APPLICATION OF REAL-TIME PCR FOR DETECTION OF ANTIBIOTIC RESISTANT PATHOGENS AND SHIGA-TOXIN PRODUCING *ESCHERICHIA COLI*

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By

PRASHANT SINGH

Dr. Azlin Mustapha, Dissertation Supervisor

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

APPLICATION OF REAL-TIME PCR FOR DETECTION OF ANTIBIOTIC RESISTANT PATHOGENS AND SHIGA-TOXIN PRODUCING *ESCHERICHIA COLI*

presented by Prashant Singh,

a candidate for the degree of doctor of philosophy of Food Science, and hereby certify that, in their opinion, it is worthy of acceptance.

Professor Azlin Mustapha, Food Science

Associate Professor Mengshi Lin, Food Science

Associate Adjunct Professor Guolu Zheng, Food Science

Professor Sheila Grant, Bioengineering

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Application of Real-Time PCR for Detection of Antibiotic Resistant Pathogens and Shiga-Toxin Producing *Escherichia coli*

Prashant Singh

Dr. Azlin Mustapha, Dissertation Supervisor

ABSTRACT

Salmonella and Shiga toxin producing *Escherichia coli* are among the most important food pathogens of concern. Culture based method for the identification of these food pathogens takes 4-5 days for the final confirmation. Alternatively, real-time PCR based methods targeting specific genes of the pathogen are highly specific and sensitive method for the detection of foodborne pathogens. Real-time PCR based methods for pathogen detection most commonly uses dual-labeled fluorescent probe or double stranded DNA binding fluorescent dye. Dual-labeled fluorescent probe probes are well known for providing high specificity to real-time PCR assays, whereas the use of High Resolution Melting (HRM) Dyes has been advocated for real-time PCR melting curve based pathogen detection assays. HRM dyes bind to the PCR amplicons in high concentration, completely saturate the amplicons without inhibiting the PCR reaction in the process, thus generating a melt curve of higher resolution.

Increasing use of antibiotics for treatment and as a therapeutic agent on food animals has been proposed as a reason for the emergence of multiple drug resistant (MDR) strains of food pathogens. In the past few years, higher incidences of outbreaks caused by MDR *Salmonella* have been increasingly documented. Numerous multiplex real-time PCR methods have been published for the detection of *Salmonella* but there is

lack of methods for the rapid detection of antibiotic resistance strains of *Salmonella*. A multiplex TaqMan® real-time PCR was designed by targeting the invasin virulence gene (*inv*A), and four commonly found antibiotic resistance genes, viz. ampicillin, chloramphenicol, streptomycin and tetracycline. To avoid any false negative results and to increase the reliability of the assay, an internal amplification control (IAC) was added which was detected using a locked nucleic acid (LNA) dual-labeled probe. The assay performed equally well on artificially contaminated samples of beef trim, ground beef of different fat contents, chicken rinse, ground chicken, ground turkey, egg, spinach and tomato. The detection limit for un-enriched inoculated food samples was 10^4 CFU/g, this was improved to 10 CFU/g after a 12-h enrichment in buffered peptone water (BPW).

Another multiplex real-time PCR melt curve assay for the detection of virulent and antibiotic resistance strains of *Salmonella* was developed. The first set of the multiplex reaction targeted the virulence gene invasin (*inv*A), tetracycline (*tet*G), streptomycin (*aad*A2) and sulphonamide (*sul*I) antibiotic resistance genes, whereas the second set amplifies ampicillin (bla_{PSE}, bla_{TEM}) and chloramphenicol (*flo*R) resistance genes. This multiplex real-time PCR melt curve assay worked efficiently over a DNA concentration range of 20 ng - 200 fg and showed a sensitivity of 290 CFU/mL with serially diluted broth cultures. The detection limit for un-enriched artificially inoculated food samples was 10^4 CFU/g, but an enrichment period of 6 h using BPW as a enrichment media allowed detection of food samples artificially contaminated with 10 CFU/g.

Extended-spectrum β-lactam (ESBL) and carbapenem resistant Enterobacteriaceae are being increasingly detected in humans and a similar trend has

been also observed for food pathogens isolated from food animals. Infections caused by ESBL and carbapenem resistant food pathogens leads to treatment failure and prolonged infections that are much harder to treat. A real-time PCR melt curve assay was developed for the detection ESBL and carbapenem resistant pathogens. Two multiplex real-time PCR melt curve reactions with IAC were standardized for the identification of 10 genes (bla_{KPC}, bla_{NDM}, bla_{CTX}, bla_{CMY}, bla_{VIM}, bla_{IMP}, bla_{OXA}, bla_{SHV}, bla_{TEM}, bla_{ACC}) that confer resistance towards ESBL and carbapenem. The multiplex was evaluated using 38 DNA samples. The results of the developed multiplex PCR assay either mostly paralleled with the previous studies or presence of more antibiotic resistance genes were detected using our assay. The assay developed in this study offers a simple, low cost method for the detection of ESBL and carbapenem resistance among enteric pathogens.

Shiga toxin-producing *Escherichia coli* are pathogenic strains of *E. coli* that cause bloody diarrhea and have a high mortality rate, whereas *Salmonella* is the second most frequently reported food pathogen in the United States. The United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) declared seven STEC serogroups O157, O26, O45, O103, O111, O121 and O145 as adulterants in ground beef and beef trims. Two sets of multiplex real-time PCR melt curve assays with IAC were standardized for the detection of STEC serogroups and *Salmonella*. The first multiplex assay targeted *E. coli* O145, *E. coli* O121, *E. coli* O104, *E. coli* O157, *E. coli* O26, *E. coli* O45, *E. coli* O103 and *E. coli* O111; while the second set detected *E. coli* O145, *E. coli* O121, *E. coli* O157, *E. coli* O26, *E. coli* O45, *E. coli* O103, *E. coli* O111, *Salmonella* and Shiga toxin genes (stx_1 and stx_2). Food samples spiked with a cocktail of four STEC serogroups with a combined count of 10 CFU/25 g food, all targets of the multiplex

assays could be detected after 6 h enrichment in BPW. The assay also worked efficiently when 325 g of ground beef was spiked with 10 CFU of each STEC serotype and *Salmonella*, all targeted were detected after enrichment period of 8 h. The assay developed in this study can be used for the detection of seven STEC serogroups, STEC virulence genes (*stx*$_1$ and *stx*$_2$) and *Salmonella* and can be completed in less than 11 h. Unlike other commercially available methods, it does not require fluorescent-labeled probes or immunomagnetic beads, making it more economically feasible to execute.

Chapter 1 Introduction

The *Salmonella* family includes over 2,300 serotypes out of which *S.* Typhimurium and *S.* Enteritidis are the most common foodborne pathogens in the United States (USDA 2014). Widespread use of antibiotics in hospitals and for animal husbandry has been proposed as the possible explanation for the emergence of antibiotic resistant strains of *Salmonella*, which are resistant towards commonly used antibiotics, such as trimethoprim, sulfasulfonamides, streptomycin, ampicillin and tetracycline. These antibiotic resistant strains of *Salmonella* are also responsible for some of the major *Salmonella* outbreaks worldwide. Our first objective of this study was to isolate and characterize antibiotics resistant strains of *Salmonella* from farm animals such as cattle, chicken, turkey and from various meat and meat products and to develop a real-time PCR-based test for rapid identification of pathogenic as well as antibiotic resistant strains of *Salmonella.*

There have been several reports of *Salmonella* resistance to the cephalosporin class of antibiotics, which were recently approved for the treatment of severe cases of *Salmonella* infection in humans (Sjölund-Karlsson and others 2010). Previous studies have reported an increase in the number of extended-spectrum cephalosporin resistant strains of *Salmonella* isolated from food animals (Folster and others 2012). Carbapenems are another group of broad-spectrum β-lactam antimicrobials, which are used for the treatment of serious infections in humans. The uses of these drugs are banned for veterinary application in the European Union. However, ceftiofur, a member of

carbapenem group of antibiotics has been licensed for veterinary application in the United States since 1988 (Smet and other 2010). With use of this class of antibiotics, there have been reports of carbapenem resistant pathogens in food-producing animals and their environment. The second objective of this study was to develop a multiplex real-time PCR test, targeting the most common genes that confer resistance towards extended-spectrum beta-lactam and carbapenem group of antibiotics.

Shiga toxin producing *Escherichia coli* (STEC) strains are also known as verocytotoxin-producing *E. coli*. These pathogens are the causative agents for hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS). The majority of foodborne outbreaks by STEC are primarily caused by *E. coli* O157:H7, but the United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) surveillance data showed six non-O157 STEC serogroups (O26, O45, O103, O111, O121, and O145) that cause diseases of equal severity, are also responsible for the majority of non-O157 STEC infections in the United States. Similar to *E. coli* O157:H7, cattle are an important reservoir for non-O157 STEC serogroups. Beef, beef products and food potentially contaminated with animal feces have been associated with human illness and HUS (Bai and others 2012). In the wake of increasing incidences of non-O157 STEC infections, the USDA FSIS has recently declared *E. coli* O26, O45, O103, O111, O121, and O145 as adulterants in non-intact raw beef products. The third objective of this research was to develop a real-time PCR test for the identification of O157:H7 and non-O157 STEC.

Research Objectives

1. To isolate and characterize antibiotics resistant strains of *Salmonella* from farm animals such as cattle, chicken, turkey and from various meat and meat products and develop a real-time PCR-based test for rapid identification of pathogenic and antibiotic resistant *Salmonella.*

2. To isolate and characterize extended-spectrum beta-lactam (ESBL) resistant strains of *Salmonella* and develop a multiplex real-time PCR test, based on melt curve analysis for their identification.

3. To develop a real-time PCR test for the identification of O157:H7 and non-O157 STEC using high resolution melt (HRM) curve analysis.

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Chapter 2 Literature Review

2.1 Antibiotic Resistance in Bacteria Associated with Food Animals

In 1949, researchers noted chickens that were administered crude *Streptomyces aureofaciens* fermentations as a vitamin B12 supplement, showed a significantly higher growth rate than those fed a diet containing purified vitamin B12 (Stokstad and others 1949). Later, this growth enhancing mystery compound was identified as chlortetracycline (Stokstad and Jukes 1950). Later, the United States Food and Drug Administration (FDA) approved certain antibiotics for use in the diet of domesticated animals as a prophylactic and disease prevention agent (Kiser 1976). The use of antibiotics on animal farms serves four main purposes: (1) treatment of sick animals; (2) metaphylaxis - short-term administration of antibiotics to prevent the spread of infection among sick and healthy animals; (3) prophylactic – prevention of infection during high risk period, such as transport or weaning; (4) growth promotion – to increase the feed efficiency (McEwen and Fedorka-Cray 2002; Viola and DeVincent 2006).

With the modernization of human civilization, production of food animal moved into large organized farms, which have a greater animal density and requires a superior disease control program. In the United States, at least 17 classes of antimicrobials have been approved for use in food animals (Anderson and others 2003). To track the consumption of these antibiotics on animal farms and agricultural sectors, a reporting system has been established in several European countries but, unfortunately, no such reporting system exists in the United States. According to the Animal Health Institute (AHI) report, member companies sold more than 10,108 tons of antimicrobial products in

the United States in 2003, which comprised of ionophores/arsenicals, tetracyclines, cephalosporins, macrolides, sulfonamides, penicillins, aminoglycosides, and fluoroquinolones. It was also estimated that 92% of the total antimicrobials were used for therapeutic purposes (AHI 2005; Mathew and others 2007).

This use of antimicrobials in farm animals as a prophylactic agent resulted in improved animal health, and increased production and reduction in foodborne pathogens. However, the use of antibiotics for animal husbandry purposes has come under scrutiny, as it has been shown to increase the prevalence of antibiotic resistant pathogens, which can lead to serious foodborne outbreaks. In some cases, even though banning of growth-promoting antibiotics led to a reduction of antibiotic-resistant pathogens, it also increased animal morbidity and mortality, especially of young animals (Mathew and others 2007).

2.2 The origin of antibiotic resistance

A number of microbial species (e.g. bacteria and mold) possess an ability to produce antimicrobial compounds. This trait helps them gain a competitive advantage over other microorganisms in complex environmental conditions, such as biofilm and soil (Amábile-Cuevas and Chicurel 1992). Many antimicrobial compounds that are used today originated from organisms such as *Streptomyces*, *Bacillus*, *Pennicillium*, *Cephalosporium*, and *Pleurotus*. However, the use of antibiotics to treat human infections and as prophylactic agents for animal production may promote the selection of antibiotic resistant strains and the dissemination of antibiotic resistance genes to other closely related bacteria. D'Costa and others (2006) suggested that soil bacteria that act as a big reservoir of antibiotic resistance genes could easily distribute the resistance genes to the microbial community under the selective pressure of antibiotics, including enteric

bacteria and pathogens. The earliest report on the use of antibiotics on animal farms promoting antibiotic resistance in bacteria was published in 1951. Starr and Reynolds (1951) reported the isolation of streptomycin resistant *E. coli* strains from a turkey farm where streptomycin was administered in form of animal feed. Since then, numerous reports were published by the scientific community substantiating the link between antibiotic use on animal farms and the emergence of antibiotic resistant pathogens in food animals (Mathew and others 2007). Due to the increasing incidences of isolation of antibiotic resistant pathogens, the United States, in 1991, established the National Antimicrobial Resistance Monitoring System (NARMS) a cooperative effort of the Food and Drug Administration (FDA)- Center of Veterinary Medicine, United States Department of Agriculture (USDA), Centers for Disease Control and Prevention (CDC) and state and local health departments (http://www.cdc.gov/narms/) to perform surveillance and monitor the prevalence of antibiotic resistant pathogens. The stated objectives of the program were to provide data on the prevalence and trends of drug resistance in enteric bacteria of concern, identification of antibiotic resistance as it arises in pathogens of concern, and to provide data to veterinarians and clinicians regarding prevailing patterns of antibiotic resistance pattern of pathogens. In 2002, NARMS also started a pilot surveillance study on pathogens from retail meat isolates and food animals.

2.3 Antibiotic resistance in foodborne pathogens and reservoirs

According to the CDC, it is estimated that around 48 million people in the United States suffer from domestically acquired foodborne illness annually (Scharff 2012). The most common genera involved in these foodborne illnesses are *Salmonella* spp., *Campylobacter* spp., *Listeria* spp., *Yersinia* spp., and certain strains of *E. coli*. Food

animals are a good reservoir for these pathogens and they can be passed to human through food chain. For example, cattle, chickens, pigs, and turkeys are common reservoirs of *Salmonella*, whereas *Campylobacter* is frequently found in chickens and turkeys (Anderson and others 2003).

Severe cases of foodborne infections require the use of antibiotics for treatment, making the occurrence of antibiotic resistance in foodborne pathogens a matter of concern. Apart from antibiotic resistant foodborne pathogens of animal origin, other commensal bacteria, such as *E. faecalis* and *E. faecium*, that are part of the natural gut microflora, can also pose a zoonotic risk via environmental routes and cause infections (Mathew and others 2007). It has also been suggested that the non-target enteric microflora are regularly exposed to a wide variety of antibiotics in our lifetime. This process leads to the selection of antibiotic resistance genes, plasmids, transposons, and integrons, that act as additional reservoirs, facilitating transfer and spread of these genes (Lipsitch and others 2002; Tenover 2001).

2.4 Beef Cattle and Associated Antibiotic Resistance

Mellon and others (2001) reported that each year more than 2,000,000 kg of antimicrobial agents was administered on beef cattle. Chlortetracycline, ionophores, sulfamethazine, tylosin, and virginiamycin are the most widely used antimicrobials for domestication of beef cattle (Inglis and others 2005). Chlortetracycline is used in beef cattle to help them gain weight under abnormal respiratory conditions and to prevent diseases like liver abscesses, diarrhea, and foot rot (Troxel and Gadberry 2006). Ionophores are used with high concentration diet-mixes to increase feed efficiency of cattle. Tylosin and virginiamycin are other commonly used feed additives (Inglis and

others 2005). These antibiotics are usually administered to beef cattle in the form of animal feed, this practice helps to prevent diseases, and also improves the feed efficiency. On feedlots, medicated feed additives are sometimes applied to young calves during the weaning process to prevent coccidiosis and increase feed efficiency. Hoyle and others (2004) reported the isolation of nalidixic acid, apramycin, and ampicillin resistant strains from calves within a few weeks of birth. Administration of feed mixes with antibiotics can be one of the possible reasons behind this observation. Sulfisoxizole, followed by tetracycline, are the most commonly observed antibiotic resistant phenotypes among bacterial strains isolated from beef cattle. Resistance to tetracycline, chloramphenicol and streptomycin are other prominent antibiotic resistance phenotypes. Further, chloramphenicol-sulfisoxizole-streptomycin-tetracycline and sulfisoxizole-tetracycline are the two most commonly reported MDR pattern (Mathew and others 2007).

2.5 Poultry and Associated Antibiotic Resistance

The poultry industry is susceptible to a wide range of bacterial infections. Antibiotics on a poultry farm are used therapeutically, non-therapeutically, and for growth promotion purposes (Lu and others 2006). Chlortetracycline, bacitracin, bambermycin, tylosin, and virginiamycin are the common growth-promoting antibiotics used by the poultry industry (CVP 2006). The poultry industry suffers a severe economic toll due to diseases like colibacillosis, enteritis, and salmonellosis. Poultries are also affected by pathogens such as *Clostridium*, *Mycoplasma* and *Campylobacter* (Singer and Hofacre 2006). Common antibiotics used for the control these pathogens and diseases include sulfonamides, amoxicillin, tetracycline, tylosin, virginiamycin, neomycin, and penicillin. NARMS data indicates that the *Salmonella* strains isolated from the chicken

samples showed an increased level of resistance towards amoxicillin/clavulanic acid, ceftiofur, cefoxitin, and tetracycline. Various studies have been conducted that compared *Salmonella* isolated from conventional poultry farms and organic poultry farms. *Salmonella* isolated from conventionally raised birds were more resistant to antibiotics and they also harbored higher number of multiple-drug resistant strains. Cui and others (2005) reported that all *S* Typhimurium isolated from conventional chicken farms were multi-drug resistant strains and were resistant to at least 5 antibiotics (five or higher), whereas most *S* Typhimurium strains (79%) obtained from organic chickens were susceptible to all 17 antimicrobials tested in the study. In another similar but recent study, Sapkota and others (2014) reported the isolation of *Salmonella* from both conventional and organic poultry farms. *Salmonella* Kentucky was the most predominant *Salmonella* serovars. The *S.* Kentucky isolates obtained from organic poultry farms showed a significantly lower level of antibiotic resistance when compared with isolates obtained from conventional poultry houses. Similar results were obtained for the multi-drug resistant *Salmonella* strains isolated from the conventional poultry farms showed a high prevalence of multiple-drug resistant *Salmonella*. These studies support the claim that voluntarily withdrawal of antibiotics from poultry farms can lead to a reduction in the number of antibiotic resistant and multi-drug resistant *Salmonella*.

2.6 *Salmonella*

Salmonella is a Gram-negative, rod-shaped bacterium that was first identified by an American scientist named Salmon, after whom the bacterium was named "*Salmonella*" (CDC 2010). The *Salmonella* family includes over 2,300 serotypes out of which *S.* Typhimurium and *S.* Enteritidis are the most common and account for half of all

human infections in the U.S. (Lawley and others 2008). *Salmonella* led to a gastrointestinal illness called as salmonellosis that has symptoms of diarrhea, fever, and abdominal cramps. Non-typhoidal *Salmonella* is known to cause infection all around the world. Each year in the United States, it is estimated to cause more than 1.2 million infections, out of which 100,000 cases involves multi-drug-resistant *Salmonella*, resulting in more than 23,000 hospitalizations and 450 deaths (CDC 2013; Scallan and others 2011). *Salmonella* Typhi causes approximately 21.7 million illnesses worldwide annually. In the United States, it causes approximately 5,700 illnesses, out of which 3,800 cases are caused by multi-drug resistant strains leading to 620 hospitalizations each year (CDC 2013). These *S* Typhi related cases of salmonellosis in the United States, are generally associated with contaminated meat products, such as ground beef, turkey and eggs. These food products have been previously reported as an important source of multi-drug resistant *Salmonella* strains (Glenn and others 2013).

2.6.1 Multi-Drug Resistant *Salmonella*

In the last few decades, widespread use of antibiotics in hospitals and animal husbandry has been thought to lead to an emergence of antibiotic resistant strains of *Salmonella* in many countries, including many developed countries such as the United States, United Kingdom, France and Germany. These antibiotic resistant strains of *Salmonella* were responsible for some of the major *Salmonella* outbreaks worldwide. In addition to resistance towards commonly used antibiotics, such as trimethoprim, sulfasulfonamides, streptothricin, ampicillin and tetracycline, there have been an increasing number of reports on cephalosporin resistance in *Salmonella* which were recently approved for the treatment of severe cases of *Salmonella* infection in humans

(Sjölund- Karlsson and others 2010). Infections caused by these antibiotic resistant strains of *Salmonella* are a great public health concern, as they causes more severe infections leading to treatment failure, prolonged hospitalization and even death.

The multi-drug resistant *S* Typhimurium DT104 was first isolated in England in 1984. It has been reported to show resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline. In 1990, cases of infections by this bacterium started rising rapidly and by the year 1996, it had become the second most indicted organism causing salmonellosis after *S.* Enteritidis. In the U.S., human isolates of *S.* Typhimurium DT104 (R-type ACSSuT) increased from 9% in 1990 to 32% in 1996 when the strain led to the first major outbreak of human salmonellosis (Akkina and others 1999). In the United Kingdom, *S.* Typhimurium DT104 was never reported in cattle before 1986, after which its incidence increased to 13% in 1991 and further to 64% in 1994 (Evans 1996). Various studies conducted worldwide indicated worldwide occurrence of this pathogen (Yan and others 2010; Hur and others 2011; Daly and others 2000). Additionally, all the *S.* Typhimurium DT104 strain were also reported to be genetically identical, indicating its very recent origin and rapid spread around the world (Glynn and others 1998). Beef, dairy cattle and pet animals, once infected with this pathogen, serve as local reservoirs of the infection. This strain of *Salmonella* is more pathogenic in comparison with other *Salmonella* strains because of several reasons: 1) ability to cause cross-species infections, such as cattle, human, and pets; 2) antibiotic resistance genes can be transferred to other bacteria by conjugation and other methods of horizontal gene transfer mechanisms and 3) presence of the antibiotic resistance genes on chromosomal DNA in the form of a gene cassette in a genetic element known as an

integron, making the antibiotic resistance traits very stable that are not easily lost even when the selective pressure is removed.

Integrons are natural expression systems, which allows insertion and expression of various antibiotic resistance genes by their own promoters, thus making the inserted gene functional. Integron 1 has been shown to harbor up to 10 different antibiotic resistance gene cassettes. These gene cassettes were reported to confer resistance towards trimethoprim, aminoglycosides, sulfasulfonamides, streptothricin, spectinomycin, rifampicin, ampicillin and tetracycline (White and others 2001) (Figure 2.1).

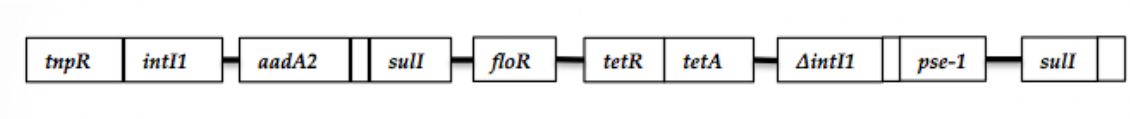

Figure 2.1 *Salmonella* Typhimurium DT104 integron resistance gene cluster (Adapted from: Carattoli 2001)

The most common serotypes of *Salmonella* causing human disease in the United States are Enteritidis, Typhimurium, Newport and Javiana. However in the recent past, antibiotic resistant strains of *Salmonella* Heidelberg and *Salmonella* Newport have become a matter of concern. *Salmonella* leads to a self-limiting injection, but severe cases of salmonellosis causing invasive disease typically require treatment with extended-spectrum cephalosporins (ESCs) or fluoroquinolones. Out of these two families of drugs, ESCs are the drug of choice for treating children (Forsythe and Ernst 2007). In 2009, cases related to *Salmonella* Heidelberg increased significantly and this pathogen became the third most common *Salmonella* serotype isolated from retail meat and food animals

(FDA 2009). In addition to that, surveillance data from NARMS has also shown an increase in the number of ESC resistant *S.* Heidelberg strains isolated from food animals at slaughterhouses, retail meat, and humans. The ESC resistance among *S.* Heidelberg has been associated with the presence of a plasmid-encoded bla$_{CMY}$ β-lactamase enzyme. Folster and others (2012) reported antimicrobial susceptibility testing of 223 *S.* Heidelberg isolated from food animals, retail meat, and strains isolated from human infections. A total of 21.1% of these isolates (n=47) displayed resistance towards ceftriaxone, ceftiofur, and amoxicillin-clavulanic acid and were also found to be positive for *bla*$_{CMY}$ β- lactamase enzyme. Almost 90% (26/29) of the strains obtained from chicken carcasses or retail chicken meat (n=29) were positive for *bla*$_{CMY}$, indicating a rise in the presence of antibiotic resistant strains of *S.* Heidelberg among food animals.

In 2000, the CDC also reported a surge in the rate of injections caused by multiple-drug resistant strains of *Salmonella* Newport. These strains were later also named Newport-MDRAmpC**.** The Newport-MDRAmpC strains showed more a enhanced level of resistance than *S.* Typhimurium DT104 strains (ACSSuT) in that they possessed the ability to degrade ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline cefoxitin, amoxicillin/clavulanic acid, cephalothin, ceftiofur, and also exhibited a decreased susceptibility to ceftriaxone. Similar to *S.* Heidelberg, the enhanced level of resistance in the Newport-MDRAmpC strain was also associated with the acquisition of a plasmid mediated *CMY-2 Amp*C-like gene. In 2001, out of all the *S.* Newport isolates submitted to NARMS for antimicrobial resistance profiling, 25% of those isolates were characterized as MDR-*Amp*C *S.* Newport.

