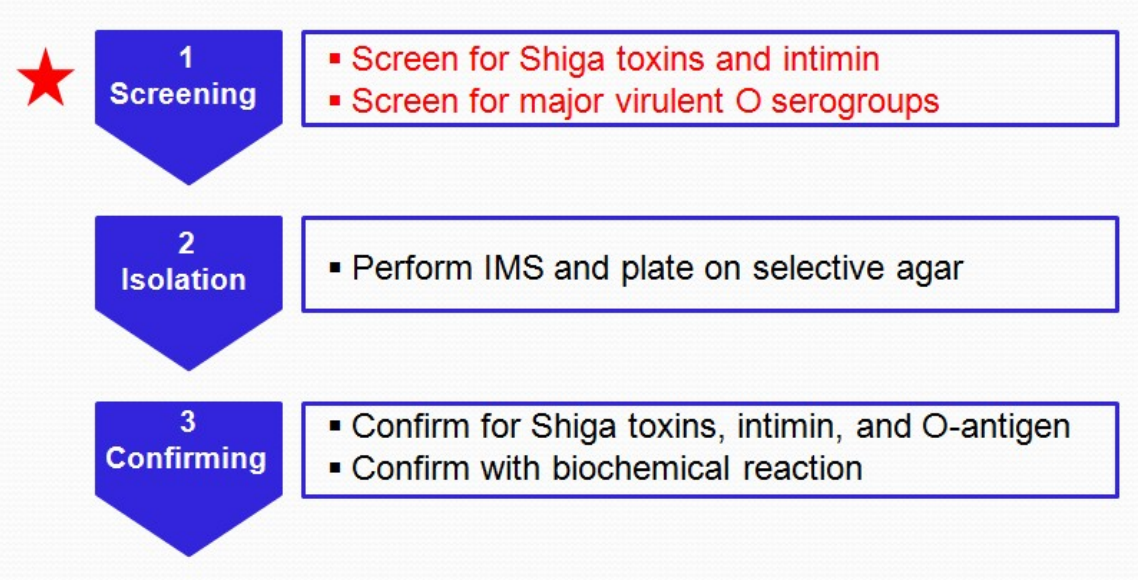

FIGURE 2.6. The flowchart of non-O157 STEC isolation by USDA (U.S. Department of Agriculture, 2010)

Loop-mediated Isothermal Amplification. Recently, a novel molecular-based assay, loop-mediated isothermal amplification (LAMP) has been developed and applied in pathogen detection as well. LAMP was developed by a group of Japanese scientists, and first published in 2000 (Notomi et al., 2000). Due to its novel design, several major advantages have been

provided in this new DNA amplification technique. Firstly, the target sequence is amplified in a single temperature incubation (60-65^{o}C) using a polymerase with high strand displacement activity, thereby avoiding the need for expensive thermal cyclers. Secondly, a set of 4 primers, two inner and two outer, is used to recognize six distinct regions in target DNA, and form a dumbbell-like structure complex with multiple amplification starting sites. Therefore, LAMP can generate larger amount of DNA than PCR based method within the same time span. All the LAMP assays can be completed within 1 hour. Additionally, many studies have also proved that most LAMP assays are 10 times more sensitive than real-time PCR method, with the lowest detection limit of less than 1 cell per reaction. Thirdly, the amplification product is detected by the visible turbidity caused by increasing quantity of Magnesium pyrophosphate in solution, or by fluorescence after the addition of SYBR-green dye (Fig. 2.7). LAMP can also be quantitative, when the real-time turbidimeter is used to correlate the turbidity signals with the number of DNA copies initially present. As a simple, rapid and cost-effective method with the high sensitivity and specificity, LAMP has great potential to be used as a simple screening assay especially in the field test. Recently, it has been extensively validated for common pathogens detection, such as *Campylobacter*, *Escherichia coli*, *Salmonella*, *Vibrio*, virus and others (Chen and Ge, 2010; Han and Ge, 2010; Hara-Kudo et al., 2007; Techathuvanan et al., 2010; Yamazaki et al., 2009; Yoda et al., 2009).

LAMP assays recognizing the *stx* genes and O157 serogroup determining gene (the *rfbE* gene) have also been developed in some studies so far (Hara-Kudo et al., 2008; Hara-Kudo et al., 2007; Kouguchi et al., 2010; Zhao et al., 2010; Zhu et al., 2009), and none of them was conducted in the United States. The most sensitive one can detect 1 dead cell per reaction, with pure isolates as the template (Hara-Kudo et al., 2007). All the studies mainly focused on the

basic characters of LAMP assay for STEC and/or O157 serogroup detection, including the sensitivity and specificity. However, the systematic study has not been done about the optimization of the assay, the quantitative application in sample, the comparison between different methods, and the suitable protocol to use. In addition, no LAMP assay is reported for STEC subtyping and virulence check. All those issues will be the subject in this study. It is believed that this assay will have the potential to be incorporated into proposed USDA protocol to replace the real-time PCR method.

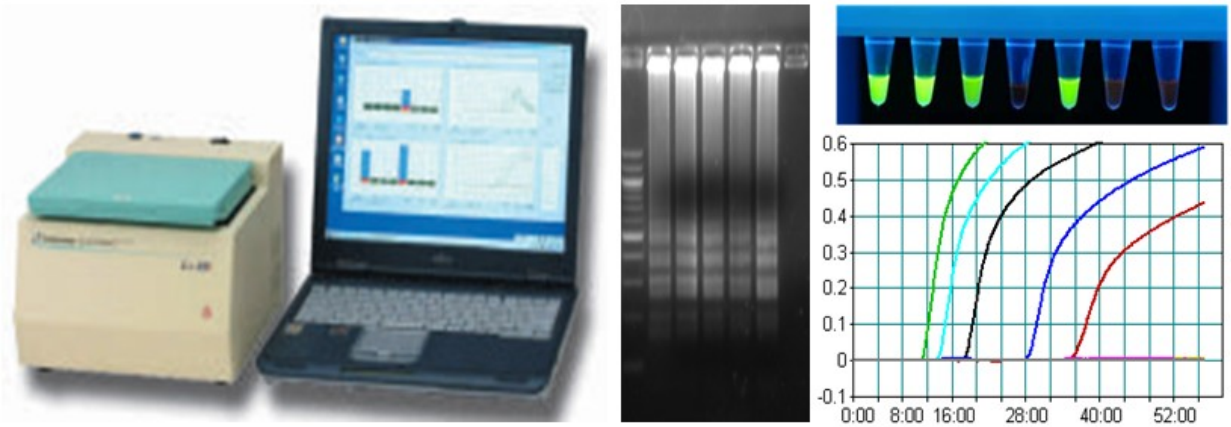

FIGURE 2.7. Loop-mediated isothermal amplification turbidimeter and three result observation methods.

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CHAPTER 3: LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAYS FOR DETECTING SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN GROUND BEEF AND HUMAN STOOLS

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is a zoonotic foodborne pathogen of significant public health concern due to its frequent involvement in outbreaks of hemorrhagic colitis (HC) and the ability to cause life-threatening complications such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Thorpe, 2004). In the United States, STEC causes an estimated 176,000 illnesses, 2,400 hospitalizations, and 20 deaths annually (Scallan et al., 2011). Ruminants particularly cattle are the major reservoirs for STEC strains (Hussein and Bollinger, 2005). STEC transmission commonly occurs through consumption of contaminated food (ground beef, produce, milk, juice) and water, contact with animals, and from person to person (Gyles, 2007). Less than 100 organisms of some STEC serotypes can lead to human illness (Thorpe, 2004).

First recognized as a foodborne pathogen in 1982 (Karmali et al., 1983), *E. coli* O157:H7 remains to be the most common STEC serotype causing human illness (Scallan et al., 2011). However, the clinical significance of non-O157 STEC is on the rise worldwide, with well over 100 serotypes associated with sporadic and epidemic human infections (Johnson et al., 2006). For the first time since 2000, FoodNet in the U.S. actually reported a higher incidence of laboratory-confirmed non-O157 STEC infections than STEC O157 in 2010 (CDC, 2011). O26, O45, O103, O111, O121, and O145 are the top 6 non-O157 serogroups in the U.S. (Brooks et al., 2005) whereas additional ones are more prevalent in other countries (Johnson et al., 2006). Since May 2011, an unprecedented large outbreak of *E. coli* O104:H4 in Germany has resulted in a total of 4,075 cases (including 908 HUS) and 50 deaths as of July 21 (WHO, 2011). Given this emerging and evolving nature of STEC serotypes involved in human illness, it is crucial that

rapid and reliable detection methods are available to screen for all STEC serotypes in food and clinical samples so that proper control and treatment can be implemented promptly.

By definition, all STEC serotypes are capable of producing at least one Shiga toxin (Stx1 or Stx2), the major virulence factors contributing to STEC pathogenicity (Thorpe, 2004). Stx1 is identical (or with only a single amino acid difference) to Shiga toxin produced by *Shigella dysenteriae* type 1 (Nataro and Kaper, 1998) whereas Stx2 shares 55-60% homology with Stx1 and is immunologically distinct (Jackson et al., 1987). In addition to Stx, many STEC strains carry a large chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE), which is responsible for producing attaching and effacing (A/E) lesions on enterocytes (Nataro and Kaper, 1998). Within the LEE region, an outer membrane protein intimin (encoded by *eae*) mediates the intimate attachment of bacteria to the enterocyte membrane (Nataro and Kaper, 1998). Although STEC virulence factors have yet to be fully elucidated, epidemiological data suggest that strains harboring both *stx2* and *eae* are strongly associated with severe human illnesses such as HC and HUS (Boerlin et al., 1999; Brooks et al., 2005; Ethelberg et al., 2004).

For STEC detection, three broad categories of assays are available. First, while traditional culture methods using sorbitol-containing selective media can readily identify *E. coli* O157:H7, currently no selective and differential media exist to culture non-O157 STEC strains (Gould et al., 2009). Second, enzyme immunoassays (EIA) for Shiga toxins and a few STEC serogroups are commercially available (Gould et al., 2009). However, false-positive results have been reported (CDC, 2001, 2006). Further, it is recommended that Shiga toxin EIA be performed on overnight (16-24 h) enrichment cultures of stools rather than direct examination (Gould et al., 2009), stretching the total assay time to days rather than hours (Ge and Meng, 2009). Third, rapid, specific, and sensitive nucleic acid amplification tests (NAAT) such as PCR and qPCR

have been developed to detect STEC by targeting genes coding for major STEC virulence factors such as Stx, intimin, or hemolysin (Fratamico et al., 2011; Paton and Paton, 1998). Nonetheless, a sophisticated thermal cycling instrument is an indispensable requirement of such tests, limiting their wide applicability.

Recently, a novel NAAT technology termed loop-mediated isothermal amplification (LAMP) has attracted great attention as a rapid, accurate, and cost-effective pathogen detection method in both food testing and clinical diagnostics (Mori and Notomi, 2009; Notomi et al., 2000). LAMP employs four to six specially designed primers and a strand-displacing *Bst* DNA polymerase to amplify up to 10^9 target DNA copies under isothermal conditions (60-65^{o}C) within an hour (Mori and Notomi, 2009). Since it is isothermal, LAMP can be performed in much simpler instruments such as a heater or water bath. To date, several LAMP assays targeting STEC Shiga toxin genes (*stx1* and *stx2*) have been developed and evaluated in food samples (Hara-Kudo et al., 2008a; Hara-Kudo et al., 2007; Hara-Kudo et al., 2008b; Kouguchi et al., 2010; Maruyama et al., 2003; Ohtsuka et al., 2010; Zhao et al., 2010; Zhu et al., 2009), as well as a few others targeting the *rfbE* gene (encoding perosamine synthetase) specific for the O157 antigen of STEC O157 (Wang et al., 2009; Zhao et al., 2010; Zhu et al., 2009). However, to our knowledge, there are no LAMP assays currently available for the *E. coli* intimin gene (*eae*). Due to the importance of STEC intimin in causing severe human illnesses (Boerlin et al., 1999; Brooks et al., 2005; Ethelberg et al., 2004), screening for both *stx* and e*ae* using qPCR is currently recommended by the U.S. Department of Agriculture's Food Safety and Inspection Service (U.S. Department of Agriculture, 2010). Additionally, none of the LAMP studies have evaluated the assay applicability in clinical samples.

The objectives of this study were to develop rapid and reliable LAMP detection assays for STEC by targeting *stx1*, *stx2*, and *eae*, and evaluate the assay performance with ground beef and human stools experimentally contaminated with low levels of STEC strains of seven major serogroups, i.e., O26, O45, O103, O111, O121, O145, and O157.

Materials and Methods

Bacterial Strains and Culture Conditions. A total of 90 strains (50 STEC and 40 non-STEC; Table 3.1) were used for specificity testing. Among these, seven STEC belonging to serogroups O26, O45, O103, O111, O121, O145, and O157 were used for sensitivity and ground beef testing. STEC O157 strain EDL933 (BEI Resources, Manassas, VA) was also used for assay optimization and application in human stools. The strains were examined for the presence of target genes (*stx1*, *stx2*, and *eae*) using previously described PCR assays (Xia et al., 2010). STEC and other Enterobacteriaceae were cultured at 35^{o}C overnight on trypticase soy agar or broth (TSA or TSB; BD Diagnostic Systems, Sparks, MD). Non-Enterobacteriaceae strains were grown on blood agar except for *Vibrio* strains for which TSA supplemented with 2% NaCl was used. *Campylobacter* strains were grown under microaerophilic conditions (85% N_2, 10% CO_2, and 5% O_2).

LAMP Primers and Reaction Conditions. The STEC *stx1*, *stx2*, and *eae* genes (GenBank accession numbers M19473, X07865, and Z11541, respectively) were selected as targets for designing LAMP primers (Table 3.2). A set of six primers, two outer (F3 and B3), two inner (FIP and BIP), and two loop (LF and LB), which recognize eight distinct regions of the target gene, were designed for each target using PrimerExplorer V4 (Fujitsu Limited, Japan).

The LAMP prototypic conditions were those recommended by the manufacturer (Eiken Chemical Co., Ltd., Tokyo, Japan). Following optimization, the final LAMP reaction mix (25 µl) for *stx1* and *stx2* consisted of 1× ThermoPol reaction buffer (New England Biolabs, Ipswich, MA), 6 mM $MgSO_4$, 1.2 mM each deoxynucleoside triphosphate (dNTP), 0.1 µM F3 and B3 (Integrated DNA Technologies, Coralville, IA), 1.8 µM FIP and BIP, 1 µM LF and LB, 10 U of *Bst* DNA polymerase (New England Biolabs), and 2 µl of DNA template. The optimized *eae* reaction mix differed from those described above for the following parameters: $MgSO_4$ (8 mM), dNTP (1.8 mM each), F3 and B3 (0.3 µM each), FIP and BIP (2 µM each), and LF and LB (1.2 µM each). One positive and one negative control were included in each LAMP run.

