HIGH RESOLUTION MELTCURVE PCR ASSAY FOR DETECTION OF *SALMONELLA* AND *ESCHERICHIA COLI* O157:H7 IN FOODS

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By

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The undersigned, appointed by the dean of the Graduate School,

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and hereby certify that, in their opinion, it is worthy of acceptance.

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ABSTRACT

Foodborne illnesses associated with *Salmonella* and *Escherichia coli* O157:H7 have become world-wide public-health problems. Conventional methods for the identification of foodborne pathogens are tedious, expensive, and time-consuming. Alternatively, real-time PCR (RT-PCR) as a promising method to detect pathogens in food samples, has recently been widely applied in food safety areas.

High Resolution Meltcurve (HRM) analysis, performed immediately at the end of a real-time PCR, is able to yield a higher resolution plot compared with SYBR® Green I PCR. HRM dyes completely saturate all amplicons without showing preferential bindings, making the results more clear and distinct.

In this research, a multiplex real-time PCR targeting the *inv*A, *fim*A and *stn* genes were developed to efficiently detect *Salmonella* in foods. Furthermore, HRM analysis is sensitive to any single mutation in PCR products, thus it was also applied in this study to distinguish *E. coli* O157 from other serogroups of *E. coli* by targeting the *uid*A gene. The specificity of primers used in this study was checked using many different strains. Results of artificially contaminated foods presented a high sensitivity of the HRM detection methods.

Due to its low cost, simplicity of the approach and rapidness, HRM technology is highly competitive with relaxed-condition PCR and probe-based PCR. Besides, an HRM assay can be performed on generic real-time PCR instrumentations found in many laboratories. In conclusion, HRM-based PCR assay are proved to be efficient methods in foodborne pathogen detections.

CHAPTER 1

INTRODUCTION

During the last several decades, foodborne pathogens and diseases have drawn increasing attention from the public. Among many pathogen-associated foodborne disease outbreaks worldwide, *Salmonella* and *Escherichia coli* O157:H7 are the most notorious causes. Beside mild symptoms, such as diarrhea and vomiting, these pathogens can cause severe illnesses, including salmonellosis and hemolytic uremic syndrome (HUS) in the young, old and weak. According to the Centers for Disease Control and Prevention (CDC), *Salmonella* is estimated to cause 1.2 million cases of illness annually, which account for approximately 21,000 hospitalizations and 380 deaths during the years 2000 to 2008 (CDC, 2012). Annually, STEC are responsible for over 265,000 illnesses in the U.S., along with around 3,700 hospitalizations and 30 deaths (CDC, 2011). Hence, numerous studies have been conducted to seek an accurate and rapid detection method for pathogenic bacteria including *Salmonella* and *E. coli* O157:H7 in food.

The traditional cultured-dependent detection method involves specimen enrichment, selective agar inoculation, biochemical tests, and serotyping procedures, which are consistently tedious and time-consuming. Common problems associated with culture-based methods are cross reactivity, low specificity, laborious steps and high cost. Therefore, because of the need for a highly sensitive and easy-to-operate method, traditional culture-based methods are gradually being replaced by genotype-based methods.

Polymerase Chain Reaction (PCR) is a technique of rapidly amplifying target genes in DNA isolates. Compared with culture-based methods, a PCR-based assay requires much less time to acquire reliable results. Real-time PCR assay, which allows amplifications and quantitation of PCR products to be performed all at once, have been applied extensively in detection and characterization of target bacteria for years. High Resolution Meltcurve (HRM) assay based on real-time PCR techniques is an emerging technology. It involves the use of intercalating dyes and meltcurve analyses. Compared with non-specific detections, a HRM assay allows the dyes to equally bind to each amplicon without showing any preference. Further, HRM assays are highly sensitive to any single base change in amplified targets. A single mutation is able to induce significant differences in the melting curve, and consequently in fluorescence collection (Erali and others 2008). Therefore, a HRM assay is widely applied in gene scanning, single nucleotide polymorphism (SNP) genotyping, DNA mapping and DNA fingerprinting due to its low cost and simplicity of operation. The objective of this study was to develop reliable and rapid multiplex HRM PCR-based assays to detect *Salmonella* and *E. coli* O157:H7 within complex food matrices.

CHAPTER 2

LITERATURE REVIEW

2.1 Foodborne pathogens and related outbreaks

Foodborne outbreak is a big concern worldwide. According to the World Health Organization (WHO), approximately 600 million foodborne illnesses and 420,000 deaths caused by foodborne hazards were reported globally in 2010 (WHO, 2015). The causes of foodborne illness are from infections by pathogens, viruses, parasites and exposure to toxic chemicals. Among foodborne pathogens, several genera of bacteria, including *Campylobacter* spp., *Salmonella*, *Clostridium perfringens*, *Listeria monocytogenes*, and pathogenic *E. coli* were recorded as the main sources of food microbial contamination. Nontyphoidal *Salmonella* spp. are responsible for nearly 11% foodborne illnesses, followed by *C. perfringens* (10%), and *Campylobacter* spp. (Scallan and others 2011).

The syndromes of foodborne illness range from mild gastroenteritis to life-threatening diseases. In general, the young, the elderly, and the immunodeficient are at a high risk to be victimized. People living in low-income regions of the world such as East Africa, are highly susceptible. In fact, diarrheal diseases are the most common syndrome that happen to an infected population. According to the WHO (2015), more than 220,000 deaths were caused by foodborne diarrheal diseases from 2010 to 2015. Non-typhoidal *Salmonella enterica*, particularly *Salmonella* Typhimurium, are responsible for the majority of deaths resulting from foodborne diarrhea. Moreover, enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) *Campylobacter* spp. and *Vibrio* cholerae were other main diarrheal causes of foodborne diseases.

Previous studies have shown that numerous reported illness outbreaks were associated with consumption of fresh produce and meat products (Olaimat and Holley 2012; Niyonzima and others 2015). An estimated 20 million illnesses resulting from consuming contaminated fresh or processed produces occur in the U.S. and cause 38.6 billion dollar losses every year (Scharff 2010).

Frequently, fruits and vegetables as daily source of vitamins and fiber for people, are consumed raw (Olaimat and Holley 2012). Thus, these produce and related processed products are at a high risk of carrying foodborne pathogens. Raw sprouts, spinach and tomatoes are the prevalent produce varieties consistently responsible for foodborne diseases. During 2005-2006, four multistate outbreaks caused by consuming raw tomatoes contaminated by *Salmonella* resulted in 459 cases of salmonellosis in of U.S. (CDC, 2007).

Meat products are one of the most common sources of foodborne bacteria due to their high protein, nutrient and water content, which provides a perfect environment for microbial growth. The microorganisms cause meat spoilage and lead to some severe illnesses, including gastroenteritis. Processed meat may be cross-contaminated with bacteria transferred from raw materials, meat juices, or from food handlers with poor personal hygiene. The most prevalent and serious emerging pathogens of meat, and derived products mainly include *Campylobacter*, *Salmonella*, Shiga toxin-producing *E. coli* (STEC) and *L. monocytogenes* (Mor-Mur and Yuste 2010). The most infamous scandal in history was the *E. coli* O157:H7 outbreak from consumption of undercooked beef patties in a Jack-in-the-Box restaurant in Washington State, U.S., in 1993, which caused 732 infections and 4 deaths (Kusumoto, 1993).

It is estimated that more than 40 million cases of foodborne illness occur in the United States, along with approximately 300,000 hospitalizations up to 9,000 deaths annually in since 2005 (Scallan and others 2011). Estimates of the amount of foodborne illness and causative bacteria are helpful for minimizing foodborne contaminations and controlling outbreaks.

2.2 *Salmonella*

2.2.1 Characteristics of *Salmonella* and serotypes

Salmonella is one of the worldwide foodborne pathogens. Annually, *Salmonella* is estimated by the CDC to account for 1.2 million cases of illness, among which 21,000 hospitalizations and 380 deaths occurred during the year 2000 to 2008 (CDC, 2012). Salmonellosis has drawn increasing attention from the public during the last several decades.

Salmonella are divided into more than 2,500 serotypes. Among all the described serotypes, only less than 100 serotypes are reported to be associated with infections. They are classified based on the various antigens they carry on their cell surface (O antigen) and flagella (H antigen). The O antigen stands for the antigen located on the polysaccharide portion of the bacterial cell's lipopolysaccharide layer, and the H antigen is on the filamentous portion on the surface of flagella (D'Aoust 1991). Each serotype is determined by the combination of O and H antigens. According to the genetic similarities different serotypes share, *Salmonella*e are divided into two species groups, *Salmonella bongori* and *Salmonella enterica*, respectively. *S. enterica* accounts for more than 90% pathogenic *Salmonella* serotypes (Chiu and others 2004). Most *Salmonella* serotypes cause mild

syndromes of diarrhea, fever, gastroenteritis and abdominal cramps. However, some serotypes can cause severe illness and even deaths in vulnerable populations.

2.2.2 Infections caused by *Salmonella*

Based on the epidemiology and clinical aspects, *Salmonella* serovars are grouped as typhoidal *Salmonella* and nontyphoidal *Salmonella*. Typhoidal *Salmonella* are responsible for typhoid fever and paratyphoid fever. Typhoid fever is a worldwide life-threatening illness. It occurred approximately 21.7 million times annually, causing more than 200,000 cases of death. *S. enterica* serovars Typhi and Paratyphi account for a majority of typhoidal fever illness (Crump and others 2004).

Nontyphoidal *Salmonella* is estimated to cause 93.8 million globally diarrheal illness in 2006. In 2010, 681,000 deaths associated with nontyphoidal *Salmonella* were reported worldwide. One of the serovars to be associated with invasive disease is *Salmonella* serovar Typhimurium. Most outbreaks of typhoid fever and paratyphoid fever are reported in low-income developing countries especially sub-Saharan African countries and some Asian countries (Acheson and Hohmann 2001).

2.2.3 Foodborne Salmonellosis

Pathogenic *Salmonella* are distributed in various food, from vegetables, nuts, and eggs to meat and poultry (Pui and others 2011). *Salmonella* contamination is transmitted primarily through contaminated food and water. So far, 26 serotypes of *Salmonella* were identified by both Unites States and European Union (EU), among which *Salmonella* Typhimurium and *Salmonella* Newport were most involved in foodborne outbreaks. For example, *S.* Typhimurium is found in a wide range of meat and nuts. According to the

CDC, a total of 116 hospitalizations and 8 deaths had been reported to be associated with *Salmonella* infections due to peanut butter consumption during 2008 and 2009. A multistate outbreak due to consumption of tomatoes contaminated by *S*. Newport infections was reported in 26 states, resulting in 510 hospitalizations in total. In addition, *Salmonella* Enteritidis is mostly linked with the consumption of poultry and eggs. In Oct 2015, frozen chicken entrees produced by Barber Foods were recalled in several states due to the *S.* Enteritidis contamination in raw chicken. Fifteen cases of infections with four hospitalizations were reported from seven states (CDC 2015) between 10 and 42, highlighting increases in 2006.