2.7 *Salmonella* Isolation

The standard microbiological method for the isolation of *Salmonella* is depicted in Figure 2.2. Twenty-five grams of food sample are diluted (1:9) in a non-selective enrichment broth. Lactose broth or buffered peptone water (BPW) are the two most commonly used enrichment media. After 24 h of enrichment, samples are transferred to Rappaport-Vassiliadis medium (RV) or Tetrathionate broth (TT) for selective enrichment of *Salmonella*. Post selective enrichment step, broth samples from both RV as well as TT medium are streaked on three differential agar media (Bismuth Sulfite, Xylose Lysine Desoxycholate, Hektoen Enteric) for the isolation of presumptive/suspected *Salmonella* colonies.

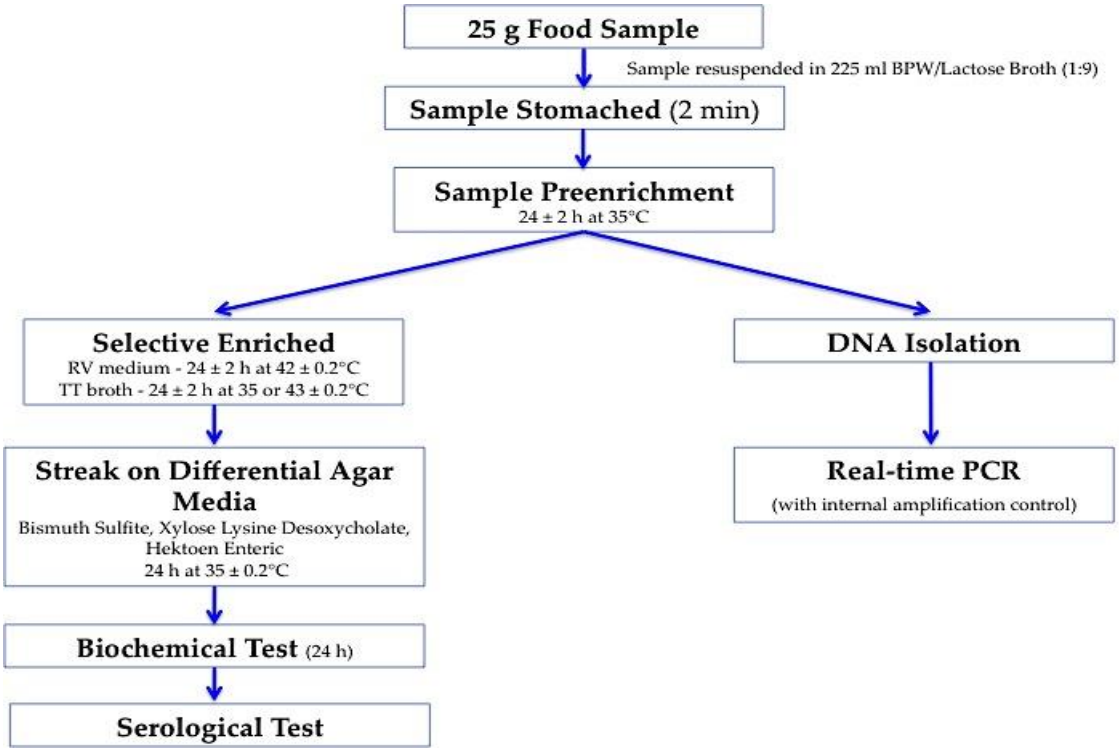

Figure 2.2 An overview of Salmonella isolation from food samples

Suspected colonies from the three differential agar medium are subjected to a wide range of biochemical test (e.g. Enterotube II, Vitek 2 GN, API 20E, MICRO-ID). Biochemically confirmed strains are further subjected to serological identification. The whole process takes between 4-6 days for final confirmation.

2.7.1 Real-Time PCR Detection of *Salmonella*

Real-time Polymerase Chain Reaction (PCR) is a method of amplifying a specific sequence in a provided DNA sample. Unlike conventional PCR where the results can only be checked at the end of the reaction, real-time PCR allows continuous monitoring of the amplification data. Real-time PCR reaction employs either fluorescent double-stranded DNA binding dye (e.g. SYBR® Green, EvaGreen®) or fluorescent-labeled hybridization probes (e.g. TaqMan, Molecular Beacon) for the quantification and specific detection of the amplicons of the PCR reaction. A wide range of regulatory and virulence genes of *Salmonella* have been previously used for developing real-time PCR assays for the detection of *Salmonella*, and a selected list of these genes and assays have been compiled in Table 2.1.

Table 2.1 Selected real-time PCR assay for the detection of *Salmonella*

Targeted Gene	**Real-time PCR type**	**Detection Limit**	**IAC**	**Inclusivity/exclusivity** **Number of strain tested**	**Sensitivity %** **Food Samples tested**	**Reference**
invA (287 bp)	TaqMan	2 cfu/ PCR reaction using pure culture of *S.* Typhimurium 3–7 cfu per PCR reaction for spiked food samples	No	100/100 164 *Salmonella* 50 non-*Salmonella*	100% 50 chicken carcass rinses and 60 raw milk samples	Chen and others 1997
invA (Unknown) Commercial kit	TaqMan	3 CFU/PCR reaction using pure *Salmonella* culture	Yes	100/100 42 serotypes of 68 *Salmonella* strains, 39 non-*Salmonella* strains	100% 100 meat and chicken	Kimura and others 1999
invA (119 bp)	TaqMan	Not tested	Yes	100/100 210 S. enterica isolates (100 problematic "rough" isolates) 120 non-Salmonella strains,	Not tested	Hoorfar and others 2000
himA (122 bp)	Molecular Beacon	2 CFU/PCR of pure culture	No	100/100 7 *Salmonella* strain 3 non-*Salmonella* strains	Not tested	Chen and others 2000
invA (285 bp)	SYBR Green1	6 CFU/ml	No	Not tested	492 intestinal homogenates and 27 drag swabs	Eyigor and others 2002

1. *sipC* 2. *invE-invA* 3. *spaQ*	TaqMan	2 CFU/PCR of pure culture of *S.* Newport	No	1. 87.5/100 2. 97/100 3. 100/100 116 *Salmonella* strain 19 non-*Salmonella* strains	1. Not tested 2. Not tested 3. 100% 230 clinical fecal specimens	Kurowski and others 2002
invA (102 bp)	TaqMan	Less than 100 fg of DNA/reaction	No	100/100 111 *Salmonella* strains 37 non-*Salmonella* strains	Food samples associated with outbreak	Daum and others 2002
ttrRSBCA (94 bp)	TaqMan	$>10^3$ CFU/ml (70% probability) $>10^4$ CFU/ml (100% probability)	Yes	100/100 110 Salmonella strains 87 non-Salmonella strains	100% 110 food samples (chicken rinses, minced meat, fish, raw milk)	Malorny and others 2004
invA (285 bp)	PCR-ELISA	10^3CFU/ml pure culture	Yes	100/100 84 *Salmonella* 44 non-*Salmonella*	100% 60 artificially-contaminated samples - fish, minced beef, raw milk	Perelle and others 2004
SipB and *SipC* (251bp)	Fluorescently-labeled hybridization probes	Approximately 6 genome equivalents/reaction	No	100/100 15 *Salmonella* Enteritidis 12 non-Enteritidis strains	spiked raw and ready-to-eat beef products	Ellingson and others 2004
invA (119 bp)	SYBR Green1	$>10^2$ and $>10^3$ in broth and milk respectively with enrichment	No	100/100 124 *Salmonella* spp. 116 non-*Salmonella* strains	100 % Lagoon water, feed/silage, bedding soil, and bulk tank milk	Nam and others 2005
fimC (102bp)	TaqMan		Yes	100/100 53 *Salmonella*	100% 36 artificially and 100	Piknová and others 2005;

				49 non- *Salmonella* strains	naturally contaminated food sample	Krascsenicsova and others 2008
ttrRSBCA (94 bp)	LNA TaqMan	fishmeal and chicken rinse (100 copies), pig feces (10 copies)	No/ Yes	Not tested	fishmeal, chicken rinse and pig feaces spiked with Salmonella genomic DNA	Reynisson and others 2006
Prot6e	TaqMan	$>10^2$ genome equivalents (100% probability) >10 genome equivalents (83% probability)	Yes	95/100 79 *Salmonella* Enteritidis 119 non-Enteritidis strains	100% 25 chicken carcass rinse, egg	Malorny and others 2007
stn (129bp)	TaqMan	3CFU/PCR reaction	No	100/96.4 269 *Salmonella* 84 non- *Salmonella* strains	Not tested	Moore and Feist 2007
bipA (65bp)	TaqMan	6CFU/25 g food (95.5 probability)	Yes	100/100 48 *Salmonella* 30 non- *Salmonella* strains	100% 120 diversified food and water sample	Calvó and others 2008
invA (68 bp)	TaqMan, enzyme-linked fluorescent assay (ELFA)	3.16pg/PCR	Yes	100/100 39 *Salmonella* strain 29 non-*Salmonella* strain	97% 20 artificially and 68 naturally contaminated chicken feces	Tomás and others 2009
ssrA	TaqMan	1-10 genome copies/PCR reaction	Yes	100/100 30 *Salmonella* 30 non- *Salmonella* strains	Swine swab at different concentrations	McGuinness and others 2009

fimY (102bp)	TaqMan	Approximately 60CFU/ml	No	100/100 6 *Salmonella* 1 non- *Salmonella* strains	biscuit, egg, juice, milk, pork, spinach	Li and others 2010
ompF (59bp)	TaqMan	2·8 or approximately 3CFU/PCR reaction	No	218 *Salmonella* 180 non- *Salmonella* strains	Total 30 samples - orange juice, mayonnaise, chicken cuts, egg salad and hamburger patty	Tatavarthy and Cannons 2010
ssaN (56bp)	MGB-TaqMan	18.6 to 41.2fg of genomic DNA/PCR reaction (serovars dependent)	Yes	100/100 40 *Salmonella* 24 non- *Salmonella* strains	Artificially contaminated chicken, liquid egg and peanut butter	Chen and others 2010
invA (261bp)	TaqMan (EMA-real-time PCR)	10^3 CFU/ml of pure culture 10^5 CFU/ml of spiked food	Yes	100 *S.* Enteritidis 13076 and *S.* Typhimurium 14028	artificially contaminated chicken rinses and egg broth	Wang and Mustapha 2010
invA (285 bp)	TaqMan	20 genome copies (100% probability)	Yes	100/100 100 *Salmonella* strain 42 non-*Salmonella* strains	100% 1,934 natural food samples	Anderson and others 2011
hilA (270bp)	Fluorescently hybridization TaqMan	10 genomic equivalent of pure culture	Yes	100/100 106 *Salmonella enterica* 30 non- *Salmonella* strains	Carcass swab, minced beef	McCabe and others 2011
invA (200bp)	Multiplex TaqMan	100-1000 genomic equivalent /PCR reaction 10^4 CFU/g of spiked food	Yes	100/100 30 *Salmonella* 73 non- *Salmonella* strains	Spiked ground beef, whole chicken, ground chicken, ground turkey, raw egg, spinach and tomato	Singh and Mustapha 2013

invA (284bp)	SYTO9 intercalating dye	20 ng–200 fg/PCR reaction 290 CFU/mL of pure culture 10^4 CFU/g of spiked food	No	100 41 *Salmonella*	Spiked ground beef, whole chicken, ground chicken, egg, produce	Singh and Mustapha 2014

2.7.2 Gene Targets for the PCR based Detection of *Salmonella*

Salmonella contain a 40-kb DNA sequence known as *Salmonella* pathogenic island 1 (SPI1). This DNA sequence (SPI1) encodes for at least 33 proteins, which include the components of a type III secretion apparatus, regulatory proteins, secreted effector proteins and their chaperones (Darwin and Miller 1999). Three proteins coded by the SPI1 are directly involved in building a supramolecular syringe-like structure, which reaches from the cytoplasmic membrane to the outer membrane. This syringe-like structure secretes effector proteins from *Salmonella*, stimulates dramatic cytoskeletal rearrangements in eukaryotic host cells called membrane ruffling. This phenomenon of membrane ruffling, which is regulated by SPI1 genes, facilitates the engulfment of the *Salmonella* by eukaryotic cells. Mutation in any of these SPI1 genes coding for apparatus proteins or the regulatory proteins greatly diminishes the ability of pathogen to cause host invasion (Galan and others 1992).

The *invA* gene is the most commonly targeted SPI1 gene for the detection of *Salmonella*. *inv*A encodes a 71kDa putative inner membrane protein and plays an important role in the internalization of *Salmonella* into epithelial cells and has been frequently targeted for PCR-based detection of *Salmonella* (Rahn and other 1992). The *hilA* (hyperinvasive locus A), is another SPI1 gene which has been used for the PCR based detection of *Salmonella* (McCabe and others 2011). The *hil*A is an upstream regulator of *invA* and, just like *invA*, it is also required for the regulation of Type III secretion system (T3SS). The *hilA* sequence has also been previously utilized as a target for the detection of *Salmonella enterica* (Fey and others 2004; McCabe and others 2011). The *Salmonella* invasion proteins or *Salmonella* secreted protein (*sipB-sipC* gene) is also

a part of SPI1 which encodes proteins required for the invasion of *S.* Typhimurium and *S.* Typhi into tissue culture cells (Darwin and Miller 1999), playing an important role in the pathogenesis of *Salmonella*. Ellingson and others (2004) developed a species-specific real-time PCR assay targeting the *sipB-sipC* genes for the detection of *Salmonella*.

The *ttrRSBCA* locus is located near the *Salmonella* pathogenicity island 2 (SPI2). The genes, *ttrA*, *ttrB*, and *ttrC*, of the ttrRSBCA locus encode the tetrathionate reductase structural proteins, whereas the *ttrS* and *ttrR* genes encode the sensor and response regulator components of the system. The end products of the *ttrRSBCA* locus is required for the tetrathionate respiration in *Salmonella* (Hensel and others 1999). This tetrathionate respiration is an important characteristic of *Salmonella*, which is also the basis for the selective enrichment of *Salmonella* using tetrathionate broth. The ability to respire tetrathionate in *Salmonella* is a significant component of the *Salmonella* life cycle. Therefore, the *ttrRSBCA* locus should be stable in *Salmonella*. It is believed that the SPI1 genes in *Salmonella* were acquired by the process of a horizontal gene transfer. Natural mutations with a deletion of the SPI1 genes (e.g. *inv*, *spa*, *hil*) can occur, leading to a false-negative result by PCR assays based on the SPI1 gene (Ginocchio and others 1997). Malorny and others (2004) developed a real-time PCR TaqMan® assay based on the *ttrRSBCA* sequence for the detection of *Salmonella*.

The *stn* gene of *Salmonella* encodes a 29-kDa *Salmonella*-specific enterotoxin protein (Chopra and others 1994). Previous researchers indicated that the *stn* is highly conserved in *S. enterica* serotypes. However, Ziemer and Steadham (2003) reported the prevalence of *stn* among *Salmonella bongori* strains. The *stn* gene sequence is genetically diverse, the percentage of sequence similarity of *S. bongori* with *S* Typhimurium is

660/750 bp (88%), whereas with *S.* Typhi is 659/750 bp (87·9%). Moore and Feist (2007) designed a conserved PCR primer and TaqMan® probe for the detection of *S. enterica* and *S. bongori* both.

Regulatory genes in a bacterial genome are considered to be more stable and are less prone to mutational changes. The *bipA* (or *typA*) gene of *Salmonella* is a member of the "GTP-binding elongation" which acts as an essential translation factor for the efficient expression of *fis* gene, thus regulating a wide variety of other global downstream processes and exhorting a global modulating properties (Owens and others 2004). The *bipA* gene product also binds to ribosomes at the elongation factor G binding site, and has additionally shown to have a GTPase activity. Therefore, considering the stable role of this gene in the *Salmonella* genome, Calvó and others (2008) developed a real-time PCR assay for the detection of *Salmonella*.

In addition to the above-mentioned genes, there have been reports of some other targets that have been also used for the detection of *Salmonella.* The *omp*F gene coding for a porin protein is present in all *Salmonella* subspecies. Tatavarthy and Cannons (2010) used the conserved region of *omp*F for the real-time PCR detection of *Salmonella*. The *Prot6e* gene is a gene located on the 60-kb virulence plasmid of *S.* Enteritidis and is considered specific and unique to *S.* Enteritidis (Chu and others 1999). Malorny and others 2007 developed real-time PCR method on *Prot6e* gene sequence for the detection of *S.* Enteritidis. A selected list of real-time PCR assays for the detection of *Salmonella* is shown in Table 2.1.

2.8 Carbapenems and Extended Spectrum beta-lactam Resistance in Enterobacteriaceae

Members of the Enterobacteriaceae family are the most common causes of community- and hospital-acquired infections. The beta-lactam, cephalosporins, carbapenems and monobactams are the most commonly used antibiotics all around the world constituting around 60% (by weight) of all antimicrobials used. Owing to their efficacy, safety and ease with which they can be chemically manipulated, they are the most preferred group of antimicrobials. (Livermore and Woodford 2006). Therefore, they are considered to be the most versatile and malleable group of antibiotics. Resistance towards beta-lactam antibiotics originated from penicillin-binding proteins of Gram-positive bacteria. These penicillin-binding proteins are an important mode of resistance in *Haemophilus* and *Neisseria* (Georgopapadakou 1993). However, in Gram-negative bacteria, synthesis of beta-lactamase enzymes, impermeability and porin loss are the most important mechanisms of resistance (Georgopapadakou 1993; Livermore and Woodford 2006).

Based on the amino acid sequence data, beta-lactamase enzymes have been divided into four major classes: Class A - serine-β-lactamases, Class B - metallo-β-lactamases, Class C - serine-β-lactamases, and Class D - serine-β-lactamases. These four classes are further classified into plasmid-mediated or chromosomal-mediated genes. However, the demarcation between the chromosomal and plasmid genes can sometimes be confusing because during the process of evolution, all plasmid genes escape from chromosomal DNA (Livermore and Woodford 2006).

In the last 60 years, the spread of beta-lactamase enzymes among pathogenic bacteria has driven the development of newer antibiotics with higher efficacy to fight the problems of antibiotic resistance. Benzylpenicillin was the first analog that penetrated Gram-negative bacteria poorly and was easily hydrolyzed by penicillinases. The resistance towards benzylpenicillin rapidly spread through *Staphylococcus aureus* and other streptococcal species. In order to counter the increasing resistance towards benzylpenicillin, semi-synthetic penicillins (e.g. ampicillin, carbenicillin, methicillin and oxacillins) were created in the 1960s. These new antibiotics possessed the ability to penetrate resistant strains of Gram-negative bacteria and were also stable against staphylococcal penicillinase. However, with widespread use of these antibiotics, new plasmid-mediated penicillinases emerged (e.g. TEM, SHV) in the Enterobacteriaceae and compromised the activity of these antibiotics. These events drove researchers to develop newer antibiotics, which led to the synthesis of 2^{nd}, 3^{rd} and 4^{th} - generation oxyimino-cephalosporins (e.g. cefuroxime, cefotaxime, ceftriaxone, ceftazidime and cefepime), and other β-lactamase inhibitors (e.g. clavulanic acid). Hereafter, oxyimino-cephalosporins became the most potent antibiotics and were commonly used all around the world, but once again, their widespread uses led to the emergence of cephalosporin and fluoroquinolones resistant (Livermore 2005), thus, driving the use of carbapenems antibiotics. Unfortunately, the practice led to a rapid selection of carbapenem-resistant Enterobacteriaceae strains (Walsh 2010). As a result of increasing rates of resistance towards carbapenem antibiotics, only a limited number of antimicrobials (e.g., colistin, fosfomycin, tigecycline) are left for the treatment of these resistant strains. Therefore, the rapid antimicrobial resistance profiling of a pathogen is of the utmost importance.

2.8.1 Extended spectrum beta lactamase

In nature, the evolution of predator and prey coincide. The emergence of resistance to beta-lactam antibiotics began even before the first beta-lactam antibiotic was identified and made available for medical use (Abraham 1940). Penicillin was one of the most widely used antibiotics for the treatment of injured soldiers during World War II. However, the age of penicillin suffered a major setback with the emergence of plasmid-encoded penicillinase in *S. aureus.* This resistance was a plasmid-borne trait that was quickly transferred to other staphylococcal species (Bradford 2001).

Many genera of soil and Gram-negative bacteria possess chromosomally mediated genes that encode for beta-lactamase enzymes. This group of enzymes that confers resistance towards beta-lactam antibiotics evolved from naturally occurring penicillin-binding proteins. One of the theories behind the evolution of these resistant strains is the selective pressure exerted by naturally occurring beta-lactam producing soil bacteria in the environment (Ghuysen 1991). The situation was later exacerbated by the excessive use of the antibiotic in clinical settings, which hastened the emergence of antibiotic resistant strains.

Datta and Kontomichalou (1965) reported the first beta-lactamase enzyme, TEM-1, in Gram-negative bacteria. This beta-lactamase enzyme was originally identified in a patient named Temoniera, hence the name, TEM (Medeiros 1984). The gene coding for TEM was located on a plasmid and transposon that facilitated rapid spread of the enzyme among other species, and very soon it was reported from all around the world. The enzyme is now commonly found in antibiotic resistant strains of members of the Enterobacteriaceae, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Neisseria*

gonorrhoeae. The SHV-1 that stands for "sulphydryl variable" is another commonly found beta-lactamase enzyme reported among the antibiotic resistance strains of *Klebsiella pneumoniae* and *E. coli.*

After the isolation of the first β-lactam resistant pathogen, more and more antibiotics were identified to counter the effects of antibiotic resistance. One of these new classes of antibiotics was called as oxyimino-cephalosporins, which in the 1980s, was widely used for the treatment of serious infections caused by Gram-negative pathogenic bacteria. However, with identification and their clinical applications, emerged the new beta-lactamase enzyme. The extensive use of the antibiotic for medical purposes exerted selective pressure for the selection of newer β-lactamase enzymes conferring resistance to newer antibiotics. The SHV-2 enzyme was the first enzyme that showed an extended spectrum β-lactam hydrolyzing capability and was isolated from a *Klebsiella ozaenae* in Germany (Kliebe and others 1985). Because of the increased spectrum of activity of these β-lactamase enzymes, especially against the oxyimino-cephalosporins, these enzymes were called extended-spectrum β-lactamases (ESBLs) (Bradford 2001). Hence, ESBLs were classified as a class of enzymes that confer resistance against penicillins, broad-spectrum cephalosporins with an oxyimino side chain (cefotaxime, ceftriaxone and ceftazidime) and the oxyimino-monobactam aztreonam antibiotics. Another common property of these ESBLs is that they can be inhibited by serine-type β-lactamase inhibitors e.g. sulbactam, clavulanate and tazobactam (Philippon and others 1989; Bradford 2001). At present, more than 150 different ESBL enzymes have been described. Today, ESBLs are one of the most influential mechanisms for cephalosporin resistance in Enterobacteriaceae, particularly in *E. coli* and *K. pneumoniae*.

2.8.1.1 Types of ESBL

The first ESBL lactamase to be identified was SHV-2 in a clinical isolate of *K. ozaenae* in Germany (Kliebe and others 1985). To date, over ten families of enzymes conferring the ESBL phenotype have been documented e.g. CTX-M, SHV, TEM, PER, VEB, BES, GES, TLA, SFO and OXA (Paterson and Bonomo 2005). Most of the ESBL enzymes found are derivatives of TEM or SHV enzymes (Bush and others 1995; Jacoby and Medeiros 1991). At present, there are more than 221 TEM-type and greater than 189 SHV-types of ESBL enzymes (http://www.lahey.org/studies/webt.htm). New types of ESBL enzymes arise by a few mutations in the DNA sequence of the gene, resulting in a change in amino acid sequence of the protein, generating new enzymatic phenotypes. These enzymes are commonly found in the Enterobacteriaceae (e.g. *E. coli* and *K. pneumoniae*). However they have been also reported in *Proteus* spp., *Providencia* spp., and *Acinetobacter* spp. (Bradford 2001).

2.8.1.1.1 TEM

The TEM-1 is one of the oldest and commonly found beta-lactamase enzymes in Gram-negative bacteria. The majority of ampicillin resistance (90%) in *E. coli* is due to the production of TEM-1 enzyme (Livermore 1995). Apart from ampicillin and penicillin resistance, the TEM-1 enzyme is responsible for hydrolysis of early cephalosporins, such as cephalothin and cephaloridine. TEM-2 was the first enzymatic variant, which originated by a single amino acid substitution in the original TEM-1 sequence (Barthélémy 1985). Another important TEM variant was the TEM-3 enzyme that was first reported in 1987, and it was also the first TEM-type lactamase that showed an ESBL phenotype (Sougakoff and others 1988). The amino acid substitution resulting

in a new enzymatic variant, occurs in the TEM protein sequence at a limited number of positions. These combinations of new amino acid substitutions in enzyme protein sequences led to subtle alternations in the enzymatic activity and substrate range that they can hydrolyze, resulting in a change in the ESBL phenotype e.g. to hydrolyze different oxyimino-cephalosporins. It has been proposed that the origin of these TEM-type ESBL enzymes was not the result of selective pressure exhorted by one single beta-lactam, but rather due to the application of several beta-lactams within institutions (Bonnet and others 1999). The TEM-type beta lactamase enzymes are commonly reported among clinical isolates of *E. coli* and *K. pneumoniae.* In addition to these, they have been also frequently reported in Enterobacteriaceae such as *Enterobacter aerogenes, Morganella morganii, Proteus mirabilis, Proteus rettgeri*, and *Salmonella* spp.