LAMP reactions were carried out at 65°C for 1 h and terminated at 80°C for 5 min in an LA-320C real-time turbidimeter (Eiken Chemical Co., Ltd.) with turbidity readings at 650 nm every 6 s. The time threshold (T_t; min) was determined when the turbidity increase measurement (differential value of moving averages of turbidity) exceeded a threshold of 0.1.

qPCR Assays. In comparison, qPCR assays (Fratamico et al., 2011) for STEC *stx1*, *stx2*, and *eae* were carried out. The mix (25 µl) contained 1× PCR buffer, 0.2 mM each dNTP, 4 mM $MgCl_2$, 0.25 µM each primer (Table 3.2), 0.1875 µM probe, 1.5 U of GoTaq Hot Start Polymerase (Promega, Madison, WI), and 2 µl of DNA template. The assays were conducted using 40 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 50 s in a SmartCycler II System (Cepheid, Sunnyvale, CA). Fluorescence readings were acquired using the FAM channel and the cycle threshold (*Ct*; cycle) was obtained when the readings crossed 30 units.

TABLE 3.1. Bacterial strains used in this study to evaluate specificity and sensitivity of LAMP assays

Strain group[a]	Serotype	Strain ID[b]	Stx	Intimin	Origin	Source[c]
STEC (*n* = 50)	O15:H27	88-1509	1, 2	-	Human	The STEC Center
	O26	MT#10	1	+	Human	The STEC Center
	O26:NM	TB352A	1	+	Human	The STEC Center
	O26:H11	97-3250[b]	1, 2	+	Human	The STEC Center
		3047-86	1	+	Human	The STEC Center
		EH1534	1	+	Human	BEI Resources
	O45:NM	DA-21	1	+	Human	The STEC Center
	O45:H2	MI01-88[b]	1	+	Human	The STEC Center
		MI03-19	1	+	Human	The STEC Center
		MI05-14	1	+	Human	The STEC Center
	O55:H7	5905	2	+	Food (meat)	The STEC Center
	O91:H21	B2F1	2	-	Human	BEI Resources
		H414-36/89	2	-	Human	BEI Resources
	O103:NM	PT91-24	1	+	Human	The STEC Center
	O103:H2	MT#80[b]	1	+	Human	The STEC Center
	O103:H6	TB154A	1	+	Human	The STEC Center
	O103:H25	8419	1	+	Human	The STEC Center
	O104:H21	G5506	2	-	Human	The STEC Center
	O111:NM	3007-85	1, 2	+	Human	The STEC Center
	O111:H2	RD8	2	-	Human	The STEC Center
	O111:H8	3215-99[b]	1, 2	+	Human	The STEC Center
	O111:H11	0201 9611	1	+	Human	The STEC Center
	O121	MT#18	2	+	Human	The STEC Center
	O121:H19	DA-5	2	+	Human	The STEC Center
		MDCH-4[b]	2	-	Human	The STEC Center
		MT#2	2	+	Human	The STEC Center
		MT#11	1, 2	+	Human	The STEC Center
	O145	EH1533	2	+	Human	BEI Resources
	O145:NM	GS G5578620[b]	1	+	Human	The STEC Center
		IH 16	2	+	Human	The STEC Center

Table 3.1 Cont.

	O145:H16	87-1713	1	+	Human	The STEC Center
	O145:H28	4865/96	2	+	Human	The STEC Center
	O157:NM	493/89	2	+	Human	The STEC Center
	O157:H7	86-24	2	+	Human	The STEC Center
		93-111	1, 2	+	Human	The STEC Center
		2886-75	1, 2	+	Human	The STEC Center
		A	1, 2	+	Human	BEI Resources
		BDMS 770	1, 2	+	Human	BEI Resources
		CoGen002096	2	+	Food (spinach)	BEI Resources
		E32511	2	+	Human	The STEC Center
		EDL931	1, 2	+	Human	BEI Resources
		EDL932	1, 2	+	Human	BEI Resources
		EDL933[b]	1, 2	+	Food (hamburger)	BEI Resources
		G5101	1, 2	+	Human	The STEC Center
		MDL 3562	2	+	Human	BEI Resources
		MDL 4444	2	+	Human	BEI Resources
		MDL 4445	2	+	Human	BEI Resources
		MDL 4572	2	+	Human	BEI Resources
		OK-1	1, 2	+	Human	The STEC Center
		RIMD 509952	1, 2	+	Human	BEI Resources
Non-STEC (*n* = 40)						
E. coli (*n* = 11)						
EAEC	O3:K2a,2b(L):H2	NCDC U14-41	-	-	Human	BEI Resources
EHEC	O55:H7	DEC5D	-	+	Human	The STEC Center
	O157:NM	94-G7771	-	+	Human	BEI Resources
EIEC	O28a,28c:K73(B18):NM	NCDC 909-51	-	-	Human	BEI Resources
	O29:NM	1885-77	-	-	Human	BEI Resources
EPEC	O126:K71(B16):NM	ATCC 12807	-	+	Human	BEI Resources
ETEC	O25:K98:NM	E2539-C1	-	-	Human	BEI Resources
	O78:H11	H10407	-	-	Human	BEI Resources
UPEC	O6:K2:H1	CFT073	-	-	Human	BEI Resources
Other *E. coli*	O9	HS	-	-	Human	BEI Resources

Table 3.1 Cont.

	OR:H48	K-12	-	-	Laboratory	BEI Resources
Salmonella enterica (*n* = 11)						
	Anatum	NR-4291	-	-	Food (tomato)	BEI Resources
	Braenderup	10 N	-	-	Food (raw chicken)	FDA CFSAN
	Enteritidis	SE 5	-	-	Food (lasagna)	FDA CFSAN
	Hartford	2807 H	-	-	Food (raw oyster)	FDA CFSAN
	Heidelberg	1364 H	-	-	Food (raw oyster)	FDA CFSAN
	Infantis	1102 H	-	-	Food (meat meal)	FDA CFSAN
	Javiana	2080 H	-	-	Food (frog legs)	FDA CFSAN
	Newport	1240 H	-	-	Food (dried yeast)	FDA CFSAN
	Saintpaul	1358 H	-	-	Food (mixed vegetables)	FDA CFSAN
	Stanley	1243 H	-	-	Food (bone meal)	FDA CFSAN
	Typhimurium	CIP 60.62	-	-	Laboratory	BEI Resources
Shigella (*n* = 7)						
boydii		NCTC 12985	-	-	Unknown	BEI Resources
dysenteriae	1	NCTC 4837	1	-	Human	BEI Resources
flexneri	2a	24570	-	-	Unknown	BEI Resources
		2457T	-	-	Laboratory	BEI Resources
	2b	ATCC 12022	-	-	Unknown	Lab collection
sonnei		NCTC 12984	-	-	Human	BEI Resources
		ATCC 25931	-	-	Human	Lab collection
Vibrio (*n* = 6)						
cholerae	O1	ATCC 14035	-	-	unknown	Lab collection
harveyi		ATCC 14126	-	-	Animal (dead amphipod)	Lab collection
fluvialis		ATCC 33809	-	-	Human	Lab collection
mimicus		ATCC 33653	-	-	Human	Lab collection
parahaemolyticus		ATCC 33847	-	-	Human	Lab collection
vulnificus		ATCC 27562	-	-	Human	Lab collection
Others (*n* = 5)						
Campylobacter jejuni		ATCC 33560	-	-	Animal (bovine feces)	Lab collection
Citrobacter freundii		ATCC 8090	-	-	Unknown	Lab collection
Enterobacter aerogenes		ATCC 13048	-	-	Human	Lab collection

Table 3.1 Cont.

Listeria monocytogenes	4b	ATCC 13932	-	-	Human	Lab collection
Staphylococcus aureus		ATCC 29213	-	-	Human	Lab collection

[a] Abbreviations are as following: STEC-Shiga toxin-producing *Escherichia coli*, EAEC-Enteroaggregative *E. coli*, EHEC-Enterohemorrhagic *E. coli*, EIEC-Enteroinvasive *E. coli*, EPEC-Enteropathogenic *E. coli*, ETEC-Enterotoxigenic *E. coli*, and UPEC-Uropathogenic *E. coli*.

[b] The seven labeled strains were used for both specificity and sensitivity evaluation of LAMP assays whereas others were used for the specificity test alone.

[c] The STEC Center is based at Michigan State University, East Lansing, MI. BEI Resources is located in Manassas, VA. FDA CFSAN stands for the U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, MD. Lab collection refers to our strain collection maintained at Louisiana State University, Baton Rouge, LA.

LAMP Specificity and Sensitivity. For LAMP specificity, DNA templates of 90 bacterial strains (Table 3.1) were prepared by heating at 95°C for 10 min as described previously (Chen et al., 2011). Aliquots (2 µl) of each template were subjected to LAMP amplification and repeated twice.

LAMP sensitivity (limit of detection) was determined by using 10-fold serial dilutions of seven STEC strains (Table 3.1). Briefly, 3-5 single colonies of each strain were inoculated separately into 8 ml of fresh TSB and incubated at 35°C for 16 h to reach the stationary phase (OD_{600} = 1, approximately 10^9 CFU/ml). The cultures were 10-fold serially diluted in 0.1% peptone water and aliquots (500 µl) of each dilution were used to prepare DNA templates similarly by heating. The exact cell numbers were determined by standard plate counting. Aliquots (2 µl) of each template were tested by LAMP and qPCR, and repeated three times.

LAMP Evaluation in Ground Beef. Ground beef (23% fat, 25 g) samples were obtained from a local grocery store and analyzed within 2 h of collection. To determine LAMP sensitivity in ground beef, each test sample (25 g) was inoculated with 2 ml of 10-fold serially diluted individual overnight STEC cultures, resulting in spiking levels between 10^9 and 10^5 CFU/25 g. Another sample was included as the uninoculated control. The samples were homogenized with 225 ml of buffered peptone water (BPW; BD Diagnostic Systems) in a food stomacher (Model 400; Tekmar Company, Cincinnati, OH) for 1 min. Aliquots (1 ml) of the homogenates were centrifuged at 16,000 × *g* for 3 min, and pellets were suspended in 100 µl of PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA). The mixtures were heated at 95°C for 10 min and centrifuged again at 12,000 × *g* for 2 min. The supernatants (2 µl) were used for both LAMP and qPCR, and repeated three times each. Aerobic plate counts were performed for the uninoculated control by standard pour plate method.

TABLE 3.2. LAMP and qPCR primers used in this study to detect STEC strains by targeting three genes (*stx1*, *stx2*, and *eae*)

Assay type	Primer/probe name	Sequence (5′-3′)	Position[a]	Amplicon size (bp)	Reference
stx1-LAMP	stx1-F3	TGATTTTTCACATGTTACCTTTC	507-529	Ladder-like bands with variable sizes for all three LAMP assays	This study
	stx1-B3	TAACATCGCTCTTGCCAC	688-705		
	stx1-FIP	CCTGCAACACGCTGTAACGT-CAGGTACAACAGCGGTTA	574-593, 530-547		
	stx1-BIP	AGTCGTACGGGGATGCAGAT-AGTGAGGTTCCACTATGC	598-617, 660-677		
	stx1-LF	GTATAGCTACTGTCACCAGACAATG	548-572		
	stx1-LB	AAATCGCCATTCGTTGACTACTTCT	618-642		
stx2-LAMP	stx2-F3	CGCTTCAGGCAGATACAGAG	812-831		
	stx2-B3	CCCCCTGATGATGGCAATT	1022-1040		
	stx2-FIP	TTCGCCCCCAGTTCAGAGTGA-GTCAGGCACTGTCTGAAACT	897-917, 840-859		
	stx2-BIP	TGCTTCCGGAGTATCGGGGAG-CAGTCCCCAGTATCGCTGA	927-947, 989-1007		
	stx2-LF	GCGTCATCGTATACACAGGAGC	860-881		
	stx2-LB	GATGGTGTCAGAGTGGGGAGAA	950-971		
eae-LAMP	eae-F3	TGACTAAAATGTCCCCGG	502-519		
	eae-B3	CGTTCCATAATGTTGTAACCAG	683-704		
	eae-FIP	GAAGCTGGCTACCGAGACTC-CCAAAAGCAACATGACCGA	581-600, 526-544		
	eae-BIP	GCGATCTCTGAACGGCGATT-CCTGCAACTGTGACGAAG	605-624, 664-681		
	eae-LF	GCCGCATAATTTAATGCCTTGTCA	545-568		
	eae-LB	ACGCGAAAGATACCGCTCT	625-643		
stx1-qPCR	stx1-150-F	GACTGCAAAGACGTATGTAGATTCG	252-276	151	(Fratamico et al., 2011)
	stx1-150-R	ATCTATCCCTCTGACATCAACTGC	379-402		
	stx1-150-P	FAM-TGAATGTCATTCGCTCTGCAATAGGTACTC-Iowa Black FQ	278-307		
stx2-qPCR	stx2-200-F	ATTAACCACACCCCACCG	425-442	206	
	stx2-200-R	GTCATGGAAACCGTTGTCAC	611-630		
	stx2-200-P	FAM-CAGTTATTTTGCTGTGGATATACGAGGGCTTG-Iowa Black FQ	445-476		
eae-qPCR	eae-170-F	CTTTGACGGTAGTTCACTGGAC	734-755	170	
	eae-170-R	CAATGAAGACGTTATAGCCCAAC	811-903		
	eae-170-P	FAM-CTGGCATTTGGTCAGGTCGGGGCG-Iowa Black FQ	789-812		

Table 3.2 Cont.

[a] The positions are numbered based on the coding sequences of STEC *stx1*, *stx2*, and *eae* genes with GenBank accession numbers M19473, X07865, and Z11541, respectively. Underlined corresponds to the F2 or B2 regions of the FIP or BIP primers, respectively.

Additionally, the capability of LAMP to detect low levels of seven STEC strains in ground beef was evaluated. For this application, ground beef samples were spiked with individual STEC cultures at two levels: 1-2 and 10-20 CFU/25 g. Another sample was included as the uninoculated control. The samples were homogenized with 225 ml of pre-warmed BPW supplemented with 8 mg/l vancomycin (Sigma-Aldrich, St. Louis, MO) in the food stomacher for 1 min, followed by incubation at 42^{o}C for up to 24 h. Aliquots (1 ml) of the enrichment broth were removed at 4, 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 both LAMP and qPCR. This experiment was independently repeated twice.