Based on the data provided by CDC, *Salmonella* spp. was responsible for 71 cases of outbreak during the year 2004-2012, which accounted for 52% of the total bacterial outbreaks associated with consumption of fresh produce in United States (CDC 2013). Likewise, in EU this percentage was approximate 50% (EFSA, 2012), followed by STEC. (Callejón and others 2015).

2.2.4 Virulence genes used in molecular detection methods

The *inv*A encodes a 71 kDa putative inner membrane protein on *Salmonella* pathogenic island I and enables *Salmonella* to invade cells of hosts' intestinal epithelium (Collazo and Galán 1997). Thus, *inv*A is the most frequently targeted gene for *Salmonella* detection through PCR-based methods (Rahn and others 1992; Chiu and Ou 1996). The *inv*A locus is present and functional in most *Salmonella* serotypes (Collazo and Galán 1997). In Chen and others' research (1997), where *inv*A was chosen as the target gene in TaqMan™ PCR, the results showed that a 287 bp length product was formed. The specificity and sensitivity both reached 100%, and less than 7 CFU per PCR reaction in

spiked food samples could be successfully detected. Similarly, Nam and others (2005) established a SYBR® Green I based RT-PCR method, that yielded a final product of 119 bp. Specificity tests on 240 *Salmonella* and non-*Salmonella* strains confirmed 100% accuracy. Similarly, Tomás and others (2009) conducted a TaqMan™ PCR, enzyme-linked fluorescent PCR assay on *Salmonella* detection with internal amplification control (IAC), and the sensitivity reached to 97%. However, some studies reported that *inv*A was absent in some strains in *S. enterica*. For instance, *S. enterica* subsp. *enterica* serovar Saint Paul is not detected by the *inv*A-based assay (Cohen et al., 1996; Malorny et al., 2004). Rahn and others(1992) found that two *Salmonella* serotypes, *Salmonella* Litchfield and *Salmonella* Senftenberg, also did not amplify with the *inv*A gene. In this study, the absence of *inv*A in *S.* Senftenberg O Group E confirmed the limitation of applying *inv*A as the single target gene in *Salmonella* detection methods.

The *fim* genes are highly conserved in *S.* Typhimurium. Among the *fim* gene groups, *fim*A is one of *Salmonella*'s fimbrial genes encoding the major fimbrial subunit, the structural and assembly components (Rossolini and others 1993). Fimbriae allow *Salmonella* to adhere to host cells for further infections. Cohen and others' (1996) showed the detection of *Salmonella* spp. by targeting the *fim*A gene yielded a specific amplicon and successfully differentiated *Salmonella* spp. from non-*Salmonella* strains.

Salmonella stn gene is a putative virulence gene which encodes for a 29-kDa enterotoxin protein (Prager and others 1995). It is highly and specifically distributed in *S. enterica* serotypes. Prager and others (1995) reported that *stn* was prevalent among strains of *S. enterica*, but absent in *Salmonella bongori*. On the other hand, Moore and Feist

(2007) developed a TaqMan™ RT-PCR assay targeting the *stn* gene and successfully detected all 267 *S. enterica* strains and *S. bongori*.

2.2.5 Limitation of single-gene detection of *Salmonella*

Single-gene PCR detection of *Salmonella* has some drawbacks, including the absence of specific target genes in some *Salmonella* strains and the lack of an IAC, both of which could lead to false-negative results.

In Moore and Feist's (2007) study, false-positive results were acquired among 4 strains of *Citrobacter amalonaticus* in TaqMan™ PCR detection of *Salmonella*, which lowered the specificity of the assay to 98.52%. Consequently, failure to find a mutual gene that all *Salmonella* strains carry has lowered the detection accuracy of such singlet PCR assays. Besides, performing TaqMan™ based PCR is not cost-effective, since manufacturing and testing desirable fluorescent probes are costly and time-consuming.

2.3 Pathogenic *E. coli*

2.3.1 STEC and foodborne outbreaks

E. coli are a group of bacteria widely found in the intestinal tracts of human and animals. *E. coli* was first discovered in human colons by a German bacteriologist Theodor Escherich in 1885, and recognized as a common foodborne pathogen in 1982 (Riley and others 1983). Most *E. coli* are harmless, however some *E. coli* are pathogenic, among which, STEC are of most concerned and widely associated with foodborne outbreaks. Shiga toxin is also referred to as verocytotoxin, which is produced most commonly by *Shigella dysenteriae* and enterohemorrhagic *E. coli*.

STEC are comprised of more than 50 serogroups, which are generally divided into *E. coli* O157 and non-O157 serogroups. In 1993, one severe outbreak of *E. coli* O157:H7 infections caused by consumption of undercooked beef patties at 73 outlets of a fast-food restaurant chain in U.S. raised public awareness of STEC (Bell and others 1994). According to the CDC, STEC are responsible for over 265,000 illnesses in the U.S. annually, along with around 3,700 hospitalizations and 30 deaths.

2.3.2 STEC infections and hemolytic uremic syndrome

STEC can cause some mild diarrhea and, sometimes, even severe illnesses, including hemolytic uremic syndrome (HUS) and bloody diarrhea (Tarr and others 2005). HUS is a life-threatening disease characterized by acute renal failure and microvascular thrombi (Cunningham and others 2000). The management of HUS remains challenging, since no specific treatments have been developed (Tzipori and others 2004). Presumably, rather than the direct injury of STEC to the intestinal epithelium, the mesenteric ischaemia is caused by the interactions between Shiga toxin and circulating leucocytes and platelets (te Loo and others 2000). *E. coli* O157 is estimated to cause one-third of the total STEC illnesses and outbreaks in the United States (Scallan and others 2011). Most cases of HUS are caused by *E. coli* O157:H7 worldwide. Therefore, so far, the most effective way to prevent HUS is to prevent the initial infection and to avoid consuming STEC-contaminated food.

2.3.3 *E. coli* O157:H7

2.3.3.1 Sorbitol fermentation ability

Phenotypic culture methods distinguish bacterial species by determining the characteristic of specific traits in that strain. One crucial phenotype of *E. coli* O157:H7 that is different from that of other *E. coli* is the absence of sorbitol fermentation ability, thus checking the ability of sorbitol fermentation has been applied to distinguish *E. coli* O157:H7 from other *E. coli* (March and Ratnam, 1986). The sorbitol-MacConkey (SMAC) agar medium has become quite commonly used to isolate and distinguish of *E. coli* O157:H7. However, some non-*E. coli* bacteria also show the same phenotype, and there are also strains of O157:H7 that can ferment sorbitol. To overcome these limitations, modified SMAC was developed to include cefixime and rhamnose (Chapman and others 1991) and cefixime and tellurite (Zadik and others 1993) in order to improve the selectivity of this medium. Typical *E. coli* O157:H7 as sorbitol-nonfermenting colonies appear colorless on the medium while other organisms form pink colonies. However, other bacteria share this phenotype, and there are strains of O157:H7 that can ferment sorbitol, thus still limiting the selectivity of this medium.

Other commercial selective media, such asRainbow agar O157 and CHROM™ agar O157, are widely applied in distinguishing *E. coli* serotypes. Rainbow Agar O157 is able to distinguish *E. coli* O103, O111, O26, O121, O48, O157 and non-toxigenic species, based on the beta-glucuronidase activity each species possesses (Bettelheim and others 1993).

2.3.3.2 MUG test and *uid*A gene

The 4-methylumbelliferyl-β-D-glucuronid (MUG) test has been frequently used in identifying *E. coli* (Feng and Hartman, 1982). The majority of *E. coli* strains were examined to be able to produce an enzyme, β-D-glucuronidase (GUD). When the compound MUG, is hydrolyzed by GUD, a fluorogenic compound, 4-methylumbelliferyl, is released and fluoresces when exposed to long-wave UV light (Feng 1993). More than 90% of *E. coli* strains are positive in the MUG assay, while *E. coli* O157:H7 is consistently negative in the test. By using this characteristic, *E. coli* O157 can be distinguished from other serogroups of *E. coli*.

The *uid*A gene controls the synthesis of the GUD enzyme. Although O157:H7 is MUG negative, it contains the *uid*A gene that is highly identical to that of other *E. coli* serotypes. The only difference is a one base substitution on the sequence of the *uid*A gene. At the +93 position of its *uid*A gene sequence, *E. coli* O157 harbors a thiamine, while in non-O157 *E. coli*, it is a guanine (Feng, 1993). This leads to an alteration in the amino acid sequence that encodes for GUD enzymes. Therefore, this mutation is used as one of the most common targets for PCR-based identification of *E. coli* O157 serogroup.

2.3.4 Prevalence of *E. coli* O157:H7 in beef produces

Beef and carcass have been implicated to be the primary vehicle for enterohemorrhagic *E. coli* O157:H7 transmission, which usually happens during the slaughter process. More than half of the *E. coli* O157:H7 infection cases were linked to foods derived from cattle. The U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) has declared a zero tolerance policy of *E. coli* O157:H7 as an

adulterant in ground beef. Therefore, intervening and controlling this pathogen at the initial stages of processing are crucial to decreasing the risk of *E. coli* O157:H7 infections. More importantly, the minimum number of *E. coli* O157:H7 that can cause illness is very low, usually less than several hundred CFU/g, necessitating a reliable detection method with a high sensitivity (Karmali 2004).

2.3.5 Need for distinguishing *E. coli* O157:H7 from STEC

Most STEC are likely to cause epidemics and associated with human gastrointestinal infections, but the syndromes and host specificities vary within species. Among disease-causing STEC, *E. coli* O157:H7 is the predominant serotype that causes HUS in humans due to the antigens it carries. Thus, identifying *E. coli* O157 from other STEC has a great significance in treating with HUS. Besides, detecting and distinguishing *E. coli* are crucial in correctly determining the food source of pathogenic *E. coli* with the intention of controlling foodborne outbreaks. It is also helpful in directing policy and interventions (Scallan and others 2011) in food safety field.

2.4 Detection methods of pathogens in foods

2.4.1 Culture-based methods

The traditional serotyping method is based on the immunological reaction of antigens located on target bacteria and antibodies specific to antigens. However, this method has a low specificity because other bacterial species sometimes also share the same antigens as the target pathogen, resulting in cross-reactions and false positive results.

Conventional phenotype-based methods are consistently time intensive because they involve specimen enrichment, selective agar inoculation, biochemical tests, and

serotyping procedures. Other problems associated with culture-based method are cross reactivity, low specificity, laborious steps and high cost. Therefore, traditional culture-based methods are gradually being replaced by genotype-based methods, which are more discriminatory.

2.4.2 Molecular-based methods

Polymerase Chain Reaction (PCR) is a technique of rapidly amplifying target genes in DNA isolates. It is widely used in distinguishing bacterial species and highly discriminatory compared with culture-based methods. The conventional PCR assay uses on agarose gel to analyze the amplified PCR products. It involves transferring amplicons from a PCR to electrophoresis device, which increases the risk of contamination. On the other hand, a real-time PCR assay, which allows amplifications and quantitation of PCR products to be performed all at once in one single tube, can avoid the cross contamination by eliminating the use of gel.