2.8.1.1.2 SHV

The SHV-1 beta-lactamases are most commonly found in *K. pneumoniae* and are responsible for more than 20% of resistance towards ampicillin. The gene responsible for the synthesis of SHV lactamase is bla_{SHV}; it can either exist as a plasmid gene or a chromosomal gene (Livermore 1995). It has been previously reported that a majority of SHV-type derivatives possess the ESBL phenotype capabilities. These ESBL variants of SHV lactamases also possess a substitution of a serine for glycine at the 238th position of the protein sequence (Bradford 2001).

2.8.1.1.3 CTX-M

In the family of ESBLs, CTX-Ms are plasmid–mediated enzymes that preferentially hydrolyze cefotaxime, and are the latest addition that have significant

clinical impacts. This new class of ESBL enzymes is commonly found among the antibiotic resistant strains of *Salmonella* and *E. coli,* but they have also been frequently reported among other members of the Enterobacteriaceae. The CTX-M enzymes are one of the biggest and most diverse groups of ESBL enzymatic variants that also include MEN-1 and Toho enzymes. The CTX-M enzymes are not closely related to the SHV and TEM beta-lactamase enzymes, and show around 40% of sequence homology with SHV and TEM, which happen to be the most frequently isolated ESBL enzymes (Tzouvelekis and others 2000). The chromosomally encoded class A cephalosporinases enzymes found in *K. oxytoca, C. diversus, Proteus vulgaris,* and *Serratia fonticola* are the most closely related to the CTX-M family enzymes. They share around 73-77% sequence homology (Bauernfeind and others 1996; Bonnet and others 1999). However, some recent studies showed that the CTX-M lactamase share a much higher degree of homology with the chromosomal mediated AmpC enzyme of *Kluyvera ascorbata,* which are designated as Klu-1 and Klu-2, suggesting that CTX-M enzymes originated from *Kluyvera ascorbata* (Decousser and others 2001). Phylogenetic analysis of all the CTX-M enzyme sequence suggest an early divergence from a common ancestor gene (Bonnet and others 2000). Most of the CTX-M type enzymes exhibit strong activity against cefotaxime and ceftriaxone but not ceftazidime. However, there are some exceptions e.g. CTX-Ms, such as CTX-M-15 (Poirel and others 2002), CTX-M-16 (Bonnet and others 2001) and CTX-M-19 (Poirel and others 2001), which exhibit enhanced catalytic activity against ceftazidime.

The CTX-M lactamases are a wide and diversified group of enzymes. To date the number of enzymatic variants of CTX-M are dramatically increasing and so far more

than 160 CTX-M variants (CTX-M-1 to CTX-M-160) have been reported by the Lahey database (Jacoby and Bush 2012). These variants have been classified into four major types: (A) CTX-M-1 type - CTX-M-1 and CTX-M-3 (B) CTX-M-2 type - that includes CTX-M-2, CTX-M-4, CTX-M-5, CTX-M-6, CTX-M-7, and Toho-1 (C) Toho-2 (D) CTX-M-8 (Bonnet and others 2000). The diversity of CTX-M enzymes varies with geographical locations, but CTX-M-15 and CTX-M-14 (followed by CTX-M-2, CTX-M-3 and CTX-M-1) are the most common enzymatic variants worldwide among all major clinically important pathogens (Zhao and Hu 2013).

Strains expressing CTX-M lactamases have been reported from many parts of the world. They also have been the focal point of outbreaks in Europe, South America and Japan (Bradford and others 1998; Ma and others 1998). The CTX-M type lactamase has also been involved in the outbreaks caused by *E. coli* and *Salmonella* (Sabaté and others 2000). According to a review paper published by Zhao and Hu (2013), CTX-M type enzymes have been detected in at least 26 bacterial species e.g. *Acinetobacter baumannii, Aeromonas caviae, A. hydrophila, Citrobacter amalonaticus, C. freundii, C. koseri, E. coli, Enterobacter cloacae, E. aerogenes, E. gergoviae, E. hormaechei, K. pneumoniae, K. oxytoca, Morganella morganii, P. mirabilis, Pantoea agglomerans, Providencia rettgeri, P. stuartii, Pseudomonas aeruginosa, S. enterica, Shigella flexneri, S. sonnei, Serratia marcescens, S. liquefaciens, Stenotrophomonas maltophilia* and *Vibrio cholera*. Out of these 26 bacterial species CTX-M ESBL are more prevalent in *E. coli, K. pneumoniae* and *P. mirabilis* (Zhao and Hu 2013). In a study conducted in 16 hospitals in England over a period of 12-weeks, a total of 19,252 clinical isolates were tested for antibiotic resistance. Out of which, *E. coli* and *Klebsiella* were the two species that

harbored the maximum number of resistant strains, and CTX-M was the most common resistance mechanism (Potz and others 2006).

2.8.1.1.4 Class C chromosomal AmpCs

Class C chromosomal AmpC (cAmpC) is a class of β-lactamases, which confer resistance towards third-generation cephalosporins and β-lactamase inhibitors. However, they are susceptible to fourth-generation cephalosporins (e.g., cefepime) and the carbapenems group of antibiotics (Jacoby 2009). These enzymes are common in some genera of Enterobacteriaceae e.g. *Citrobacter freundii*, *Enterobacter* spp., *Serratia* spp. Genes responsible for the synthesis of these enzymes (AmpC) are under the control of complex regulatory elements (ampD, ampR) (Jacoby 2009). With the widespread use of cephalosporins for treatments, an increasing number of bla_{pAmpC} positive Enterobacteriaceae strains have been isolated. There are several families of these enzymes (e.g. CMY, FOX, LAT, MIR, ACT, DHA, ACC, MOX). Out of these, CMY-2 is the most prevalent pAmpC among members of Enterobacteriaceae. However, DHA and FOX have been also reported within certain geographic regions (Jacoby 2009; Seiffert and others 2013).

2.8.2 Carbapenemase

The emergence of resistance towards carbapenems in Gram-negative bacteria especially in the Enterobacteriaceae, *Pseudomonas*, and *Acinetobacter* species (E.P.A) has become a matter of a public health crisis worldwide, and they are responsible for a large number of hospital-acquired and nosocomial infections. This scenario has been

further worsened by the rapid spread of these resistance phenotypes and the lack of development of new and effective antimicrobial drugs (Diene and Rolain 2014).

In the early 1990s, resistance towards carbapenems was reported to be a species-specific phenomenon, but resistance-conferring genes, being a plasmid borne trait, spread to all related genera and become a worldwide phenomena.

Carbapenemase are enzymes that are produced by pathogenic bacterial species to degrade and neutralize the effects of almost all beta-lactam antibiotics, including carbapenems (Cantón and others 2012). In the last two decades, a large number of different groups of enzymes possessing carbapenemase activity have emerged, and are rapidly spreading all around the world. This highly genotypically and phenotypically diversified group of carbapenemase enzymes differs in their enzymatic activity (Figure 2.3). Some of them hydrolyze carbapenems very efficiently, whereas others show weak or limited activity. Some enzymes can confer resistance towards broad-spectrum cephalosporins, while some do not (Nordmann and Poirel 2014).

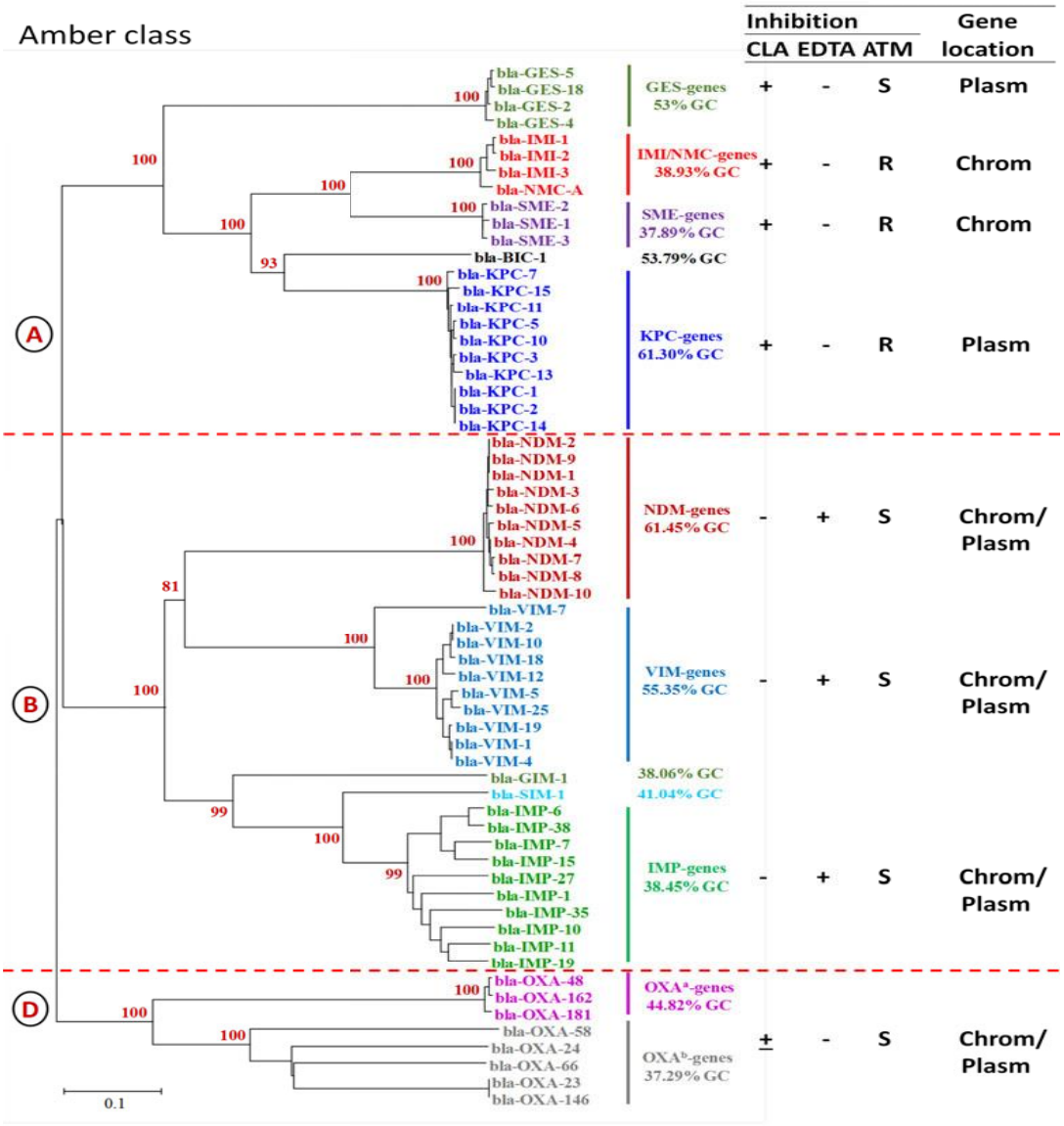

Figure 2.3 Phylogenetic tree of the metallo-carbapenemase and serine carbapenemase genes. Their mean percentage GC content, according to groups and phenotypic properties. The tree was constructed from the amino acid sequences aligned with the free ClustalX software version 2.0 and MEGA software version 6.06, by use of the neighbour-joining method with the amino acid Poisson correction model with 1000 bootstrap replicates. Bootstrap values are expressed as percentage of the 1000 replicates, and only those up to 50% are kept and shown at branch points. [a]OXA genes described overall in Gram-negative bacteria. [b]OXA genes described only in Acinetobacter species. The percentage GC values correspond to the averages of each subgroup. ATM, aztreonam; CLA, clavulanic acid; R, resistant; S, susceptible. Adapted from Diene and Rolain (2014); License Number: 3505650398577; License Date: Nov 10, 2014

2.8.2.1 Classification of carbapenemases

The carbapenemases group of enzymes can be either classified using its genetic makeup or they can be also categorized based on the functional characteristics of their active sites (Table 2.2).

Table 2.2 Classification of β-lactamases enzymes

Class	Enzymes that are plasmid-, transposon- or integron-mediated	Chromosomal and ubiquitous in species or group
Class A- Serine-β-lactamases	TEM, SHV, VEB, PER, CTX-M penicillinases and ESBLs; KPC, IMI/NMC and SME carbapenemases	SHV-1, LEN-1 and K1 in *Klebsiella* spp.; chromosomal cefuroximases of *Proteus vulgaris*; chromosomal, β-lactamases of *Bacteroides* spp.
Class B- Metallo-β-lactamases	IMP, NDM, VIM, SPM	L1 enzyme of *Stenotrophomonas maltophilia*; chromosomal enzymes of some *Chryseobacterium* spp. and *Aeromonas* spp.; CcrA enzyme found in 1–3% of *Bacteroides fragilis* isolates
Class C- Serine-β-lactamases	CMY-1, LAT-1, BIL, MOX, ACC, FOX, DHA	Chromosomal AmpC enzymes of *Escherichia coli*, *Shigella* spp., *Enterobacter* spp., *Citrobacter freundii*, *Morganella morganii*, *Providencia* spp., *Serratia* spp.
Class D - Serine-β-lactamases	Most OXA types, excluding those detailed here as chromosomal	Chromosomal (along with other b-lactamases) in *Acinetobacter* spp. (OXA-51-like); *P. aeruginosa* (OXA-50) chromosomal and some *Aeromonas* spp. (e.g. OXA-12).

(Adapted from Livermore and Woodford 2006)

Based on the composition of the active site, carbapenemases enzymes can be broadly classified into two major groups: (1) Serine Carbapenemases: This group consists of a serine amino-acid in their active site e.g. class A penicillinases and class D

oxacillinases. These enzymes can be inactivated with the use of the β- lactamase inhibitors e.g. clavulanic acid and tazobactam. (2) Metallo-betalactamases: This group of enzymes is also known as Class B carbapenemases. Enzymes of this group have one or more zinc atoms in the active site of the enzyme. The activity of these enzymes can be inhibited by ethylenediaminetetraacetic acid (EDTA) (Queenan and Bush 2007).

2.8.2.1.1 Class A carbapenemases

Enzymes of this class are coded by IMI, NMC, SME, KPC, and GES genes, which confer resistance to the carbapenems group of antibiotics at various levels, ranging from complete resistance to reduced susceptibility (Queenan and Bush 2007). Out of the above-mentioned five enzymes IMI, NMC and SME are encoded by chromosomal genes, whereas KPC and GES are encoded by genes located on plasmids. Further, SME enzymes are mostly species-specific and are restricted to *Serratia marcescens,* whereas IMI and NMC enzymes have been sporadically reported only in *E. cloacae* (Queenan and Bush 2007). Therefore the chromosome-mediated genes (e.g. IMI, NMC and SME) are species-specific genes enzymes, are rarely reported and are also less prevalent all around the world. However, other two Class A carbapenemases enzymes, KPC (*K. pneumoniae* carbapenemases) and GES (Guiana extended-spectrum β-lactamase) enzymes are located on transferrable plasmids, so they are more widely distributed among the Enterobacteriaceae and other pathogenic genera. The KPC enzymes are mostly prevalent in *K. pneumoniae* but they could be also found in other members of the Enterobacteriaceae, *Pseudomonas* and *Acinetobacter* (Cantón and others 2012). Similarly, the GES enzymes are associated with integrons (mobile genetic element) and are prevalent in *P. aeruginosa* and *K. pneumoniae* (Queenan and Bush 2007).

2.8.2.1.2 Class D carbapenemases

These groups of enzymes are also called Class D metallo-beta-lactamases or OXA beta-lactamases (owing to their ability to hydrolyze oxacillin). The OXA-23, OXA-24, and OXA-58 genes are the most dominant genetic determinants encoding this enzyme in *A. baumannii* whereas OXA-48 is are being increasingly reported in *K. pneumoniae*. The gene coding for this group of enzymes is found on chromosomal as well as plasmid DNA facilitating their spread among related genera (Queenan and Bush 2007).

2.8.2.1.3 Class B carbapenemases or class B metallo-beta-lactamase

This group includes the GIM, IMP, NDM, SIM and VIM enzymes that are commonly found on the transferrable plasmids of Enterobacteriaceae with addition to *P. aeruginosa* and *A. baumannii*. Out of the five above-mentioned enzymes; IMP, NDM and VIM are the most prominent enzymes.

The IMP enzyme was first reported in Japan in a clinical isolate of *S. marcescens,* but has now spread all around the world and is commonly found in the Enterobacteriaceae, *P. aeruginosa* and *A. baumannii* (Osano and others 1994; Cornaglia and others 2011).

The VIM-type of enzymes, so far, comprise of around 43 variants (VIM-1 to VIM-43). Out of these enzymatic variants, VIM-1 is the most commonly found variant. The gene encoding for the synthesis of VIM-1 enzyme (bla_{VIM-1}) was first reported in 1997 in a clinical strain of *P. aeruginosa* inVerona, Italy. This gene was integrated as a gene cassette in a Class I integron along with the aminoglycoside resistance gene, *aacA4* (Lauretti and others 1999). The Class I integron is a mobile genetic elements that can

harbor multiple drug resistance genes as a cassette and are responsible for multiple drug-resistance phenotypes in Enterobacteriaceae. The presence of *bla*$_{VIM-1}$ in *E. coli* was first reported in Greece in 2001, and it later on spread all round the world. The next common enzymatic variant of this group is VIM-2, it was first reported in a clinical strain of *P. aeruginosa*, and now it is endemic to many countries of the world (Cornaglia and others 2011).

NDM is the most recent addition to the Class B carbapenemases group of enzymes. The enzyme was reported in the year 2009 from clinical isolates obtained from Asia and India (Yong and others 2009; Kumarasamy and others 2010). Since the first report of NDM-1 in the year 2009, it has spread worldwide and become the most common carbapenemases enzyme in all the Enterobacteriaceae (Rolain and others 2010).

2.8.2.2 Distribution of carbapenemase enzymes

In the last 24 years, since the first description of the carbapenemase enzyme in *S. marcescens* in 1990 (Yang and others 1990), the occurrence of carbapenemase producing Enterobacteriaceae has increased worldwide.

2.8.2.2.1 Serine carbapenemases group

KPC enzymes are most important due to their most frequent and worldwide occurrence, especially in *K. pneumonia* and *E. coli*. Other Enterobacterial species harboring these enzymes include *Citrobacter*, *Enterobacter*, *Serratia*, and *Proteus* (Queenan and Bush 2007). The widespread occurrence of KPC enzymes in EPA species has been associated with the occurrence of the KPC enzyme-producing gene (*bla*$_{KPC}$) on transposon Tn4401 (a mobile genetic element) (Cuzon and others 2010). At present, there

are 22 enzymatic variants of the KPC (KPC-1 to KPC-22), and all of them originated as point-mutation variants of a common amino acid sequence (Nordmann and Poirel 2014). Out of these 19 enzymatic variants, KPC-2 and KPC-3 producing *K. pneumoniae* clones have been most extensively identified all around the world (Cuzon and others 2010). Other serine carbapenemases enzymes (e.g. BIC, GES, IMI, NMC, and SME) also share a common ancestry to the KPC enzyme (Walther-Rasmussen and Høiby 2007). Out of them, bla_{SME}-genes are restricted to only *S. marcescens* species whereas IMI and NMC-A enzymes which are more similar to each other, are mainly distributed among *E. cloacae* and *E. coli* (Walther-Rasmussen and Høiby 2007; Queenan and Bush 2007). GES enzymes are mostly reported among *P. aeruginosa* species, but they are also found in the Enterobacteriaceae. There are around 26 (GES-1 to GES-26) enzymatic variants of GES, out of which GES-2, GES-4, GES-5, GES- 6, GES-14 and GES-18 have been reported to exhibit an enzymatic activity against carbapenems (Walther-Rasmussen and Høiby 2007; Queenan and Bush 2007). The enzymatic variant, GES-5 has been reported in *K. pneumoniae, E. coli* and *E. cloacae* (Girlich and others 2012).

2.8.2.2.2 Class D metallo-beta-lactamases or OXA beta-lactamases

It is one of the most genetically diversified groups of carbapenemase enzymes. The first OXA-48 enzyme-producing strain was identified in a *K. pneumoniae* isolate recovered in Turkey. Later, the KPC enzyme successfully spread to all important members of the Enterobacteriaceae. To date, more than 435 (OXA-1 to OXA-435) sequence types of bla_{OXA} gene have been reported (Diene and Rolain 2014). All these enzymatic variants possess some level of enzymatic activity against carbapenems, and do not provide high levels of resistance against carbapenems (Nordmann and Poirel 2014).

The OXA gene variants *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{OXA-58}}$, and some orthologs of these genes have been reported in *Acinetobacter,* whereas *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-162}}$ and *bla*$_{\text{OXA-181}}$ have been reported in the Enterobacteriaceae (Walther-Rasmussen and Høiby 2006). The most dominant variant *bla*$_{\text{OXA-48}}$ was for the first time isolated in 2004 from *K. pneumoniae* in Turkey (Poirel and others 2004). The OXA-48 enzyme has now been reported among all major enterobacterial species, which include *E. coli*, *S. marcescens*, *E. cloacae*, *K. oxytoca*, *Providencia rettgeri*, and *C. freundii* (Poirel and others 2012). Other equally important enzymatic variants of this group are OXA-181, OXA-204, and OXA-232 (Poirel and others 2012).

2.8.2.2.3 Class B carbapenemases or the Class B metallo-beta-lactamase

This group of enzyme comprises of GIM, IMP, NDM, SIM and VIM. These enzymes were historically associated with *P. aeruginosa* and *Acinetobacter* spp.; however in last two decades they have widely spread across the enterobacterial species (Walther-Rasmussen and Høiby 2006). Out of these five Class B metallo-beta-lactamase enzymes NDM, VIM and IMP are the most commonly encountered enzymes in clinical pathogens. The IMP-1 enzyme was the first metallo-carbapenemase enzyme reported in the Enterobacteriaceae (Osano and others 1994). Therefore, based on its early existence, it has been reported to be in almost all enterobacterial species, e.g *E. coli, C. freundii, C. koseri, K. pneumoniae, K. oxytoca, E. aerogenes, E. cloacae, P. mirabilis, P. rettgeri, M. morganii, and Shigella flexneri* (Queenan and Bush, 2007). The VIM-2 enzyme was first discovered in 2000 from a *P. aeruginosa* isolate, but now the enzyme has been widely distributed in Gram-negative bacteria (Poirel and others 2000; Walsh and others 2005). The last addition to the Class B metallo-beta-lactamase group of enzymes was NDM-1

(New Delhi metallo-β- lactamase-1). The NDM enzyme was first reported in the Indian subcontinent (Yong and others 2009). The enzyme has around 10 enzymatic variants and has rapidly spread from the Indian subcontinent to other parts of the world. The enzyme has been identified in all major enterobacterial species viz. *K. pneumoniae, K. oxytoca, E. coli, P. mirabilis, C. freundii, E. cloacae, and M. morganii* (Nordmann and others 2011). The GIM- 1 and SIM-1 enzymes are less commonly reported. The $bla_{\text{GIM-1}}$ gene has been reported from *E. cloacae, K. oxytoca, S. marcescens, E. coli*, and *C. freundii* (Wendel and others 2013), whereas the SIM-1 enzyme has not yet been reported among enterobacterial species (Diene and Rolain 2014).

2.8.3 Epidemiology and spread of these enzymes

2.8.3.1 Primary Reservoir

These enzymes have the ability to degrade/hydrolyze antibiotics emerge in geographical regions where selective pressure for evolution is high e.g. highly populated area, poor hygiene, over use of antibiotics for human treatments, agriculture and animal husbandry purposes.

2.8.3.2 Mobile Genetic Elements

There are genetic elements in the bacterial genome that increase the plasticity or movement of antibiotic resistance genes. These genetic elements are integrons, transposons, and plasmids that facilitate horizontal gene transfer between closely related bacteria. Some plasmids have a broader-host range, allowing them to replicate in a wide range of bacterial genera, which translates into the ability to transfer antibiotic resistance genes to a broad range of bacteria. On the contrary, narrow-host range plasmids have the

ability to transfer and replicate only in closely related bacterial genera. Therefore, the genetic makeup of antibiotic resistance strains plays a vital role in the clonal spread of a particular antibiotic resistance genotype and phenotype.

2.8.3.3 Exchange among human population

Once the antibiotic resistance genotype and phenotype have evolved and are established in a particular population group due to a particular selective pressure, then it acts as a reservoir. Mobility of the population of that reservoir determines the spread of the antibiotic resistance trait around the world. Antibiotic resistant traits that have a reservoir in places with higher mobility of the population (e.g. tourist spots, hospitals, medical-tourism) results in a faster spread of the antibiotic resistant traits e.g. clonal spread of NDM enzyme from the Indian subcontinent to Europe and American continents (Nordmann and others 2011).

2.8.4 Need for detection of antibiotic resistance

Standard microbiological procedures can take up to several days for the isolation and characterization of pathogens from clinical samples. For the diagnosis of bacteremia or sepsis, clinical laboratories commonly use an automated system that can monitor the growth of the pathogen in the blood culture bottles (Riedel and Carroll 2010). In case of positive growth (e.g. members of the Enterobacteriaceae take around 24-48 h to grow), the samples can be plated on agar media for cultivation and isolation of pure culture of the pathogenic strain of bacteria. It takes around another 24 h for the bacteria to grow on the agar media. If, there is growth on the agar medium, an antimicrobial susceptibility test can be performed that requires an additional 24 h. Therefore, for non-multidrug

resistant strains it often takes around 3-4 days, while for a resistant strain, it can take even longer. However, in some cases, the antimicrobial susceptibility test of carbapenemase, ESBL or plasmid-mediated AmpC (pAmpC) producers can be inaccurate (Livermore and others 2011; Poirel and others 2012; Luzzaro and others 2006, Doi and Paterson 2007). These ambiguities and difficulties can be resolved by performing additional tests e.g. clavulanate for ESBL detection, boronic acid for pAmpCs and KPCs detection, and modified Hodge test (mHT) for detecting carbapenemases. Performing these tests takes additional time, which adds up to 4-6 days for getting accurate antimicrobial susceptibility test results (Lupo and others 2013).