LAMP Application in Human Stools. Human stool specimen was obtained from donor and processed immediately. Each stool sample (0.5 g) was inoculated with 1 ml of 10-fold serially diluted STEC O157 strain EDL933 overnight culture, resulting in spiking levels of 10^3 and 10^4 CFU/0.5 g stool. The samples were mixed with 5 ml of TSA, and aliquots (1 ml) were removed for direct testing. The remaining mixtures were incubated at 35^{o}C and aliquots (1 ml) were removed at 4, 6, and 8 h for further analysis. For both direct stool testing and testing after enrichment, the samples were treated with PrepMan Ultra Sample Preparation Reagents as described above and subjected to both LAMP and qPCR. This experiment was independently repeated twice.

Data Analysis. Means and standard deviations of *Tt* for LAMP or *Ct* for qPCR were calculated by Microsoft Excel (Seattle, WA). The detection limits (CFU/reaction in pure culture or CFU/g in spiked ground beef) were presented as the lowest numbers of STEC cells that could be detected by the assays. In spiked ground beef, CFU/reaction was calculated by using CFU/g ×

25 g ÷ 250 × 10 × 2 × 10^{-3}, i.e., CFU/g × 2 × 10^{-3}. Similarly, in spiked human stools, CFU/reaction was converted by using CFU/g × 2 × 10^{-3}. Standard curves to quantify STEC in pure culture and spiked ground beef were generated by plotting *Tt* values against log CFU/reaction or log CFU/g, respectively, and quantitative capabilities of the LAMP assays were derived based on the coefficient of determination (R^2) values from the standard curves.

In spiked ground beef and human stool experiments, *Tt* and *Ct* values sorted by target gene, spiking level, and enrichment time were compared by using the analysis of variance (ANOVA; SAS for Windows version 9; SAS Institute Inc., Cary, NC). Differences between the mean values were considered significant when $P < 0.05$.

Results

LAMP Specificity. Among 90 bacterial strains (Table 3.1) used to determine specificity of the three LAMP assays (*stx1*-LAMP, *stx2*-LAMP, and *eae*-LAMP), false positive or false negative results were not observed, i.e., LAMP results matched 100% with known strain characteristics for the three target genes. Using *stx1*-LAMP, mean *Tt* values for 30 STEC strains harboring the *stx1* gene ranged from 11.4 to 14.5 min and one *stx1*-positive *Shigella dysenteriae* strain NCTC 4837 also gave positive LAMP result with a mean *Tt* value of 13.2 min. Similarly, by *stx2*-LAMP, mean *Tt* values for 35 STEC strains containing *stx2* fell between 13.1 to 19.7 min, whereas mean *Tt* values for 47 *eae*-positive *E. coli* strains fell between 13 to 25.2 min by *eae*-LAMP. In contrast, for strains lacking any or all of the three target genes, no *Tt* value was obtained by corresponding LAMP assays, suggesting negative LAMP results.

LAMP Sensitivity and Quantitative Capability. Table 3.3 summarizes LAMP sensitivity when testing 10-fold serial dilutions of individual STEC strains of seven serogroups

in three repeats. In pure culture testing, all three LAMP assays consistently detected down to 10^1 CFU/reaction of the seven STEC strains except for *eae*-LAMP when STEC O26 strain 97-3250 and O111 strain 3215-99 were tested. Further, in one to two out of three repeats, all three LAMP assays detected several STEC strains at concentrations 10-fold lower (i.e., 10^0 CFU/reaction). It is noteworthy that LAMP assays proceeded faster in some strains than others. For example, at the 10^5 CFU/reaction level, the mean *Tt* values by *stx1*-LAMP ranged from 15 min for STEC O103 strain MT#80 to 19.6 min for O157 strain EDL933. Similar variations in amplification speed among the seven STEC strains were also observed for *stx2*-LAMP and *eae*-LAMP. Regardless of target genes, the detection limits for qPCR were between 10^0-10^1 CFU/reaction (data not shown, see appendix).

Fig. 3.1 shows a typical LAMP amplification graph and a standard curve generated for pure culture sensitivity testing of STEC O157 strain EDL933 by *stx2*-LAMP. The *Tt* values ranged from 21.4 to 45.2 min for cell concentrations between 1.6×10^5 and 1.6 CFU/reaction. Excluding data for 1.6 and 16 CFU/reaction, the quantification equation for this assay was determined to be $y = -2x + 31.2$, and the coefficient of determination (R^2) was 0.997. Similar quantification equations were obtained for other assay/strain pairs and the overall R^2 values ranged between 0.933 and 0.997 (data not shown, see appendix).

LAMP sensitivity in spiked ground beef is also summarized in Table 3.3. For the uninoculated control sample, APC averaged 2×10^5 CFU/g and all three target genes tested negative by LAMP and qPCR (data not shown, see appendix). Using *stx1*-LAMP and *stx2*-LAMP, the lower limits of detection were at the 10^3 CFU/g level, equivalent to 8-14 CFU/reaction. While by *eae*-LAMP, at least 10-fold higher cell concentrations (i.e., 10^4 CFU/g) were needed in three strains (Table 3.3).

TABLE 3.3. Sensitivity of the three LAMP assays when testing 10-fold serial dilutions of individual STEC strains of seven serogroups in pure culture and spiked ground beef samples

Strain ID	Serotype	Stx	Intimin	Detection limit (CFU/reaction or CFU/g)					
				stx1-LAMP		*stx2*-LAMP		*eae*-LAMP	
				Culture	Ground beef	Culture	Ground beef	Culture	Ground beef
97-3250	O26:H11	1, 2	+	10	4×10^{3}-4×10^{4}[b]	1-10[b]	4×10^{3}-4×10^{4}[b]	10-100[b]	4×10^{4}-4×10^{5}[b]
MI01-88	O45:H2	1	+	1.6-16[a]	7×10^{3}	N/A	N/A	1.6-16[b]	7×10^{3}-7×10^{4}[a]
MT#80	O103:H2	1	+	1.6-16[b]	6.5×10^{3}	N/A	N/A	16	6.5×10^{4}
3215-99	O111:H8	1, 2	+	1.1-11[a]	5×10^{3}	1.1-11[b]	5×10^{3}	110-1,100[a]	5×10^{4}-5×10^{5}[a]
MDCH-4	O121:H19	2	-	N/A	N/A	1.2-12[b]	4×10^{3}	N/A	N/A
GS G5578620	O145:NM	1	+	17	4×10^{3}	N/A	N/A	1.7-17[b]	4×10^{3}
EDL933	O157:H7	1, 2	+	1.6-16[a]	6.5×10^{3}	1.6-16[b]	6.5×10^{3}	1.6-16[a]	6.5×10^{3}

[a] One out of three repeats was positive for the lower detection limit.

[b] Two out of three repeats were positive for the lower detection limit. In ground beef testing, CFU/reaction equals to CFU/g$\times2\times10^{-3}$.

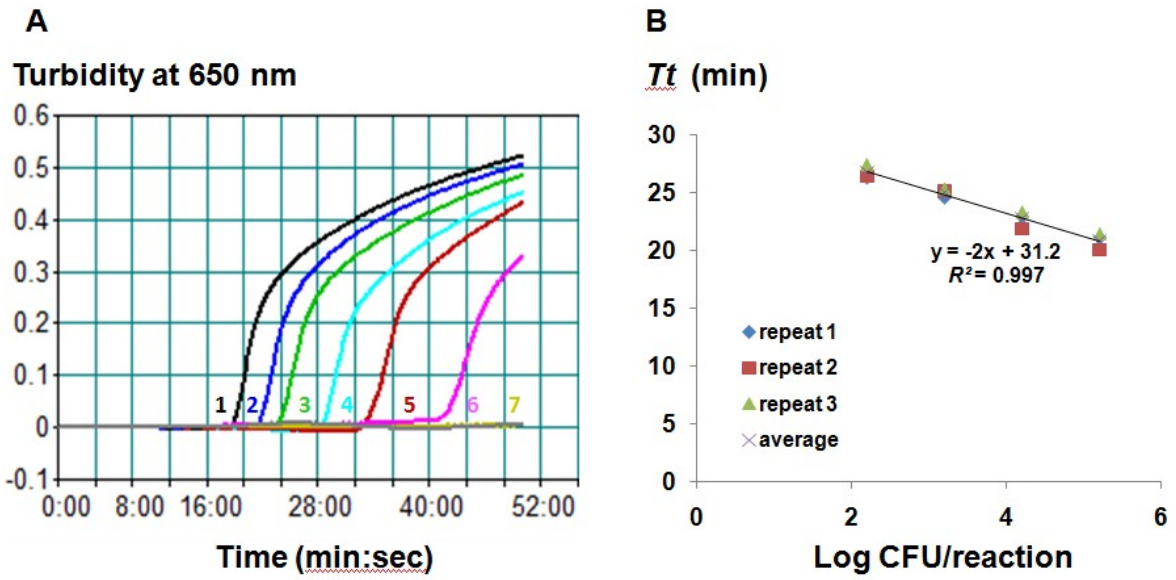

FIGURE 3.1. A typical LAMP amplification graph (A) and a standard curve generated for pure culture sensitivity testing of STEC O157 strain EDL933 by *stx2*-LAMP (B). Samples 1-6 correspond to 10-fold serial dilutions of *E. coli* O157:H7 EDL933 cells ranging from 1.6×10^5 to 1.6 CFU/reaction; sample 7 is water. The standard curve was drawn based on three independent repeats and excluding data for cell concentrations of 1.6 and 16 CFU/reaction.

In comparison, the majority of qPCR assays had detection limits of 10^4 CFU/g for *stx1* and *stx2* and 10^3 CFU/g for *eae* in spiked ground beef (data not shown, see appendix). Similar to pure culture testing, quantification equations were generated based on ground beef sensitivity data and R^2 ranged between 0.904 and 0.994 (data not shown, see appendix).

Rapid Detection of Low Levels of STEC in Ground Beef. Table 3.4 shows LAMP and qPCR results in ground beef samples spiked with two low levels (1-2 and 10-20 CFU/25 g) of individual STEC strains of seven serogroups after various enrichment periods. A typical LAMP amplification graph generated for ground beef enrichment samples is shown in Fig. 3.2. Regardless of spiking levels, none of the 4-h enrichment samples tested positive by either LAMP or qPCR. Positive LAMP results appeared at 6 h with significantly larger *Tt* values ($P < 0.05$),

and for samples enriched for 8, 10, 12, and 24 h, stable and lower *Tt* values were observed with no significant differences among different enrichment periods ($P > 0.05$) (Table 3.4). A similar trend of detection was observed for qPCR. At the 6-h enrichment point, the only LAMP-negative sample was the one spiked with STEC O157 strain EDL933 and tested by *stx1*-LAMP, which was confirmed by qPCR. However, STEC O45 strain MI01-88 tested positive by LAMP was negative by qPCR (Table 3.4). Additionally, qPCR results were presented by cycles, which were approximately 2 min/cycle. Therefore, additional 30-55 minutes of amplification time were needed for qPCR with the same enrichment sample.

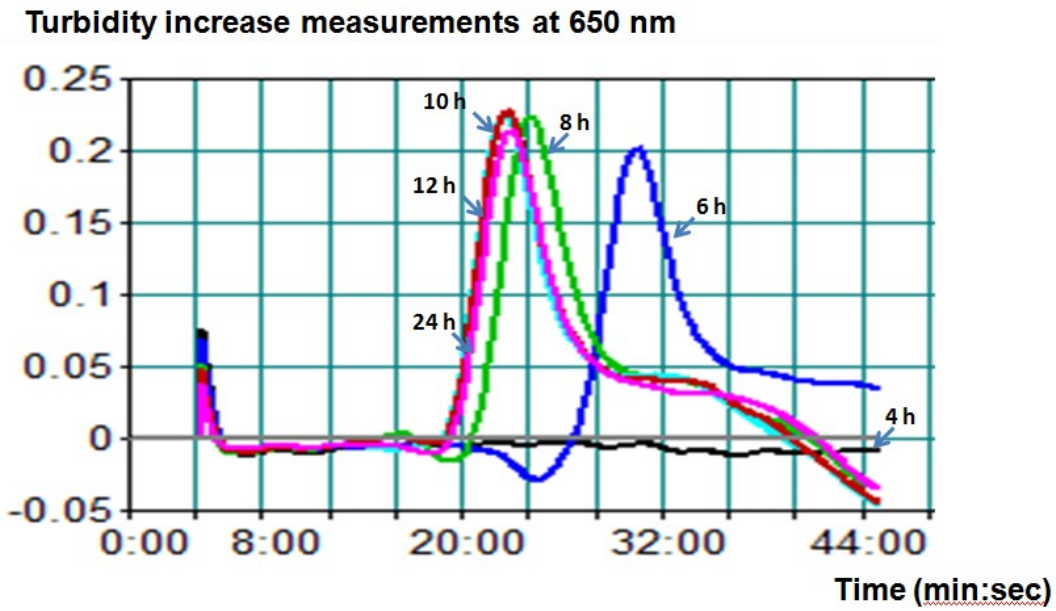

FIGURE 3.2. A typical LAMP amplification graph generated when testing ground beef samples spiked with two low levels of individual STEC strains of seven serogroups after various enrichment periods (4, 6, 8, 10, 12, and 24 h). In this graph, the ground beef samples were spiked with 1.2 CFU of STEC O111 strains 3215-99 and the enrichment samples were tested by *stx2*-LAMP.

Rapid Diagnostic of STEC in Human Stools. Table 3.5 shows LAMP and qPCR results in human stool specimen spiked with 10^3 and 10^4 CFU/0.5 g of STEC O157 EDL933 cultures based on two independent repeats. For direct stool testing, all samples were negative except for

the 10^4 CFU/0.5 g level tested by *eae*-LAMP in one repeat. Regardless of spiking levels, after 4, 6, and 8 h of enrichment, all samples were positive by LAMP. However, several negative qPCR results were observed at the 4-h enrichment point at the 10^3 CFU/0.5 g spiking level (Table 3.5). Noticeably, both *Tt* and *Ct* values decreased as the enrichment proceeded with significantly higher *Tt* values observed at the 4-h enrichment point ($P < 0.05$). Similar to ground beef testing, qPCR (*Ct* approximately 30 cycles) required additional 40 min to generate positive results compared to LAMP (*Tt* around 20 min).