2.5 Application of Real-time PCR assay in bacterial detections

2.5.1 SYBR® Green I

Due to purposes of PCR assays, different dyes are applied in specific studies. The most common and basic one for non-specific detection involves the use of the fluorophore SYBR® Green I. SYBR® Green I is an asymmetrical cyanine dye that would bind to any amplified double-strained DNA (Ishiguro and others 1995). The binding is not specific for one target but to all amplicons in the PCR reaction. The distinctive advantages of nonspecific DNA binding PCR contain the inexpensiveness and convenience. Thus, it is

used mostly in testing pure bacterial DNA samples, but barely applied in multiple target detection.

The advent of SYBR® Green I RT-PCR provides a rapid detection method for pathogens in food (Mackay 2004). In Liu and others' (2006) study, a simple and reproducible SYBR® Green PCR method was developed to identify *Enterobacter sakazakii* in infant formula, and this assay was validated on 35 strains of *E. sakazakii* and 88 other bacterial strains, which acquired a 100% specificity. Similarly, a PCR method based on SYBR® Green I fluorescence for detection of *Salmonella* in milk was reported (Nam and others 2005). This assay successfully reproduced target genes and reduced the total time required for testing to 23 h.

2.5.2 TaqMan™ probe-based PCR

To detect single base mutations, probe-based PCR and HRM-based PCR assays are commonly applied. Unlike nonspecific DNA amplification PCR, probes only bind to specific sequences of target amplicons. Probe-based RT-PCR involves the use of a probe that is a single-strained DNA or RNA with a strong affinity to a specific region on the target sequence. During the reaction, the TaqMan™ probe is cleaved and then the fluorescent reporter is released. The quantitation of target PCR products is measured by the accumulation of fluorescence of reporters. Therefore, TaqMan™ probe-based PCR method is more specific in binding with target DNA. The applications of probes enable different single nucleotide polymorphisms (SNPs) to be detectable. However, designing a viable probe is usually time-consuming since it requires numerous and repeated tests. Also, probe manufacturing is costly due to the rigorous process of probe production.

Piknová and others (2005) developed a TaqMan™ PCR assay with a probe oriented to the *fim*C gene. The specificity and sensitivity of the assay have been confirmed by using 53 *Salmonella* and 49 non-*Salmonella* DNA isolates, both of which reached 100%. Other study also reported the high selectivity of TaqMan™ probe-based PCR. In Tomás and others' study (2009), TaqMan™ PCR assay based on the *inv*A gene was proved to be an effective method for detection of *Salmonella* serotypes, thus it could avoid yielding false-positive from SYBR® Green I based method.

2.5.3 High Resolution Meltcurve PCR

In comparison, the HRM-based PCR assay is specific and requires no application of probes to obtain desired results. HRM dyes can completely saturate amplicons, thus yielding a meltcurve with higher resolution. This property has made HRM assays applicable in multiplex PCR as well, since HRM dyes would bind each type of amplicons equally without showing any preference. The final products with different GC contents, lengths, and sequences will present distinctive peaks in the resulting HRM plot. Therefore, HRM analysis can acquire the same precision as probe-based method with less expense and time. Owing to the simplicity, low cost and ease of use, the application of HRM has also increased in the areas of clinical diagnostics and food safety. One of the earliest research reported that HRM analysis was used to discriminate among different species groups between *Mycobacterium* chelonae and *M*. abscessus (Odell and others 2005). Likewise, in Merchant-Patel's (2010) study, a single-locus HRM analysis was applied to distinguish *Campylobacter jejuni* and *Campylobacter coli* genotypes based on the mutation on the *fla*A fragment. Both studies demonstrated that HRM play an important role in resolving significant numbers of alleles of individual loci.

2.5.4 Multiplex PCR

2.5.4.1 Principals of multiplex PCR

Multiplex PCR, which allows for the simultaneous detection of several target genes in one PCR assay, has been commonly applied in foodborne pathogen detections. Multiplex PCR is used to detect multiple microorganisms in a single reaction or detect several target genes of one microbial strain. In conventional multiplex PCR, the separation of different products is based on the differences in molecular weight, thus these amplicons with different sizes are visualized via agarose gel electrophoresis. In RT-PCR, diverse melting temperature (Tm) values of double-strained DNA targets are crucial in distinguishing different products, because the target genes will form peaks in different positions (Figure 1).

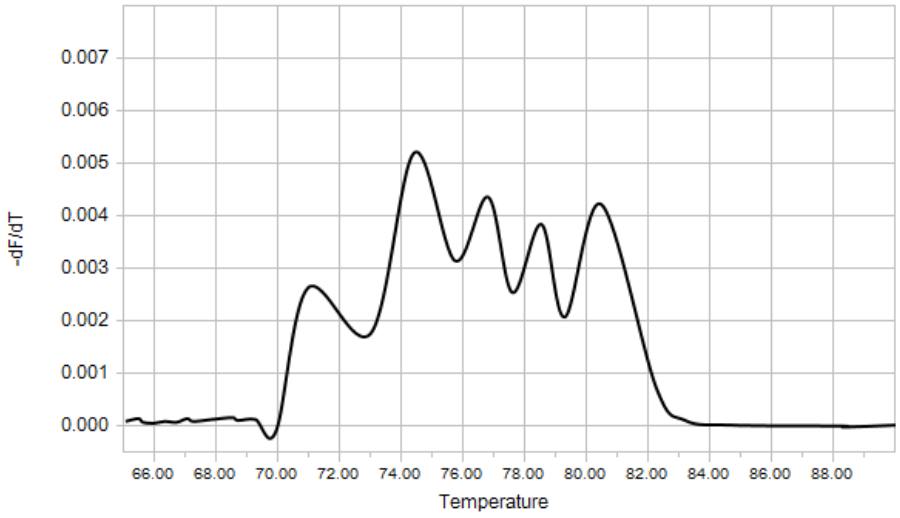

Figure 2.1 A result of Multiplex PCR plot in Real-time PCR

The HRM assay based on real-time PCR techniques has been commonly applied in multiplex PCR. Due to the equal binding of HRM dyes to each amplicon, HRM-based assays do not show any preferential affinity towards an amplicon. Also, the application of

multiplex PCR technique could avoid false positive or negative results from single-gene detections. Therefore, an HRM assay is able to generate a melt curve of high resolution, which can accurately be used to differentiate several target genes.

2.5.4.2 Application of multiplex PCR in pathogen detection

Melting curve based RT-PCR assay have been previously used for pathogen detections in foods (Nam and others, 2005). Compared with a culture-based method which is extremely time-consuming, this assay requires less than 12 h, including the enrichment time and PCR operating time, to obtain results. Singh and Mustapha (2014) developed a multiplex PCR assay for detection of virulent and antibiotic resistant *Salmonella* using one primer set. In their assay, multiple target genes including invasin (*inv*A), and three antibiotic resistance genes, *tet*G, *aas*A2 and *sul*I were successfully amplified and formed distinctive peaks in one meltcurve assay. Similarly, Lim et al. (2003) developed a multiplex PCR assay with primers targeting three genes-- *rfb*J, *fli*C and *flj*B encoding flagellin in *S.* Typhimurium strains. After testing on *S*. Typhimurium, other *Salmonella* serovars, and non-*Salmonella* enteric pathogens, this multiplex PCR assay was proven to be practical in the identification of *S*. Typhimurium.

Holland and others (2000) performed a multiplex PCR targeting *stx*1, *stx*2 and *eae*A genes in *E. coli* O157:H7. The presence of one or both *stx* genes and *eae*A gene were considered as positive for *E. coli* O157:H7 among 153 *E. coli* samples tested in this assay. Likewise, Lin and others (2011) built a probe-based PCR method specific for differentiating *E. coli* serotypes. With *wzx* and *wzy* as target genes, 10 *E. coli* serogroups were able to be identified.

CHAPTER 3

Multiplex High Resolution Melt-Curve Real-time PCR Assay for Reliable Detection of *Salmonella*

Abstract

A multiplex high resolution melt-curve (HRM) PCR assay was developed to detect the majority of *Salmonella* serotypes in general. This assay used a HRM multiplex PCR rather than a singleplex PCR to detect *Salmonella* serotypes, which reduced the chance of getting false-negative results from *Salmonella* serotypes and false-positive results from non-*Salmonella* strains. Three genes -- *inv*A, *stn* and *fim*A were selected and primers pairs were designed for each target gene. This method was tested on 82 *Salmonella* strains and applied in various food samples. PCR result showed three specific and separated peaks were formed for most *Salmonella* serotypes, which indicated that all three products were formed. Several *Salmonella* strains showed the absence of one peak, but gave positive results for the other two target genes. 30 isolates of non-*Salmonella* yielded negative for all three genes, except *Citrobacter freundii* which was false-positive in single gene detection. After a 6-h enrichment, target genes could be detected in 25 g of most food samples inoculated with 10^3 CFU/g of *Salmonella.*

3.1 Materials and methods

3.1.1 Bacterial DNA extraction

Bacterial strains were obtained from the culture collection of the Food Microbiology Laboratory, University of Missouri, or procured from the American Type Culture Collection (ATCC, Rockville, MD). Genomic DNA from all tested strains was isolated from overnight cultures in Tryptic Soy broth (TSB) (Difco Labs, Sparks, MD, USA). One milliliter of the overnight cultures in TSB was centrifuged at 6,000 ×*g* for 2 min. The obtained cell pellet was extracted by using PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The concentration and purity of the obtained DNA samples were measured by a Nanodrop Lite Spectrophotometer (Thermos Fisher Scientific, Wilmington, DE, USA).

3.1.2 Primer design

Primer sets for *inv*A, *fim*A and *stn* genes were designed using the Primer3 software (Untergasser and others 2012). In order to clearly distinguish peaks for each target gene, the T_m of amplicons should have an approximately 3 ^oC interval between them (Singh and Mustapha 2014). The oligonucleotides were commercially synthesized (IDT, Coraville, IA, USA).

The melting temperature of each product was determined in singlet PCR with SYBR® Green. To ensure that the difference in the T_m values between the primers was at least 2 ^oC in order to avoid overlapping of peaks, different combinations of primers for each of three genes were chosen. All the combination groups were tested in multiplex PCR

using HRM curve analysis. Due to the uneven distributions of each target in the DNA samples, the relative concentrations of primer sets were adjusted with the purpose of forming equal amplifications. One combination that yielded the best result was ultimately picked. The sequences of tested primers are shown in Table 3.1.

Table 3.1 Primers used for real-time PCR melt curve assay

Primer	**Sequence (5'-3')**	**Product size (bp)**	**Primer conc.**
***stn*-1F**	GCCATGCTGTTCGATGATATT	100	0.2 µM
***stn*-1R**	GGATCAGTTGGAGGCGATTT		0.2 µM
inv-558F	TGACGGTGCGATGAAGTTTA	95	0.25 µM
inv-558R	GAGTCATCCCCACCGAAATA		0.25 µM
fimA -225F	GCAGGTGCCTTTCTCCATC	243	0.1 µM
fimA -225R	AGCGTATTGGTGCCTTCAAC		0.1 µM
IAC	CATATATGACGGTGCGATGAAGTTTAA TATATATATTATCAAAATAAGACTAAT AAAGTATTTCGGTGGGGATGACTCCAT AC		5 fg/ uL

3.1.3 Standardization of IAC concentration

An internal amplification control (IAC) is a non-target product incorporated into an assay to prevent false negatives in case of a PCR reaction failure. In this assay, the chosen IAC amplified simultaneously with the target sequence in the same reaction. A positive IAC peak and missing target amplicons in one assay would indicate that target products were not successfully amplified. A PCR accompanied with a negative IAC and missing

targets usually indicate that the reaction was inhibited as a result of a malfunctioned PCR running program, or the presence of inhibitory materials.