Delays in identification and characterization of antimicrobial resistance profiling can be of grave consequences. Longer diagnosis time for a patient infected with multi-drug resistant pathogens can have a less favorable outcome. For a patient suffering from a multi-drug resistant infection, a delay in diagnosis can lead to treatment failure, which translates into higher mortality rates. Hence, rapid antimicrobial susceptibility profiling is of the utmost importance. As initially stated, any delay in antimicrobial susceptibility testing results can lead to treatment failure, which can make a big difference in final hospital bills. A rapid and accurate method for antimicrobial susceptibility testing can reduce the duration of hospitalization and consequently allows reduction in the healthcare cost (e.g. by avoiding use of expensive carbapenems, shorten duration of hospital stay). Lee and others (2006) reported that the mean length of a hospital stay for a person with an ESBL infection was 21 days, which was much higher than a patient with an infection with a non-ESBL strain (11 days), which leads to an additional $16,450 per patient. In another study, Schwaber and others (2006) reported that the average cost of treatment for

a bloodstream infection caused by an ESBL-producing strain was $46,970, which was around four times higher ($16,877) than the treatment cost of infection caused by non-ESBL producers.

2.8.4.1 Need for molecular methods for antibiotic susceptibility testing

Determination of minimum inhibitory concentrations (MIC) using automated systems is commonly used in clinical laboratories and is considered to be accurate, but these systems are also associated with reproducibility issues (Miriagou and others 2010). Giakkoupi and others (2005) compared antimicrobial susceptibility results for five VIM-1 producing *K. pneumoniae* isolates towards imipenem and meropenem antibiotics using broth microdilution, Etest, disc diffusion and three automated system (Vitek 2, Phoenix, and MicroScan). These five VIM-1 producing *K. pneumoniae* isolates showed major variations among susceptibility results especially when the automated systems were used (Giakkoupi and others 2005). Similarly in another study, 15% of KPC-producing *K. pneumoniae* isolates were found to be susceptible to imipenem using automated broth microdilution methods, while only 4% isolates were found susceptible by the Etest method (Bratu and others 2005). Tenover and others (2006) tested MicroScan, Phoenix, and Sensititre automated systems using 15 KPC-producing *K. pneumonia* isolates against imipenem antibiotic. The number of susceptible isolates obtained using MicroScan, Phoenix, and Sensititre, varied between 1, 2 and 13, respectively. Variation in the inoculum concentration has been suggested as one of the possible reasons for the disparity among the results obtained from different automated systems. Samples with lower inoculum concentrations appear as susceptible, whereas, sample with higher inoculum count can appear to be resistant.

Antimicrobial susceptibility testing for ESBL- or carbapenemase-producing Enterobacteriaceae are also performed using commercially available selective agar plates. However, many recent comparative studies have shown that the commercially available selective agars have lower sensitivity than the PCR-based methods. This is especially true for clinical isolates with lower gene expression leading to a low-level of MIC (e.g., OXA-48-producing *E. coli*) (Nordmann and others 2012). Singh and others (2012) reported that the real-time PCR method for the detection of bla_{KPC} in clinical samples showed a sensitivity of 97%, which was much higher then the sensitivity achieved by CHROMagar plates (77%). In another study, Naas and others (2011) reported that a real-time PCR method for the detection of bla_{NDM} had a limit of detection of 10 CFU/mL, where ChromID ESBL and CHROMAgar KPC plates required a pathogen concentration of 10^2-10^3 CFU/mL. Therefore, as the phenotypic methods can either lead to variation in results or lower sensitivity, the molecular-based methods are currently considered as "gold standards" for antibiotic susceptibility testing (Hammoudi and others 2014).

2.8.4.2 Ideal gene targets for rapid detection of antibiotic resistant pathogens

Resistance towards some antibiotics is more wide spread than others and has more serious clinical implications than others. Hence, while developing a diagnostic assay for the detection of antibiotic resistance, some antibiotic resistant genes deserve more attention than others (Table 2.3). In the Enterobacteriaceae family, bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{CMY}, bla_{KPC}, bla_{NDM}, bla_{OXA-48}, the 16S rRNA methylases genes and mutations in *gyr*A and *par*C are the most important genes that should be targeted for the development of a diagnostic assay (Lupo and others 2013). However, other gene targets

with limited global impact, which are found only in limited geographical locations (e.g. *bla*$_{PER}$, *bla*$_{VEB}$, *bla*$_{GES}$, *bla*$_{SPM}$, *bla*$_{GIM}$, *bla*$_{SME}$, and plasmid-mediated quinolone resistance determinants) can be also targeted (Lupo and others 2013). The ideal characteristics of a diagnostic assay for detection of antibiotic resistance are as follows: (a) it should target all resistance genes in the same assay; (b) generate results same day; (c) the data should be easy to analyze and interpret; (d) preferably should be able to differentiate between allelic variants, as protein encoded by different allelic variants have different clinical impacts; (e) its method should be easy to perform, cheap and accurate (Lupo and others 2013).

Table 2.3 List of most important antibiotic resistance trait and genes that molecular methods should be able to detect for the identification of Extended-spectrum cephalosporins and Carbapenems resistance in Enterobacteriaceae. Adapted from Lupo and others (2013) (License Number: 3506120612617; License Date: Nov 11, 2014)

Antibiotics	Mechanism of resistance	Most frequent resistance traits	Importance	Ideal level of detection/characterization
Extended-spectrum cephalosporins	Extended-spectrum β-lactamases (ESBLs)	CTX-M	++++	Generic
		TEM and SHV	++++	Distinguishing ESBLs from non-ESBLs
		PER	++	Generic
		GES	++	Distinguishing ESBLs (e.g., GES-1) from carbapenemases (e.g., GES-5)
		SFO	+	Generic
	Plasmid-mediated AmpCs (pAmpCs)	CMY	++++	Generic
		DHA	+++	Generic
		FOX	++	Generic
		LAT, MIR, ACT, MOX	+	Generic
	Chromosomal AmpCs (cAmpCs)	Mutations in the promoter (*E. coli*)	++	Identifying substitution (e.g., at -32/-42) and insertions (between -10 and -35)
		Mutations in ampD (*Enterobacter* spp.)	+	Unknown specific target
Carbapenems	Carbapenemases Class A	KPC	++++	Generic
		GES	++	Distinguishing ESBLs (e.g., GES-1) from carbapenemases

			(e.g., GES-5)[b]
Class B (MBL)	NDM	++++	Generic
	SME	+	Generic
	VIM	++++	Generic
	IMP	+++	Generic
	SPM and GIM	++	Generic
	AIM, SIM	+	Generic
Class D	OXA-48	++++	Specific
	OXA-181, -162, -163	+++	Specific
Impermeability (porins)	OmpK-35 and -36 (*K. pneumoniae*)	++	Identifying insertions and/or frame-shift mutation(s)
	OmpF/C (*E. coli* and *Enterobacter* spp.)	+	Unknown specific mutation/target

2.8.4.3 PCR based method for ESBL and carbapenems detection

2.8.4.3.1 Multiplex end-point PCR for the detection of ESBL and carbapenems resistance in Enterobacteriaceae

The multiplex PCR, unlike the single end-point PCR, uses a multiple set of primer-pairs in a single reaction, allowing amplification and detection of multiple targets in the same reaction. The design of a multiplex PCR reaction is more complex than a single end-point PCR reaction. The following points should be considered while designing a multiplex PCR reaction: (a) high specificity of each primer set; (b) no self-reactivity or primer-dimer formation; (c) similar PCR cycling condition for all primer-pairs being used in the assay; and (d) each amplicon of the multiplex PCR reaction should differ from other amplicons by at least 30 bp, enabling visible band separation on a regular agarose gel.

Pérez-Pérez and Hanson (2002) developed a multiplex assay for the detection of bla_{AmpC} genes. The PCR assay uses six sets of *Amp*C-specific primers generating amplicons between 190-520 bp. The size of the amplicons generated by this assay was very similar, thus requiring the WAVE technology, a high-pressure liquid chromatography-based separation system. However the primer-pairs used in the assay suffered from cross reactivity. Dallenne and others (2010) reported a multiplex PCR assay for the detection of the most frequent and widespread β-lactamase genes. The assay used seven multiplex reactions and three different cycling conditions for the detection of OXA-1-like broad-spectrum β-lactamases, extended-spectrum β-lactamases (ESBLs),

plasmid-mediated AmpC β-lactamases and class A, B and D carbapenemases. Voets and others (2011) further improved the assay described by Dallenne and others (2010). The assay used a large number of primers in seven multiplex PCR reactions for the detection of plasmid-mediated AmpC β-lactamases (ACC, ACT, DHA, CMY, FOX, LAT, MIR and MOX), metallo-carbapenemases (GIM, NDM, SIM and SPM), serine carbapenemases (IMI, SME and NMC-A) and OXA β-lactamases (OXA groups 23, 24, 48, 1, 2, 51, 4 and 58). The PCR conditions were further optimized to eliminate cross reactivity of the primer. Ellington and others (2007) published a multiplex PCR assay for the detection of metallo-β-lactamases (MBLs). The assay was designed to target five different families of MDLs e.g. IMP, VIM, SPM-1, GIM-1 and SIM-1. Hong and others (2012) reported a multiplex assay for the detection of four families of class A carbapenemases (e.g. SME, IMI/NMC-A, KPC, and GES). Another multiplex PCR assay for the simultaneous detection of bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and $bla_{\text{OXA-48-like}}$ was reported by Doyle and others (2012). The method was evaluated using 142 antibiotic-resistant Enterobacteriaceae strains obtained from the Study for Monitoring Antimicrobial Resistance Trends (SMART; 2008-2009). In the comparative study, this PCR assay showed 100% sensitivity and specificity.

2.8.4.3.2 Commercially available multiplex PCR assay for the detection of ESBL and carbapenems resistance in Enterobacteriaceae

Amplex Diagnostics (Germany) launched three PCR-ELISA kits for the detection of carbapenems and extended spectrum beta-lactam resistance in clinical isolates of Enterobacteriaceae (www. hyplex.info/). The assay targets are first amplified using gene-

specific primers, and the labeled amplicons are reverse-hybridized in a 96-well plate, and the amplicons detected after the addition of a conjugate (peroxidase) and substrate (tetra-methyl-benzidin). The final data is photometrically measured at 450 nm. The three PCR-ELISA assay targets the following genes: (a) Hyplex ESBL ID – *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, and *bla*$_{OXA}$ (b) Hyplex SuperBug ID - *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{OXA-48-like}$ (c) Hyplex CarbOXA ID - *bla*$_{OXA-23}$, *bla*$_{OXA-24}$, and *bla*$_{OXA-51-like}$. Hyplex SuperBug ID PCR-ELISA assay was evaluated by using 132 carbapenem-resistant Enterobacteriaceae isolates. The results showed a 97% (128/132) accuracy (Kaase and others 2012).

2.8.4.3.3 Multiplex real-time PCRs assay for the detection of extended-spectrum cephalosporins and carbapenems resistance

Bisiklis and others (2007) reported a multiplex real-time PCR melt-curve analysis assay for the detection of the most commonly found *bla*$_{VIM}$ (*bla*$_{VIM-1}$, *bla*$_{VIM-2}$, *bla*$_{VIM-3}$, *bla*$_{VIM-4}$, *bla*$_{VIM-5}$, *bla*$_{VIM-6}$, *bla*$_{VIM-10}$, *bla*$_{VIM-11}$, *bla*$_{VIM-12}$) and *bla*$_{IMP}$ (*bla*$_{IMP-1}$, *bla*$_{IMP-2}$, *bla*$_{IMP-6}$, *bla*$_{IMP-8}$, *bla*$_{IMP-10}$, *bla*$_{IMP-15}$, *bla*$_{IMP-19}$, *bla*$_{IMP-20}$) genes in a single reaction. Brolund and others (2010) modified the end-point multiplex PCR assay reported by Pérez-Pérez and Hanson (2002) into a real-time melt-curve analysis PCR assay using SYBR Green® dye with the same PCR cycling conditions. The assay was divided into two triplex PCR reactions. The first reaction targeted the MOX, CIT and FOX genes whereas the second reaction targeted the DHA, ACC and EBC genes. Geyer and others (2012) developed a multiplex TaqMan® assay (two color FAM and HEX) for the detection of pAmpCs. The assay targeted MOX, FOX, CMY-2, DHA, ACT, ACC and 16S rRNA gene as internal amplification control (IAC). All probes targeting pAmpCs

gene targets in the assay were labeled with FAM dye and the IAC probe was labeled with HEX dye. The assay showed 100% specificity and sensitivity. Swayne and others (2011) designed a real-time PCR multiplex TaqMan® assay for the detection simultaneous detection of five types of serine carbapenemases (e.g. GES, IMI/NMC, KPC, OXA-48 and SME). The assay was highly specific, and the amplicons can also be distinguished by performing a melt-curve analysis. A multiplex assay for the detection of different classes of carbapenemases assay was designed by Monteiro and others (2012). The single multiplex real-time melt-curve analysis PCR assays targeted six different genetic types of carbapenemases commonly reported in the Enterobacteriaceae (KPC, GES, NDM, IMP, VIM and OXA-48). Chen and others (2011) designed a multiplex real-time PCR assay, for the detection of $bla_{\text{KPC-2}}$ to $bla_{\text{KPC-11}}$. The assay utilized one pair of conserved primer and six molecular beacon probes. A total of 457 Gram-negative clinically isolates were successfully characterized using this Molecular Beacon real-time PCR assay.

2.8.4.3.4 Commercially available multiplex real-time PCRs Assay

NucliSENS EasyQ KPC (bioMérieux, France) is a commercial real-time PCR assay for the detection of bla_{KPC} in *K. pneumoniae*. It is designed to work with the bioMérieux NucliSENS platform. The result of the assay is analyzed using an automated NucliSens EasyQ analyzer. Spanu and others (2012) evaluated 300 members of the Enterobacteriaceae using the NucliSENS EasyQ KPC kit. The assay correctly detected all of the 111 isolates harboring bla_{KPC} genes, with no false positive results within 2 h. Check-Points Health BV (Netherlands) developed a ligation-mediated multiplex real-time PCR assay (Check-MDR ESBL kit) for the detection of SHV/TEM

variants (ESBL and non-ESBL) and CTX-M groups. This ligation-mediated multiplex real-time PCR assay can be performed in 3.5 h. Nijhuis and others (2012) compared the Check-MDR ESBL kit with a combination disc test (CDT). The Check-MDR ESBL kit showed sensitivity and specificity of 99.0, 92.2, respectively. The same company launched another commercial multiplex real-time PCR assay (Check-MDR Carba) for rapid detection of carbapenems resistance. The assay detects the presence of bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and $bla_{\text{OXA-48}}$ resistance genes.

2.9 Shiga Toxin-Producing *Escherichia Coli*

Shiga toxin-producing *Escherichia coli* (STEC) are a group of pathogenic *E. coli* that produces Shiga toxin. This group of pathogenic bacteria causes illnesses ranging from mild diarrhea to hemorrhagic colitis and a life-threatening hemolytic uremic syndrome (HUS) (Tarr and others 2005). The STEC group is comprised of more than 50 serogroups, and they are broadly divided into *E. coli* O157 and non-O157 serogroups. *E. coli* O157 was first recognized as a foodborne pathogen in 1982 (Riley and others 1983) and so far, is responsible for causing more than one-third of the total (estimated) STEC illnesses in the United States (Scallan and others 2011). Due to an increasing number of deaths and HUS cases, on September 28, 1994, the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) declared *E. coli* O157:H7 an adulterant, enforcing their zero tolerance policy in ground beef (Taylor 1994). Since then, surveillance activities conducted by the USDA-FSIS at federally inspected plants and retail stores have resulted in 234 recalls. This increase in active surveillance activities may be partly responsible for the recent decline in the incidence of *E. coli* O157 infections (CDC 2011).

Non-O157 STEC serogroups in the last decade have emerged as major food-borne pathogens of concern worldwide (Smith and others 2014). In Canada, they were involved in 63% of the total STEC infections (Thompson and others 2005)**;** while in Denmark, 74% of cases have been related to non-O157 STEC (Nielsen and others 2006) and data from Germany showed the highest incidence rate (82%) (Werber and others 2008). The infection rate in the Netherlands was reported to be 80% (Van Duynhoven and others

2008). Hale and others (2012), reported that around 231,157 cases of infections are caused by this group of pathogens in the United States annually. Out of these, *E. coli* O157 led to 40.3% of infections; whereas, non-O157 serogroups caused the remaining 59.7% of cases. In another study that analyzed the data collected from 2000 to 2010 by the USDA-FSIS, a total of 7694 STEC-related cases was reported, out of which 5688 (73.9%) were linked to the O157 serogroup; whereas, 2006 (35.2%) cases were caused by non-O157 STEC. Therefore, in light of these new data, the USDA, in 2012, declared these six non-O157 STEC serogroups, also known as the "big six non-O157" (O26, O103, O111, O121, O45, and O145) as adulterants (zero tolerance policy) in non-intact raw beef products (USDA 2011). In 2010, for the first time in the United States, the number of infections caused by non-O157 STEC was higher than the *E. coli* O157 (CDC 2011). This observed increase in the number of infections caused by non-O157 STEC is also attributed to the increasing availability and use of diagnostic assays for the detection of STEC (Gould and others 2013).

In a recent study, Luna-Gierke and others (2014) compiled all the data concerning outbreaks caused by non-O157 STEC in the USA (up to 2010). According to the study, a total of 46 outbreaks (38 were single-etiology outbreaks) were reported in 26 states, which resulted in 1727 illnesses and 144 hospitalizations. Out of these 38 single-etiology outbreaks, 66% was caused either by STEC O111 (n=14) or O26 (n=11), followed by O45 (n=4), O103 (n=2), O121 (n=2), O145 (n=2), O104 (n=1), O165 (n=1) and O undetermined (n=1). Eighty-four percent of these infections was either transmitted by food (n=17) or via person-to-person (n=15) contacts. The study also noted dairy products,

produce, meat and childcare centers to be the most common sources of non-O157 STEC infections. Eight (17%) out of 46 outbreaks were multiple-etiology outbreaks. The most common serogroups involved in these multiple-etiology outbreaks were O111 (n=4), followed by O26 (n=2). Apart from these two, O69, O84, O121, O141, O145, and O undetermined were involved in one outbreak each. In addition to non-O157 STEC, other pathogens involved in multiple-etiology outbreaks were *Cryptosporidium* (n=3), STEC O157: H7 (n = 3), *Campylobacter* (n = 3), *Shigella* (n = 1), *Salmonella* serotype Typhimurium (n=1), and norovirus (n=1) (Luna-Gierke and others 2014).

2.9.1 Foodborne non-O157 STEC outbreaks

Forty-five percent (n=17) of all outbreaks caused by non-O157 STEC were foodborne. Out of all non-O157 infections that led to HUS (n=39), 92% (n=36) were caused by non-O157 STEC of food origin. A food vehicle was reported for 12 outbreaks, and 10 were classified in commodities: dairy (n=3), leafy vegetables (n=2), game meat (n=2), beef (n=1), pork (n=1), and fruits or nuts (n=1) (Luna-Gierke and others 2014).

2.9.2 Virulence factors of STEC

Several virulence factors have been reported in STEC, which allow them to infect and cause disease in their hosts. Shiga toxins (*stx*$_1$ and *stx*$_2$) are the most prominent virulence genes found in this group of pathogens. *Escherichia coli stx*$_1$ and Shiga toxin of *Shigella dysenteriae* have almost identical amino acid sequences, and they are also serologically very similar. However, *E. coli stx*$_2$ are a genetically as well as an antigenically diverse group of toxins, which cannot be neutralized by antibodies of either *stx*$_1$ or Shiga toxin antibodies of *S. dysenteriae* (Boerlin and others 1999). Some STEC

strains have the ability to tightly adhere to the epithelial cells of the intestine through the use of an adhesin protein called intimin**.** Such strains induce profound structural modifications in the underlying cells, which is referred to as attaching and effacing lesions. The *eae* (for *E. coli* attaching and effacing) genes encode the intimin protein and are clustered in a pathogenicity island named locus for enterocyte effacement (LEE) (McDaniel and others 1995). Apart from Shiga toxin and *eae*, some pathogenic strains of STEC also contain a plasmid-encoded hemolysin gene (*ehx*A gene), which is called the enterohemorrhagic *E. coli* hemolysin (EHEC hemolysin). This virulence gene is often associated with severe STEC infections in humans (Schmidt and others 1995).

According to a recent review that compiled outbreak data caused by non-O157 STEC, all but one strain in each group (single-etiology and multiple-etiology outbreaks) for which information was available was positive for the *eae* virulence gene, and all strains were also *ehx*A positive (Luna-Gierke and others 2014). Outbreaks caused by stx_2 positive strains (with or without stx_1) were more likely to cause HUS; whereas, the outbreaks due to stx_1-only strains were significantly less likely to end up in HUS (32% vs. 4% of outbreaks, P=0·02) (Luna-Gierke and others 2014).

2.9.3 Methods for the detection of *E. coli* O157 and non-O157 STEC

A partial list of commercially available culture, immunological and DNA-based assays for detecting non-O157 Shiga toxin–producing *Escherichia coli* has been compiled (Table 2.4).

2.9.3.1 Culture-based methods

Culture-based methods for the isolation of *E. coli* O157 and non-O157 STEC is roughly a four-day process that requires multiple incubation steps: pre-enrichment, selective enrichment, selective and differential plating, bacterial isolation, additional biological, serological, or molecular tests for confirmation (Wang and others 2013). Many of the culture media, which are presently being used for the isolation of non-O157 STEC, are modified from those originally developed for the isolation of *E. coli* O157:H7. Novobiocin is used for the selective enrichment of *E. coli* O157:H7 (20 μg/mL), because non-O157 STEC have a lower tolerance towards novobiocin when it is used at a lower concentration (16 μg/mL) (Vimont and others 2007). Sorbitol MacConkey (SMAC) is a commonly used culture medium for the isolation of *E. coli* O157:H7, but recently rhamnose-MacConkey (RMAC) agar was developed that facilitated differential isolation of *E. coli* O26 due to its inherent inability to ferment rhamnose (Hiramatsu and others 2002). Similar to *E. coli* O157:H7, other STEC (e.g. *E. coli* O26, *E. coli* O111, and *E. coli* O145) are also resistant to high-level tellurite, which is used as a selective agent in some commercial agar media for isolation of non-O157 STEC (Hiramatsu and others 2002). At present, there are several commercially available chromogenic agar media for the isolation of non-O157 STEC; for example, modified Rainbow agar (Biolog, Inc.,

Hayward, CA), CHROMagar STEC (CHROMagar Microbiology, France), Chromocult agar (Merck, Germany) and chromID EHEC (bioMerieux, France).

Posse´ and others (2008) described a differential plating medium for the characterization of some non-O157 STEC. This differential medium was based on a complex mixture of carbohydrate sources (sucrose and sorbose), β-D-galactosidase activity, and selective compounds (e.g. bile salts, novobiocin, and potassium tellurite). The medium can be used for the color-based differentiation of STEC O26, O103, O111, and O145. The isolation efficiency of this medium for low levels of non-O157 STEC inoculated food samples (≤100 CFU 25 g^{-1}) ranged from 64.3-100% (Posse´ and others 2008). FDA's Bacteriological Analytical Manual (BAM) has adopted STEC heart infusion washed blood agar with mitomycin C (SHIBAM agar) medium for the differential identification of non-O157 STEC (Wang and others 2013). In a study, SHIBAM agar was tested for accuracy of the medium; 365 of 410 (89%) STEC strains were found to be hemolytic on SHIBAM, whereas 63 of 73 (86.3%) *E. coli* strains that did not produce the Shiga toxin were not hemolytic (Lin and others 2012).

2.9.3.2 Immunological methods

Serotype-specific or Shiga toxin antibodies bind to their targets with very high specificity and aid in the detection of the STEC. The identification of STEC using antibodies can be performed using various methods such as Shiga toxin enzyme immunoassay (EIA), latex agglutination kits, immunoblots, enzyme-linked immunosorbent assays (ELISA), immunomagnetic separation (IMS) and flow cytometry. EIA was quickly accepted in the clinical setting for the identification of Shiga toxin in the

clinical samples. The time required to perform one EIA varies from 20 min to 4 h and it may also involve an overnight enrichment step. To date, the FDA has approved a few Shiga toxin EIA commercial kits, such as the Duopath Verotoxins Gold Labeled Immunosorbent Assay (GLISA) (Merck, Germany) and ImmunoCard STAT! EHEC (Meridian BioScience, Inc., USA).

O-antigens of *E. coli* are the most reliable markers for the detection and identification of non-O157 STEC serogroups. The latex agglutination assay uses antibodies targeting specific O-antigens of non-O157 STEC. These rapid and convenient assays are commonly used for the identification of non-O157 STEC e.g. SDIX beads (Newark, USA), Abraxis (Philadelphia, USA). IMS is a very simple and effective method for selective enrichment of specific pathogens from a complex sample matrix with high background microflora. IMS uses microscopic paramagnetic beads that are coated with specific antibodies and can be separated by the application of a magnetic field. The process helps in concentrating specific pathogens and eliminating inhibitors from the complex sample matrix. The IMS efficiency of pathogen recovery varies depending on the location of an antigen on the pathogen, the antigenic expression, the antigen-antibody affinity and the physicochemical properties of the food matrix (Dwivedi and Jaykus 2011). Flow cytometry and ELISA are other less commonly used immunological methods used for the detection of different STEC serogroups (Hegde and others 2012a, b).