Discussion

The three LAMP assays (*stx1*-LAMP, *stx2*-LAMP, and *eae*-LAMP) developed in the present study were rapid (11-45 min), specific (100% inclusivity and 100% exclusivity among 90 strains tested), sensitive (1-20 CFU/reaction in pure culture and 10^3-10^4 CFU/g in spiked ground beef), and accurate (R^2= 0.904-0.997). With 6-8 h of enrichment, the assays accurately detected two low levels (1-2 and 10-20 CFU/25 g) of STEC in ground beef samples. In human stool specimen, the assays also consistently detected STEC spiked at 10^3 or 10^4 CFU/0.5 g stool after 4 h enrichment. To our knowledge, this is the first study applying the novel LAMP NAAT technology to detect STEC in food and clinical samples by targeting both *stx* and *eae*. Previously, LAMP assays have been developed for the detection of generic (Hill et al., 2008) and pathogenic *E. coli*, including enteroaggregative *E. coli* (Yokoyama et al., 2010), enteroinvasive *E. coli* (Song et al., 2005), enterotoxigenic *E. coli* (Yano et al., 2007), STEC (Hara-Kudo et al., 2007; Kouguchi et al., 2010; Maruyama et al., 2003), and more specifically *E. coli* O157:H7 (Wang et al., 2009; Zhao et al., 2010; Zhu et al., 2009).

TABLE 3.4. Comparison of effect of enrichment time on LAMP or qPCR assays in ground beef samples spiked with low levels (1-2 CFU and 10-20 CFU/25 g) of individual STEC strains of seven serogroups

Gene[b]	Average LAMP *Tt* (min) after enrichment of[a]					Average qPCR *Ct* (cycles) after enrichment of[a]				
	6 h	8 h	10 h	12 h	24 h	6 h	8 h	10 h	12 h	24 h
stx1-l	20.5 ± 4.3^{A}	17.0 ± 3.5^{AB}	15.8 ± 3.1^{B}	15.9 ± 2.7^{B}	16.1 ± 3.3^{B}	37.4 ± 2.2^{A}	32.5 ± 3.3^{B}	29.4 ± 2.2^{B}	30.2 ± 1.9^{B}	30.5 ± 3.3^{B}
stx2-l	28.2 ± 3.3^{A}	22.4 ± 0.8^{B}	20.4 ± 0.6^{B}	20.3 ± 0.3^{B}	20.5 ± 1.1^{B}	37.7 ± 1.5^{A}	30.1 ± 3.4^{B}	28.4 ± 1.6^{B}	29.4 ± 1.4^{B}	29.0 ± 2.6^{B}
eae-l	27.7 ± 12.4^{A}	19.6 ± 4.8^{B}	18.8 ± 4.2^{B}	18.6 ± 4.0^{B}	18.9 ± 4.2^{B}	34.7 ± 2.2^{A}	28.3 ± 2.9^{B}	26.1 ± 2.0^{B}	27.3 ± 2.1^{B}	27.1 ± 2.6^{B}
stx1-h	22.0 ± 7.7^{A}	15.9 ± 3.4^{B}	15.1 ± 3.4^{B}	15.1 ± 3.0^{B}	15.2 ± 2.8^{B}	36.0 ± 3.5^{A}	28.9 ± 3.6^{B}	26.4 ± 3.5^{B}	27.2 ± 2.8^{B}	26.2 ± 3.4^{B}
stx2-h	23.4 ± 0.6^{A}	19.4 ± 0.4^{B}	18.5 ± 0.6^{B}	19.1 ± 0.7^{B}	19.2 ± 0.7^{B}	35.4 ± 2.3^{A}	27.8 ± 3.9^{B}	25.9 ± 3.0^{B}	26.8 ± 2.1^{B}	26.1 ± 3.0^{B}
eae-h	23.2 ± 7.1^{A}	18.5 ± 4.2^{AB}	18.0 ± 4.1^{AB}	17.2 ± 4.0^{B}	17.6 ± 4.0^{AB}	31.8 ± 4.4^{A}	26.0 ± 2.8^{B}	24.0 ± 3.0^{B}	24.4 ± 3.3^{B}	23.7 ± 3.4^{B}

[a] None of the 4-h enrichment samples tested positive by either LAMP or qPCR. After 6 h enrichment, one out of six *stx1*-positive strains were negative for LAMP whereas two were negative for qPCR. In each row within LAMP or qPCR, *Tt* or *Ct* values followed by different upper case letters are statistically significant ($P < 0.05$).

[b] *l* and *h* means low inoculation level (1-2 CFU/25 g) and high inoculation level (10-20 CFU/25 g), respectively.

TABLE 3.5. Comparison of effect of enrichment time on LAMP or qPCR assays in human stool specimen spiked with 10^3 and 10^4 CFU/0.5 g of STEC O157 strain EDL933 based on two independent repeats

Cell level	Target	LAMP *Tt* (min) after enrichment of				qPCR *Ct* (cycles) after enrichment of			
(CFU/0.5 g)	gene	0 h	4 h	6 h	8 h	0 h	4 h	6 h	8 h
10^3	*stx1*	-	32.0 ± 2.6^{A}	20.9 ± 1.5^{B}	17.3 ± 1.6^{B}	-	37.9[a]	34.1 ± 1.5^{A}	29.1 ± 1.4^{A}
	stx2	-	33.9 ± 2.3^{A}	23.9 ± 1.1^{B}	21.0 ± 1.3^{B}	-	-	34.3 ± 1.5^{A}	29.4 ± 1.5^{A}
	eae	-	27.8 ± 2.1^{A}	19.0 ± 2.0^{B}	15.9 ± 1.8^{B}	-	37.2[a]	32.3 ± 1.4^{A}	26.6 ± 1.5^{A}
10^4	*stx1*	-	22.9 ± 0.8^{A}	18.9 ± 0.4^{B}	18.2 ± 0.5^{B}	-	34.5 ± 1.4^{A}	31.3 ± 1.5^{A}	30.2 ± 1.4^{A}
	stx2	-	26.6 ± 1.3^{A}	23.2 ± 0.5^{AB}	21.5 ± 1.3^{B}	-	34.5 ± 1.2^{A}	31.2 ± 1.4^{AB}	30.3 ± 1.3^{B}
	eae	26.9[a]	19.8 ± 3.0^{A}	17.5 ± 1.4^{A}	17.0 ± 1.1^{A}	-	33.5 ± 1.8^{A}	29.7 ± 1.5^{A}	28.8 ± 1.1^{A}

[a] only one repeat generate positive result. In each row within LAMP or qPCR, *Tt* or *Ct* values followed by different upper case letters are statistically significant ($P < 0.05$).

With 35 min to 1 h of reaction time, these LAMP assays were capable of detecting between 0.7 and 100 CFU of *E. coli* per reaction, 10-100 fold more sensitive than conventional PCR (Hara-Kudo et al., 2007; Hill et al., 2008; Kouguchi et al., 2010; Song et al., 2005; Wang et al., 2009; Yokoyama et al., 2010; Zhu et al., 2009). The three LAMP assays developed here fell within these detection ranges in terms of speed and sensitivity. Numerous other studies also reported the superior sensitivity of LAMP in comparison with PCR (Chen et al., 2011; Han and Ge, 2008; Han et al., 2011); however, few comparisons were made between LAMP and qPCR. Similar to findings of the present study, a recent study on LAMP detection of *Salmonella* also reported comparable sensitivities between LAMP and qPCR (Chen et al., 2011). It is noteworthy that the LAMP assays reported here were markedly faster than qPCR assays developed by USDA scientists (Fratamico et al., 2011) by at least 30 min, therefore significantly shortening the total assay time.

Among the three target genes, *stx1* does not possess sequence heterogeneity, but multiple distinct variants of either *stx2* or *eae* have been identified (Gyles, 2007; Zhang et al., 2002). In this study, sequence alignments of several Stx2 and intimin variants were conducted before suitable regions were chosen for LAMP primer design. Consequently, all of the three LAMP assays possessed 100% inclusivity and 100% exclusivity among 50 STEC and 40 non-STEC strains tested, a specificity similar to that reported previously for LAMP assays targeting *stx1* and *stx2* (Hara-Kudo et al., 2007). Noticeably, *eae*-LAMP showed inferior sensitivity in detecting two strains (O26 97-3250 and O111 3215-99) compared to others (Table 3), which may be partially explained by sequence variations of the *eae* gene (Zhang et al., 2002).

LAMP positive reactions are commonly detected by gel electrophoresis, visual endpoint judgment of turbidity or color change, and real-time turbidity/fluorescence analysis (Mori and

Notomi, 2009). Through real-time turbidity analysis, the quantitative capability of LAMP has been demonstrated previously (Chen and Ge, 2010; Chen et al., 2011; Han and Ge, 2010; Han et al., 2011; Mori et al., 2004). Other studies also showed LAMP to be quantitative using fluorescence-based platforms (Ahmad et al., 2011; Chen and Ge, 2010; Han and Ge, 2010). In the present study, R^2 fell between 0.933 and 0.997 for STEC cells ranging from 10^5 to 10^2 CFU/reaction in pure culture and 0.904-0.988 for cells between 10^7 and 10^4 CFU/g (10^5 and 10^2 CFU/reaction) in spiked ground beef, suggesting good quantitative capabilities. However, for STEC cells lower than 10^2 CFU/reaction, the quantitative capability of LAMP was poor, indicated by much delayed *Tt* values (Fig.3.1A). Similar findings regarding the poor quantification of LAMP at low cell levels were reported previously (Aoi et al., 2006; Francois et al., 2011). It is important to note that whenever enrichment was incorporated in the detection steps, quantification is out the picture.

To date, application of LAMP assays for the detection of STEC and *E. coli* O157:H7 has been reported exclusively in food samples (Hara-Kudo et al., 2008a; Hara-Kudo et al., 2007; Hara-Kudo et al., 2008b; Ohtsuka et al., 2010; Wang et al., 2009). Only STEC O157 and O26 strains have been used for inoculation, and the spiked samples were usually enriched overnight without characterizing the effects of different enrichment time on the detection outcomes (Hara-Kudo et al., 2008a; Hara-Kudo et al., 2007; Hara-Kudo et al., 2008b; Ohtsuka et al., 2010; Wang et al., 2009). For instance, a recent study reported that 45-50% of liver samples inoculated with 1-4 CFU/25 g of *E. coli* O157:H7 strains tested positive by LAMP after overnight enrichment, compared to 10-35% detection rate by culture (Ohtsuka et al., 2010). Two earlier studies by the same group reported that for ground beef samples inoculated with approximately 10 CFU/25 g of *E. coli* O157 or O26 strains, 100% detection rates were observed after 24 h enrichment (Hara-

Kudo et al., 2008a; Hara-Kudo et al., 2007), whereas culture methods detected 100% of ground beef samples spiked with STEC O157 but only 50-80% of those spiked with STEC O26 (Hara-Kudo et al., 2008a). In raw milk, a detection limit of 4.1×10^4 CFU/ml of *E. coli* O157 was reported (Wang et al., 2009).

In the present study, STEC strains of seven major serogroups were used in ground beef experiments and STEC O157 EDL933 was also used to spike human stool specimen. The three LAMP assays had 10^3-10^4 CFU/g detection limits in ground beef, which were comparable to previously reported LAMP and qPCR assays (Fratamico et al., 2011; Wang et al., 2009). For the ground beef samples spiked with two low levels (1-2 and 10-20 CFU/25 g) of STEC, positive detection occurred at 6 h enrichment and consistently thereafter, which were superior than results obtained in the liver study (Ohtsuka et al., 2010) mentioned above. We also found LAMP performed better than qPCR in terms of positive detection rate and assay speed in spiked ground beef. In human stool experiments, consistent detection of samples spiked with 10^3 and 10^4 CFU/0.5 of STEC O157 EDL 933 culture after 4 h enrichment were observed by LAMP in the present study. Again, qPCR failed to detect several samples positive for LAMP after 4 h enrichment. In general, molecular-based detection methods such as PCR and LAMP are subject to various inhibitors present in food and clinical samples. However, LAMP has been confirmed previously to be more robust than PCR with regards to tolerance to inhibitors in clinical samples and other biological substances (Francois et al., 2011; Kaneko et al., 2007).

Currently, *E. coli* O157:H7 is regulated as an adulterant in raw beef in the U.S. The growing clinical importance of non-O157 *E. coli* also warrants the development of rapid, sensitive, and specific methods for detection. However, to meet the goal of detecting very low levels of these pathogens in food, enrichment is essential (Ge and Meng, 2009). For example, in

the newly updated USDA protocol for *E. coli* O157:H7 and non-O157 STEC detection in ground beef and beef trimmings, enrichment is an indispensable step followed by initial screening of Shiga toxins and intimins by qPCR and a second screening of O157 and top six STEC serogroups by another set of qPCR assays (U.S. Department of Agriculture, 2010). Given the rapidity, sensitivity, specificity, and robustness of LAMP assays demonstrated in the present study, these assays may effectively serve as serogroup-independent screening of STEC strains in ground beef samples, which is to be followed with serogroup-specific tests and virulence characterizations to ascertain the food safety and public health relevance of the STEC-positive samples. In conclusion, the LAMP assays developed in this study may facilitate rapid and reliable identification of STEC contaminations in high-risk food commodities and also facilitate prompt diagnosis of STEC infections in clinical laboratories

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CHAPTER 4: RAPID AND SPECIFIC DETECTION OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* O26, O45, O103, O111, O121, O145 AND O157 SEROGROUPS IN GROUND BEEF BY LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

Introduction

Shiga toxin-producing *E. coli* (STEC) distinguish themselves from other pathogenic *E. coli* by their potential to excrete the Shiga toxin and cause disease ranging from mild diarrhea to life-threatening complications such as hemorrhagic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Brooks et al., 2005; Johnson et al., 2006). Among more than 250 STEC serogroups identified to date (Johnson et al., 2006), STEC O157:H7 is the most frequently isolated one in the United States since 1982, and highly associated with bloody diarrhea and HUS. Meanwhile, approximately 100 other non-O157 STEC serogroups also contribute to the burden of comparable disease and are always involved in foodborne outbreaks (Johnson et al., 2006). It is estimated that STEC, including all serogroups, may annually cause 176,000 illnesses, 2,400 hospitalizations, and 20 deaths in the United States (Elaine Scallan et al., 2011). STEC strains are mainly carried on by ruminants particularly the cattle, and transferred through contaminated food (ground beef, produce, milk, and juice), water, or contact with animals and infected person (Johnson et al., 2006).