An 83-base long DNA oligo was specifically designed to serve as the IAC in this assay. The IAC molecule was diluted in nuclease-free water to obtain the final concentration of 100 fg/uL, 50 fg/uL, 20 fg/uL, 15 fg/uL, 10 fg/uL, 5 fg/uL, and 1 fg/uL. The ideal dilution of IAC is the minimum concentration that is able to yield reproducible results without inhibiting the amplification of any target gene. The optimal dilution of IAC was determined by testing the primers of each dilution in multiplex format.

3.1.4 Real-time PCR

Real-time PCR assay was conducted using the Meltdoctor^{TM} HRM Master Mix (Applied Biosystem, Foster City, CA, USA) in a LightCycler® 96 Real-time PCR (Roche Diagnostics Corp., Indianapolis, USA). PCR amplification was performed in a 10 µL reaction volume containing 5 µL Master Mix and 30 ng of genomic DNA. The primer sets and IAC concentrations are listed in Table 3.1.

A two-step amplification PCR assay was performed on a LightCycler® 96 instrument (Roche Diagnostics Corp., Indianapolis, USA) using SYBR Green dye. The amplification starts with an initial denaturation at 95°C for 780s, followed by 40 cycles of 95°C for 20s, and 60°C for 40 s. One cycle of melt curve step was conducted by ramping the temperature from 60°C to 90°C. Fluorescence signal was collected in the Resolight channel on the PCR instrument. The melt curve plot was prepared by plotting the negative derivative of fluorescence (-Rn) versus temperature.

3.1.5 Specificity of the real-time PCR assay

The specificity of the three primer pairs was determined by testing with 84 *Salmonella* strains comprising 42 serovars and 20 non-*Salmonella* species listed in Appendix 1. Gram-negative non-*Salmonella* isolates included *Pseudomonas fluoresecens*, *Proteus mirabilis*, *Enterobacter cloacae, Enterobacter aerogenes, Klebsiella pneumoniae*, *Escherichia coli* O157:H7, *E. coli* O111: NM, *E. coli* O26:H11, *E. coli* O103:H2, *E. coli* O121:H19, *E. coli* O145:NM, *E. coli* K12, and *E. coli* P24, Gram-positive isolates included *Bacillus megaterium, Bacillus sphaericus, Shigella sonnei, Shigella flexneri, Listeria innocua, Listeria ivanovii, Staphylococcus aureus, Lactobacillus plantarum,* and *Streptococcus pyogenes*.

3.1.6 Sensitivity of the real-time PCR assays

To determine the working efficiency of each primer in the developed multiplex assay, DNA isolate of *S.* Typhimurium 788, *S.* Enteritidis, *S.* Arizonae and *S. enterica* 6539 were serially diluted, starting from 5 nanograms to 5 picograms (e.g. 5 ng, 500 pg, 50 pg, 5 pg). Two microliter of each serially diluted DNA was applied in triplicate with each primer in singleplex for the real-time PCR. PCR efficiency was calculated from the slope of the standard curve using the formula: $E = 10^{(-1/slope)}-1$. The primer efficiency range between 90%-110% was considered to be reliable. In this study, the efficiency of each gene was calculated by LightCycler® 96 Software 1.1.

To determine the detect limit of this assay, *S.* Typhimurium var. Copenhagen LJH 788 overnight cultures were serially dilute using 9 mL peptone water (1.0 g/L). One mililiter of each dilution tube was plated on TSA for enumeration, and another 1mL from

the same dilution tube was isolated for genomic DNA using PrepMan™ Ultra. Two microliter of the obtained DNA sample was performed with the multiplex assay. The obtained PCR results were then correlated with corresponding bacterial count (CFU/mL) to estimate the limit of detection of the assays.

3.1.7 Preparation of *Salmonella* cocktail

According to the association and frequency of presence in foodborne outbreaks, *Salmonella* Typhimurium 788, *Salmonella* Enteritidis, *Salmonella* Arizonae and *Salmonella enterica* 6539 were selected to make a *Salmonella* cocktail as the inocula for food samples. Cultures were grown overnight in TSB plus yeast (TSBY) at 37°C. One hundred microliter broth from each culture were taken and mixed in a new tube with TSBY. After incubating at 37°C overnight, serial dilutions and standard plating were conducted with the *Salmonella* cocktail. Plates were incubated at 37°C for 24-48h before counting. Corresponding dilutions and volumes were determined to create concentrations of 10^2 CFU/mL and 10^3 CFU/mL as spiking stocks.

3.1.8 Preparation of food samples

Food samples, including tomato, green pepper, spinach, cucumber, ground turkey, turkey sausage, apple juice, orange juice, chicken meat, shelled peanut, shelled pistachio, nuts mix, peanut butter, raw egg, whole milk, fat-free milk, infant formula, were purchased from local groceries. Ground beef was purchased from the University of Missouri Meat Lab.

Twenty-five grams of each food sample, except ground beef, were placed in filtered stomacher bags. Each food sample was spiked with 10 CFU/g, 100 CFU/g and 10^3 CFU/g of *Salmonella* cocktail, respectively. After standing for 15 min, 225 mL Buffered Peptone Water (BPW) was added and the mixture homogenized by stomaching for 2 min. Samples were incubated at 37 °C, and sampling was conducted at intervals of 4 h, 5 h, 6 h, and 8 h. One milliliter of broth was taken from enriched samples. DNA isolation was conducted by the protocol of PrepMan™ Ultra Reagent. Three twenty-five grams of ground beef were placed in 69 oz. Whirl-Pak™ filtered stomacher bags (NASCO, MI, USA) and diluted with 925 mL BPW. The rest of the steps were the same as the above.

The DNA isolates from samples were diluted to 50-70 ng/µL before conducting the PCR assay.

3.2 Results

3.2.1 Melt curve real-time PCR

As illustrated in Figure 3.1, this melt curve assay showed three distinctive and well separated peaks. These separated peaks of *inv*A, *stn* and *fim*A were formed with the average melting temperature of amplicons of 77.21°C, 81.43°C and 85.44°C, respectively. After adjusting the concentration of each primer pair, MeltDoctor™ HRM dyes bound equally to each amplicon without showing preferential bindings to specific amplicons.

According to Figure 3.1, the reaction with only IAC and other reagents except DNA samples formed a distinctive peak whose melting temperature is 69.10°C, which is

different from all three products generated from target genes. The presence of IAC indicated the reaction was not inhibited by any inhibitory substances in the reaction. Further, the IAC peak was not detectable when amplicons peaks of *inv*A, *stn* and *fim*A were present, which indicated the target sequences were successfully reproduced.

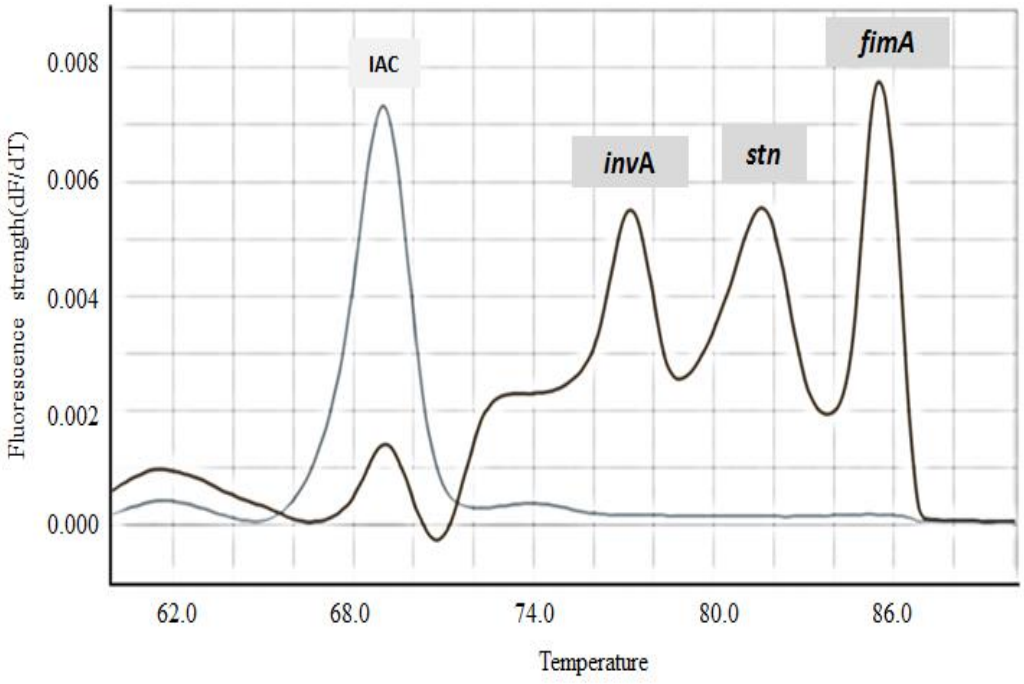

Figure 3.1 Multiplex Real-time PCR assay for detection of *Salmonella* and IAC.

3.2.2 Specificity of the PCR assay

As shown in Appendix 1, 79 of 82 isolates of *Salmonella* strains were found to be positive with all three *inv*A, *stn* and *fim*A genes. Two *Salmonella* Arizonae strains were negative with *fim*A, and a *Salmonella* Senftenberg was *inv*A-negative. *Citrobacter freundii* was examined to be *stn* positive and negative for *inv*A and *fim*A, thus *Citrobacter freundii* was excluded from *Salmonella* serotypes.

Therefore, this three-gene multiplex PCR assay was considered to be reliable since the presence of two or more target genes indicates the incidence of pathogenic *Salmonella*.

3.2.3 Sensitivity of the real-time PCR assay

The obtained primer efficiency were as follows *inv*A efficiency =108%, *stn* efficiency =101.2% and *fim*A efficiency =97.6%. The sensitivity of the multiplex assay was found to be 10 pictogram/reaction as shown in Figure 3.2.

The limit of detection would be 900 CFU/mL, corresponding to a Ct value of 27.33. All targets were successfully detected at this cell concentration.