2.9.3.3 Nucleic acid amplification based assay

The nucleic acid amplification assay targets and amplifies specific genes of the *E. coli* O157:H7 and non-O157 STEC serogroups and facilitates their detection. Apart from conventional end point PCR assay, a few other assays based on nucleic acid amplification have been described (e.g. qPCR assay, loop-mediated isothermal amplification assay, Luminex xTAG assay). The serotype-specific genes *wzx* and *wzy* that encodes the O-antigen flippase and the O-antigen polymerase are the most commonly used for the development of a PCR-based assay for the identification of non-O157 STEC serogroups (DebRoy and others 2011) whereas, the *uid*A gene is the most common target for the identification of *E. coli* O157:H7 (Cebula and others 1995). Additional genes, such as *wbd* and *wzm* from the O-antigen cluster can also be targeted for the identification of non-O157 STEC (DebRoy and others 2011). In addition to the serotype-specific genes, nucleic acid amplification assays are also used for the identification of virulence genes. The most frequently targeted virulence genes among STEC are stx_1, stx_2, and intimin. Over the years, many variants of these three virulence genes have been identified e.g. stx_1 subtypes (stx_{1a}, stx_{1c}, and stx_{1d}), stx_2 subtypes (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, and stx_{2g}) (Feng and others 2011) and 21 intimin subtypes (Feng and others 2011; Blanco and others 2010). Hence, the newer PCR assays were designed to accommodate more numbers of virulence gene variants. The hemolysin gene (*hly*A) is another virulent gene present in the pathogenicity islands and has been associated with STEC strains isolated from outbreaks. Multiplex PCR and multiplex qPCR assays are frequently performed for the simultaneous detection of these increasing numbers of STEC serogroups and their

virulence genes. Most nucleic acid amplification based assays can be performed in between 2-4 h and their sensitivity is in the range of $10–10^3$ CFU/reaction, but the performance of each assay varies when they are evaluated independently.

Table 2.4 Partial list of commercially available assays for detecting non-O157 Shiga toxin–producing *Escherichia coli*

Method	Product name	Non-O157 STEC	Manufacturer
Culture - Media	CHROMagar STEC	Top 6 and other	CHROMagar Microbiology, Paris, France
	Rainbow Agar O157	Top 6 and others	Biolog, Inc., Hayward, CA
	SHIBAM agar	All STEC producing enterohemolysin	Hardy Diagnostics, Santa Maria, CA
	ChromID EH EC	Top 6 and others	bioMérieux, France
PCR	IEH Non-O157 STEC detection and identification method [a]	Top 7	IEH Laboratories, WA, USA
Real-time PCR	MLG 5B.05	Top 6	United States Department of Agriculture
	Assurance GDS Top 7 STEC [a]	Top 7	BioControl Systems, Inc., Bellevue, WA
	Assurance GDS MPX Top 7 STEC method [a]	Top 7	BioControl Systems, Inc., Bellevue, WA
	BAX System Real-Time PCR STEC Suite[a]	Top 6	DuPont Qualicon, Wilmington, DE
	GeneDisc	Top 7	Pall Corporation, Port Washington,

	STEC Top 7 [a]		NY
	GeneDisc EHEC 5 ID[b]	H7, O103, O111, O26, O145	Pall Corporation, Port Washington, NY
	iQ Check VirX and iQ Check SerO STEC [a]	Top 6	Bio-Rad Laboratories, Hercules, CA
	VTEC MP-0510	O26, O103, O111, O145	Multiplicom N.V., Niel, Belgium
LAMP	Loopamp VTEC Detection Kit	All STEC	Eiken Chemical Co., Tokyo, Japan
PCR-mass spectrometry	NeoSEEK STEC Confirmation[a]	Top 6	Neogen Corp., Lansing, MI

[a]No-objection letters were issued by U.S. Department of Agriculture Food Safety and Inspection Service for these non-O157 STEC assays as of January 8, 2013 (USDA, 2012d).

[b]The assays have been validated by the AOAC's Performance Tested Methods Program.

2.9.3.3.1 Advantages and limitations of nucleic acid amplification assays

When nucleic acid amplification assays are compared with immunological assays, PCR based methods are more superior as they allow multiplexing of a higher number of genes (virulence and species-specific genes), generating much-detailed data in a similar amount of time. However, there are a few limitations of PCR-based methods. Detection of a virulence gene by a PCR assay is not a guarantee of gene expression (Feng and others 2011). Further, PCR results also require isolation of the contagion by standard culture-based methods. Another major drawback of PCR based methods is their inherent

inability to differentiate between live and dead cells. The use of viability dyes (e.g. ethidium monoazide, propidium monoazide) has helped to address this problem (Liu and Mustapha 2014; Wang and others 2009). In addition to the above-mentioned limitations, food samples contain a large number of PCR inhibitors. Hence, in the absence of a proper sample preparation, the nucleic acid amplification assay can generate false negative results.

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Chapter 3 Multiplex TaqMan® Detection of Pathogenic and Multi-Drug Resistant *Salmonella*

(Adapted from International Journal of Food Microbiology)

ABSTRACT

Overuse of antibiotics in the medical and animal industries is one of the major causes for development of multi-drug-resistant (MDR) food pathogens that are often difficult to treat. In the past few years, higher incidences of outbreaks caused by MDR *Salmonella* have been increasingly documented. The objective of this study was to develop a rapid multiplex real-time polymerase chain reaction (PCR) assay for simultaneous detection of pathogenic and MDR *Salmonella* spp. A multiplex TaqMan® real-time PCR was designed by targeting the invasin virulence gene (*inv*A), and four commonly found antibiotic resistance genes, *viz*. ampicillin (bla_{PSE}), chloramphenicol (*flo*R), streptomycin (*aad*A2) and tetracycline (*tet*G). To avoid false negative results and to increase the reliability of the assay, an internal amplification control (IAC) was added which was detected using a locked nucleic acid (LNA) probe. In serially diluted (5 ng-50 fg) DNA samples, the assay was able to detect 100 genomic equivalents of *Salmonella*, while in a multiplex format, the sensitivity was 1000 genomic equivalents. The assay performed equally well on artificially contaminated samples of beef trim, ground beef of different fat contents (73% lean: 27% fat, 80% lean: 20% fat, 85% lean: 15% fat and 93% lean: 7% fat), chicken rinse, ground chicken, ground turkey, egg, spinach and tomato.

While the detection limit for un-enriched inoculated food samples was 10^4 CFU/g, this was improved to 10 CFU/g after a 12-h enrichment in buffered peptone water, with 100% reproducibility. The multiplex real-time assay developed in this study can be used as a valuable tool to detect MDR virulent *Salmonella*, thus enhancing the safety of food.

3.1 INTRODUCTION

Salmonella outbreaks in the U.S. are generally associated with contaminated products of animal origin, such as ground beef, turkey and eggs (CDC 2010). *Salmonella* leads to the second highest number of foodborne infections in the United States, making it a pathogen of major concern. On top of that, the emergence of antibiotic resistant strains of *Salmonella* is an even greater challenge. In the last few decades, widespread use of antibiotics in the medical field and animal husbandry has been thought to lead to an emergence of antibiotic resistant strains of *Salmonella* that were responsible for some of the major *Salmonella* outbreaks worldwide. Antimicrobials administered to dairy or meat cattle frequently belong to the same class as those used in humans (Aarestrup and others 2008). Alarmingly, *Salmonella* Enteritidis and *Salmonella* Typhimurium isolated from animal feed have been reported to be highly resistant to antimicrobials (Li and others 2012). In addition to resistance towards commonly used antibiotics, such as ampicillin, streptomycin, sulfonamides, trimethoprim and tetracycline, there have been several reports of resistance to the cephalosporin class of antibiotics which were recently approved for the treatment of severe cases of *Salmonella* infection in humans (Sjölund-Karlsson and others 2010). Antibiotic resistant *Salmonella* can cause severe infections that could lead to prolonged hospitalizations or even death. The multiple drug resistant

(MDR) *S.* Typhimurium DT104 is known to show resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline. In the U.S., human isolates of *S.* Typhimurium DT104 (R-type ACSSuT) increased from 9% in 1990 to 32% in 1996 when this strain led to the first major outbreak of human salmonellosis (Akkina and others 1999). Apart from an increasing rate of antibiotic resistance among *Salmonella*, there has also been an increase in the rate of antibiotic resistance in other members of the Enterobacteriaceae family. These MDR members of Enterobacteriaceae have become one of the biggest challenges among hospital-acquired infections (Cohen 2013). The Study for Monitoring Antimicrobial Resistance Trends (SMART) has also shown an increase in cephalosporin resistance in *Escherichia coli* and *Klebsiella pneumoniae* (Hoban and others 2012), making this a critical problem for vulnerable patients.

Multidrug resistance in bacteria is frequently linked to genetic elements called integrons. These genetic elements play an important role in dissemination of a number of antibiotic resistance genes. There are three major classes of integrons, of which the class I integrons are most commonly found and extensively studied in *Salmonella* serovars of animal origin (Molla and others 2007, Zhao and others 2007). Integrons are natural expression systems that allow insertion and expression of various antibiotic resistance genes by their own promoters, thus making the inserted gene functional. Integron 1 has been shown to harbor up to 10 different antibiotic resistance gene cassettes. These gene cassettes were reported to confer resistance towards aminoglycosides, ampicillin, rifampicin, spectinomycin, streptothricin, sulfasulfonamides, tetracycline and

trimethoprim (White and others 2001). Apart from integrons, all pathogenic strains of *Salmonella* have also been reported to possess the *inv*A virulence gene. This gene is located on the *Salmonella* pathogenicity island 1 (SPI-1) of *Salmonella* spp., which encodes Type III secretion system proteins. This, along with other genes regulates the expression of other virulence genes present on SPI-1 involved in pathogenicity (Boyd and others 1997).

Real-time PCR methods have been increasingly used as a rapid and sensitive technique for detection of food pathogens. With the use of TaqMan® probes, real-time PCR offers an advantage of rapid, sensitive and specific detection of food pathogens, while avoiding cross-contamination from other closely related bacteria (Malorny and others 2007). TaqMan® real-time PCR assay, in addition to the use of a specific primer pair, also employs a single stranded fluorescent labeled molecule called a TaqMan® probe. A TaqMan® probe is usually 10-15 bp long and is labeled with a fluorescent dye (also known as reporter) on the 5´ end and a quencher molecule on the 3´ end. When a reporter dye of the TaqMan® probe is excited with a light source of appropriate wavelength, and the reporter dye absorbs the light energy and emits a fluorescent signal. If the probe is intact, the quencher molecule absorbs the emitted fluorescent signal resulting in generation of no fluorescent signal. In a TaqMan® assay, a target region of genomic DNA is amplified using a specific primer pair; during the denaturation step amplicons generated in the last PCR cycle is converted into single-stranded DNA. The TaqMan® probe hybridizes to these single-stranded PCR products. In the subsequent PCR cycle, primers anneal to the single stranded DNA templates and are extended by the Taq

polymerase. The 5´– 3´exonuclease activity of Taq polymerase cleaves the TaqMan® probe, separating the reporter dye and quencher molecules leading to an increase in the fluorescent signal. As the amount of amplicons exponentially increases in the PCR reaction, so does the amount of fluorescent signal generated in the PCR reaction tube. At end of the PCR reaction, the obtained fluorescent signal is used to construct an amplification plot (ΔRn vs cycle) and can be used for the quantification of the target pathogen. In a multiplex TaqMan® assay, multiple targets can be simultaneously amplified and detected using probes labeled with different fluorescent dyes.

Incorporation of locked nucleic acids (LNA) molecules in the probe has further helped to increase the sensitivity of these assays. LNA are nucleic acid analogs containing a locked bicyclic furanose unit in an RNA-mimicking sugar conformation. These molecules allow for better base stacking and, therefore, show a higher stability and affinity towards LNA, DNA and RNA targets. LNA residues confer a relative degree of resistance towards exo- and endonucleases (Kumar and others 1998; Whitcombe and others 1999). LNA modification in a 5′-nuclease probe leads to a significant increase in the melting temperature (T_m) of the probe, allowing for designing of shorter fluorescent probes with higher specificity (Tolstrup and others 2003).

To date, limited studies have been done on rapid detection of antibiotic resistant *Salmonella*. Chiu and others (2006) described a multiplex PCR method for the detection of antibiotic resistant strains of *S.* Typhimurium. Khan and others (2011) developed intercalating dyes-based multiplex real-time PCR for detection of bacteria showing resistance towards quaternary ammonium compounds, tetracycline, gentamicin and

chloramphenicol. Because of the importance of MDR *Salmonella* in food safety, a rapid and specific method for simultaneous detection of pathogenic and antibiotic resistant strains of *Salmonella* is much needed. The aim of this study, therefore, was to develop a novel multiplex TaqMan® real-time PCR based method for the detection of pathogenic as well as antibiotic resistant strains of *Salmonella*.

3.2 MATERIAL AND METHODS

3.2.1 Bacterial strains

Standard cultures of *S.* Typhimurium 14028, *S.* Typhimurium 13311 and *S.* Enteritidis 13076 were procured from ATCC (Rockville, MD), *S.* Typhimurium LJH 666, *Salmonella* Newport 692, *Salmonella* Agona LJH 1132, *S.* Agona LJH 1122 and *S.* Typhimurium var. Copenhagen LJH 788 were generously provided by Dr. Linda Harris, University of California-Davis. Cultures were grown at 37 °C in Tryptic Soy broth supplemented with 0.5% yeast extract (TSBY) (Difco Labs., MD, USA). Twenty-two *S.* Enteritidis isolates were from the University of Missouri Food Microbiology Lab culture collection.

3.2.2 Phenotypic and genotypic determination of antibiotic resistance

All *Salmonella* cultures were screened for antibiotic resistance by the standard agar disk diffusion assay according to the criteria suggested by the Clinical Laboratory Standards Institute (Watt and others 2008), with the following quality control strains: *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* 25923. Isolates were also

tested for the presence of ampicillin, chloramphenicol, streptomycin and tetracycline resistance genes by conventional PCR, as described by Chiu and others (2006).

3.2.3 Bacterial DNA isolation

Genomic DNA was isolated from actively growing cells in TSBY broth. One milliliter of an overnight culture was centrifuged at 6,000 ×*g* and the obtained cell pellet was resuspended in 100 µl of sterile MilliQ water. The DNA from the obtained cell pellet was isolated using a Generation Capture Column Kit (Qiagen, Hilden, Germany). Concentrations and purity of thus obtained DNA was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington).

3.2.4 Primers and probes

Primers for the antibiotic resistance genes, chloramphenicol (*flo*R), ampicillin (*pse*), streptomycin (*aad*A2) and tetracycline (*tet*G) were designed on GenBank accession No. AY339985.2 and GU987052, as described by Chiu and others (2006). The *inv*A gene primer was modified using GenBank accession No U43239.1. Primers and TaqMan® probes for the amplification of antibiotic resistance and virulence genes were designed using Primer Express software version 3.0 (Applied Biosystems, Foster City, CA, USA) and Primer3 software (http://primer3.wi.mit.edu) (Rozen and Skaletsky 2000). The specificity of the designed primers was tested using NCBI's Primer BLAST tool. The melting temperature (T_m) of the LNA probe was estimated using the Exiqon T_m prediction program (Tolstrup and others 2003). Fluorophores for labeling the probes were selected based on two criteria described by Molenkamp and others (2007). First, each fluorophore was detected by separate detectors and secondly, the excitation and

emission wavelengths of each fluorophore were separated by 30 nm. This approach allowed minimal cross-talk between the different detectors and provided a platform for quantitative detection of multiple targets in the sample.

Dual-labeled probes, minor groove binding (MGB) TaqMan® probes and LNA-dual labeled probe were commercially synthesized by IDT (Coraville, IA, USA), Applied Biosystems and Sigma Life Science (Sigma Aldrich, TX, USA), respectively. PCR amplification was performed in a 20 µL reaction volume using 2X TaqMan® Universal PCR Master Mix with no AmpErase UNG, 20 ng of genomic DNA, and the respective primer concentrations, as shown in Table 3.1, on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A three-step protocol was used for DNA amplification: initial denaturation at 95°C for 10 min, and 40 cycles of 95°C for 30 s, 58°C for 45 s and 72°C for 45 s. An amplification plot was constructed between ΔRn versus cycles. Because of the limited number of channels available on the ABI 7500 instrument, this multiplex assay was designed in two sets: the first set amplifying the *inv*A virulence gene, *aad*A2 streptomycin resistance gene, *bla*PSE ampicillin resistance gene and an internal amplification control (IAC); and the second targeting *floR* chloramphenicol resistance gene, *tet*G tetracycline resistance gene and the IAC.

3.2.5 Standardization of IAC concentration

A 98-base long single stranded DNA sequence was designed to serve as the IAC for the multiplex real-time TaqMan® assay developed in this study. The IAC molecule was initially dissolved in nuclease-free water to a concentration of 100 ng/µL. It was further 10-fold serially diluted to a concentration of 1 fg/µl. Multiplex real-time PCR was

performed with each dilution to optimize IAC concentration required for the assay. The minimum concentration of IAC molecule that gave reproducible results, without inhibiting the amplification of any targets of the multiplex real-time PCR reaction was selected for further studies.

3.2.6 Specificity of the invasin (*inv*A) primer

The specificity of the modified invasin primer set was validated using 73 bacterial strains spread across 20 genera and 31 species of Gram-negative and Gram-positive bacteria, including *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Bifidobacterium*, *Citrobacter*, *Clostridium*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Lactobacillus*, *Leuconostoc*, *Listeria*, *Micrococcus*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Staphylococcus* and *Yersinia.*

Table 3.1 Primers and probes for real-time PCR assays.

Gene	Primer Sequence (5'-3')	Product size (bp)	Primer conc.	Reference
Str F	CAGCCA(T/c)GATCGACATTGATCT	66	0.16 μM	
Str R	CCAAGGCAACGCTATGTTCTC			This study
Str-Probe	VIC-CTGCTTACAAAAGC-NFQ		0.17 μM	
Tet F	CGGTACTTCTGGCTTCTCTT	148	0.22 μM	
Tet R	GAATCGGCAATGGTTGAG			This study
Tet-Probe	FAM-CAGGAGCCG/ZEN/CAGTCGATTACACG-Iowa Black® FQ		0.1 μM	
Chl F	GGCAGGCGATATTCATTACT	197	0.12 μM	
Chl R	CGAGAAGAAGACGAAGAAGG			This study
Chl-Probe	VIC- CTAAAGCCGACAGTGTA-NFQ		0.12 μM	
Amp F	GATTTGGTGCTCGGAGTATT	92	0.12 μM	
Amp R	CATTGAAGCCTGTGTTTGAG			This study
Amp-Probe	NED-CTTGATGCTCACTCCA-NFQ		0.1 μM	
Inv F	CCAGTTTATCGTTATTACCAAAGG	200	0.15 μM	This study
Inv R	ATCGCACCGTCAAAGGA(A/g)C			
Sal-invA-SO-WH Probe	FAM-CTCTGGATGGTATGCCCGGTAAACA-BHQ1		0.2 μM	Anderson and others 2011
IAC-Probe	Cy5-CTC[+T]TGCTC[+T][+C]T[+T]C[+C][+T]-BHQ3		0.1 μM	This study
IAC	TCGCCTGTATTTCCAGTTTATCGTTATTACCAAAGGT TTGATCTCTTGCTCTCTTCCTACCGTTCAAGYTCCTTT GACGGTGCGATGATTAACGGCAG		0.1 pg	This study

3.2.7 Sensitivity of the real-time PCR assay

To determine the sensitivity of the developed multiplex assay, genomic DNA from a MDR isolate, *S.* Typhimurium DT104 strain LJH 788, was isolated using Generation Capture Column Kit (Qiagen). DNA stock solution (50 ng/µL) was ten-fold serially diluted from 5 ng/reaction to 50 fg/reaction of DNA. *Salmonella* genomic copy number equivalents were calculated based on the *S.* Typhimurium genome size. McClelland and others (2001) reported the genome size of *S.* Typhimurium as 4951 kilobase pairs. The *Salmonella* genomic equivalent can be calculated using the formula: m × n (1.013×10^{-21} g/bp), where "m" is the mass and "n" is the number of base pairs in the *Salmonella* genome. Using the aforementioned equation, one *S.* Typhimurium double-stranded genomic DNA weighs about 5.0 fg. Thus, one genomic equivalent of *Salmonella* DNA is equal to 5.0 fg of *Salmonella* genomic DNA (McClelland and others 2001). Each serially diluted DNA was used in duplicates in the real-time PCR for construction of standard curves and determination of the assay's amplification performance. The reaction was performed in duplex format with the specific gene targets and a specially designed IAC (Table 3.1). Because the amount of PCR product is doubled at the end of each cycle in a PCR reaction, the PCR efficiency should ideally be 100%. PCR efficiency is calculated from the slope of the standard curve using the formula: E = 10(-1/slope)-1.

A PCR reaction is considered good if the reaction efficiency ranges between 90% and 110%, which corresponds to a standard curve slope between -3.58 and -3.10. In this study, the PCR efficiency of each gene was calculated using the StepOne® Software

V2.2.2. Microsoft Excel software was used to compute the regression coefficients and standard deviations for the standard curves.

3.2.8 Preparation of artificially spiked samples

Ground beef of different fat contents (73% lean: 27% fat, 80% lean: 20% fat, 85% lean: 15% fat and 93% lean: 7% fat), whole chicken, ground chicken, ground turkey, raw egg, spinach and tomato were purchased from local supermarkets. Beef trim was obtained from the University of Missouri Meat Laboratory. These food products were tested for the presence of *Salmonella* using a standard culture-based protocol (Andrews and others 2007). Chicken rinse was prepared as described by Wang and Mustapha (2010). Whole pre-cut chickens were purchased from a local supermarket. Each of the chickens were sampled and tested for the presence of *Salmonella* by standard culture-based methods (Andrews and others 2007) and real-time PCR using *Salmonella* specific primers designed in this study. Each of the whole chicken was washed with 500 mL of buffered peptone water (BPW) (Remel, Lenexa, KS, USA) to obtain a chicken rinse. The chicken rinse was distributed in 50 mL portions in separate pre-sterilized tubes and was used to perform experiment. The aerobic plate count of each food sample was estimated using standard serial dilution and the pour plate method on plate count agar (Difco Labs., MD, USA).

Twenty-five grams of ground beef with the highest fat content (27%) was mixed with 225 mL of buffered peptone water. The homogenate was inoculated with 10 to 10^7 CFU/g of MDR isolate *S*. Typhimurium var. Copenhagen LJH 788 and stomached for 2 min. Samples were used for construction of a standard curve and determination of the

sensitivity of the assay. Upon inoculation of cells at 0 h, 2 mL of broth was withdrawn from each stomacher bag and briefly centrifuged (0.1 ×*g* for 1 min) to separate out beef particles from the broth. The supernatant was transferred to a fresh centrifuge tube and further centrifuged at 16,100 ×*g* for 3 min to separate the bacterial cells from the enrichment broth. The obtained cell pellets were resuspended in 100 µL of PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) and heated at 100 °C for 10 min in a dry bath, which resulted in lysis of bacterial cells. Samples were later removed from the dry bath and cooled at room temperature for 2 min and centrifuged at 13,200 ×*g* (2 min) to sediment the cell debris. Fifty microliters of supernatant (containing DNA) was transferred to a fresh eppendorf tube and was used for the real-time PCR to construct a standard curve for each target in duplex format with the IAC.

Twenty-five grams each of beef trim, ground beef of different fat contents (73% lean: 27% fat, 80% lean: 20% fat, 85% lean: 15% fat and 93% lean: 7% fat), chicken, ground chicken, ground turkey, raw egg, spinach and tomato were placed in filtered stomacher bags (Fisherbrand, Houston, TX) and mixed with 225 mL BPW. BPW has been reported to be better than universal pre-enrichment broth for enrichment of food samples contaminated with *Salmonella* (Hein and others 2006). The samples were inoculated with *S*. Typhimurium LJH 788 at different concentrations ranging from 1-10^5 CFU/mL. One negative (un-inoculated) control for each sample was also included in the study. To determine the enrichment time required for detection of food samples contaminated with low levels of *Salmonella*, 1 mL of spiked samples was withdrawn

after intervals of 0, 12 and 24 h. DNA was isolated from the enriched sample using PrepMan® Ultra Sample Preparation Reagent, and used for the detection of *Salmonella* by TaqMan® real-time PCR.

3.3 RESULTS

3.3.1 Antibiotic resistance of tested *Salmonella* strains

Standard agar disk diffusion assay results showed that *S.* Typhimurium 14028, *S.* Typhimurium 13311, *S.* Typhimurium LJH 666, *S.* Newport 692, *S.* Agona LJH 1132 and *S.* Agona LJH 1122 were resistant to streptomycin and tetracycline. *S.* Typhimurium var. Copenhagen LJH 788 was found to be resistant to all four antibiotics tested, *viz* streptomycin, ampicillin, chloramphenicol and tetracycline (Table 3.2). This strain was, thus, considered to be an MDR isolate. On the other hand *S.* Enteritidis 13076 was found to be sensitive to all four mentioned antibiotics. The antibiotic resistance profiles of other *Salmonella* isolates are shown in Table 3.2. These isolates also tested positive for the presence of the respective antibiotic resistance genes by PCR, which mostly paralleled their respective antibiotic resistance phenotypic patterns. Based on these results, *S.* Typhimurium var. Copenhagen LJH 788 was subsequently used for the standardization of a multiplex 5′ nuclease assay for the detection of virulent and antibiotic resistant *Salmonella*.