Recently, the number of non-O157 STEC infection has been on the rise since it became nationally notifiable in 2000, and a ten-fold increase in incidence was recorded in CDC FoodNet Report between 2000 and 2010 (0.12 cases per 100,000 to 1 case per 100,000 people). Conversely, the incidence of STEC O157:H7 infection has dropped by 44% (3 cases per 100,000 to 0.9 cases per 100,000 people) over the similar time span (Centers for Disease Control and Prevention, 2010). The top six most common non-O157 STEC serogroups (O26, O45, O103, O111, O121, O145) account for 71% of non-O157 STEC infection in the U.S., and O111 is the second serogroup most frequently linked with HUS after STEC O157 (Brooks et al., 2005). Other STEC serogroups may be more prevalent in continental Europe, South America, Australia,

and New Zealand (Johnson et al., 2006). For example, since May 2011, an unprecedented large outbreak of *E. coli* O104:H4 in Germany has resulted in a total of 4,075 cases (including 908 HUS) and 50 deaths as of July 21(WHO, 2011).

Although current methods like enzyme immunoassay (EIA) and molecular technique targeting the common virulent factors (Shiga toxin or the *stx*, *eae*, and *hlyA* genes) (Centers for Disease Control and Prevention, 2009) successfully provide the tool for early STEC detection in food matrix or human stool specimen, the isolation and serotyping of pure culture is still critical for timely and reliable identification of infection source, which in turn enable the implementation of appropriate public health action. However, the effective isolation method for non-O157 STEC is still problematic due to their same phenotypic characteristics as generic *E. coli* (Johnson et al., 2006). Traditional non-O157 STEC isolation relies on random picking from less selective agar followed by serotyping confirmation, leading to low recovery rates in *stx*-positive samples. A recent survey in the U.S. found 65% of labs conducting *E. coli* testing practice are only culturing for O157, so non-O157 strains would not be detected initially (Hoefer et al., 2011). One latest strategy for non-O157 STEC strains detection and isolation proposed an additional serotyping step before immuno magnetic separation (IMS), therefore, the target bacteria can be selected and concentrated before plating on selective agar and the recovery rate is increased (Fratamico et al., 2011; Perelle et al., 2007).

For STEC serotyping, two broad categories of assays are available. Previously, it mainly uses agglutination reactions between antisera and specific O-antigen; however, the process is time consuming and labor intensive, and generally can only be conducted in specialized laboratories (Centers for Disease Control and Prevention, 2009). Further, the cross-reaction of antisera with multiple O serogroups often occurs (Fratamico et al., 2005). Now rapid, specific,

and sensitive nucleic acid amplification tests (NAAT) such as PCR and qPCR have been developed to identify main virulent STEC O serogroups (O26, O45, O55, O91, O103, O104, O111, O121, O145, and O157) by targeting the *wzx* and *wzy* genes (coding for flippase and polymerase in O-antigen synthesis), which locate on a 10 kb O-antigen gene cluster fragment and are demonstrated to be specific to each O serogroup (D'Souza et al., 2002; DebRoy et al., 2005; Feng et al., 2005; Fratamico et al., 2003; Fratamico et al., 2005; Perelle et al., 2002; Wang et al., 2001; Wang et al., 1998; Wang and Reeves, 1998). Nonetheless, a sophisticated thermal cycling instrument is an indispensable requirement of such tests, limiting their wide applicability.

Recently, a novel NAAT technology termed loop-mediated isothermal amplification (LAMP) has attracted great attentions as a rapid, accurate, and cost-effective pathogen detection method in both food testing and clinical diagnostics (Mori et al., 2001; Notomi et al., 2000). LAMP employs four to six specially designed primers and a strand-displacing *Bst* DNA polymerase to amplify up to 10^9 target DNA copies under isothermal conditions (60-65^{o}C) within an hour (Mori et al., 2001). Since it is isothermal, LAMP can be performed in much simpler instruments such as a heater or water bath. To date, a few of LAMP assays targeting the *rfbE* gene (encoding perosamine synthetase) specific for the O157 antigen of STEC O157 have been developed (Wang et al., 2009; Zhao et al., 2010; Zhu et al., 2009). However, to our knowledge, there are no LAMP assays currently available for the top non-O157 STEC serogroups.

The objectives of this study were to develop rapid and reliable LAMP detection assays for 7 main STEC O-serogroup (O26, O45, O103, O111, O121, O145, and O157) typing by targeting the *wzx or wzy* genes, and evaluate the assay performance with ground beef experimentally contaminated with low levels of STEC strains of these seven major serogroups.

Materials and Methods

Bacterial Strains and Culture Conditions. Seven STEC clinical or food strains (Table 4.1) respectively representing serogroups O26, O45, O103, O111, O121, O145, and O157 were used for sensitivity testing and ground beef experiments. An additional 84 *E. coli* strains belonging to 19 different O serogroups and 29 non-*E. coli* strains (Table 4.1) were used for specificity testing. The strain serogroup information was provided by the donor institute. STEC and other *Enterobacteriaceae* strains were cultured at 35^{o}C overnight on trypticase soy agar or broth (TSA or TSB; BD Diagnostic Systems, Sparks, MD). Non-*Enterobacteriaceae* strains were grown on blood agar except for *Vibrio* strains for which TSA supplemented with 2% NaCl was used. *Campylobacter* strains were grown under microaerophilic conditions (85% N_2, 10% CO_2, and 5% O_2).

LAMP Primers and Reaction Conditions. The specific *wzx* gene for O103 and O145 (GenBank accession numbers AY532664 and AY647260), and *wzy* genes for O26, O45, O111, O121, and O157 (GenBank accession numbers AF529080, AY771223, AF078736, AY208937 and AF061251, respectively) were selected as targets for designing LAMP primers (Table 4.2). A set of six primers, two outer (F3 and B3), two inner (forward inner primer [FIP] and backward inner primer [BIP]), and one or two loop (LF and LB), which recognize eight distinct regions of the target gene sequence, were designed for each target using PrimerExplorer V4 (Fujitsu Limited, Japan). The primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Following the optimization of prototype LAMP condition recommended by the manufacturer (Eiken Chemical Co., Ltd., Tokyo, Japan), the final LAMP reaction mix (25 µl) for

TABLE 4.1. Bacterial strains used in this study to evaluate specificity and sensitivity of LAMP assays

Strain group[a]	Serotype	Strain ID[b]	Origin	Source[c]
STEC (*n* = 80)	O15:H27	88-1509	Human	The STEC Center
	O26	MT#10	Human	The STEC Center
		VP30	Human	The STEC Center
	O26:NM	TB352A	Human	The STEC Center
		DEC9F	Human	The STEC Center
	O26:H2	TB285A	Human	The STEC Center
	O26:H11	97-3250[b]	Human	The STEC Center
		3047-86	Human	The STEC Center
		EK29	Human	The STEC Center
		H19	Human	The STEC Center
		DEC10C	Human	The STEC Center
		EH1534	Human	BEI Resources
	O45:NM	DA-21	Human	The STEC Center
		5431-72	Human	The STEC Center
		4309-65	Human	The STEC Center
		D88-28058	Cow	The STEC Center
		B8026-C1	Cow	The STEC Center
		2566-58	Pig	The STEC Center
	O45:H2	MI01-88[b]	Human	The STEC Center
		MI03-19	Human	The STEC Center
		MI05-14	Human	The STEC Center
		DEC11C	Human	The STEC Center
	O55:H7	5905	Food (meat)	The STEC Center
	O91:H21	B2F1	Human	BEI Resources
		H414-36/89	Human	BEI Resources
	O103:NM	PT91-24	Human	The STEC Center
	O103:H2	MT#80[b]	Human	The STEC Center
		87-2931	Human	The STEC Center
		EK30	Human	The STEC Center
		107-226	Human	The STEC Center

Table 4.1 Cont.

	RW1372	Cow	The STEC Center
O103:H6	TB154A	Human	The STEC Center
O103:H25	8419	Human	The STEC Center
	MT#82	Human	The STEC Center
O104:H21	G5506	Human	The STEC Center
O111	TB226A	Human	The STEC Center
	412/55	Human	The STEC Center
	ED-31	Human	The STEC Center
	C412	Cow	The STEC Center
O111:NM	3007-85	Human	The STEC Center
	DEC8C	Cow	The STEC Center
O111:H2	RD8	Human	The STEC Center
O111:H8	3215-99[b]	Human	The STEC Center
	CL-37	Human	The STEC Center
	EK35	Human	The STEC Center
O111:H11	0201 9611	Human	The STEC Center
O121	MT#18	Human	The STEC Center
O121:NM	DA-1	Human	The STEC Center
	DA-69	Human	The STEC Center
O121:H7	87-2914	Human	The STEC Center
O121:H19	DA-5	Human	The STEC Center
	MT#2[b]	Human	The STEC Center
	MT#11	Human	The STEC Center
	F6173	Human	The STEC Center
	3-524	Human	The STEC Center
O145	EH1533	Human	BEI Resources
	TB269C	Human	The STEC Center
	0 2-3422	Rabbit	The STEC Center
O145:NM	GS G5578620[b]	Human	The STEC Center
	MT#66	Human	The STEC Center
	BCL73	Cow	The STEC Center
	IH 16	Human	The STEC Center

Table 4.1 Cont.

	O145:H28	4865/96	Human	The STEC Center
	O157:NM	493/89	Human	The STEC Center
	O157:H7	86-24	Human	The STEC Center
		93-111	Human	The STEC Center
		A	Human	BEI Resources
		BDMS 770	Human	BEI Resources
		CoGen002096	Food (spinach)	BEI Resources
		E32511	Human	The STEC Center
		EDL931	Human	BEI Resources
		EDL932	Human	BEI Resources
		EDL933[b]	Food (hamburger)	BEI Resources
		G5101	Human	The STEC Center
		MDL 3562	Human	BEI Resources
		MDL 4444	Human	BEI Resources
		MDL 4445	Human	BEI Resources
		MDL 4572	Human	BEI Resources
		OK-1	Human	The STEC Center
		RIMD 509952	Human	BEI Resources
Non-STEC (*n* = 40)				
E. coli (*n* = 11)				
EAEC	O3:K2a,2b(L):H2	NCDC U14-41	Human	BEI Resources
EHEC	O55:H7	DEC5D	Human	The STEC Center
	O157:NM	94-G7771	Human	BEI Resources
EIEC	O28a,28c:K73(B18):NM	NCDC 909-51	Human	BEI Resources
	O29:NM	1885-77	Human	BEI Resources
EPEC	O126:K71(B16):NM	ATCC 12807	Human	BEI Resources
ETEC	O25:K98:NM	E2539-C1	Human	BEI Resources
	O78:H11	H10407	Human	BEI Resources
UPEC	O6:K2:H1	CFT073	Human	BEI Resources
Other *E. coli*	O9	HS	Human	BEI Resources
	OR:H48	K-12	Laboratory	BEI Resources
Salmonella (n=11)	Anatum	NR-4291	Food (tomato)	BEI Resources

Table 4.1 Cont.

	Braenderup	10 N	Food (raw chicken)	FDA CFSAN
	Enteritidis	SE 5	Food (lasagna)	FDA CFSAN
	Hartford	2807 H	Food (raw oyster)	FDA CFSAN
	Heidelberg	1364 H	Food (raw oyster)	FDA CFSAN
	Infantis	1102 H	Food (meat meal)	FDA CFSAN
	Javiana	2080 H	Food (frog legs)	FDA CFSAN
	Newport	1240 H	Food (dried yeast)	FDA CFSAN
	Saintpaul	1358 H	Food (mixed vegetables)	FDA CFSAN
	Stanley	1243 H	Food (bone meal)	FDA CFSAN
	Typhimurium	CIP 60.62	Laboratory	BEI Resources
Shigella (n=7)				
boydii		NCTC 12985	Unknown	BEI Resources
dysenteriae	1	NCTC 4837	Human	BEI Resources
flexneri	2a	24570	Unknown	BEI Resources
		2457T	Laboratory	BEI Resources
	2b	ATCC 12022	Unknown	Lab collection
sonnei		NCTC 12984	Human	BEI Resources
		ATCC 25931	Human	Lab collection
Vibrio (n=6)				
cholerae	O1	ATCC 14035	unknown	Lab collection
harveyi		ATCC 14126	Animal (dead amphipod)	Lab collection
fluvialis		ATCC 33809	Human	Lab collection
mimicus		ATCC 33653	Human	Lab collection
parahaemolyticus		ATCC 33847	Human	Lab collection
vulnificus		ATCC 27562	Human	Lab collection
Others (n = 5)				
Campylobacter jejuni		ATCC 33560	Animal (bovine feces)	Lab collection
Citrobacter freundii		ATCC 8090	Unknown	Lab collection
Enterobacter aerogenes		ATCC 13048	Human	Lab collection
Listeria monocytogenes	4b	ATCC 13932	Human	Lab collection
Staphylococcus aureus		ATCC 29213	Human	Lab collection

Table 4.1 Cont.

[a] Abbreviations are as following: STEC-Shiga toxin-producing *Escherichia coli*, EAEC-Enteroaggregative *E. coli*, EHEC-Enterohemorrhagic *E. coli*, EIEC-Enteroinvasive *E. coli*, EPEC-Enteropathogenic *E. coli*, ETEC-Enterotoxigenic *E. coli*, and UPEC-Uropathogenic *E. coli*.

[b] The seven labeled strains were used for both specificity and sensitivity evaluation of LAMP assays whereas others were used for the specificity test alone.