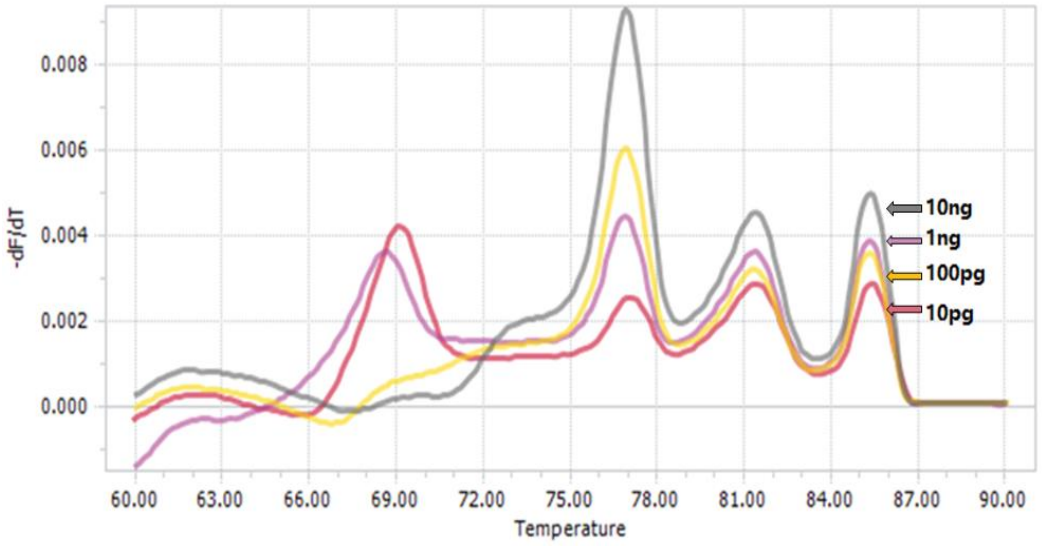

Figure 3.2 Sensitivity of the multiplex PCR assay as DNA concentration ranging from 10ng to 10 pg using MeltDoctor™ master mix.

3.2.4 Detection of *Salmonella* in spiked food samples

According to Table 3.2, all food samples, except infant formula, whole milk and chicken meat, needed at least a 5-h enrichment to be detectable regardless of the *Salmonella* spike level. For infant formula, whole milk and chicken meat, a detection limit of 10^3 CFU/g was achieved after a 4-h enrichment. At low spike levels, such as 4-10 CFU/g, most food samples required an 8-h enrichment period. However, *Salmonella* in chocolate and green onion were not able to be detected at 4-10 CFU/g spike level with an 8-h enrichment. Meat samples usually involve a shorter enrichment period when spiked at level of 10^3 CFU/g compared with other food categories.

Table 3.2 Detection of *Salmonella* in spiked food samples.

Food sample	***Salmonella* inoculum level (CFU/g)**	**Inoculation time**				
		0 h	**4 h**	**5 h**	**6 h**	**8 h**
Ground turkey	4-10	-	-	-	+	+
	10^2	-	-	+	+	+
	10^3	-	-	+	+	+
Turkey sausage	4-10	-	-	+	+	+
	10^2	-	-	+	+	+
	10^3	-	-	+	+	+
Peanut with shells	4-10	-	-	-	-	+
	10^2	-	-	-	-	+
	10^3	-	-	+	+	+
	4-10	-	-	-	-	+

Pistachio with shells	10^2	-	-	-	-	+
	10^3	-	-	+	+	+
Raw Egg	4-10	-	-	-	-	+
	10^2	-	-	+	+	+
	10^3	-	-	+	+	+
Cucumber	4-10	-	-	-	+	+
	10^2	-	-	+	+	+
	10^3	-	-	+	+	+
Fat-free milk	4-10	-	-	-	-	+
	10^2	-	-	-	-	+
	10^3	-	-	-	+	+
Nuts mix	4-10	-	-	-	+	+
	10^2	-	-	-	+	+
	10^3	-	-	+	+	+
Infant formula	4-10	-	-	-	-	+
	10^2	-	+	+	+	+
	10^3	-	+	+	+	+
Peanut butter	4-10	-	-	-	-	+
	10^2	-	-	-	+	+
	10^3	-	-	+	+	+
Apple juice	4-10	-	-	-	+	+
	10^2	-	-	-	+	+
	10^3	-	-	+	+	+
Whole milk	4-10	-	-	-	-	+

	10^2	-	+	+	+	+
	10^3	-	+	+	+	+
Tomatoes	4-10	-	-	-	-	+
	10^2	-	-	-	+	+
	10^3	-	-	-	+	+
Ground beef	4-10	-	-	-	+	+
	10^2	-	-	+	+	+
	10^3	-	-	+	+	+
Almonds	4-10	-	-	-	-	+
	10^2	-	-	+	+	+
	10^3	-	-	+	+	+
Chicken meat	4-10	-	-	-	-	+
	10^2	-	-	+	+	+
	10^3	-	+	+	+	+
Chocolate	4-10	-	-	-	-	-
	10^2	-	-	-	-	+
	10^3	-	-	-	-	+
Turkey slice	4-10	-	-	-	-	+
	10^2	-	-	+	+	+
	10^3	-	-	+	+	+
Orange juice	4-10	-	-	-	-	+
	10^2	-	-	-	+	+
	10^3	-	+	+	+	+
Turkey Bacon	4-10	-	-	-	-	+

	10^2	-	-	+	+	+
	10^3	-	-	+	+	+
Green onion	4-10	-	-	-	-	-
	10^2	-	-	-	-	-
	10^3	-	-	-	+	+
Cantaloupe	4-10	-	-	-	-	+
	10^2	-	-	-	+	+
	10^3	-	-	-	+	+
Spinach	4-10	-	-	-	+	+
	10^2	-	-	-	+	+
	10^3	-	-	+	+	+
Bell pepper	4-10	-	-	-	-	+
	10^2	-	-	-	+	+
	10^3	-	-	-	+	+

3.3 Discussion

Melting curve based real-time PCR assay have been previously used for pathogen detections in foods (Nam and others 2005; Singh and Mustapha 2015). Compared with a culture-based method which is extremely time-consuming, this assay only requires less than 12 h including the enrichment time and PCR operating time, to obtain the final result. Singh and Mustapha (2014) developed a multiplex PCR assay for detection of virulent and antibiotic resistant *Salmonella* using one primer set. In their assay, multiple target genes including invasin (*inv*A), and three antibiotic resistance genes, *tet*G, *aas*A2

and *sul*I were successfully amplified and formed distinctive peaks in one meltcurve assay (Singh and Mustapha 2014). Similarly, Lim et al. (2003) developed a multiplex PCR assay with primers targeting three genes-- *rfb*J, *fli*C and *flj*B encoding flagellin in *Salmonella* Typhimurium strains. After testing on *S*. Typhimurium, other *Salmonella* serovars, and non-*Salmonella* enteric pathogens, this multiplex PCR assay was proven to be practical in the identification of *S*. Typhimurium.

False-positive results were repeatedly reported in previous single-locus PCR assays (Rahn and others 1992; Moore and Feist 2007). Since the development of multiplex PCR assays, their application in detection of pathogens has been widely used. Therefore, quite a few researchers have developed multiplex PCR assays for the detection of *Salmonella* (Kim and others 2006; Ali and others 2006). The correct gene targets were critical for building the PCR assay, in order to detect most pathogenic *Salmonella* serotypes.

The gene, *inv*A, enables *Salmonella* to invade cells of hosts' intestinal epithelium. The *inv*A locus is present and functional in most *Salmonella* serotypes. In Chen's research (Chen and others 1997), the *inv*A was picked as the target gene in TaqMan™ PCR, and the results showed that a 287 bp length product was formed. The specificity and sensitivity of the assay both reached 100%, and less than 7 CFU per PCR reaction in spiked food samples was successfully detected. However, some studies reported that *inv*A was absent in some strains in *S. enterica*. For instance, *S. enterica* subsp. *enterica* serovar Saint Paul is not detected by the *inv*A-based assay (Malorny and others 2004; Cohen and others 1996). In Rahn and others' research (1992), two *Salmonella* spp.-- *Salmonella* Litchfield and *Salmonella* Senftenberg, did not amplify with the *inv*A gene. In this study, the absence of *inv*A in *S.* Senftenberg O Group E confirmed the limitation

of applying *inv*A as the single target gene in detection methods. Besides, performing TaqMan™ based PCR is not cost-effective, since manufacturing and testing desirable fluorescent probes are costly and time-consuming.

The *fim*A gene is highly reserved in *S.* Typhimurium. Among 376 strains of *Salmonella* being tested, all isolates were found to be positive with the *fim*A gene in the assay (Cohen and others 1996). In our current study, two strains of *Salmonella* Arizonae failed to yield a fragment when *fim*A was targeted, but were positive for both *invA* and *stn*. In Aabo's research (Aabo and others 1993), with yielding a 429-bp amplicon in PCR, all *Salmonella* except two strains of *S.* Arizonae were successfully detected.

In this assay, the inclusion of *invA* and *fim*A has eliminated the possibility of getting false-positive results from a uniplex PCR. Hence, the presence of the two target genes in the assay can confirm the incidence of *Salmonella.*

Salmonella contamination is transmitted primarily through contaminated food and water. When *Salmonella* contamination in food products happens, it is usually in a low concentration. Therefore, direct detection from the original sample using PCR is not feasible due to the low sensitivity of the assay when food matrices are involved. Therefore, an enrichment step prior to following experiments is necessary. Enrichment allows for the amplification of microbes in food sample, in order to reach their detectable level.

Usually, the infective dose of *Salmonella* in food is more than 10^5 CFU, but a low ingested dose of less than 10^3 CFU has been involved in some foodborne *Salmonella* outbreaks (Kothary 2001). Food contaminated by low doses of *Salmonella* serovars

requires a high sensitivity of detection method. Therefore, in this study, 4-10, 10^2 and 10^3 CFU/g ingested concentrations were employed as inocula for testing food samples.

Salmonella in food matrices containing low moisture and high fat content are challenging to detect. These factors place a major effect on bacterial recovery during enrichment and may decrease detection sensitivity (Podolak and others 2010). In this assay, the chocolate samples were confirmed positive for *Salmonella* only after 8 h of enrichment and with a spike level > 100 CFU/g. Also, some food debris, including fat, enzyme, polysaccharides and tiny insoluble substances, may remain with DNA isolates during sample processing. This represents major inhibitors that limit the sensitivity and accuracy of the PCR assay.

An appropriate DNA concentration helps to enhance the efficiency of a PCR assay (Demeke and Jenkins 2010). The initial concentration can reach 900 ng/μL, which possibly inhibits the accurate amplification of target genes. However, a DNA concentration that is too low may reduce PCR sensitivity. A concentration between 50 ng/μL to 70 ng/μL is suitable. Thus, diluting samples to a desired level before conducting PCR is required.

Pathogenic *Salmonella* distribute in various food commodities from vegetables, nuts, and eggs to meat and poultry. Meat and meat products, especially poultry, are the main reservoirs of various *Salmonella* serovars. In this study, meat products which included ground turkey, turkey bacon, turkey sausage, turkey slice and chicken meat, usually required a lower spike level and less enrichment time to be detectable, due to the rich nutrient and high water content. Thus, the component of meat provides a favorable environment for the growth of bacteria. Besides meat and dairy products, fruits and

vegetables that are usually consumed raw are also the common vehicle for *Salmonella* transmission. *Salmonella* may get on these produces while growing, during harvesting and during distribution. In fact, fresh produce such as green onion and lettuce have been implicated in documented produce-related outbreaks in the US (Ge and others 2012).