Table 3.2 Phenotypic and genotypic antibiotic resistance profile of *Salmonella* isolates.

Isolate	**Antimicrobial susceptibility[1] and real-time PCR[2] results**							
	STR[1]	***aadA2*[2]**	**TET[1]**	***tetG*[2]**	**AMP[1]**	***Pse*[2]**	**CHL[1]**	***floR*[2]**
S. Enteritidis F3-1	R	+	R	+	R	-	S	-
S. Enteritidis F3-2	R	+	R	+	R	-	S	-
S. Enteritidis F3-6	R	+	R	+	R	-	S	-
S. Enteritidis F3-7	R	+	R	+	S	-	S	-
S. Enteritidis F3-8	R	+	R	+	R	-	S	-
S. Enteritidis F3-9	R	+	R	+	S	-	S	-
S. Enteritidis F3-10	R	+	R	+	S	-	S	-
S. Enteritidis G5-02	R	+	R	+	R	+	R	+
S. Enteritidis G5-08	R	+	R	+	R	-	S	-
S. Enteritidis G5-09	R	+	R	+	S	-	S	-
S. Enteritidis G5-10	R	+	R	+	S	-	S	-
S. Enteritidis I4-1	R	+	R	+	S	-	S	-
S. Enteritidis I4-2	R	+	R	-	S	-	R	-
S. Enteritidis I4-3	R	+	R	-	S	-	S	-
S. Enteritidis I4-4	R	+	R	-	R	+	R	-
S. Enteritidis I4-5	R	+	R	+	R	+	S	-
S. Enteritidis I4-6	R	+	R	+	S	-	S	-
S. Enteritidis I4-7	R	+	R	+	S	-	S	-
S. Enteritidis I4-8	R	+	R	+	S	-	S	-
S. Enteritidis I4-9	R	+	R	+	S	-	S	-
S. Enteritidis I4-10	R	-	R	-	S	-	S	-
S. Enteritidis I4-11	R	+	R	+	S	-	S	-
S. Typhimurium 13311	R	+	R	+	S	-	S	-
S. Typhimurium 14028	R	+	R	+	S	-	S	-
S. Enteritidis 13076	S	-	S	-	S	-	S	-
S. Typhimurium LJH 666	R	+	S	-	S	-	S	-
S. Newport 692	R	+	R	+	S	-	S	-
S. Agona LJH 1132	R	+	R	+	S	-	S	-
S. Agona LJH 1122	R	+	R	+	S	-	S	-
S. Typhimurium LJH 788	R	+	R	+	R	+	R	+

3.3.2 Specificity of the assay

The PCR cycling conditions of the primers designed for this assay were standardized using Veriti gradient thermal cycler (Applied Biosystems, Foster City, CA, USA). All the primers were found to perform efficiently at an annealing temperature of 58 °C. A multiplex real-time PCR assay was standardized for the multiplex detection of virulent as well as antibiotic resistant *Salmonella*. The specificity of the modified *inv*A gene primers was tested with genomic DNA of a wide range of Gram-negative and Gram-positive bacteria. No cross-reactivity was observed in any of the other 73 bacterial strains evaluated while all 30 *Salmonella* strains tested were positively detected by the assay (data not shown).

3.3.3 Sensitivity of the assay

The duplex assay for the detection of virulent strains of *Salmonella*, using the *inv*A gene primer/probe with IAC was able to successfully detect as low as 100 genomic equivalents of *Salmonella* from a 10-fold serially diluted DNA sample. The standard curve for this assay was found to be linear from 2-6 log genomic equivalents of *Salmonella* with a regression coefficient of 0.98 and a reaction efficiency of 97.948% (Figure 3.1). For other antibiotic resistant genes being targeted in this assay, the detection limit was found to be 500 fg/reaction (100 genomic equivalents). The obtained regression coefficient and reaction efficiency of these targets, as shown on Figure 3.1, were as follows: ampicillin (R^2 = 0.97, PCR efficiency = 97.51%), chloramphenicol (R^2 = 0.97, PCR efficiency = 96.03%), streptomycin (R^2 = 0.99, PCR efficiency = 91%) and

tetracycline (R^2 = 0.93, PCR efficiency = 110%). The sensitivity of the multiplex assay was found to be 5 pg/reaction (1000 genomic equivalents).

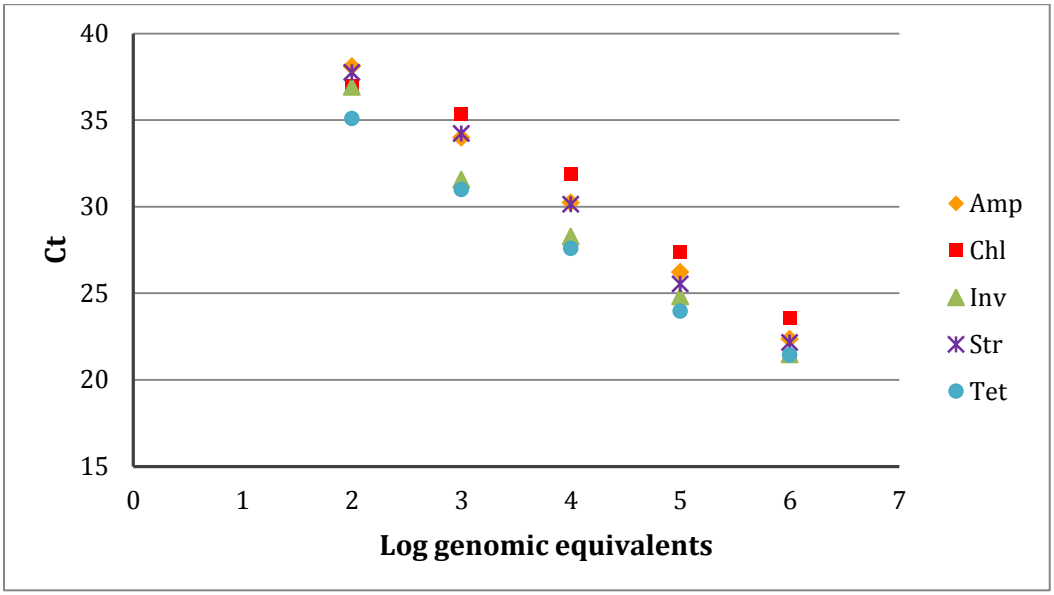

Figure 3.1 Standard curve constructed using serially diluted DNA: ampicillin (Amp), chloramphenicol (Chl), invasin (Inv), streptomycin (Str) and tetracycline (Tet) constructed using serially diluted DNA. 1 Genomic equivalent = 5 fg of *Salmonella* genomic DNA.

A standard curve constructed from spiked beef sample for each of the five targets in duplex format with IAC was found to be linear from log 2 to log 7 CFU/g (Figure 3.2). The obtained regression coefficient and reaction efficiency of these targets were as follows: invasin (R^2 = 0.97, PCR efficiency = 72.34%), ampicillin (R^2 = 0.94, PCR efficiency = 66.81%), chloramphenicol (R^2 = 0.97, PCR efficiency = 75.59%), streptomycin (R^2 = 0.99, PCR efficiency = 106%) and tetracycline (R^2 = 0.93, PCR efficiency = 114.89%).

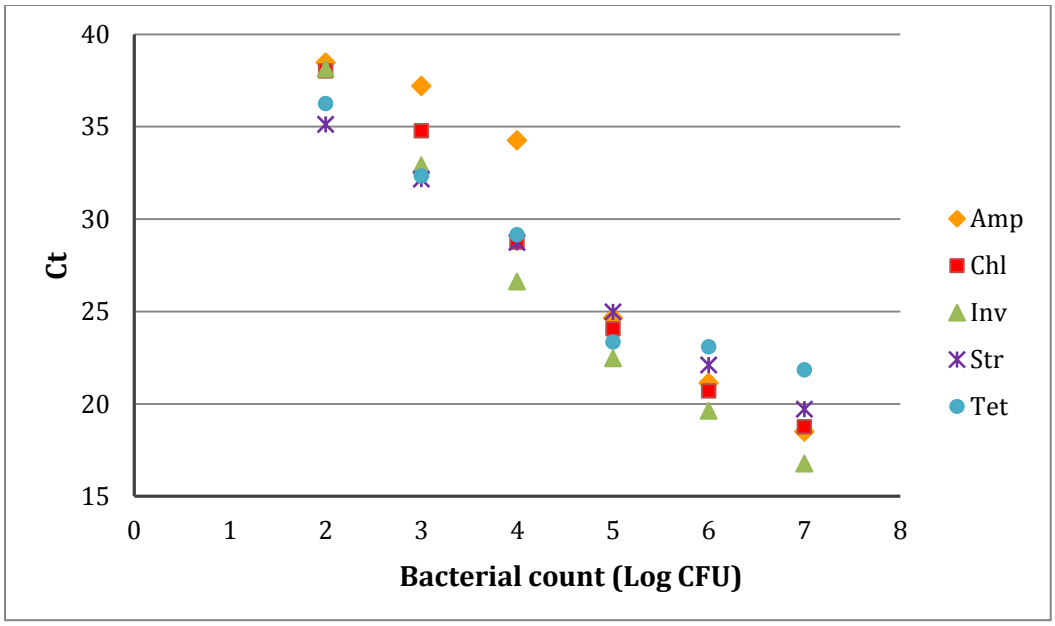

Figure 3.2 Standard curve constructed using a spiked beef sample: (Amp), chloramphenicol (Chl), invasin (Inv), streptomycin (Str) and tetracycline (Tet).

3.3.4 Detection of antibiotic resistant *Salmonella* in spiked samples

Except for ground turkey, all other food samples obtained from local supermarkets were found to be negative for *Salmonella*. Standard aerobic plate count of food samples used in this study were as follows: beef trim (5.2×10^4 CFU/g), ground beef (73% lean: 27% fat) (4.5×10^2 CFU/g), ground beef (80% lean: 20% fat) (8.2×10^3 CFU/g), ground beef (85% lean: 15% fat) (8.3×10^3 CFU/g), ground beef (93 % lean: 7% fat) (7.0×10^2 CFU/g), ground chicken (6.2×10^7 CFU/g), ground turkey (1.1×10^3 CFU/g), egg (> 10 CFU/g), spinach (3.1×10^8 CFU/g) and tomato (1.1×10^7 CFU/g).

Table 3.3 Multiplex real-time PCR results of artificially contaminated food samples.

Sample	**Spiked *Salmonella* concentration (CFU/mL)**	**Real-time PCR result after enrichment**		
		0 h	12 h	24 h
Beef trim	10	-	+	+
	10^2	-	+	+
	10^3	-	+	+
	10^4	+	+	+
	10^5	+	+	+
Ground beef (73% lean: 27%fat)	10	-	+	+
	10^2	-	+	+
	10^3	-	+	+
	10^4	+	+	+
	10^5	+	+	+
Ground beef (80% lean: 20%fat)	10	-	+	+
	10^2	-	+	+
	10^3	-	+	+
	10^4	+	+	+
	10^5	+	+	+
Ground beef (85% lean: 15%fat)	10	-	+	+
	10^2	-	+	+
	10^3	-	+	+
	10^4	+	+	+
	10^5	+	+	+
Ground beef (93% lean: 7%fat)	10	-	+	+
	10^2	-	+	+
	10^3	-	+	+
	10^4	+	+	+
	10^5	+	+	+
Ground chicken	10	-	+	+
	10^2	-	+	+
	10^3	-	+	+

	10^4	+	+	+
	10^5	+	+	+
Egg	10	-	+	+
	10^2	-	+	+
	10^3	-	+	+
	10^4	+	+	+
	10^5	+	+	+
Spinach	10	-	+	+
	10^2	-	+	+
	10^3	-	+	+
	10^4	+	+	+
	10^5	+	+	+
Tomato	10	-	+	+
	10^2	-	+	+
	10^3	-	+	+
	10^4	+	+	+
	10^5	+	+	+

All *Salmonella*-negative food samples were artificially inoculated with the MDR isolate, *S.* Typhimurium var. Copenhagen LJH 788 at five different concentrations, starting from 10 CFU/mL to 10^5 CFU/mL. The multiplex assay for all five targets was able to detect *Salmonella*, in samples spiked with10^4 CFU/mL or higher without any enrichment (Table 3.3). Food samples spiked with 10 to 10^3 CFU/mL required an enrichment steps to allow the multiplication of *Salmonella* in the spiked food samples and were detected after an enrichment period of 12 h. Different fat percentages in ground beef samples were not an inhibitory factor for this assay (Table 3.3).

3.4 DISCUSSION

Multiplex real-time PCR has the ability to detect more than one target in a single reaction, thus saving time and cost. The *inv*A primer set designed by Rahn and others

(1992) is generally considered as a standard set of primers for detection of pathogenic strains of *Salmonella* spp. by the FOOD-PCR project (Malorny and others 2003). The primer set has been validated to show 99.4% specificity with 630 *Salmonella* strains and 142 non-*Salmonella* strains (Rahn and others 1992) and has also been tested with 100 other *Salmonella* strains from both *Salmonella enterica* and *Salmonella bongori* species (Anderson and others 2011). This published *inv*A primer-probe set was initially tested for its compatibility with other primers and probes being used in our multiplex assay. However, the results with this *inv*A primer and probe set were not reproducible in either uniplex or multiplex format. This might have been due to two reasons: first, the T_m difference in the forward and reverse primers is very high, and secondly, the annealing temperature of the probe was lower than that of the forward primers. As a rule of thumb, the probe annealing temperature should be 5-10 °C higher than the primer annealing temperature. This T_m mismatch between the primers and probe is not ideal for real-time PCR amplification. Even the amplicon size of this *inv*A gene primer set (284 bp) was on the higher side of the generally recommended amplicon size for real-time PCR (100-200 bp). As this primer and probe set was already validated by various labs for their specificity towards *Salmonella*, some minor changes in the primer set were made in our study, to allow for the real-time assay to be more thermodynamically stable. The annealing temperature of the reverse primer was adjusted by reducing it by one base from the 3′-end and a new forward primer was designed using Primer3 software (Rozen and Skaletsky 2000). This modified *inv*A primer pair generated a 200 bp amplicon and also the primer pair had a lower annealing temperature than the *inv*A probe (Sal-invA-SO-

WH). The primer-pair was validated with 73 bacterial strains and found to be highly specific for *Salmonella* (data not shown).

Malorny and others (2007) reported a 5′ nuclease uniplex assay for detection of *Salmonella* with a sensitivity of 100 genomic equivalents. Wang and Mustapha (2010) reported a duplex TaqMan® assay for detection of viable *Salmonella* with a sensitivity of 100 CFU/mL and 1000 CFU/mL in uniplex and multiplex assays, respectively. Hein and others (2006) and Anderson and others (2011) reported a lower detection limit of 10 CFU/mL. In the present study, a detection limit of 100 and 1000 genomic equivalents was achieved in uniplex and multiplex assay format, respectively. The detection limit of the real-time PCR assay developed in this study for detection of antibiotic resistant *Salmonella* was comparable to results obtained by Malorny and others (2007) and Wang and Mustapha (2010). However, the assay demonstrated a lower sensitivity when compared to real-time multiplex PCR assays described by Hein and others (2006) and Anderson and others (2011). This might be due to a higher number of primers and probes used in this multiplex assay. Food samples artificially contaminated with *Salmonella* concentrations lower than 10^4 CFU/mL could not be detected without an enrichment step. A detection limit of 10 CFU/g was achieved after an enrichment period of 12 h, for all the artificially contaminated food samples tested (Table 3.3). The use of a sterile filter stomacher bag for the enrichment of artificially contaminated food samples allowed for good separation of fat and other debris from the enriched food sample. Thus, ground beef samples with a higher fat content showed no inhibitory effects on the assay. A 12-h enrichment period for food samples contaminated with lower concentration of *Salmonella*

(10 to 10^3 CFU/mL) facilitated luxuriant growth of the pathogen, aiding their detection irrespective of the fat content of the ground beef. When the sensitivity of the spiked samples was compared, a sample artificially contaminated with 10 CFU/g of *Salmonella* could be detected after a 12-h enrichment. This enrichment time is much shorter than the 16-20 h enrichment period reported by previous researchers (Anderson and others 2011; Hein and others 2006; Malorny and others 2007).

Food samples contain many inhibitory factors that include organic and inorganic substances, such as phenolic compounds, fats, enzymes, polysaccharides, proteins, and salts. These inhibitory substances can either inhibit PCR amplification, leading to false-negative results, or they may cause a reduction in the amplification efficiency of the reaction, leading to a reduction in the detection limit of the assay (Španová and others 2000). These inhibitory compounds could be one of the reasons for the low PCR efficiency observed in the standard curve constructed using ground beef (73% lean: 27%fat) (Figure 3.2).

An IAC is a non-target DNA sequence present in the sample reaction mixture that is co-amplified with the target sequence in the reaction. The IAC is used to overrule chances of false negative results in a PCR assay. In this assay, a 98-base long single-stranded DNA sequence was uniquely designed to serve as the IAC, which has a primer-binding site for the *inv*A gene primers and a different probe binding sequence. Thus, the IAC target DNA could be co-amplified with the same primer set but detected using a different dual-labeled probe. This approach helped to reduce the number of primers used in this multiplex assay. Because the detection of an IAC is an important part of any real-

time PCR assay, a LNA dual-labeled probe was used for the detection of the IAC target in this study. Probes with LNA bases have the property of binding to complementary target molecules (DNA, RNA or LNA) with higher affinity (Kumar and others 1998; Whitcombe and others 1999). The probe worked efficiently even at a starting IAC concentration of 0.1pg/20 μL reaction. The LNA-dual labeled probe used for the detection of the IAC target was labeled with Cy5 fluorophore and BHQ-3 quencher. BHQ-2 quencher is generally recommended for use in a dual-labeled probe with Cy5 fluorophore (Marras 2006). In a study done by Reynisson and others (2006), Cy5–BHQ3 dual-labeled probes showed a lower cycle threshold (Ct) value and stronger fluorescence signal than Cy5–BHQ2 labeled probe. This might be because of a better overlap in the fluorescence spectra of Cy5 dye and BHQ-3 quencher than other quenchers.

Other real-time assays only detected the presence of *Salmonella* in a food sample but, to our knowledge, the assay described in this study is the first to successfully identify both virulent and antibiotic resistant strains of *Salmonella* in a single reaction. The primer set developed in this study, which uses the same set of primers for amplification of the IAC and was detected with a LNA dual-labeled probe, demonstrated that such combinations do work robustly in multiplex PCR. Aslam and others (2012) reported an unconditional association between antibiotic resistances genes, which can be co-selected and transferred through mobile genetic elements to other members of Enterobacteriaceae family by horizontal gene transfer mechanisms. Hence, the assay developed in this study might also be used to detect antibiotic resistant strains of other members of Enterobacteriaceae. The assay can be performed in less than 16 h and, has the potential to

be applied for pathogenic and antibiotic resistant pathogen testing in the food industry to ensure food safety and minimize the associated health hazards.

3.5 CONCLUSION

A multiplex real-time PCR TaqMan® assay was standardized for the detection of antibiotic and virulent strains of *Salmonella*. The assay can assist as a useful tool for screening of strains of *Salmonella* in various settings.

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Chapter 4 Development of a real-time PCR melt curve assay for simultaneous detection of virulent and antibiotic resistant *Salmonella*

(Adapted from Food Microbiology)

ABSTRACT

Multiple drug resistance in *Salmonella* is an emerging problem in the area of food safety. Depending on the virulence and antibiotic resistance characteristics of the *Salmonella* strain, infections of varying severity could result. In this study, a multiplex melt curve real-time PCR assay for the detection of virulent and antibiotic resistance strains of *Salmonella* was developed with two primer sets. The first set targets the virulence gene, invasin (*inv*A), and tetracycline (*tet*G), streptomycin (*aad*A2) and sulphonamide (*sul*I) antibiotic resistance genes, and the second set amplifies ampicillin ($bla_{PSE,}$ bla_{TEM}) and chloramphenicol (*flo*R) resistance genes. The multiplex assay was evaluated using 41 *Salmonella* strains and was further tested on eight different artificially inoculated food samples. The fluorescent DNA intercalating dye, SYTO9, generated high resolution melt curve peaks and, hence, was used for the development of the assay. This multiplex assay worked efficiently over a DNA concentration range of 20 ng to 200 fg and showed a sensitivity of 290 CFU/mL with serially diluted broth cultures. The detection limit for un-enriched artificially inoculated food samples was 10^4 CFU/g, but an enrichment period of 6 h allowed for detection of 10 CFU/g of cells in the samples.

4.1 INTRODUCTION

In the United States, around 42,000 cases of salmonellosis are reported every year, while in the European Union, *Salmonella* caused 283 foodborne-associated outbreaks in the year 2011 alone (EFSA 2013). On a global scale, non-typhoidal *Salmonella* causes around 93.8 million gastroenteritis-related infections, resulting in 155,000 deaths each year (Majowicz and others 2010). According to the Centers for Disease Control and Prevention (CDC), this pathogen results in 1.4 million cases of salmonellosis, and causes around 400 deaths annually in the United Sates (CDC 2009). Many *Salmonella* outbreaks and infection in humans have been traced to consumption of contaminated food of animal origin, such as eggs, chicken, turkey and beef (Favier and others 2013). At the production level, inadequate sanitation in slaughterhouses and cross contamination through processing equipment, are one of the most frequent causes of the spread of *Salmonella* (Bertrand and others 2010, EFSA 2012, Podolak and others 2010,). Further, although *Salmonella* in and of itself has been one of the major problems for the food industry, the emergence of multiple-drug-resistance (MDR) strains of this organism has compounded the problem (Koluman and Dikici 2013). Antibiotics are commonly used as prophylactic agents and for the treatment of animal diseases on farms. The antibiotics administered to farm animals are frequently the same as or belong to the same class of antibiotics that are used for treatment of human diseases (Aarestrup and others 2008). *Salmonella* strains isolated from food of animal origin have been shown to possess a high level of resistance to antibiotics, such as streptomycin, tetracycline and sulphonamide (Li and others 2012). Infections caused by MDR strains of *Salmonella* are

more severe and usually result in prolonged hospitalizations and even death. In addition to resistance towards commonly used antibiotics, such as ampicillin, streptomycin, sulfonamides, trimethoprim and tetracycline, there have also been several reports of increasing resistance towards extended spectrum β-lactam (ESBL) antibiotics, including fourth-generation cephalosporins in *Salmonella.* These antibiotics were recently approved for the treatment of invasive cases of *Salmonella* infection in humans (Sjölund-Karlsson and others 2010). The increasing incidence of MDR among *Salmonella* isolates towards newly introduced antibiotics poses a serious human health threat by limiting treatment options. The MDR phenotype is frequently linked to integrons, which are DNA elements that allow insertion and expression of multiple antibiotic resistance genes by their own promoters. These mobile genetic elements have been reported to harbor up to 10 different antibiotic resistance gene cassettes and help in the dissemination of antibiotic resistance genes from extra-chromosomal DNA to chromosomal DNA or to other closely related bacteria (White and others 2001). Recently, these antibiotic resistance determinants have been targeted for the development of PCR assays for the detection of MDR pathogens, as discussed in the following paragraph.

Rapid and cost-effective detection of *Salmonella* are important for clinical diagnosis as well as for the food industry. The gold standard for the identification of *Salmonella* is based on serologic identification of the O (somatic) and H (flagellar) antigens (Brenner and others 2000), utilizing more than 150 O and H antisera for the characterization of over 2,500 serotypes. This traditional method of *Salmonella* identification is time consuming and labor intensive and can take up to five days or more

(Van der Zee 2000). On the other hand, PCR and real-time PCR methods are rapid and sensitive and have become the method of choice for the identification of *Salmonella* during surveillance and outbreak investigations (Agarwal and others 2002; de Freitas and others 2010; Hein and others 2006; Josefsen and others 2007; O'Regan and others 2008).

Thus far, one real-time melt-curve PCR assay, targeting tetracycline, gentamicin and chloramphenicol, has been reported for the detection of antibiotic resistant foodborne pathogens (Khan and others 2011) and one TaqMan® assay for the detection of antibiotic resistance and virulent strains of *Salmonella* (Singh and Mustapha 2013) has been published. In this study, we describe a melt curve multiplex real-time PCR assay for the detection of pathogenic as well as ampicillin, streptomycin, sulphonamide, tetracycline, and chloramphenicol resistant *Salmonella*. A melt curve real-time PCR assay is a type of assay that uses a fluorescent DNA intercalating dye, such as SYBR® Green I, SYTO9, and Eva Green. In a multiplex real-time PCR melt curve assay, multiple primer pairs are used for the simultaneous amplification of multiple targets from a DNA sample. As the amount of amplicons increases exponentially during the PCR reaction, the fluorescent DNA intercalating dye binds to the amplified PCR products, leading to an exponential increase in the fluorescence signal generated by the PCR reaction tube. After completion of the PCR reaction, a melt curve analysis is performed where the obtained PCR product is heated from 65 °C to 95 °C with a gradual increment of temperature (0.1 – 0.3 °C/sec). Fluorescent data is collected for each temperature point. As the temperature reaches the amplicon melting, or denaturation temperature (T_m), the PCR products melts, and is converted into single-stranded DNA leading to liberation of the dye that was bound to the

amplicon, and causing a reduction in fluorescence signal. Each amplicon generated in the multiplex PCR reaction has a fixed GC content, which causes melting of each amplicon only at a fixed temperature. The obtained fluorescence data recorded by the real-time PCR instrument is used to plot a derivatized melt curve (fluorescence vs temperature), in which each amplicon of the multiplex assay is represented by a separate peak corresponding to the T_m of the amplicon. Unlike a TaqMan® real-time PCR assay, a melt curve multiplex assay does not require separate dual-labeled probes for the detection of each target in the assay, making it more economical, while still ensuring a high sensitivity of the assay.