[c] The STEC Center is based at Michigan State University, East Lansing, MI. BEI Resources is located in Manassas, VA. FDA CFSAN stands for the U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, MD. Lab collection refers to our strain collection maintained at Louisiana State University, Baton Rouge, LA

TABLE 4.2. LAMP primers used in this study to detect STEC serogroups by targeting 7 genes (*O26-wzy, O45-wzy, O103-wzx, O111-wzy, O121-wzy, O145-wzx, O157-wzy*)

Target	Primer name	Sequence (5′-3′)	Position[a]
O26	O26-F3	GACTATGAAGCGTATGTTGAT	136-156
	O26-B3	TCCTGATTTGAACAATGTCAAT	352-373
	O26-FIP	ACCGCCTAAATACTTAACACCATAA-TTAATGTCAATGAACTTTATGCC	207-231, 161-183
	O26-BIP	TTCCTTGGGACCACATTCCT-ACATGTAAAGCAGCAAACC	265-284, 319-337
	O26-LF	ACCAGCGATAACCAATCTC	184-202
	O26-LB	TACAATACAGTAAGTATACAGCATT	293-317
O45	O45-F3	AATGTCCCCAGGGTTTGT	15-32
	O45-B3	TTTAGTCGCTCGCCAAGA	217-234
	O45-FIP	AGCGGGCTAATATTAGTAGTCACTC-GTATGCTTCAATTTGGCTGT	77-101, 33-52
	O45-BIP	ACTCTGGGTTTGATTTTTTCACTTC-ATAATTTCATCCAGACGAACG	139-163, 192-212
	O45-LB	TTATTACTCCTGGCAGTATTAATCG	167-191
O103	O103-F3	ACTCAGTGGTGTAGTAACATG	33-53
	O103-B3	TCACCTTGATTTTCTGCTGA	205-224
	O103-FIP	ATTTGCTATTCCAATTGGACCAGTA-CTTTAGACTAATTTGTGGCCTTC	102-126, 54-76
	O103-BIP	TTGGGACAATTGCAAAATTTTGTGG-ATCTATTAACTCCTTGTGAAACTTG	127-151, 178-202
	O103-LF	AATTGCAACAACTTTTGAAATAA	77-99
	O103-LB	CCTTTATAAATGGATTCATTTCATC	152-176
O111	O111-F3	AAGGCGTAACTTTTTTTGAAC	623-643
	O111-B3	TCATGAGGGTCATTAGGAATT	786-806
	O111-FIP	TCACCAAGCTGTGAAACCAAA-CTACAGCAAGTAATATTGAACGT	684-704, 644-666
	O111-BIP	TCCATGGTATGGGGACATTAAATTT-TGATGGAAGTCCATATAACGT	713-737, 763-783
	O111-LB	CTTAAATAACGGCGGACAAT	738-757
O121	O121-F3	GCTCAGCTTTTATCTTGTTCAA	864-885
	O121-B3	ATAGGCTCCCAACCATCC	1087-1104
	O121-FIP	ACGCAAAAAGTATGGATTCATACCT-GATATAACAGAACCGACTTGG	955-979, 895-915
	O121-BIP	TGTTGCTGGTTCCTTATTATGTAGT-AAAAGCAAGCCAAAACACTC	995-1019, 1047-1066
	O121-LF	TAAAGCCATCCAACCACGC	929-947
O145	O145-F3	TTTGTAAGACAAGGTGTATGG	433-453
	O145-B3	GCATTGGTACAGACAGCTTTA	632-652

Table 4.1 Cont.

	O145-FIP	CACAGTACCACCAAACCAAAAAATA-TTGGTTAGCTATAGCTGTGA	516-540, 456-475
	O145-BIP	AGTGTGCTTGGAGTGGCTTA-CAATCCCAGTTTGTAATATCGC	547-566, 590-611
	O145-LF	TTCTTAAGTTCGGATACACTAGCA	476-499
O157	O157-F3	TCCCTTTAGGGATATATATACCTT	935-958
	O157-B3	ATAACTGATATTTTCATTTCGTGAT	1146-1170
	O157-FIP	TTCCCAGCCACTAAGTATTGCAATA-TGAAAAAAACCCATAGCTCGA	1034-1058, 977-997
	O157-FIP	TGCATCGGCCTTCTTTTTTGG-AACGTATCATGCAATAAGATCA	1059-1079, 1115-1136
	O157-LF	ATAATGATATATGAATAGAATGCGC	1004-1028
	O157-LB	TCCTTTTCTCTCCGTATTGAT	1080-1100

[a] The positions are numbered based on the coding sequences of STEC O-antigen specific *O26-wzy*, *O45-wzy*,*O103-wzx*, *O111-wzy*, *O121-wzy*, *O145-wzx*, and *O157-wzy* genes with GenBank accession numbers AF529080, AY771223, AY532664, AF078736, AY208937, AY647260 and AF061251, respectively.

all seven targets consisted of 1× ThermoPol reaction buffer (New England Biolabs, Ipswich, MA), 6 mM $MgSO_4$, 1.2 mM each deoxynucleoside triphosphate (dNTP), 0.1 μM F3 and B3, 1.8 μM FIP and BIP, 1 μM LF and LB, 10 U of *Bst* DNA polymerase (New England Biolabs), and 2 μl of DNA template. The LAMP reactions were carried out at 63°C for 1 h for O157 STEC, or at 65°C for the other six STEC serogroups. Then it was terminated at 80°C for 5 min in an LA-320C real-time turbidimeter (Eiken Chemical Co., Ltd.), which acquired turbidity readings at 650 nm every 6 s. The time threshold (*Tt*; min) values were determined when the turbidity increase measurements (the differential value of the moving average of turbidity) exceeded a threshold of 0.1.

qPCR Assays. In comparison, qPCR assays designed in one recently published study targeting O-serogroup determining genes were also carried out with minor modification. The qPCR reagent mix (25 μl) contained 1× PCR buffer, 0.2 mM each dNTP, 4 mM $MgCl_2$, 0.25 μM each primer, 0.1875 μM probe (Integrated DNA Technologies), 1.5 U of GoTaq Hot Start Polymerase (Promega, Madison, WI), and 2 μl of DNA template. The assays were conducted using 40 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 50 s in a SmartCycler II System (Cepheid, Sunnyvale, CA). Fluorescence readings were acquired using the FAM channel (excitation at 450-495 nm and detection at 510-527 nm). The cycle threshold (*Ct*; cycle) values were obtained when the fluorescence readings crossed a threshold of 30 units.

LAMP Specificity and Sensitivity. A total of 120 bacterial strains (Table 4.1) were used to determine LAMP specificity. DNA templates were prepared by heating at 95°C for 10 min as

described previously. Aliquots (2 µl) of each DNA template were subjected to LAMP amplification. Specificity testing was repeated twice for each strain.

LAMP sensitivity (limits of detection) was determined by using 10-fold serial dilutions of seven individual STEC strains (Table 4.1). Briefly, 3-5 single colonies of each strain were inoculated separately into 8 ml of fresh TSB and incubated at 35°C for 16 h to reach stationary phase (optical density at 600 nm [OD_{600}] = 1, approximately 10^9 CFU/ml). The cultures were 10-fold serially diluted in 0.1% peptone water and aliquots (500 µl) of each dilution were used to prepare DNA templates similarly by heating. The exact cell numbers were determined by standard plate counting. Aliquots (2 µl) of the sensitivity templates were tested by LAMP and qPCR, and repeated five times each.

LAMP Evaluation in Ground Beef. Ground beef (23% fat, 25 g) samples were obtained from a local grocery store and analyzed within 2 h of collection. To determine LAMP sensitivity in ground beef, each test sample (25 g) was inoculated with 2 ml of 10-fold serially diluted individual overnight STEC cultures, resulting in spiking levels between 10^9 and 10^5 CFU/25 g. Another sample was included as the uninoculated control. The samples were homogenized with 225 ml of buffered peptone water (BPW; BD Diagnostic Systems) in a food stomacher (Model 400; Tekmar Company, Cincinnati, OH) for 1 min. Aliquots (1 ml) of the homogenates were centrifuged at 16,000 × *g* for 3 min, and pellets were suspended in 100 µl of PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA). The mixtures were heated at 95°C for 10 min and centrifuged again at 12,000 × *g* for 2 min. The supernatants (2 µl) were used for both LAMP and qPCR, and repeated three times each. Aerobic plate counts were performed for the uninoculated control by standard pour plate method.

Additionally, the capability of LAMP to detect low levels of seven STEC strains in ground beef was evaluated. For this application, ground beef samples were spiked with individual STEC cultures at two levels: 1-2 and 10-20 CFU/25 g. Another sample was included as the uninoculated control. The samples were homogenized with 225 ml of pre-warmed BPW supplemented with 8 mg/l vancomycin (Sigma-Aldrich) in the food stomacher for 1 min, followed by incubation at 42^{o}C for up to 24 h. Aliquots (1 ml) of the enrichment broth were removed at 4, 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 both LAMP and qPCR (in single target format). This experiment was independently repeated twice.

Data Analysis. Means and standard deviations of *Tt* for LAMP or *Ct* for qPCR were calculated by Microsoft Excel (Seattle, WA). The detection limits (CFU/reaction in pure culture or CFU/g in spiked ground beef) were presented as the lowest numbers of STEC cells that could be detected by the assays. In spiked ground beef, CFU/reaction was calculated by using CFU/g × 25 g ÷ 250 × 10 × 2 × 10 $^{-3}$, i.e., CFU/g × 2 × 10^{-3}. Standard curves to quantify STEC in pure culture and spiked ground beef were generated by plotting *Tt* values against log CFU/reaction or log CFU/g, respectively, and linear regression was calculated using Microsoft Excel. Quantitative capabilities of the LAMP assays were derived based on the coefficient of determination (R^2) values from the standard curves.

In spiked ground beef experiments, *Tt* and *Ct* values sorted by target gene, spiking level, and enrichment time were compared by using the analysis of variance (ANOVA; SAS for Windows, version 9; SAS Institute Inc., Cary, NC). Differences between the mean values were considered significant when $P < 0.05$

Results

LAMP Specificity. Among 120 bacterial strains (Table 4.1) used to evaluate the seven LAMP assays specificity, no false-positive or false-negative results were observed after comparing LAMP results with known strain serogroup characteristics, indicating 100% inclusive and exclusive accuracy (Table 4.3). Using O26-LAMP assay, the *Tt* value for 11 STEC strains belonging to O26 serogroup ranged from 15.6 to 20.4 min, with an average of 16.9 ± 1.4 min. Similarly, the average *Tt* values determined by the other six O serogroup specific LAMP assays for 10 O45-STEC, 9 O103-STEC, 11 O111-STEC, 9 O121-STEC, 8 O145-STEC, and 18 O157-*E. coli* strains (17 O157 STEC and 1 O157 EHEC) were 19.8 ± 0.8 min, 19.8 ± 1.2 min, 21.7 ± 0.9 min, 19.5 ± 1.5 min, 17.5 ± 1.3 min, and 16.7 ± 1.3 min, respectively. In contrast, no *Tt* values were obtained from either LAMP assay for the other 43 strains consisting of 15 *E. coli* from 12 other different O serogroups and 29 non-*E. coli* strains (Table 4.1), suggesting negative LAMP results.

TABLE 4.3. Inclusive and exclusive studies of O serogroup specific LAMP assays

O serogroups	Inclusive[a]	Exclusive[b]	
		Other *E. coli*	Microorganisms[c]
O157	18/18	0/73	0/29
O26	11/11	0/80	0/29
O45	10/10	0/81	0/29
O103	9/9	0/82	0/29
O111	11/11	0/80	0/29
O121	9/9	0/82	0/29
O145	8/8	0/83	0/29

[a] Inclusive studies are listed as number of positive results/number of inclusive strains tested
[b] Exclusive studies are listed as number of false positive results/number of exclusive strains tested
[c] Microorganisms include *Shigella*, *Salmonella*, *Vibrio*, *Staphylococcus*, *Campylobacter*, *Listeria*, *Citrobacter* and *Enterobacter*

LAMP Sensitivity and Quantitative Capability. Table 4.4 summarized LAMP sensitivity when testing 10-fold serial dilutions of individual STEC strains of seven serogroups in five repeats. For the pure culture templates, all seven O-antigen specific LAMP assays were cable to consistently detect down to 10^1 CFU/reaction in all tests. Further, O45-based LAMP assay had a even better detection limit of 1.6 CFU/reaction, and in two out of five repeats, O26-based LAMP can detect several targets at 10-fold lower template concentration (i.e., 1 CFU/reaction). It is noteworthy that O111-based LAMP assay proceeded slightly slower than the other six LAMP assays. When testing the pure culture templates ranging from 10^5 to 10^1 CFU/reaction level, the average *Tt* value for O111-based LAMP assay fell between 25.7 min and 41.8 min, which was approximately 5 minutes later than other assays. In comparison with corresponding qPCR assay, the similar detection limit (10^0-10^1 CFU/reaction) was achieved for each serogroup testing.

TABLE 4.4. Sensitivity of LAMP and qPCR assays when testing 10-fold serial dilutions of individual STEC strains of seven serogroups in pure culture and spiked ground beef

Strain ID	Serotype	Detection limit of LAMP		Detection limit of qPCR	
		Culture (CFU/R)	Ground beef (CFU/g)	Culture (CFU/R)	Ground beef (CFU/g)
EDL933	O157:H7	16	6.5×10^3-10^4 [a]	16	6.5×10^4
97-3250	O26:H11	1-10 [a]	4×10^4	10	4×10^4
MI01-88	O45:H2	1.6	7×10^3	1.6	7×10^3
MT#80	O103:H2	16	6.5×10^3	16	6.5×10^3
3215-99	O111:H8	11	4×10^4	11	4×10^4
MT#2	O121:H19	18	9×10^3	1.8	9×10^3
GS G5578620	O145:NM	17	4×10^3-10^4 [b]	17	4×10^4

[a] Two out of five repeats were positive for the lower detection limit. [b] Three out of five repeats were positive for the lower detection limit. In ground beef testing, CFU/reaction equals to CFU/g $\times 2 \times 10^{-3}$.

Fig 4.1. shows a typical LAMP amplification graph and standard curve generated when 10-fold serially diluted STEC O26 strain 97-3250 was tested by O26-based LAMP in pure culture format. The average *Tt* value based on five repeats ranged from 20.9 min to 35.2 min for cell concentrations between 1×10^5 and 10 CFU/reaction. Within this template range, the quantification equation was determined to be y = -3.556 x + 37.636 with the coefficient of determination (R^2) at 0.964. Similar quantification equations were obtained for other assay/serogroup combinations in its detection limit range, and the overall R^2 values ranged between 0.945 and 0.993 (data not shown, see appendix).