In Singh and Mustapha's (2014) study, two poultry samples-- whole chicken and ground chicken with a 6-h enrichment treatment yielded positive results with all targets through an established multiplex PCR assay (Singh and Mustapha 2014). Likewise, Hein and others (2006) established a real-time PCR assay in detection of *Salmonella* spp. in foods. After an enrichment period of 16 h with BPW, chicken meat samples spiked with low doses of *Salmonella* in the range of 0.5 to 6.4 CFU/g all yielded positive results.

3.4 Conclusions

In this work, we described a three-gene HRM-multiplex PCR assay with an IAC, which is highly reliable, rapid and cost-effective to detect a majority of pathogenic *Salmonella* serotypes. This assay avoids false-positive results from non-*Salmonella* strains that possess one of the three target genes, and reduces the chances of achieving false-negative resulting from a single-gene based PCR. Food samples initially spiked with 4-10 CFU/g *Salmonella* could be successfully detected within a 10-h (including enrichment) period. Hence, this method has a good potential to be used in epidemiological, clinical and laboratory settings as a robust method for *Salmonella* detection.

CHAPTER 4

High-Resolution Melt Curve PCR Assay for Specific Detection of *Escherichia* coli O157:H7 in Beef

Abstract

According to the CDC, among the disease-causing Shiga toxin producing *Escherichia coli* (STEC), *E. coli* O157:H7 is estimated to cause one-third of the total STEC illnesses and the most cases of hemolytic uremic syndrome (HUS) in the U.S. The *uid*A gene which is present in the majority of *E. coli* strains, codes for the synthesis of the β-D-glucuronidase (GUD) enzyme. In *E. coli* O157:H7, the *uid*A gene has a single point mutation at the +93 position that leads to an alteration in the amino acid sequence encoding the GUD enzyme. The aim of this study was to distinguish *E. coli* O157:H7 from other *E. coli* using a high resolution melt curve (HRM) PCR assay. Based on the *uid*A mutation in *E. coli* O157:H7, a reliable PCR assay targeting the *uid*A gene was developed to differentiate *E. coli* O157:H7 from other STECs and the closely related *Shigella* sp. The assay was validated using a set of 120 bacterial strains and spiked ground beef and beef trim. Isolates of *E. coli* O157:H7 formed distinctive melt peaks that were easily distinguishable from those of non-O157 isolates in the PCR plot. Therefore, this assay was verified to be able to clearly discriminate *E. coli* O157 strains from other *E. coli* and *Shigella*. With a 6-h enrichment, 10 CFU *E. coli* O157:H7 were detectable in

325 g spiked beef samples. This assay will help in correctly determining the food source of *E. coli* O157:H7 for preventing and controlling foodborne outbreaks.

4.1 Materials and methods

4.1.1 Bacterial DNA extraction

STEC strains were obtained from the University of Missouri, Food Microbiology Lab culture collection. Cultures were grown overnight at 37 °C in Tryptic Soy broth (TSB) (Difco Labs, Sparks, MD, USA). Genomic DNA of pure STEC strains was isolated from overnight cultures in TSB broth. One milliliter of the overnight cultures in TSB broth was centrifuged at 6,000 ×*g* for 2 min. The obtained cell pellet was extracted by using PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The concentrations and purity of the obtained DNA samples were measured by a Nanodrop Lite Spectrophotometer (Thermos Fisher Scientific, Wilmington, DE, USA).

4.1.2 Primer and HRM-PCR assay design

The primer pairs, HRM-F and HRM-R, was designed using the Primer3 software (Untergasser and others 2012). The specificity of the designed PCR primers was tested using NCBI/Primer-BLAST, and all oligonucleotides were commercially synthesized (IDT, Coraville, IA, USA) as shown in Table 4.1.

Real-time PCR assay was performed using 2× LightCycler® 480 High Resolution Melting Master (Roche Diagnostics Corp., Indianapolis, USA). The HRM assay was standardized on a LightCycler® 96 real-time PCR. PCR reaction was performed in a 10

μL reaction volume in duplicate, with 30 ng of genomic DNA, 0.5 μM primers and 2.5 mM $MgCl_2$. A two-step amplification protocol included an initial denaturation step at 94 ^oC for 10 min, followed by 40 cycles of 95 ^oC for 15 s, 64 ^oC for 30 s. A high-resolution melt curve was added at the end of the PCR amplification (from 60 ^oC to 95 ^oC, with gradual temperature increments of 0.04 ^oC/s) A HRM analysis was performed with a pre-melt region 76.9 -77.9 ^oC and a post-melt region 87.7-88.7 ^oC.

128 *E. coli* O157 and non-O157 DNA isolates were tested in the PCR to validate this assay (Appendix 2).

Table 4.1 The uidA primer details

Primer	**Sequence**	**Gene**	**Product Size (bp)**	**$MgCl_2$ concentraio-n**	**Primer concentraion**	**Reactio n volume**
HRM-F	GCCCGGC TTTCTTGT AAC	*uid*A	62 bp	2.5 mM	0.5 μM	10 μl
HRM-R	GATCGCG AAAACTG TGGAAT				0.5 μM	

4.1.3 Food sample preparation

Ground beef and beef trim were purchased from the University of Missouri Meat Lab. Five *E. coli* O157:H7 strains were used to spike samples respectively (Table 4.2). Three hundred and twenty-five grams of ground beef were placed in one filtered

stomacher bags and spiked with 10 CFU *E. coli* O157:H7. After 15 min in room temperature, the spiked ground beef was placed in 4 °C for 72 h, in order to cold stress the *E. coli* O157:H7. After that, 975 mL Buffered Peptone Water (BPW) with 8 mg/L vancomycin was added into the bag and the sample was homogenized by stomaching for 2 min. Samples were incubated at 37 °C for 8 h, 10 h and 12 h. Two milliliters of homogenate were transferred from the enriched sample into a centrifuge tube. After centrifuging at 3800 ×*g* for 1.5 min, 1 mL of the supernatant (without drawing the fat content on top) was removed and transferred into a new centrifuge tube.

Table 4.2 *E. coli* O157:H7 strains used in spiked beef samples and enrichment period needed.

Strains	**Sources**	**Enrichment time**	
		Ground beef	**Beef trim**
E. coli O157:H7 EDL-933	Food (hamburger)	8 h	6 h
E. coli O157:H7 505B	Beef (FRI)	6 h	5 h
E. coli O157:H7 3178-85	Human (CDC)	8 h	6 h
E. coli O157:H7 C7927	Human (CDC)	8 h	5 h
E. coli O157:H7 MF1847	Beef (FSIS)	8 h	6 h

4.1.4 Biochemical confirmation tests

Immunomagnetic separation (IMS) was conducted by using Dynabeads® *E. coli* anti-O157 (Thermos Fisher Scientific, Wilmington, DE, USA), followed by DNA isolation with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Another 1.0 mL supernatant was treated with IMS, and the collected beads were diluted to 10^{-4} using peptone water. One hundred microliters of diluted broth were transferred and spread-

plated on Rainbow Agar (Biolog, Hayward, CA) plate. Plates were incubated at 37 °C for 24 h. The distinctive black or gray colonies on Rainbow Agar were suspected to be *E. coli* O157:H7.

A single colony of suspect *E. coli* O157:H7 from the Rainbow Agar plate was picked and tested with DrySpot™ *E. coli* O157 Latex Agglutination Test (Oxoid Diagnostic Reagents, Hampshire, England) according to the manufacturers' instructions. A positive result was interpreted as large clumps of agglutination with partial or complete clearing of the background latex within 1 to 2 min.

4.1.5 DNA isolation of *E. coli* O157:H7 from beef samples

Due to the high amount content of fat and impurities of beef samples, the extraction of DNA was challenging. Our preliminary studies found that the DNA extraction using PrepMan™ Ultra was not effective due to a wide variety of inhibitory factors in samples. On the other hand, DNeasy Blood & Tissue Kit was much more sensitive than PrepMan™ Ultra. It relies on the binding of DNA to silica in the presence of a high concentration of chaotropic salts, which delivers a stable DNA extract. Thus, DNeasy Blood & Tissue Kit was employed in beef samples.

Genomic DNA of *E. coli* was isolated using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the instructions of the purification of Total DNA from Animal Tissues.

4.2 RESULTS AND DISCUSSION

A high efficient laboratory detection method of pathogen is critical for the rapid and accurate diagnostics among patients, and timely infection controlling (Mandal and others 2011). The conventional culture-dependent methods remain the gold standard and have been extensively used in foodborne pathogen detections worldwide (Gracias and McKillip 2004). However, conventional methods are tedious and require at least 4-5 working days to complete the whole process. In this study, a HRM real-time PCR method was developed to distinguish *E. coli* O157:H7 from other *E. coli* in ground beef and beef trim. The obvious advantage of this assay over phenotypic assays is to shorten the whole process to less than 24 h. Moreover, the HRM assay does not require special equipment other than a real-time PCR machine, thereby making it accessible and affordable for most of laboratories.

The HRM assay was standardized using pure DNA samples of *E. coli* O157:H7 and other *E. coli*. According to Figures 4.1 and 4.2, isolates of *E. coli* O157:H7 form melting peaks in different positions than those of other *E. coli*, and their melting profiles are also different, making this serotype distinguishable from other *E. coli*. Besides, *Shigella*, which can sometimes be difficult to distinguish from *E. coli* O157 was found to form the same peak as *E. coli* non-O157.

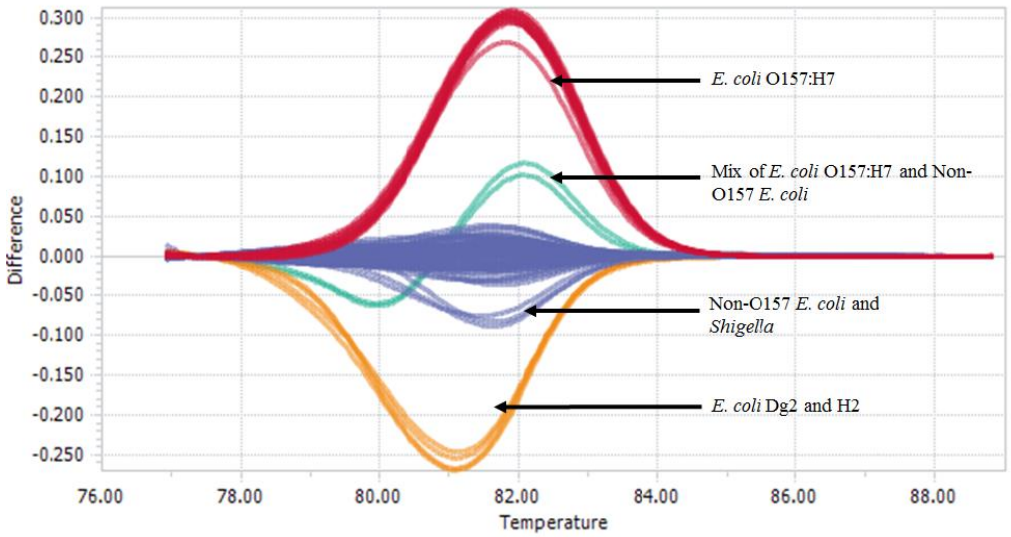

Figure 4.1 High-resolution melting plots for the identification of *E. coli* O157:H7.