4.2 MATERIAL AND METHODS

4.2.1 Bacterial strains

Standard cultures of *Salmonella* Enteritidis 13076, *Salmonella* Typhimurium 13311 and *S.* Typhimurium 14028 were procured from ATCC (Rockville, MD, USA). *S.* Typhimurium LJH 666, *Salmonella* Newport 692, *Salmonella* Agona LJH 1132, *S.* Agona LJH 1122 and *S.* Typhimurium var. Copenhagen LJH 788 were generously provided by Dr. Linda Harris, University of California-Davis, USA. Other cultures used in the study were from the culture collection of the Food Microbiology Laboratory, University of Missouri, Columbia, MO, USA. Cultures were grown at 37°C in Tryptic Soy broth (Difco Labs., MD, USA) supplemented with 0.5% yeast extract (Difco Labs., MD, USA) (TSBY). The isolates were preserved as glycerol stocks in TSBY broth supplemented with 30% glycerol at -60°C.

4.2.2 Identification of antibiotic resistance and antibiotic resistance genes

All cultures of *Salmonella* were screened for antibiotic resistance phenotypes by the standard agar disk diffusion assay on Mueller Hinton agar according to the criteria suggested by the Clinical and Laboratory Standards Institute (CLSI) (2012) with *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 as quality control strains. Isolates were further tested for the presence of ampicillin, chloramphenicol, streptomycin, tetracycline and sulphonamide resistance genes by conventional PCR, as described by Chiu and others (2006).

4.2.3 Bacterial DNA extraction

Genomic DNA from all bacterial isolates was isolated from actively growing cells in TSBY broth. One milliliter of an overnight culture was centrifuged at 16,100 ×*g* for 3 min and the obtained cell pellet was resuspended in 100 µL of PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA). Samples were heated at 100 °C for 10 min in a dry bath, to initiate cell lysis and release of bacterial genomic DNA. Samples were later removed from the dry bath, cooled at room temperature for 2 min and centrifuged at 13,200 ×*g* (2 min) to sediment the cell debris. Fifty microliters of the supernatant containing the genomic DNA was transferred to a fresh microcentrifuge tube. The concentration and purity of the obtained DNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

4.2.4 Primer design

Specific primers for amplification of virulence and antibiotic resistance genes were designed using the Primer3 software (Untergasser and others 2012) (Table 4.1) and

commercially synthesized (IDT, Coraville, IA, USA). The primers were designed such as to keep the melting temperature (T_m) of the PCR amplicons between 70 °C to 87 °C and each amplicon T_m separated by approximately 3 °C from the melting peak of the neighboring amplicon. The specificity of the designed primers was tested using the NCBI Primer BLAST tool. The T_m of all amplicons was calculated using the BioEdit software (Hall 1999). The multiplex real-time melt curve assay was designed as two sets based on the compatibility of primers in the multiplex PCR reaction and the location occupied by each amplicon peak on the melt curve plot to avoid any overlapping of peaks. The first multiplex set amplified the *Salmonella* invasion virulence gene (*inv*A), with three other commonly found antibiotic resistances genes, *viz*. tetracycline (*tet*G), streptomycin (*aad*A2) and sulphonamide (*sul*I); while the second multiplex reaction included primers targeting the ampicillin (bla_{PSE} and bla_{TEM}) and chloramphenicol (*floR*) resistance genes.

Table 4.1 Primers used for real-time PCR melt curve assay.

Primer	Primer sequence (5'-3')	Target gene	Product size (bp)	Primer Conc. (µM)	References
Str F	CAGCCA(T/C)GATCGACATTGATCT	*aad*A2	66	0.16	Singh and Mustapha 2013
Str R	CCAAGGCAACGCTATGTTCTC				
Tet F	GTTCTGTTACGGCTGGATGATG	*tet*G	66	0.22	This study
Tet R	AATAATGAAGAAAACTGCGAAAAGC				
Chl F	GGCAGGCGATATTCATTACT	*floR*	197	0.12	Singh and Mustapha 2013
Chl R	CGAGAAGAAGACGAAGAAGG				
Amp F	GATTTGGTGCTCGGAGTATT	bla_{PSE}	92	0.12	Singh and Mustapha 2013
Amp R	CATTGAAGCCTGTGTTTGAG				
Inv A1	GTGAAATTATCGCCACGTTCGGGCAA	*inv*A	284	0.15	Rahn and others 1992
Inv A2	TCATCGCACCGTCAAAGGAAC				
Sul I-F	CGGATCAGACGTCGTGGATGT	*sul*I	351	0.16	Chiu and others 2006
Sul I-R	TCGAAGAACCGCACAATCTCGT				
bla_{TEM-1}	CAGCGGTAAGATCCTTGAGA	bla_{TEM}	323	0.15	Chen and others 2004
bla_{TEM-R}	TTACATGATCCCCCATGTTG				This study

4.2.5 Real-time PCR

In this study, we evaluated the suitability of two real-time PCR master mixes (Applied Biosystems, Foster City, CA, USA) for the development of a multiplex assay: (1) SYBR® Green I-based Power SYBR® Green master mix, and (2) SYTO9-based MeltDoctor™ HRM master mix. PCR amplification was performed in duplicate in a 20 µL reaction volume using 2× of each master mixes with 20 ng of genomic DNA, using the primer concentrations mentioned in Table 4.1. A StepOnePlus™ real-time PCR (Applied Biosystems, Foster City, CA, USA) instrument was utilized. A two-step amplification protocol included initial denaturation at 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 45 s; and a melt curve step at the end of the PCR (from 60 °C to 95 °C, with gradual temperature increments of 0.1 °C/s). A melt curve plot was prepared by plotting the negative derivative of fluorescence (–Rn) versus temperature. Fluorescence signal for the MeltDoctor™ HRM master mix was detected in the FAM channel of the real-time PCR instrument. The mean T_m values for each product was calculated by averaging the T_m values obtained after amplification using the MeltDoctor™ HRM or Power SYBR® Green master mix in uniplex reactions, respectively.

4.2.6 Sensitivity of the real-time PCR assay

To determine the sensitivity of the developed multiplex assay, DNA from MDR isolate *S.* Typhimurium var. Copenhagen LJH 788 was isolated using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Stock DNA sample was standardized to 10 ng/µL and ten-fold serially diluted from 10 ng/µL to 0.1 fg/µL of DNA. Two microliters of each

serially diluted DNA, in triplicate, was used in the reactions for the construction of a standard curve. Microsoft Excel software was used to compute regression coefficients and standard deviations for the standard curves.

To further validate the sensitivity of the assay, an overnight culture of *S.* Typhimurium var. Copenhagen LJH 788 was serially diluted in peptone water (1.0 g/L) and enumerated using Tryptic Soy Agar (Difco Labs., MD, USA). DNA was isolated from 1 mL of each serially diluted peptone water tube using PrepMan® Ultra Sample Preparation Reagent. Real-time PCR was performed using 2 µL genomic DNA isolated from each dilution tube.

4.2.7 Preparation of artificially spiked food samples

Ground beef of different fat contents (73% lean/27% fat, 80% lean/20% fat, 85% lean/15% fat and 93% lean/7% fat), whole chicken, ground chicken, egg, spinach, apple cider, green peppers and tomato were purchased from a local supermarket. These food products were tested for the presence of *Salmonella* using standard culture-based protocols (Andrews and others 2007). Chicken rinse was prepared as previously described (Wang and Mustapha 2010). Twenty-five grams of food sample was diluted in 225 mL buffered peptone water (BPW) (Remel, Lenexa, KS, USA) and inoculated with MDR isolate *S.* Typhimurium var. Copenhagen LJH 788 at different levels, starting from 10 CFU/g to 10^5 CFU/g. Positive and negative process controls were also included in this study as described by Malorny and others (2007)[a]. The negative process control consisted of sterilized enrichment media with the food sample and was enriched for 8 h. The positive process control comprised of enrichment media, food sample and *S.*

Typhimurium var. Copenhagen LJH 788 inoculated at 10 CFU/g food sample. Enrichment of spiked samples was performed in sterile filter stomacher bags (Fisherbrand, Houston, Texas, USA) containing BPW (Remel, Lenexa, KS, USA) as the enrichment medium in a shaking incubator at 35 ºC. The DNA from spiked food samples was isolated from 2 mL of enriched broth withdrawn at intervals of 0, 4, 6 and 8 h. Samples were centrifuged at 0.1 ×*g* for 1 min to separate suspended food particles from the media, and DNA was isolated from the obtained supernatant using PrepMan® Ultra Sample Preparation Reagent according to the manufacturer's instructions.

4.3 RESULTS

4.3.1 Antibiotic resistance of *Salmonella* strains

Standard agar disk diffusion assay results showed that *S.* Typhimurium strains 14028 and 13311 were resistant to streptomycin, tetracycline and sulphonamide, and *S.* Enteritidis 13076 was found to be sensitive to all antibiotics, except sulphonamide (Table 2). *S.* Typhimurium var. Copenhagen var. LJH 788 was found to be resistant to all five antibiotics used in this study (Table 4.2). Thus, it was considered to be a MDR isolate and was subsequently used for standardization of the melt curve multiplex real-time PCR for simultaneous detection of virulent and antibiotic resistant *Salmonella.*

All isolates used to validate this assay was also tested for the presence of the respective antibiotic resistance genes by conventional PCR. The antibiotic resistance profiles of all *Salmonella* isolates used in this study are listed in Table 4.2 as presence or absence of the respective antibiotic resistance gene. Results of the agar disk diffusion assay were mostly consistent with the genetic profile of antibiotic resistance genes of the

Salmonella isolates as determined by PCR. However, there were some instances where the phenotypic and genotypic traits did not match. One of the reasons for this observed variation may be due to the extensive diversity of antibiotic resistance genes found in members of the Enterobacteriaceae family. The other reason for the observed variation in phenotypic traits was the fact that some strains may have lost their ability to express resistance phenotype during storage, as was found when compared with our previous publication (Singh and Mustapha 2013). Similar findings where some antibiotic resistance genes were present in the bacterial genome but were not phenotypically expressed were also reported by Perreten and others (2005).

Table 4.2 Phenotypic and genotypic antibiotic resistance profile of *Salmonella* isolates.

Isolate	**STR**[a]	***aad*A2**	**TET**[b]	***tet*G**	**CHL**[c]	***flo*R**	**AMP**[d]	***bla*$_{PSE}$**	***bla*$_{TEM}$**	**Sulfisoxazole**	***sul*I**
S. Typhimurium 13311	R	+	R	+	S	-	S	-	-	R	+
S. Typhimurium 14028	R	+	R	+	S	-	S	-	-	R	+
S. Enteritidis 13076	S	-	S	-	S	-	S	-	-	R	+
S. Typhimurium 788	R	+	R	+	R	+	R	+	-	R	+
S. Typhimurium LJH 666	R	+	S	-	S	-	S	-	-	R	+
S. Newport LJH 692	R	+	R	+	S	-	S	-	-	R	+
S. Agona LJH 1122	R	+	R	+	S	-	S	-	-	R	+
S. Agona LJH 1132	R	+	R	+	S	-	S	-	-	R	+
S. Berta D(O), G comp H	S	-	S	-	S	-	S	-	-	R	+
S. Drypool E_2(O), G comp H	S	-	S	-	S	-	S	-	-	R	+
S. Rubislaw	S	-	S	-	S	-	S	-	-	R	+
S. Group B(4,5)	S	-	S	-	S	-	S	-	-	S	+
Salmonella Spp	S	-	S	-	S	-	S	-	-	R	+

S. Arizona	S	+	S	-	S	-	S	-	-	R	+
S. Thompson B&B3	R	+	R	+	R	+	R	-	+	R	+
S. Typhimurium 13311	S	-	S	-	S	-	S	-	-	R	+
S. Pulloram 13036	S	-	S	-	S	-	S	-	-	R	+
Salmonella API 670752	S	-	S	-	S	-	S	-	-	R	+
S. Seftenberg O Group E	S	-	S	-	S	-	S	-	-	R	+
S. Enteritidis F3-1	R	+	S	+	S	-	S	-	-	R	+
S. Enteritidis F3-2	R	+	R	+	S	-	S	-	-	R	+
S. Enteritidis F3-6	R	+	R	+	S	-	S	-	-	R	+
S. Enteritidis F3-7	R	+	R	+	S	-	S	-	-	R	+
S. Enteritidis F3-8	R	+	S	+	S	-	S	-	-	R	+
S. Enteritidis F3-9	R	+	S	+	S	-	S	-	-	R	+
S. Enteritidis F3-10	R	+	R	+	S	-	S	-	-	R	+
S. Enteritidis G5-02	R	+	S	+	S	-	S	+	-	R	+
S. Enteritidis G5-08	R	+	S	+	R	+	S	-	-	R	+

S. Enteritidis G5-09	R	+	R	+	S	-	S	-	-	R	+
S. Enteritidis G5-10	R	+	S	+	S	-	S	-	-	R	+
S. Enteritidis I4-1	R	+	S	-	S	-	S	-	-	R	+
S. Enteritidis I4-2	R	+	R	+	R	-	S	-	-	S	+
S. Enteritidis I4-3	R	+	R	+	S	-	S	-	-	S	+
S. Enteritidis I4-4	R	+	S	+	R	-	S	+	-	R	+
S. Enteritidis I4-5	R	+	S	+	S	-	S	+	-	R	+
S. Enteritidis I4-6	R	+	R	+	S	-	S	-	-	R	+
S. Enteritidis I4-7	R	+	R	+	S	-	S	-	-	R	+
S. Enteritidis I4-8	R	+	R	+	S	-	S	-	-	R	+
S. Enteritidis I4-9	R	+	R	-	S	-	S	-	-	R	+
S. Enteritidis I4-10	R	+	S	-	R	-	S	-	-	R	+
S. Enteritidis I4-11	R	+	R	+	S	-	S	-	-	R	+

[a] Streptomycin, [b] Tetracycline, [c] Chloramphenicol, [d] Ampicillin

4.3.2 Real-time PCR

The suitability of two real-time PCR master mixes (SYBR® Green I and MeltDoctor™ HRM) was evaluated for the detection of MDR *Salmonella*. The average melting temperatures (T_m) of all amplicons generated from the targeted genes are outlined in Table 4.3. In the developed multiplex assay, SYBR® Green I showed preferential binding to the smaller amplicons generated in the reaction. The melt curve of the tetraplex assay using this dye generated only two peaks for the streptomycin (66 bp) and tetracycline (66 bp) resistance genes (Figure 4.1A). This preferential binding to smaller amplicons was expected because of the manner in which SYBR® Green intercalates to the minor-groove region of the DNA. Therefore, the dye was not further investigated for the development of the assay. The MeltDoctor™ HRM master mix, however, showed no preferential binding and generated all four peaks in the same assay (Figure 4.1B). The SYTO9 dye in the MeltDoctor™ HRM master mix also produced a melt curve with a higher resolution. For the second set of reactions, all targets gave defined peaks irrespective of the master mix used (Figure 4.1C). Because the melt curve step was performed at the slowest ramp rate (gradual temperature increments of 0.1 °C/s), the melt curve peaks obtained using both master mixes were equally sharp.

Table 4.3 Melting temperature (T_m) of the amplicons.

Target	Mean T_m (^oC)	
	SG I	**SYTO9**
bla$_{Pse}$	78.28 ± 0.10	78.62 ± 0.00
*flo*R	83.21 ± 0.00	83.41 ± 0.20
*inv*A	84.11 ± 0.40	84.37 ± 0.30
*aad*A2	74.80 ± 0.30	75.47 ± 0.21
*sul*I	86.95 ± 0.07	87.37 ± 0.03
*tet*G	77.96 ± 0.20	78.86 ± 0.48
bla$_{TEM}$	81.91 ± 0.53	81.96 ± 0.16

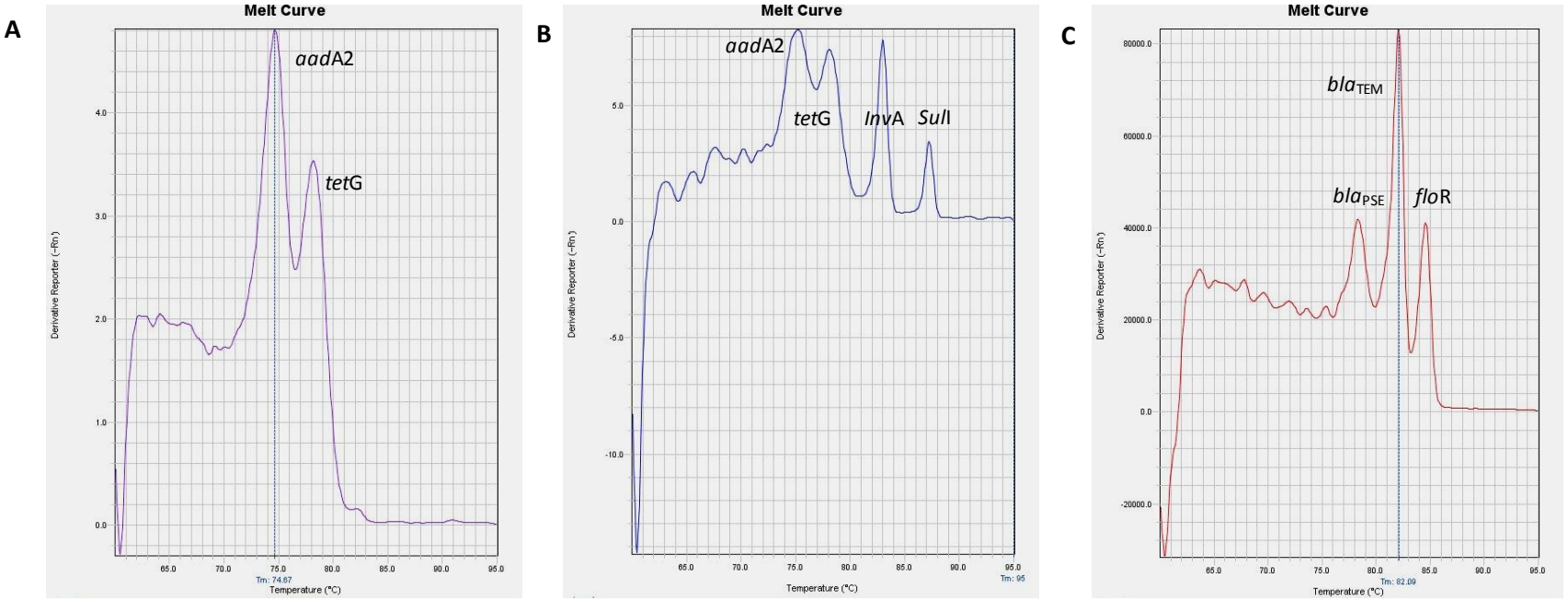

Figure 4.1 Multiplex assay for the detection of virulent and antibiotic resistant *Salmonella*.: A) streptomycin (*aad*A2), tetracycline (*tet*G), invasin (*inv*A), sulphonamide (*sul*I) multiplex using SYBR® Green I dye-based master mix; B) *aad*A2, *tet*G, *inv*A, *sul*I multiplex using SYTO9-based MeltDoctor™ master mix; C) ampicillin (bla_{PSE} and bla_{TEM}) and chloramphenicol (*flo*R) resistance gene multiplex using MeltDoctor™ master mix.

4.3.3 Sensitivity of the assay

The multiplex assays using the MeltDoctor™ HRM master mix developed in this study was found to be working efficiently over a DNA concentration range of 20 ng to 200 fg (Figure 4.2A, 4.2B). The obtained standard curve for each targeted gene with its respective regression coefficient (r^2 value) is represented in Figure 4.3. At 20 fg (4 genomic equivalent) DNA per reaction, only *aad*A2, *sul*I and bla_{PSE} genes were detected. The starting cell concentration of *Salmonella* broth culture was 2.9×10^9 CFU/mL. DNA isolated from this decimally diluted broth culture was used to validate the sensitivity of the assay, and a detection limit of 290 CFU/mL was obtained for both the tetraplex and triplex assays, *viz*. all targets were successfully detected at this cell concentration.

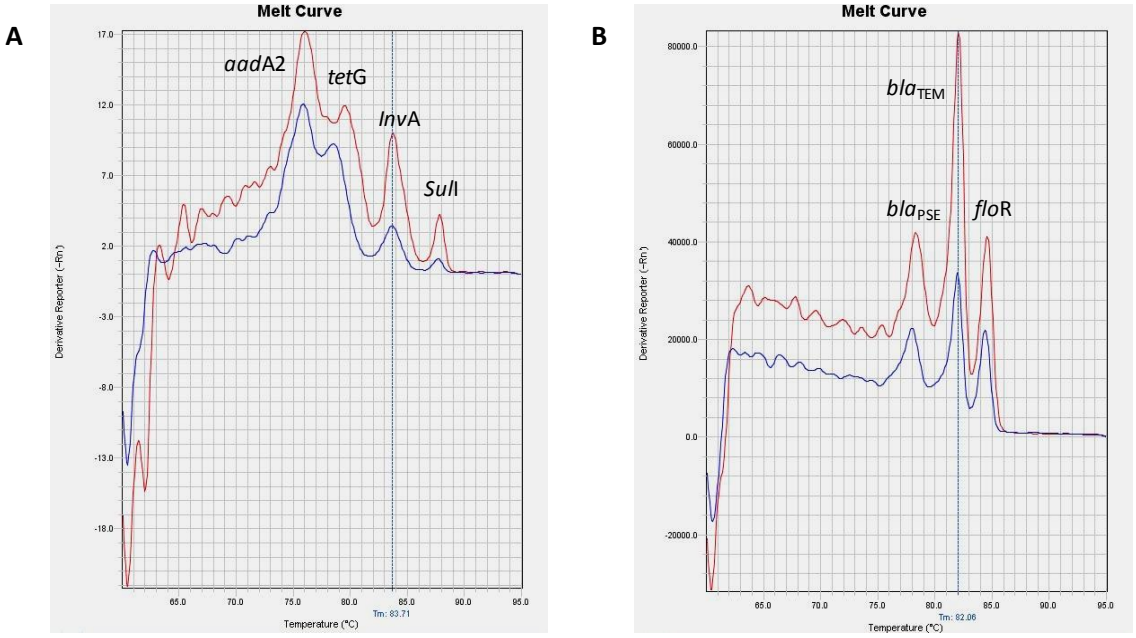

Figure 4.2 Sensitivity of multiplex assay: DNA concentrations ranging from 20 ng to 200 fg using MeltDoctor™ master mix. A) sensitivity of streptomycin (*aad*A2), tetracycline (*tet*G), invasin (*inv*A), and sulphonamide (*sul*I) resistance gene multiplex; B)

sensitivity of ampicillin (*bla*$_{PSE}$ and *bla*$_{TEM}$) and chloramphenicol (*flo*R) resistance gene multiplex.

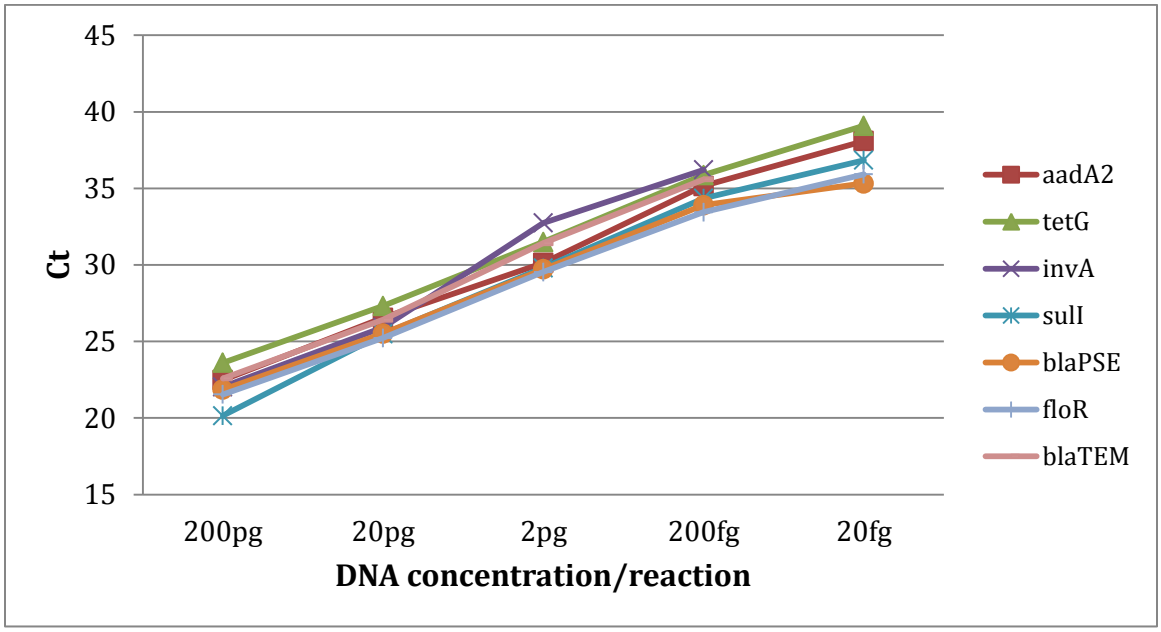

Figure 4.3 Standard curve constructed using serially diluted DNA: streptomycin (*aad*A2, R^2 =0.9954), tetracycline (*tet*G, R^2 =0.9979), invasion (*inv*A, R^2 =0.9837), sulphonamide (*sul*I, R^2 =0.9866), ampicillin (*bla*$_{PSE}$, R^2 =0.9798), chloramphenicol (*floR*, R^2 =0.9928) and ampicillin (*bla*$_{TEM}$, R^2 =0.9976).