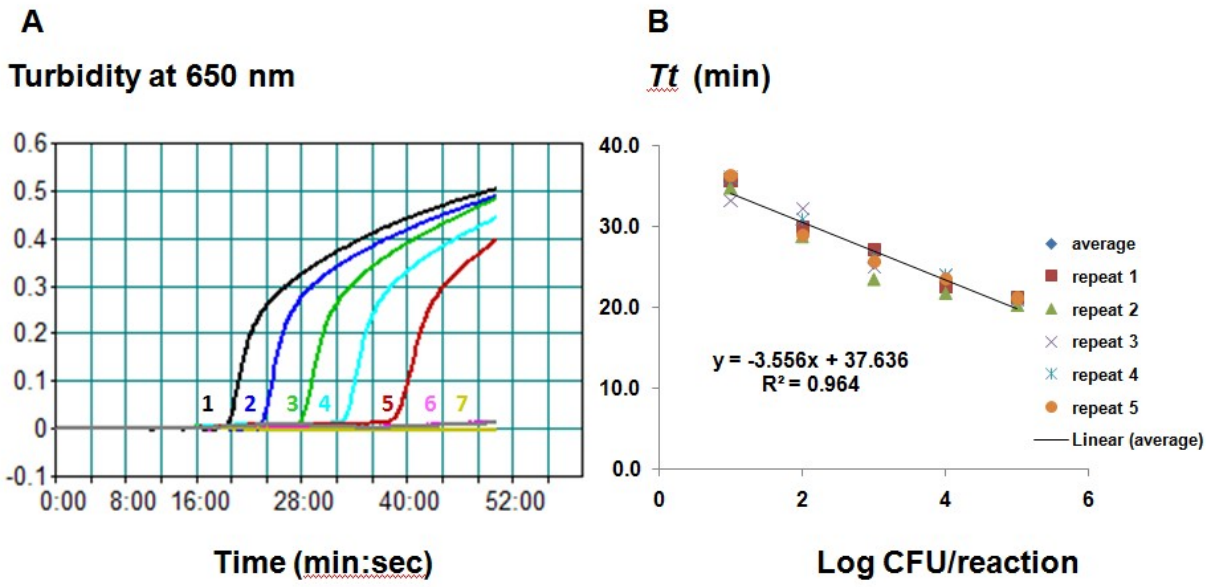

FIGURE 4.1. A typical LAMP amplification graph (A) and a standard curve generated for pure culture sensitivity testing of STEC O26 strain 97-3250 by O26-LAMP (B). Samples 1-6 correspond to 10-fold serial dilutions of *E. coli* O26:H11 97-3250 cells ranging from 1×10^5 to 1 CFU/reaction; sample 7 is water. The standard curve was drawn based on five independent repeats.

LAMP sensitivity result in spiked ground beef is also summarized in Table 4.4. For the uninoculated control sample, APC averaged 2×10^5 CFU/g and all seven target genes tested

negative by LAMP and qPCR. Among 7 O serogroup specific LAMP assays, three assays (O45, O103, and O121-based) consistently detected their targets in five repeats at the 10^3 CFU/g level, equivalent to 13-18 CFU/reaction, whereas two assays (O145 and O157-based) only partially recognized their target genes at this level, and another two assays (O26 and O111-based) required 10-fold higher cell concentrations (i.e., 10^4 CFU/g) to achieve positive result. In comparison, qPCR assays had detection limits of 10^4 CFU/g for O26, O111, O145 and O157 serogroups, and 10^3 CFU/g for the other three ones, including O45, O103, and O121. Similar to pure culture testing, quantification equations were generated based on ground beef sensitivity data and R^2 ranged between 0.932 and 0.982 in their detection range (data not shown, see appendix).

Rapid Detection of Low Levels of STEC in Ground Beef. Table 4.5 shows LAMP and qPCR results for ground beef samples inoculated with low level (1-2 and 10-20 CFU/25 g) of individual STEC strains of seven serogroups after various enrichment periods. Fig.4.2 shows a typical LAMP amplification graph generated for *E. coli* O26:H11 strain 97-3250 by O26-based LAMP in ground beef enrichment samples. All of the samples tested negative after 4 h enrichment by either LAMP or qPCR. Positive LAMP results appeared at 6 h with significantly larger *Tt* values ($P < 0.05$), and for samples enriched for 8, 10, 12, and 24 h, stable and lower *Tt* values were observed with no significant difference among different enrichment periods ($P > 0.05$) (Table 4.5). A similar trend of detection was observed for qPCR with 6 h enrichment being the starting point for positive results. However, qPCR results were presented by cycles, which were approximately 2 min/cycle. Therefore, additional 25 to 40 minutes of amplification time were needed for qPCR with the same sample.

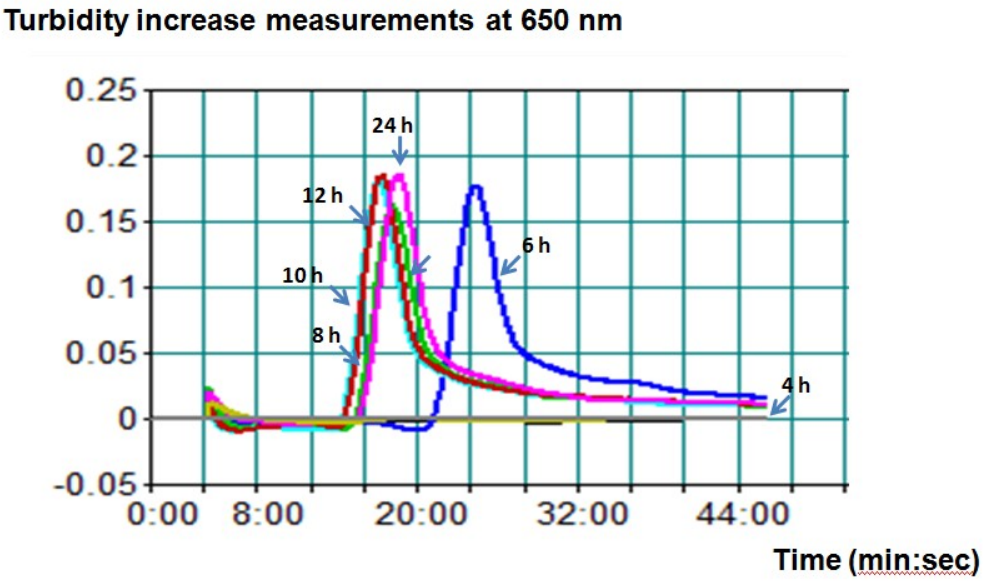

FIGURE 4.2. A typical LAMP amplification graph generated when testing ground beef samples spiked with two low levels of individual STEC strains of seven serogroups after various enrichment periods (4, 6, 8, 10, 12, and 24 h). In this graph, the ground beef samples were spiked with 10 CFU of STEC O26 strain 97-3250 and the enrichment samples were tested by O26-LAMP.

TABLE 4.5. Comparison of effect of enrichment time on LAMP or qPCR assays in ground beef samples spiked with low levels (1-2 and 10-20 CFU/25 g) of individual STEC strains of seven serogroups

Target	Level	LAMP *Tt* (min) after enrichment of[a]					qPCR *Ct* (cycles) after enrichment of[a]				
	CFU/25g	6 h	8 h	10 h	12 h	24 h	6 h	8 h	10 h	12 h	24 h
O157	1-2	34.2 ± 0.1^{A}	26.7 ± 0^{B}	21.2 ± 3.2^{C}	20.7 ± 3.5^{C}	23.5 ± 0.3^{BC}	37.2 ± 0.8^{A}	30.8 ± 1.1^{B}	27.1 ± 2.4^{B}	26.1 ± 4^{B}	29 ± 1.8^{B}
	10-20	31.3 ± 2^{A}	20.4 ± 1.9^{B}	17.9 ± 3.3^{B}	17.9 ± 3.3^{B}	20.9 ± 1.1^{B}	33.4 ± 2.4^{A}	27.2 ± 3.3^{AB}	24.4 ± 4.5^{AB}	23.6 ± 5.5^{B}	25.5 ± 1.9^{AB}
O26	1-2	29.3 ± 3.3^{A}	18.9 ± 0.5^{B}	17.4 ± 0.9^{B}	17.7 ± 1.3^{B}	18.5 ± 0.3^{B}	34.8 ± 0.7^{A}	27.4 ± 1.1^{B}	23.8 ± 3.4^{B}	24.2 ± 3^{B}	25.5 ± 1.1^{B}
	10-20	23.6 ± 0.9^{A}	18 ± 1.4^{B}	17 ± 1.5^{B}	16.9 ± 1.1^{B}	17.7 ± 0.7^{B}	33 ± 1^{A}	25.4 ± 2.9^{AB}	22.5 ± 3.8^{B}	22.2 ± 4.3^{B}	23.8 ± 2.7^{B}
O45	1-2	36.6 ± 0.2^{A}	26.2 ± 0.2^{B}	23.5 ± 0.1^{C}	22.6 ± 0.1^{C}	24.5 ± 2.1^{BC}	35.2 ± 1.1^{A}	28 ± 2.3^{B}	23.2 ± 2.6^{B}	23.2 ± 3.3^{B}	24.8 ± 2.3^{B}
	10-20	30.6 ± 0.9^{A}	24.4 ± 0.1^{B}	21.2 ± 1^{C}	21 ± 0.6^{C}	22.6 ± 1.4^{BC}	31.2 ± 0.9^{A}	24.3 ± 2.2^{B}	19.8 ± 2^{B}	19.9 ± 2.8^{B}	22 ± 0.2^{B}
O103	1-2	31.7 ± 7.6^{A}	20.6 ± 1.3^{B}	18.6 ± 0^{B}	18.5 ± 0.8^{B}	19.2 ± 0.3^{B}	33.6 ± 3.5^{A}	25.8 ± 2.5^{B}	21 ± 2.1^{B}	21.3 ± 3.0^{B}	22.1 ± 0.4^{B}
	10-20	29.2 ± 8.7^{A}	20.1 ± 1.6^{AB}	17.7 ± 0^{B}	17.5 ± 1^{B}	17.9 ± 0.3^{B}	32.1 ± 4.1^{A}	24.7 ± 3.8^{B}	19.3 ± 1.6^{B}	18.9 ± 2.6^{B}	19.6 ± 0.3^{B}
O111	1-2	30.1 ± 1.9^{A}	25.7 ± 0.8^{B}	24.4 ± 0.1^{B}	24.1 ± 0.9^{B}	26.3 ± 1.5^{B}	33.9 ± 0.3^{A}	25.6 ± 0^{B}	21.8 ± 2.2^{B}	22.5 ± 4.5^{B}	24.9 ± 0.6^{B}
	10-20	28.7 ± 1.3^{A}	24.2 ± 0.9^{B}	22.9 ± 0.1^{B}	23.2 ± 0.5^{B}	25 ± 2.1^{B}	31.6 ± 0.4^{A}	24.2 ± 0.7^{B}	20.4 ± 1.8^{B}	21.1 ± 3.8^{B}	23.2 ± 1.9^{B}
O121	1-2	27.6 ± 0.6^{A}	21.2 ± 0.6^{BC}	19.2 ± 0.4^{C}	19 ± 0.7^{C}	21.7 ± 1.6^{B}	32.5 ± 0.6^{A}	24.8 ± 0.4^{B}	19.6 ± 0.1^{C}	19.4 ± 0.1^{C}	24.2 ± 0.1^{B}
	10-20	25.4 ± 1.6^{A}	20.7 ± 0.5^{BC}	18.8 ± 0.7^{C}	18.7 ± 1.3^{C}	21.9 ± 0.6^{B}	30.9 ± 0.2^{A}	24.1 ± 0.5^{B}	19.1 ± 0^{C}	18.3 ± 0.2^{D}	23.6 ± 0.2^{B}
O145	1-2	32.7 ± 3.9^{A}	22.8 ± 3.2^{B}	21.1 ± 3^{B}	21.6 ± 4.5^{B}	22.1 ± 1.1^{B}	35.3 ± 1.6^{A}	28.4 ± 0.3^{B}	26.3 ± 1.7^{B}	26 ± 2.8^{B}	27.4 ± 1.4^{B}
	10-20	27.5 ± 1.6^{A}	20.7 ± 2.2^{B}	19.1 ± 2.3^{B}	19.3 ± 2.1^{B}	20.9 ± 0.4^{B}	32.7 ± 1.2^{A}	25.5 ± 0.5^{B}	22.3 ± 1.4^{B}	22.1 ± 2.6^{B}	24.3 ± 1.2^{B}

[a] None of the 4-h enrichment samples tested positive by either LAMP or qPCR. In each row within LAMP or qPCR, *Tt* or *Ct* values followed by different upper case letters are statistically significant ($P < 0.05$).

Discussion

LAMP technology has been applied previously to detect generic and pathogenic *E. coli* (Hara-Kudo et al., 2007; Hill et al., 2008; Kouguchi et al., 2010; Song et al., 2005; Yano et al., 2007; Zhao et al., 2010; Zhu et al., 2009), including STEC, however, O-serogroup specific LAMP assays are still unavailable except for O157 (Wang et al., 2009; Zhao et al., 2010; Zhu et al., 2009), and the quantitative capability of LAMP assay were not evaluated. The STEC serogroup-based LAMP assay developed in present study was rapid (19 - 45 min), specific (no false positive or false negative results for 120 strains tested), sensitive (1-20 CFU/ reaction in pure culture and 10^3-10^4 CFU/g in spiked ground beef, based on testing 1 typical outbreak STEC strain in each of the 7 main virulent serogroups), and quantitative (R^2= 0.932-0.993). Coupled with 6 h enrichment, it can detect STEC strain in ground beef at a low level of 1-2 CFU/25 g. To our knowledge, this is the first report applying LAMP to detect and quantify main virulent STEC serogroups in ground beef by targeting *wzx/wzy* genes.

In this study, the *wzx* and *wzy* genes were selected as targets to design primers for 7 main virulent STEC serogroups detection. Both genes, along with several other genes/fragments (*rfb*, *rfbE*, *wbsD*, *wbdI*, and *ihp1* gene) used in recently published O157-LAMP studies (Wang et al., 2009; Zhao et al., 2010; Zhu et al., 2009) and numbers of O serogroup specific PCR studies (Fratamico et al., 2011; Madic et al., 2011; Valadez et al., 2011), are located together on a 10-15 kb O-antigen gene cluster, and encode the protein carrying out specific assembly or processing steps in conversion of the O unit to the O antigen (Wang and Reeves, 1998). It is noteworthy that the *rfbE* gene was more frequently used for O157 serogroup detection than others. In fact, this gene (GenBank accession #: S83460) is right after the O157-*wzx* gene (GenBank accession #: AF061251) in sequence and even has 14 overlapped bases with each other. According to

previous O-antigen cluster sequencing studies in various serogroups (O26, O45, O55, O91, O103, O104, O111, O121, O145, and O157), the *wzx* and *wzy* genes were proved to be highly group specific (D'Souza et al., 2002; DebRoy et al., 2005; Feng et al., 2005; Fratamico et al., 2003; Fratamico et al., 2005; Perelle et al., 2002; Wang et al., 2001; Wang et al., 1998; Wang and Reeves, 1998). The attempt to design LAMP primer sets based on O103 and O145 *wzy* genes was also made in preliminary test, but failed for the unpredictable false positive result or slow amplification speed.