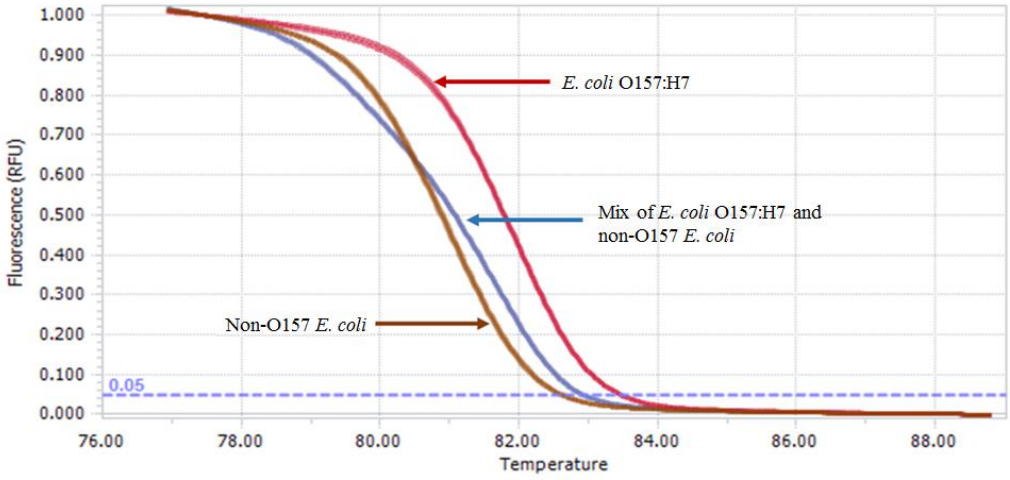

Figure 4.2 Normalized melting curves showing three different melt profile obtained using Standard *E. coli* O157 ATCC 43894, *E. coli* and mixture of two DNA samples.

The optimal concentration of $MgCl_2$ for the PCR was determined to be 2.5 mM. A proper $MgCl_2$ concentration in a PCR reaction mix is one of the most important factors in building a distinguishable plot for determining *E. coli* genotypes. In addition to Taq DNA polymerase, which is a magnesium-dependent enzyme, template primers and

deoxynucleotide triphosphates (dNTPs) need to bind with $MgCl_2$ in order to replicate target genes. Inadequate $MgCl_2$ concentration will cause the reduction of products while excessive $MgCl_2$ will prevent complete denaturation of DNA and result in primers to bind to incorrect template sites.

The optimized assay was validated using 120 bacterial stains, comprising 21 strains of *E. coli* O157, 9 of *E. coli* O26, 10 of *E. coli* O45, 10 of *E. coli* O103, 6 of *E. coli* O104, 10 of *E. coli* O111, 10 of *E. coli* O121, 9 of *E. coli* O145, 3 *Shigella* strains including *S. dysenteriae, S. flexneri* and *S. sonnei*, and 40 non STEC *E. coli* strains isolated from the feces of eight different animals (chicken, cattle, duck, dog, goose, goat, human, pig) The detail information was listed in Appendix 2. An equal concentration mixture of *E. coli* O157 and *E. coli* DNA was used to create a heterozygous genotype. This HRM assay was able to clearly discriminate *E. coli* O157 strains from other *E. coli* and *Shigella*.

Based on the single point mutation at position +93 of the *uid*A in *E. coli* O157, Cebula et al. (1995) designed a mismatch amplification mutation assay (MAMA) for the detection of this serotype. It was one of the most commonly used methods for the identification of *E. coli* O157:H7. It was found that under relaxed PCR conditions, the assay amplified *uid*A gene of other *E. coli* and *Shigella*, generating a false positive result. Hence, the HRM assay developed in this study was further validated using *Shigella* strains. The melt profile generated by the *Shigella uid*A gene amplicon grouped with other *E. coli*, proving the specificity of the assay towards the *E. coli* O157 serogroup.

In this study, out of 40 *E. coli* strains isolated from feces of 8 different animals, the melt curve profile of 38 strains aligned with one another but two strains, *E. coli* Dg2 and

E. coli H2 generated a separate profile in the differential plot (Figure 4.2). The formation of a separate melt curve profile in the differential plot indicates the presence of mutations in the amplicon region of targeted *uid*A gene sequences. GenBank nucleotide data search on the *Shigella uid*A gene revealed the presence of two single nucleotide polymorphism (SNP) in the amplicon region. Due to the high sensitivity of the HRM assay, the presence of any other SNP is expected to form a separate melt curve profile other than *E. coli* O157:H7 on the differential plot.

Raw ground beef is a type of food material that has a high level of background microflora, which makes the isolation of target bacteria difficult, especially target bacteria that are present in low numbers. In this study, artificially inoculated ground beef was detected to be positive with *E. coli* O157:H7 after N 8-h enrichment. However, spiked beef trim was found to be positive with only a 6-h enrichment (Table 4.2). That was probably because the beef trim carried less amounts of initial microflora than ground beef. The black colonies on Rainbow Agar was suspected to be *E coli* O157:H7 (Fig 4.3) and was positively confirmed by DrySpot™ *E. coli* O157 Latex Agglutination Test (Figure 4.4).

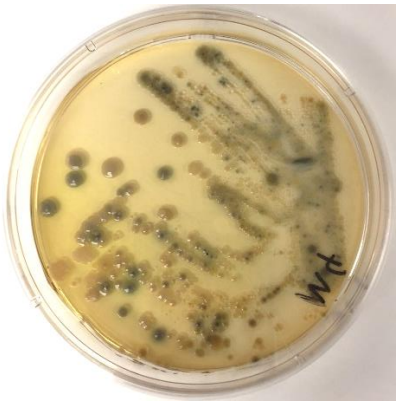

Figure 4.3 Growth of colonies from spiked ground beef with 6 h enrichment on Rainbow Agar

Figure 4.4 Latex agglutination test on susceptive colony from Rainbow Agar

IMS as a tool to isolate *E. coli* O157:H7 has shown its high sensitivity and specificity in capturing this target bacterium. It is reported that IMS can reach a detection limit of less than 1 CFU/g when applied to spiked animal feces and meat samples (Islam and others 2006). Other studies also proved IMS as a sensitive tool in isolating *E. coli* O157:H7 from pathogen cocktail, food samples and bovine feces (Chapman and others 1994). Therefore, conducting IMS prior to PCR increases the isolation efficiency in the application of HRM assay for food samples. In Weagant(Weagant and Bound 2001)'s (2001) study, the recovery of *E. coli* O157:H7 after a 5-h enrichment period was significantly improved after the addition of IMS beads. Likewise, Fedio and others. (Fedio and others 2011) found that the introduction of IMS into food samples could prompt culture recovery from 56% without IMS to 100% after IMS within a 24-h enrichment.

After dispensing and packing, processed meats are typically stored at refrigerated temperatures to extend the shelf life before shipping to markets. In this study, cold stressing at 4 ^{o}C was performed to simulate a typical storage procedure. Some studies

found that a cold shock of food samples could affect the enrichment period for sensitive pathogen detection. Uyttendaele et al. (1998) claimed in their findings that ground beef inoculated with *E. coli* O157:H- after a cold stress at 4 ^{o}C for 14 days required 3.5 h longer enrichment time. While in another study (Fratamico and others 2000), when the cold stress period was shortened to 48h, the enrichment period required to reach to the detectable level was the same as with samples without a cold stress treatment.

Buffered peptone water (BPW) is a common pre-enrichment broth used for pathogen recovery in a various range of foods (Baylis and others 2000). A previous study verified that BPW provided appropriate conditions for bacterial resuscitation and recovered suppressed cells during enrichment (Thomason and others 1977). Vancomycin as an antibiotic could suppress the growth of Gram-positive bacteria, especially *Staphylococcus aureus,* which is a prevalent pathogen associated with ready-to-eat meat products (Williams and Bardsley 1999). The addition of vancomycin in BPW inhibited replications of *S. aureus* and avoided the interference of those organism with our assay.

Enrichment period is another factor that influences the recovery of *E. coli* O157:H7 from foods. The complexity and high count of background microflora were inhibitory in *E. coli* O157:H7 recovery (Johnson and others 1998). When testing foods with high levels of non-target microflora, a low enrichment period was not sufficient to bring target bacteria to a detectable level. However, after IMS was employed, enrichments of 5-h and 24-h had no significant effect in *E. coli* O157:H7 recovery (Fedio and others (Fedio and others 2011).

The applications of different DNA isolation kits would affect DNA yielding rate and purity in a HRM assay. As a sensitive method, a tiny shearing of DNA and different resolution buffer could cause significant bias in PCR amplification. In the current study, PrepMan™ Ultra was employed on pure cultures while food samples were treated with DNeasy Blood & Tissue Kit. Despite the higher cost compared with PrepMan™ Ultra, DNeasy could deliver a stable and pure DNA extract with less inhibitors, which is crucial in acquiring a reproductive result during PCR (ELIZAQUÍVEL and AZNAR 2008).

Due to the complex and high counts of microflora in ground beef, distinguishing *E. coli* O157:H7 from the background microflora is challenging. This assay involves IMS in combination with HRM-PCR, making the rapid and reliable detection method feasible. However, further improvements on rapid capture of target bacteria are necessary to lessen the enrichment time and improve the detection limit.

4.3 CONCLUSIONS

The HRM-PCR assay was able to distinguish *E. coli* O157:H7 from other *E. coli* and *Shigella* and obtain a low detection limit when applied to beef samples. The advantages of low cost, easiness, rapidness and non-destructive nature would make this HRM assay a reliable method in distinguishing *E. coli* O157:H7 from other *E. coli* serotypes and background microflora. This assay may aid in epidemiological studies and infection control.

CHAPTER 5
CONCLUSIONS

Real-time PCR technique as an alternative confirmation test for gene detection has been widely employed in detections of foodborne pathogen during recent decades, as it offers accurate, rapid and low-cost detection.

In the first study, a multiplex high resolution melt-curve (HRM) PCR assay for the detection of *Salmonella* by targeting three virulence genes-*inv*A, *fim*A and *stn* genes was developed. An 83-base long DNA oligo was uniquely designed to serve as the internal amplification control (IAC). The HRM dyes have the property of binding to each amplicon equally, thus it could be used in this assay to detect several target genes simultaneously. Through presenting two or more types of target amplicons, the DNA isolate can be confirmed as positive for *Salmonella*, while the presence of only one target was determined to be *Salmonella* negative. Therefore, this assay overcome the limitation of generating false-negative and false-positive results from using single-gene detection method. Validation on 82 *Salmonella* and 21 non-*Salmonella* strains showed a 100% specificity and a detection limit of 900 CFU for culture only. For most food samples spiked with a cocktail of four *Salmonella* strains, a detection limit of 10^3 CFU/g was achieved after a 4-h enrichment. An 8-h enrichment was required for a detection limit of 10 CFU/g in PCR assay. The developed HRM assay is easy-operating and requires no use of probe, which makes it a useful tool for epidemiology analysis and laboratory use.

The second study was to developed a reliable HRM assay targeting *uid*A for differentiation of *E. coli* O157:H7 from other *E. coli*. *E. coli* O157:H7 is one of the most notorious foodborne pathogens responsible for over 73,000 illnesses and life-threatening diseases in the U.S. annually. A zero-tolerance policy of *E. coli* O157:H7 as an adulterant in ground beef has been declared by The U.S. Department of Agriculture (USDA). Based on the *uid*A mutation in *E. coli* O157:H7, a reliable HRM PCR assay targeting the *uid*A gene was developed to differentiate *E. coli* O157:H7 from other STECs and the closely related *Shigella* serogroups. Via this assay, *E. coli* O157:H7 isolates could be distinguished from isolates of non-O157 *E. coli* and *Shigella* according to the melt cure plots. DNA isolates of *E. coli* yielded a distinctive peak which is different from that of non-O157 *E. coli* isolates and *Shigella* in this assay. Ground beef samples spiked with 10 CFU/325g required an enrichment period of 8-h followed by antibody-based immunomagnetic separation (IMS) respectively, whereas beef trim needed only 6-h because of the low amount of background microflora. The whole process can be performed in less than 20h, including sample preparation, enrichment, DNA isolation and Real-time PCR, making it competitive to culture-based detection methods. This study will also help in dealing with epidemiological analysis and traceback.

Overall, HRM-based PCR methods worked effectively in detecting *Salmonella* and *E. coli* O157:H7 in various food categories. Hence, this study provided rapid detection methods for the food industry to detect *Salmonella* and *E. coli* O157:H7 in food products. Owing to the simplicity and ease of use, HRM application in the areas of clinical diagnostics and food safety has also increased. This assay can be further tested with a large amount of pure or mixed strains and contaminated food samples.

APPENDIX 1

Salmonella isolates test on the specificity of PCR primers of *inv*A, *stn* and *fim*A.

Salmonella isolate	*inv*A	*fim*A	*stn*
S. Typhimurium 14028	+	+	+
S. Typhimurium 788	+	+	+
S. Enteritidis	+	+	+
S. Thompson B&B3	+	+	+
S. Drypool	+	+	+
S. Typhimurium LJH 666	+	+	+
S. Arizonae	+	-	+
S. Berta	+	+	+
S. Choleraesuis	+	+	+
S. Agona LJH 1122	+	+	+
S. Agona LJH 1132	+	+	+
S. Agona LJH 692	+	+	+
S. Enteritidis I4-2	+	+	+
S. Enteritidis I4-7	+	+	+
S. Enteritidis I4-8	+	+	+
S. Enteritidis I4-9	+	+	+
S. Enteritidis I4-10	+	+	+
S. Enteritidis I4-11	+	+	+
S. Diarizona 12325	+	+	+

S. Paratypia A(A)	+	+	+
S. Poona NCTC 4840	+	+	+
S. Enteritidis F3-8	+	+	+
S. Arizonae 13314	+	-	+
S. Dublin 15408	+	+	+
S. Typhimurium 1527	+	+	+
S. Derby 6960	+	+	+
S. Enteritidis I4-5	+	+	+
S. BO-3	+	+	+
S. Enteritidis G5-08	+	+	+
S. Bongori 43975	+	+	+
S. Enteritidis LO-4	+	+	+
S. LJH 788	+	+	+
S. API 670752	+	+	+
S. Senftenberg O Group E	-	+	+
S. Rubislaw	+	+	+
S. LO-1	+	+	+
S. LO-2	+	+	+
S. LO-3	+	+	+
S. Paratyphi 77/08	+	+	+
S. Kentucky 184/10	+	+	+
S. Bareilly 73	+	+	+

S. Binza MH06736	+	+	+
S. Binza MH44056	+	+	+
S. Cerro 1768	+	+	+
S. Cerro MH02659	+	+	+
S. Derby MH81226	+	+	+
S. Harder 14145-L	+	+	+
S. Harder 17888-k	+	+	+
S. Harder MH44684	+	+	+
S. Harder VA07170803	+	+	+
S. Havana 99-109840	+	+	+
S. Heidelberg 18213	+	+	+
S. Heidelberg 6316-J	+	+	+
S. Heidelberg MH95276	+	+	+
S. Infantis MH953828	+	+	+
S. Javanicus 17339-0	+	+	+
S. Javanicus 49005	+	+	+
S. Lexington 9492-M	+	+	+
S. Mbandaka 74	+	+	+
S. Meleagridis 92	+	+	+
S. Michigan	+	+	+
S. Monophasic 103	+	+	+
S. Monophasic 102	+	+	+

S. Montevideo VA07123001	+	+	+
S. Montevideo VA7171801	+	+	+
S. Montevideo VA07172202	+	+	+
S. Montevideo VA07172205	+	+	+
S. Montevideo VA07211202	+	+	+
S. Montevideo	+	+	+
S. Newport 55	+	+	+
S. Newport 57	+	+	+
S. Newport 78	+	+	+
S. Newport 88	+	+	+
S. Senftenberg 15106q	+	+	+
S. Thompson 11842M	+	+	+
S. Thompson 15371-K	+	+	+
S. Typhimurium DT104 9115199 97-18448	+	+	+
S. Gaminara	+	+	+
S. Typhimurium	+	+	+
S. Montevideo	+	+	+
S. Newport 19930-N	+	+	+
S. Newport MH57137	+	+	+

APPENDIX 2

Escherichia coli and *Shigella* strains used in *uid*A study

O-Serogroup	**Strains**	**Source**
E. coli O157	93-111	Human (USA)
E. coli O157	EDL-933	Food (hamburger)
E. coli O157	OK-1	Human (Japan)
E. coli O157	2886-75	Human (USA)
E. coli O157	86-24	Human (USA)
E. coli O157	G5101	Human (USA)
E. coli O157	ATCC 43894	Human (USA)
E. coli O157:H7	505B	Beef (FRI)
E. coli O157:H7	3178-85	Human (CDC)
E. coli O157:H7	43894	Human
E. coli O157:H7	C7927	Human (CDC)
E. coli O157:H7	MF 1847	Beef (FSIS)
E. coli O157:H7	E19	Calf feces isolate
E. coli O157:H7	H1730	Human feces, lettuce-associated outbreak
E. coli O157:H7	CDC-658	Human feces; Cantaloupe-associated outbreak
E. coli O157:H7	LJH557	Apple cider isolate
E. coli O157:H7	E009	Beef
E. coli O157:H7	932	Human
E. coli O157:H7	3	Beef
E. coli O157:H7	E0018	Cattle
E. coli O157:H7	F4546	Human feces, alfalfa sprout outbreak
E. coli O26	DEC10C	Human Infant USA
E. coli O26	DEC9F	Human (USA)
E. coli O26	TB285C	Human (USA)
E. coli O26	VP30	Human (Chile)
E. coli O26	DEC9A	Human (USA)
E. coli O26:H11	DEC10B	Human (Australia)
E. coli O26:H11	97-3250	Human (USA)
E. coli O26:H	MT#10	Human (USA)
E. coli O26:H N	TB352A	Human (USA)
E. coli O45	DEC11C	Human (USA)
E. coli O45	5431-72	Human (Canada)

E. coli O45	4309-65	Human (USA)
E. coli O45	88-4110-H	Cow (USA)
E. coli O45	D88-28058	Cow (USA)
E. coli O45	2566-58	Pig (UK)
E. coli O45:H2	M103-19	Human (USA)
E. coli O45:H2	MI01-88	Human (USA)
E. coli O45:H2	MI05-14	Human (USA)
E. coli O45:H NM	DA-21	Human (USA)
E. coli O103:H2	MT#80	Human (USA)
E. coli O103:H6	TB154A	Human (USA)
E. coli O103:H25	8419	Human (USA)
E. coli O103:H N	PT91-24	Human (USA)
E. coli O103	DA-41	Human (USA)
E. coli O103	6:38	Human (USA)
E. coli O103	PT91-24	Human (USA)
E. coli O103	DA-55	Human (USA)
E. coli O103	87-2931	Human (Canada)
E. coli O103	GS G5550637	Human (USA)
E. coli O104:H	ECOR-28	Human (USA)
E. coli O104:H	G5506	Human (USA)
E. coli O104:H	G5508	Human (USA)
E. coli O104:H	TW01435	Cow (Germany)
E. coli O104	O104 standard	
E. coli O104	E28	Human (USA)
E. coli O111	CL-37	Human (Canada)
E. coli O111	DEC8B	Human (USA)
E. coli O111	TB226A	Human (USA)
E. coli O111	928/91	Human (Germany)
E. coli O111	412/55	Human (Germany)
E. coli O111	DEC8C	Cow (USA)
E. coli O111:H2	RD8	Human (France)
E. coli O111:H8	3215-99	Human (USA)
E. coli O111:H11	0201 9611	Human (USA)
E. coli O111:H NM	3007-85	Human (USA)
E. coli O121:H19	3377-85	Human (USA)
E. coli O121:H19	MT#2	Human (USA)
E. coli O121:H	MT#18	Human (USA)
E. coli O121:H[19]	DA-5	Human (USA)
E. coli O121	87-2914	Human (Canada)
E. coli O121	DA-1	Human (USA)

E. coli O121	7927+++	
E. coli O121	5518	
E. coli O121	O121 standard	
E. coli O121	PT91-4	
E. coli O145	70300885	
E. coli O145	MT#66	Human (USA)
E. coli O145	6940	
E. coli O145	TB269C	Human (USA)
E. coli O145	DEC10I	Human (Canada)
E. coli O145:H NT	D177	
E. coli O145:H[28]	4865/96	Human (Germany)
E. coli O145:H NM	GS G5578620	Human (USA)
E. coli O145:H NT	IH 16	Human (Uruguay)
S. dysenteriae	ATCC 29028	
S. flexneri		
S. sonnei		
E. coli	C1	Chicken
E. coli	C2	Chicken
E. coli	C3	Chicken
E. coli	C4	Chicken
E. coli	C5	Chicken
E. coli	Ct1	Cattle
E. coli	Ct2	Cattle
E. coli	Ct3	Cattle
E. coli	Ct4	Cattle
E. coli	Ct5	Cattle
E. coli	D1	Duck
E. coli	D3	Duck
E. coli	D4	Duck
E. coli	D5	Duck
E. coli	D6	Duck
E. coli	Dg2	Dog
E. coli	Dg3	Dog
E. coli	Dg4	Dog
E. coli	Dg5	Dog
E. coli	Dg6	Dog
E. coli	G2	Goose
E. coli	G4	Goose
E. coli	G5	Goose
E. coli	G8	Goose

E. coli	G9	Goose
E. coli	Gt2	Goat
E. coli	Gt3	Goat
E. coli	Gt4	Goat
E. coli	Gt5	Goat
E. coli	Gt6	Goat
E. coli	H2	Human
E. coli	H3	Human
E. coli	H10	Human
E. coli	H11	Human
E. coli	H12	Human
E. coli	P10	Pig
E. coli	P17	Pig
E. coli	P22	Pig
E. coli	P23	Pig
E. coli	P24	Pig

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