The 7 O serogroup specific LAMP assays developed in present study consistently detected down to 1-20 cells of STEC strains per reaction in pure culture, similar as the limit reported in those three LAMP assays targeting O157 (3 to 20 CFU/reaction) (Wang et al., 2009; Zhao et al., 2010; Zhu et al., 2009). This level of sensitivity was also comparable to that of qPCR assay run in parallel, but around 10-100 folds superior to those of two PCR assays targeting O111, O113, and O157 (Paton and Paton, 1998, 1999). The increased sensitivity of LAMP (by at least 10 folds) compared to that of PCR agreed with findings from many previous studies (Chen and Ge, 2010; Han et al., 2011; Hara-Kudo et al., 2007). On the other hand, the comparison between LAMP and qPCR for STEC serotyping has not been made before. Without enrichment, the detection limit of 7 LAMP assays for STEC strain in spiked ground beef was 6.5×10^3-10^4 CFU/g (13-130 CFU/reaction), the similar range reported in a qPCR study for same targets (Fratamico et al., 2011). The slightly inhibition of food matrix on detection limit (10 folds higher) was found for O45 and O111 when testing the assay sensitivity in ground beef without enrichment procedure. This inhibition caused by DNA polymerase inhibitors (tissue, fat, acid, blood, and salt) may also happen for other molecular detection methods, including PCR and qPCR (Chen et al., 2011; Lin et al., 2011).

LAMP amplicons were commonly detected by gel electrophoresis, naked eye observation of turbidity or color change, and real-time turbidimeter monitoring, and among those, real-time turbidimeter monitoring is the only one that is potentially quantitative (Han and Ge, 2010).The quantitative capability of LAMP assay was not evaluated in other two O157-LAMP assays, but was reported in a few of studies detecting *Vibrio parahaemolyticus*, *V. vulnificus*, and *Salmonella* in spiked oyster and produce (Chen and Ge, 2010; Chen et al., 2011; Han et al., 2011). A strong linear coefficients (R^2 = 0.94 to 0.99) was demonstrated, indicating the good quantitative capability of LAMP at between 10^1 and 10^5 DNA copies/reaction. In our study, the R^2 value were found to be 0.945-0.993 for STEC cell concentrations between 10^5 and 10^0 CFU/reaction in pure culture, and 0.932-0.982 for cells ranging from 10^7 to 10^3 CFU/g in spiked ground beef homogenates, suggesting excellent quantitative capabilities.

Due to the stringent zero-tolerance policy for STEC O157:H7 in ground beef in the U.S., together with its extremely low infectious dose (~100 cells) to cause severe illness in human (Pennington, 2010), the detection method is required to be sensitive enough to accurately recognize its targets at low level. In present study, all 7 LAMP assays were capable to consistently detect an initial spiking of 1-2 CFU/25g of STEC in ground beef after 6 h of enrichment, and this was the shortest enrichment procedure among other published detection methods (PCR or qPCR) when testing STEC in ground beef (8 h, 20h, or overnight) (Fratamico et al., 2011; Lin et al., 2011; Pina M. Fratamico and DebRoy, 2010; Valadez et al., 2011). The qPCR assay run in parallel also generated the positive result in 6 h enrichment broth, whereas, the 30-40 min delays were needed. This LAMP assay evaluation test in ground beef contaminated with low level STEC was not conducted in other two O157-LAMP assays, but described in similar LAMP assays screening the STEC *stx* gene in beef products (ground beef

and beef liver), where 18 h enrichment was tried. To ensure the quality of templates, it is recommended to use simplified DNA extraction Kits rather than directly boiling method, which may also delay the assay process by 2 hours (data not shown). Taken together, the short-period enrichment procedure combined with simplified sample processing steps and rapid LAMP confirmation (< 35 min) would make it possible to complete the analysis within an 8-h workday.

The ability of PCR and qPCR assays to simultaneously identify more than one target (Fratamico et al., 2011; Madic et al., 2011; Pina M. Fratamico and DebRoy, 2010; Valadez et al., 2011) gives them advantage over LAMP assays, particularly in this STEC serotyping case as no related information is available before test. Multiplex LAMP assay has been conducted in one study targeting both *stx1* and *stx2* genes, but with relatively sacrificed sensitivity (10^2 CFU/reaction) (Kouguchi et al., 2010). However, LAMP assay is still a promising technology in many other respects. Except for the same high sensitivity and specificity as PCR, it has the highest gene amplification speed among other NAAT technology. A new LAMP format referred as microRT-LAMP can achieve accurate result within 10 min at the initial cell concentration of 10^5 CFU/reaction (Farhan Ahmad et al., 2011). Further, the isothermal condition and various amplicon detection methods make it adaptable to different platforms (PCR, qPCR, heating block, water bath, and real-time turbidimeter) (Chen and Ge, 2010; Farhan Ahmad et al., 2011; Han and Ge, 2010). Now it has been selected by the Foundation for Innovative New Diagnostics (FIND) in collaboration with the World Health Organization (WHO) to develop diagnostic tests that are simple to use and effective in Africa remote area for tuberculosis and malaria (Fondation for Inovative New Diagnostics, 2011).

From a public health perspective, the development of rapid and reliable method capable of identifying both virulent genes and serogroup specific genetic determinants holds promise for

a more comprehensive characterization of STEC strains in food, for timely outbreak responses, and to monitor trends in disease epidemiology. The 7 STEC main virulent O serogroup specific LAMP assays developed in this study are rapid, specific, sensitive, and cost-effective, and can be used for detection and characterization of O26, O45, O103, O111, O121, O145, and O157 STEC in ground beef. This set of assays may present a valuable tool for the meat industry and regulatory agencies to better control the STEC risks associated with ground beef consumption. Future combination with IMS method for STEC isolation and testing with natural ground beef samples are desired for further evaluate the performance of LAMP in a setting closer to application.

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CHAPTER 5: CONCLUSIONS

In this dissertation research, two sets of LAMP assays, consisting of 10 individual ones, were developed and evaluated for the detection of three STEC key virulent factors (the *stx1*, *stx2* and *eae* genes) and seven O antigens of major STEC serogroups (O26, O45, O103, O111, O121, O145, and O157) in food and/or human stool. All of the assays were rapid (11 - 45 min), specific (no false positive or false negative results for 120 STEC and non-STEC strains tested), sensitive (1-20 CFU/ reaction in pure culture and 10^3-10^4 CFU/g in spiked ground beef), and accurate (R^2 = 0.904-0.997). Coupled with 4-8 h enrichment, the assays detected STEC strains spiked in ground beef at a very low level of 1-2 CFU/25 g, and in human stools at 10^3 CFU/0.5 g. LAMP also possessed superior sensitivity and rapidity compared with qPCR. To our knowledge, this is the first study applying the novel LAMP technology to detect major STEC serogroups by targeting the *wzx/wzy* genes, as well as the first study to develop and evaluate an LAMP assay targeting the *eae* gene in food and clinical samples.

Currently, *E. coli* O157:H7 is regulated as an adulterant in raw beef in the U.S. The growing clinical importance of non-O157 *E. coli* also warrants the development of rapid, sensitive, and specific methods for detection. In the newly updated USDA protocol for *E. coli* O157:H7 and non-O157 STEC detection in ground beef and beef trimmings, enrichment is an indispensable step followed by initial screening of Shiga toxins (encoded by *stx1* and *stx2*) and intimin (encoded by *eae*) by qPCR and a second screening of O157 and top six STEC serogroups by another set of qPCR assays. Given the rapidity, sensitivity, specificity, and robustness of LAMP assays demonstrated in the dissertation research, these assays may effectively serve as promising alternatives to qPCR in the two stage screening, one is serogroups-independent and the other one serogroups-specific, which is to be followed by culture isolation and further virulence characterizations to ascertain the food safety and public health relevance of the LAMP-

positive samples. The three serogroups-independent LAMP assays also had superior performance than qPCR in human stools.

The LAMP assays may be advantageous over other techniques, particularly in resource-limited regions due to the simplicity (isothermal amplification requires simple instrument, high tolerance to biological inhibitors eases DNA extraction requirement, turbidity and fluorescence changes simplify results reading), sensitivity, specificity, and rapidity. Although all of the assays developed and evaluated in this dissertation research were performed on the LAMP turbidimeter platform, they can be similarly conducted under much simpler settings (such as a water bath or a heating block) or existing settings (PCR and qPCR) without modifying the reagents and reaction conditions. Meanwhile, LAMP detection kits (Eiken Chemical Co., Ltd, Tokyo, Japan) have been manufactured to detect several foodborne pathogens (*Campylobacter, Salmonella,* and *Vibrio*), and research is also ongoing on LAMP reagent lyophilization. Taken together, the wide application of LAMP assays in food safety arena is anticipated.

Further testing of the assays developed in this dissertation research may include tolerance to various conditions and inhibitors, including temperature, pH, salts, soil, plant tissue, and others, as well as evaluation in additional food items (produce, milk, juice), as matrix effect may affect the detection outcomes. Finally, the assays need to be validated in multiple laboratories to become a standardized method.

In conclusion, the LAMP assays developed in this study are rapid, sensitive, specific, and quantitative for STEC, which may facilitate rapid and reliable identification of STEC contaminations in high-risk food commodities and also facilitate prompt diagnosis of STEC infections in clinical laboratories.

APPENDIX: SUPPLEMENTAL RESULTS

1. Sensitivity of the three qPCR assays when testing 10-fold serial dilutions of individual STEC strains of seven serogroups in pure culture and spiked ground beef samples

Strain ID	Serotype	Stx	Intimin	Detection limit (CFU/reaction or CFU/g)					
				stx1-qPCR		*stx2*-qPCR		*eae*-qPCR	
				Culture	Ground beef	Culture	Ground beef	Culture	Ground beef
97-3250	O26:H11	1, 2	+	1	4×10^4	1	4×10^4	1	4×10^4
MI01-88	O45:H2	1	+	1.6	7×10^3	N/A	N/A	16	7×10^3
MT#80	O103:H2	1	+	1.6	6.5×10^3	N/A	N/A	1.6	6.5×10^3
3215-99	O111:H8	1, 2	+	11	5×10^3	1.1	5×10^4	11	5×10^3
MDCH-4	O121:H19	2	-	N/A	N/A	1.2	4×10^4	N/A	N/A
GS G5578620	O145:NM	1	+	17	4×10^4	N/A	N/A	17	4×10^4
EDL933	O157:H7	1, 2	+	1.6	6.5×10^4	1.6	6.5×10^3	1.6	6.5×10^3

2. Quantitative capabilities of serogroup-independent LAMP assays when testing serially diluted STEC cells in pure culture and spiked ground beef samples

Targets	Strain	Quantification capability of LAMP assay			
		Pure culture		Ground beef	
		Quantification equation	R^2	Quantification equation	R^2
stx1	EDL933	y = -2.39x + 31.618	0.977	y = -1.57x + 26.78	0.973
	97-3250	y = -2.43x + 29.33	0.986	y = -2.59x + 33.567	0.913
	MI01-88	y = -2.31x + 27.872	0.983	y = -1.98x + 29.934	0.952
	MT#80	y = -2.94x + 29.728	0.973	y = -2.98x + 36.494	0.916
	3215-99	y = -2.67x + 29.32	0.983	y = -3.34x + 39.322	0.958
	GS G5578620	y = -2.99x + 30.838	0.933	y = -2.22x + 31.313	0.95
stx2	EDL933	y = -1.98x + 31.109	0.997	y = -1.64x + 28.763	0.956
	97-3250	y = -1.84x + 27.993	0.963	y = -1.2x + 26.267	0.931
	3215-99	y = -2.23x + 26.027	0.989	y = -1.35x + 27.886	0.971
	MDCH-4	y = -2.18x + 25.9	0.970	y = -1.98x + 32.177	0.974
eae	EDL933	y = -1.79x + 23.115	0.978	y = -1.18x + 21.141	0.965
	97-3250	y = -4.24x + 39.44	0.957	y = -3.43x + 45.656	0.904
	MI01-88	y = -3.13x + 39.077	0.976	y = -3.13x + 44.775	0.965
	MT#80	y = -4.367x + 38.92	0.959	y = -2.05x + 35.875	0.994
	3215-99	y = -4.22x + 39.667	0.99	y = -2.78x + 42.674	0.978
	GS G5578620	y = -1.81x + 21.335	0.977	y = -1.4x + 23.493	0.988

3. Quantitative capabilities of serogroup-specific LAMP assays when testing serially diluted STEC cells in pure culture and spiked ground beef samples

Strain ID	Serotype	Quantification capability of LAMP assay			
		Pure culture		Ground beef	
		Quantification equation	R^2	Quantification equation	R^2
97-3250	O26:H11	y = -3.556x + 37.636	0.964	y = -4.248x + 51.322	0.932
MI01-88	O45:H2	y = -3.77x + 38.497	0.973	y = -2.508x + 39.534	0.953
MT#80	O103:H2	y = -4.19x + 41.793	0.945	y = -4.286x + 53.21	0.958
3215-99	O111:H8	y = -4.2x + 46.498	0.991	y = -4.382x + 62.329	0.947
MDCH-4	O121:H19	y = -3.598x + 38.516	0.993	y = -2.542x + 40.788	0.947
GS G5578620	O145:NM	y = -3.116x + 36.646	0.996	y = -3.704x + 51.932	0.963
EDL933	O157:H7	y = -4.636x + 42.362	0.988	y = -4.426x + 56.186	0.982

VITA

Fei Wang was born in Anyang, Henan Province, People's Republic of China. He was awarded Bachelor and Master Degree of Engineering in Food Science and Engineering in China Agricultural University, Beijing, in 2006 and 2008, respectively. After that, he came to Louisiana State University for his doctoral study in the Department of Food Science in August 2008. Currently, he is a candidate for the degree of Doctor of Philosophy in food science in the College of Agriculture. He will receive the doctoral degree in December 2011.

During his graduate study, he has been actively involved in multiple research projects under Dr. Beilei Ge, and also an active member in the Food Science Club. Now he has six manuscripts published in highly ranked scientific journals in food safety and microbiology, and another two under review. He was awarded the LSU Graduate School Supplement Scholarship in 2008-2011, LSU Food Science Department Grodner Scholarship in 2010, and both Gamma Sigma Delta LSU Chapter Outstanding Ph.D. Student and LSU Graduate School Dissertation Year Fellowship in 2011.