4.3.4 Detection of antibiotic resistant *Salmonella* in spiked samples

Using standard culture methods, all food samples, except ground turkey, were found to be negative for *Salmonella*. These *Salmonella*-negative samples were artificially inoculated with MDR *S.* Typhimurium var. Copenhagen LJH 788. The developed multiplex assay in this study was able to detect as low as 10^4 CFU/g in the food samples without an enrichment step (Table 4.4). Following an enrichment step, DNA isolated from enriched samples was directly used for real-time PCR. Initially, no amplification

was observed from these DNA samples. The concentration of the DNA samples following enrichment ranged between 470 to 923 ng/µL, which is too high for accurate detection by real-time PCR. This high concentration of DNA might be the reason for our initial PCR detection failure. When the DNA was diluted (1:2) with nuclease-free water, the real-time PCR could detect all targets of the multiplex after an enrichment period of 6 h (Table 4.4).

Table 4.4 Multiplex real-time PCR results of artificially contaminated food samples.

Food Samples	**Spiked *Salmonella* concentration (CFU/g)**	**Real-time PCR result after enrichment**			
		0 h	**4 h**	**6 h**	**8 h**
Ground beef (73% lean)	10	-	-	+	+
	10^2	-	-	+	+
	10^3	-	-	+	+
	10^4	+	+	+	+
	10^5	+	+	+	+
Ground beef (80% lean)	10	-	-	+	+
	10^2	-	-	+	+
	10^3	-	-	+	+
	10^4	+	+	+	+
	10^5	+	+	+	+
Ground beef (85% lean)	10	-	-	+	+
	10^2	-	-	+	+
	10^3	-	-	+	+
	10^4	+	+	+	+
	10^5	+	+	+	+
Ground beef (93% lean)	10	-	-	+	+
	10^2	-	-	+	+
	10^3	-	-	+	+
	10^4	+	+	+	+
	10^5	+	+	+	+

Whole chicken	10	-	-	+	+
	10^2	-	-	+	+
	10^3	-	-	+	+
	10^4	+	+	+	+
	10^5	+	+	+	+
Ground chicken	10	-	-	+	+
	10^2	-	-	+	+
	10^3	-	-	+	+
	10^4	+	+	+	+
	10^5	+	+	+	+
Egg	10	-	-	+	+
	10^2	-	-	+	+
	10^3	-	-	+	+
	10^4	+	+	+	+
	10^5	+	+	+	+
Spinach	10	-	-	+	+
	10^2	-	-	+	+
	10^3	-	-	+	+
	10^4	+	+	+	+
	10^5	+	+	+	+
Apple cider	10	-	-	+	+
	10^2	-	-	+	+
	10^3	-	-	+	+
	10^4	+	+	+	+
	10^5	+	+	+	+
Green pepper	10	-	-	+	+
	10^2	-	-	+	+
	10^3	-	-	+	+
	10^4	+	+	+	+
	10^5	+	+	+	+
Tomato	10	-	-	+	+
	10^2	-	-	+	+
	10^3	-	-	+	+
	10^4	+	+	+	+
	10^5	+	+	+	+

Fat contents of the food samples tend to affect the efficiency of the reaction. Beef samples with lower fat contents gave a lower Ct value. The obtained Ct values of the tetraplex assay (first set) for 7% fat, 15% fat, 20% fat and 27% fat beef samples were 23.30, 25.70, 27.64 and 30.83, respectively, and for the triplex assay (second set), they were 25.01, 26.92, 29.09 and 31.67, respectively. The inhibitory effect of these different fat contents was also tested in uniplex format with beef samples spiked at 10 CFU/25 g (Table 4.5).

Table 4.5 Mean Ct values of each PCR primer in uniplex format, using ground beef of different fat contents.

Target	**Mean C_t value**			
	Ground beef (93% lean)	**Ground beef (85% lean)**	**Ground beef (80% lean)**	**Ground beef (73% lean)**
bla$_{Pse}$	24.6	28.1	28.7	33.4
*flo*R	26.5	30.9	31.6	33.6
*inv*A	26.7	28.5	29.3	32.1
*aad*A2	20.4	22.7	23.7	27.9
*sul*I	18.2	21.1	24.7	30.5
*tet*G	21.6	24.0	24.3	29.0
bla$_{TEM}$	22.3	24.9	30.4	33.7

4.4 DISCUSSION

In recent years, melt curve-based real-time PCR assays have emerged as a powerful tool for pathogen detection. In this study, we developed a multiplex real-time PCR assay for the detection of pathogenic as well as antibiotic resistant strains of *Salmonella* using melt curve technology. Based on previously published literature, the *inv*A gene was selected as the marker for virulent strains of *Salmonella*. This gene is located on the *Salmonella* pathogenicity island 1 (SPI1) and it encodes a protein for the type III secretion system. The gene, *inv*A, encodes a 71 kDa putative inner membrane protein and plays an important role in the internalization of *Salmonella* into the mammalian epithelial cells. The absence of this gene in *Salmonella* indicates that the strain is either non-invasive or the strain possesses an alternate mechanism for invading mammalian cells (Galán and Curtiss 1991). The *inv*A primer set used in this study had previously been tested on 630 *Salmonella* strains and 142 non-*Salmonella* strains with a 99.4% specificity (Rahn and others 1992). The assay developed in this study showed a sensitivity of 200 fg/reaction (40 genomic equivalents) for the DNA sample obtained from pure cultures and a sensitivity of 10 CFU/g after a 6 h enrichment period for spiked food samples. A duplex melt curve assay for the simultaneous detection of *Salmonella* and *Listeria* was reported with a detection limit of 1 log CFU/mL (Singh and others 2012). Another study (Nam and others 2005) reported a uniplex melt curve assay for the detection of *Salmonella* with a detection limit of 10 CFU/mL. Even though the assay developed in our study is a multiplex one, the sensitivity of the assay is comparable to

that reported by Nam and others (2005) for the detection of *Salmonella* in artificially contaminated food samples.

Wang and Mustapha (2010) reported a duplex TaqMan® assay for detection of live *Salmonella* with a sensitivity of 100 CFU/mL and 1000 CFU/mL in uniplex and multiplex format, respectively. Anderson and others (2011), Hein and others (2006) and Malorny and others (2007b) reported 5′ nuclease assays with a detection limit of 10 genomic equivalents. The *Salmonella* genomic equivalent can be calculated using the formula: m × n (1.013×10^{-21} g/bp), where "m" is the mass and "n" is the number of base pairs in the *Salmonella* genome. McClelland and other (2001) reported the genome size of *S.* Typhimurium 4951 kilobase pairs. Using the aforementioned equation, the weight of one *S.* Typhimurium double stranded genomic DNA is about 5.0 fg. Thus, one genomic equivalent of pure *Salmonella* DNA is equal to 5.0 fg of *Salmonella* genomic DNA (McClelland and others 2001). In this present melt curve PCR study a detection limit of 40 genomic equivalents was achieved. This detection limit is also comparable with the abovementioned 5′ nuclease assays. However, it demonstrated a lower sensitivity when compared to the assays described by Hein and others (2006) and Anderson and others (2011), which could be attributed to the higher number of primers used.

Food samples artificially contaminated with *Salmonella* concentrations lower than 4 log CFU/mL could not be detected without enrichment, thus necessitating the addition of an enrichment step. For all the artificially contaminated food samples tested in this study, a detection limit of 10 CFU/g was achieved after an enrichment period of 6 h,

which is a much shorter time than the 16-20 h enrichment periods reported by Malorny and others (2007b), Hein and others (2006) and Anderson and others (2011).

Food samples contain many organic and inorganic substances, such as phenolic compounds, fat, enzymes, polysaccharides, proteins and salts which can either inhibit PCR amplification or may lead to a reduction in amplification efficiency of the reaction (Demeke and Jenkins 2010; Španová and others 2000). However, all these PCR inhibitors cannot be detected spectrophotometrically (A_{280}), which is commonly done to check the purity of isolated DNA samples (Rossen and others 1992). Ground beef samples have higher fat and protein concentrations that affect the quality of the isolated DNA and which, in turn, affect the PCR amplification efficiency. In this study, an increase in Ct values was observed with an increase in fat content of beef samples. The increasing fat content seems to exert some inhibitory effect on the amplification of the reaction. The use of filter stomacher bags and low temperature centrifugation in this study facilitated separation of the fat from enriched broth samples during the DNA isolation process. Other measures that could also be taken to reduce the level of these PCR inhibitors in the DNA samples might include a low speed (0.1 $\times g$ for 1 min) centrifugation step to sediment large fat and protein particles, coupled with the use of a sterile cotton swab to physically remove the fat in enriched broth sample that tends to stick on the wall of the tubes. A recent study conducted by the USDA (Bainet and others 2014), reported that most DNA samples obtained using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) were not very pure and harbored PCR inhibitors, effecting outcome of the multiplex PCR reaction. A DYNAL® Magnetic Bead based

DNA purification kit (e.g. DYNAL DNA Direct, Dynabeads MyOne SILANE) or a silica column based sample preparation kit **(**e.g. Genomic DNA Clean & Concentrator™, PureLink® Genomic DNA Kit,**)** can also be employed for the removal of these impurities from the DNA obtained from enriched food samples.

Although it is commonly accepted that probe-based real-time PCR assays have a higher specificity than melt curve real-time PCR assays. However, it can be debated, specificity of a hybridizing probe-based assay is bestowed by the specificity of the primer-pair as well as the probe. On the other hand, in melt curve real-time PCR assays, the PCR reaction specificity is conferred by the use of a specific primer pair and a unique melting peak generated by the amplicons during the melt-curve analysis. It is very rare that a non-specific amplicon generated in real-time PCR reaction can produce a melt-curve peak similar to that of a target amplicon. Even in some cases, a high-resolution melt (HRM) analysis can be performed to resolve the ambiguity of the melting curve peaks, without additional costs. The hybridizing probe-based assays are commonly used for pathogen detection but these assays have a higher running cost. Probe-based real-time chemistry requires licensed softwares for probe design, multiple probes for multiplexing, and calibration of multiple detectors in the real-time PCR instrument. Further, the probe itself has a limited storage life. Thus, intercalating dyes, such as SYBR® Green I have become the preferred alternative for performing real-time PCR and are being increasingly used for bacterial identification (Tong and Giffard 2012). However, the SYBR® Green I dye also has some limitations. It has been reported that this dye can inhibit PCR reactions in a concentration-dependent manner (Nath and others 2000). The degradation product of

this dye has also been reported to be inhibitory for the PCR reaction (Karsai and others, 2002). Another crucial drawback of this dye is its limited application in multiplex real-time PCR. It has been reported to show preferential binding to PCR products with higher T_m or higher GC content in a multiplex reaction, thus allowing for the detection of only a limited number of targets in a multiplex reaction format (Giglio and others 2003). Unlike other dyes like SYTO9, SYBR® Green I binds to the minor groove region of DNA (Dragan and others 2012), and is reported to exhibit PCR inhibitory effect at higher dye concentrations. SYBR® Green I also suffers from the dye-jumping phenomenon whereby, upon melting of a bound double stranded DNA, it may be reincorporated into regions of the DNA that have not yet melted (Reed and others 2007). Hence, the resolution of a melt curve obtained using the SYBR® Green I dye is inferior when compared with a saturating fluorescent dye. SYTO9 dye is a saturating dye, which can be used at a broad range of dye concentrations without inhibiting the PCR reaction. This dye also does not show any preferential binding of PCR amplicons and, thus, is more suitable for multiplex melt curve real-time PCR assays (Monis and others 2005).

Good primer design, the real-time PCR instrument and the instruments analysis software all play a crucial role in the development of multiplex melt curve real-time PCR assays. Out of the various software's used for the estimation of melting temperatures of PCR products (BioEdit, Oligo Calc: Oligonucleotide Properties Calculator, Primer Express Version 2.0, Primer3) for the development of this melt curve multiplex assay, BioEdit Sequence Alignment Editor (Hall 1999) was found to be the most accurate in predicting the melt temperatures of the amplicons (data not shown).

Initial attempts were made to standardize this assay on the ABI 7500 platform with SDS software version 1.4 (Applied Biosystems, Foster City, CA, USA). However, only two peaks were seen for the tetraplex assay using both the master mixes. The SDS software 1.3 has a fixed ramp rate for the melt curve step, which cannot be changed. The StepOnePlus™ Software v2.1 (Applied Biosystems, Foster City, CA, USA) allows for manually changing the default ramp rate during the melt curve step (from 0.3 °C/s to 0.1 °C/s), leading to a much slower rate of melting of the PCR amplicons and generation of data with a higher resolution.

While traditional culture-based methods are still the gold standard, they require 3-5 days for the confirmation of *Salmonella* in food samples. Samples containing antibiotics, such as animal feed or blood drawn after administration of antibiotics, cannot reliably be tested with culture-based methods because the presence of antibiotics will inhibit the growth of bacteria (Jeng and others 2012). The current assay with an enrichment step of 6 h, followed by 4 h for DNA isolation and real-time PCR enables rapid detection of MDR and virulent *Salmonella* in 10 h. This process can be further shortened by using fast real-time PCR machine (e.g. 7500 Fast real-time PCR instrument) that allows for a faster DNA amplification and melt curve analysis. Another advantage of this melt curve assay is that it does not require any post-PCR processing, such as gel electrophoresis or DNA sequencing, which, in turn, helps to save time and to avoid post-PCR contaminations. The assay can also be used in a clinical setting as an adjunctive test to provide physicians with an early detection tool of pathogens causing bacteremia.

Apart from the above-mentioned advantages, there are also a few limitations of genotype- based assays. A limitation of the method described in this study is that out of a vast number of antibiotic resistance genes found in members of the Enterobacteriaceae family, the assay only targets the most frequently reported antibiotic resistance genes. Hence, *Salmonella* isolates tested negative by this assay might be positive for other less frequently occurring antibiotic resistance genes. Another common drawback of all genotypic methods is that they cannot differentiate the origin of genetic markers in environmental samples, including food, which might lead to false positive results.

The melting profile of the amplicons generated in a melt curve- based real-time PCR assay is dependent on the quality of the initial DNA template used for the reaction (Ajitkumar and others 2012). DNA isolated from samples containing PCR inhibitors or high salt contents might lead to a T_m shift or reduction in efficiency of the PCR amplification. Therefore, the inclusion of a positive process control is a very important component of melt curve assays. Positive and negative process controls were included in this study, as described by Malorny and others (2007)[a]. The negative process control consisted of sterilized enrichment media containing the food sample, which was enriched for 8 h. The positive process control comprised of enrichment media and food sample inoculated with 10 CFU/g of *S.* Typhimurium var. Copenhagen LJH 788. The method described in this study targeted more number of antibiotic resistance genes, required a shorter enrichment time, and was also more cost effective than our previously published TaqMan® assay for the detection of antibiotic and virulence strains of *Salmonella* (Singh and Mustapha 2013).

4.5 CONCLUSIONS

The multiplex melt curve real-time PCR assay described in this study offers a simple work flow with a total turn-around time of 10 h for the detection of MDR and virulent strains of *Salmonella* from food samples with an initial count of 10 CFU/g. This multiplex assay can be a useful tool for epidemiologic, laboratory, and traceback investigations of tainted food samples during an outbreak caused by MDR *Salmonella*. Further, this assay is an economical alternative to the hybridization probe-based real-time PCR assay, which is currently more widely used for the detection of *Salmonella*.

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Chapter 5 Multiplex real-time PCR assay for the Detection of Extended-Spectrum β-Lactamases and Carbapenemase Producing Genes Using Melting Curve Analysis

ABSTRACT

Antibiotic resistance among Gram-negative pathogens is a matter of great concern. Out of the different antibiotic groups, resistance towards extended-spectrum β-lactamase (ESBL) and carbapenem antibiotics in Enterobacteriaceae Family has more severe consequences and recent surveillance data have shown their increasing trend. The objective of this study was to develop a real-time PCR melt curve assay for the detection of extended-spectrum β-lactam and carbapenem resistant pathogens. Two multiplex real-time PCR melt curve assays were standardized for the detection of 10 most common ESBL and carbapenem resistance genes: bla_{KPC}, bla_{NDM}, bla_{CTX}, bla_{CMY}, bla_{VIM}, bla_{IMP}, bla_{OXA}, bla_{SHV}, bla_{TEM}, bla_{ACC}. The multiplex was evaluated using a total of 38 DNA samples obtained from previous studies. The results of the multiplex PCR assay either mostly paralleled the previous studies or the presence of more antibiotic resistance genes were detected using our assay. The assay developed in this study offers a simple, low cost method for the detection of ESBL and carbapenem resistance among enteric pathogens.

5.1 INTRODUCTION

Broad-spectrum β-lactamase genes coding for extended-spectrum β-lactamases and enzymes conferring resistance towards others antibiotics have been frequently isolated from the microbiota of food-producing animals. Food animals colonized with strains of ESBL-producing *Escherichia coli* and *Salmonella* have been reported as important sources of community infections. ESBL- and carbapenemase-producing members of the Enterobacteriaceae have been increasingly isolated from food animals and have gained considerable attention worldwide (Smet and others 2010). Recent data from the National Antimicrobial Resistance Monitoring System (NARMS) have shown an increase in extended-spectrum cephalosporins resistance among *Salmonella* Heidelberg isolated from food animals at slaughter, retail meat, and humans (Folster and others 2012). β-lactams are one of the most important groups of antimicrobial agents used in veterinary medicine on food animals. These antimicrobial agents can be divided into three major groups: the penicillins, the first- to fourth-generation cephalosporins and the β-lactamase inhibitors. Cephalosporins have been approved for treatment of various diseases in food animals such as foot rot and mastitis in cattle, and respiratory diseases in ruminants (Smet and others 2010). Additionaly, these antibiotics are also mixed with animal feed to improve feed efficiency and avoid animal disease in cattle feedlots.

The extensive use of antibiotics for various purposes on animal feedlots exerted selective pressure leading to evolution and selection of newer beta-lactamase enzymes conferring resistance to a broad range of antibiotics. Datta and Kontomichalou (1965) reported the first beta-lactamase enzyme, TEM-1, in Gram-negative bacteria. The SHV-2

enzyme was the first enzyme that showed ESBL-hydrolyzing capability and was isolated from a *Klebsiella ozaenae* in Germany (Kliebe and others 1985). Because of the increased spectrum of activity of these beta-lactamase enzymes, especially against the oxyimino-cephalosporins, these enzymes were called ESBLs (Bradford 2001). Hence, ESBLs were classified as a class of enzymes that confer resistance against penicillins, broad-spectrum cephalosporins with an oxyimino side chain (cefotaxime, ceftriaxone and ceftazidime) and the oxyimino-monobactam aztreonam antibiotics. Another common property of these ESBLs is that they can be inhibited by serine-type β-lactamase inhibitors, such as sulbactam, clavulanate and tazobactam (Philippon and others 1989; Bradford 2001). At present, ESBL enzymes are spread over ten families and have been documented all around the world e.g. CTX-M, SHV, TEM, PER, VEB, BES, GES, TLA, SFO and OXA (Paterson and Bonomo 2005). Today, ESBLs are one of the most influential mechanisms for cephalosporin resistance in Enterobacteriaceae, particularly in *E. coli* and *K. pneumoniae*.

In the family of ESBLs, CTX-Ms are plasmid–mediated enzymes that preferentially hydrolyze cefotaxime, and are the latest addition that have significant clinical impacts. This new class of ESBL enzymes is commonly found among the antibiotic resistant strains of *Salmonella* and *E. coli*, but they have also been frequently reported among other members of the Enterobacteriaceae (Sabaté and others 2000). The CTX-M enzymes are one of the biggest and most diverse groups of ESBL enzymatic variants (CTX-M-1 to CTX-M-160) and exhibit strong activity against cefotaxime and ceftriaxone, but not ceftazidime. The diversity of CTX-M enzymes varies with

geographical locations, but CTX-M-15 and CTX-M-14 (followed by CTX-M-2, CTX-M-3 and CTX-M-1) are the most common enzymatic variants worldwide among all major clinically important pathogens (Zhao and Hu 2013). Class C chromosomal AmpC (cAmpC) is a class of β-lactamases that confers resistance towards third-generation cephalosporins and β-lactamase inhibitors. However, they are susceptible to fourth-generation cephalosporins, such as cefepime and the carbapenem group of antibiotics (Bush and Jacoby 2010). There are several families of cAmpC enzymes e.g. CMY, FOX, LAT, MIR, ACT, DHA, ACC, MOX. Out of these, CMY-2 is the most prevalent pAmpC among members of the Enterobacteriaceae.

Carbapenemase enzymes are produced by pathogenic bacteria to degrade and neutralize the effects of almost all beta-lactam antibiotics, including carbapenems (Cantón and others 2012). Based on the composition of the active site, carbapenemases enzymes can be broadly classified into two major groups: (1) Serine carbapenemases: consist of a serine amino-acid in their active site and are subdivided into two classes *viz*. class A penicillinases and class D oxacillinases. The class A enzymes are coded by IMI, NMC, SME, KPC, and GES genes, which confer resistance to the carbapenems group of antibiotics at various levels, ranging from complete resistance to reduced susceptibility (Queenan and Bush 2007). On the other hand, OXA-48, OXA-23, OXA-24, and OXA-58 genes are the most dominant genetic determinants encoding Class D oxacillinases enzyme in *A. baumannii* and *K. pneumoniae*. (2) Metallo-betalactamases: also known as Class B carbapenemases. Enzymes of this group have one or more zinc atoms in their active site. This group includes the GIM, IMP, NDM, SIM and VIM enzymes that are

found on the transferrable plasmids of Enterobacteriaceae members, *P. aeruginosa* and *A. baumannii* (Nordmann and others 2011). Out of the five above-mentioned enzymes; IMP, NDM and VIM are the most prominent enzymes. NDM is the most recent addition to the class B carbapenemases group of enzymes. Since the first report of NDM-1 in the year 2009, it has spread worldwide and become the most common carbapenemases enzyme in all of the Enterobacteriaceae (Rolain and others 2010).

Antimicrobial susceptibility testing for ESBL or carbapenemase producing Enterobacteriaceae is generally performed using commercially available selective agar plates. Standard microbiological procedures can take up to several days for the culture, isolation and characterization of antibiotic resistance profile of pathogens from a provided sample. The total time required for this process can add up to 4-6 days for getting an accurate antimicrobial susceptibility test results (Lupo and others 2013). However, many recent comparative studies have shown that the commercially available selective agars have lower sensitivity than PCR-based methods (Naas and others 2011; Singh and others 2012). The objective of this study was to develop a multiplex real-time melt curve assay for the detection of genes coding for ESBL and carbapenemase enzymes.

5.2 MATERIALS AND METHODS

5.2.1 Procurement of standard DNA sample and cultures

DNA of antibiotic resistant *Salmonella* and members of the Enterobacteriaceae was obtained from Robert Koch Institute, (Berlin, Germany), Calgary Laboratory Services,

(Calgary, AB, Canada), Institute of Microbiology, University of Lausanne and University Hospital Center, (Lausanne, Switzerland), Infectious Diseases, VA Medical Center (Minneapolis, MN, USA) and University of Missouri Hospital (Columbia, MO, USA).

5.2.2 Bacterial DNA extraction

Genomic DNA from all bacterial isolates used in the study and from enriched food samples was isolated using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The concentrations and purity of the obtained DNA samples was determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

5.2.3 Primer design

A total of 85 primer pairs targeting 10 antibiotic resistance genes (bla_{KPC}, bla_{NDM}, bla_{CTX}, bla_{CMY}, bla_{VIM}, bla_{IMP}, bla_{OXA}, bla_{SHV}, bla_{TEM}, bla_{ACC}) were designed and evaluated for the standardization of the multiplex real-time PCR melt curve assay. The primers were designed such as to keep the melting temperature (T_m) of the PCR amplicons between 67 °C to 87 °C and each amplicon T_m separated from neighboring amplicon T_m by approximately 2-3 °C. The specificity of the designed PCR primers was tested using the NCBI/Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The designed oligonucleotides were commercially synthesized (IDT, Coraville, IA, USA). PCR amplification conditions of each primer pair were standardized using conventional gradient PCR. Real-time PCR was performed using MeltDoctor™ HRM master mix (Applied Biosystems, Foster City, CA, USA) in singleplex format for the estimation of the T_m of each PCR amplicon. Based on the obtained T_m values of the amplicons in

singleplex reaction, multiplex real-time melt curve assay was standardized in a stepwise manner. PCR primers generating low T_m amplicons (e.g. 70 °C) were tested for their ability to work in a multiplex reaction with primer-pairs of another gene generating amplicons of T_m values 2-3 °C higher and subsequently targeting primers with higher amplicon T_m in a step-wise manner.

5.2.4 Internal amplification control (IAC) design

Two reaction-specific, single-stranded, 75-100 base long DNA sequences were designed to act as IAC in the multiplex real-time PCR reaction. These IAC molecules could be amplified using one of the primer pairs of the multiplex assay (but generating a separate melt curve peak). The IAC concentrations of each multiplex assay was optimized and kept as low as possible to prevent any possible competition for primers and facilitating the preferential amplification of the antibiotic resistant genes.

5.2.5 Real-time PCR

All primers generating specific amplicons in the conventional PCR and real-time PCR reactions were tested for their suitability for the development of the multiplex real-time melt curve assay. The final primer sets for the assay were selected based on the following criteria: (a) PCR reaction efficiency: A reaction efficiency around 90% or higher in singleplex reaction was preferred (b) T_m difference between amplicons: a minimum of 2-3 °C difference in melting temperature between neighboring peaks was preferred, this allowed us to achieve a better resolution between melting peaks and avoid any overlap of melting peak (c) Product size: amplicon size between 51 - 250 bp was more desirable.

The multiplex assay for the detection of ESBL and carbapenemase resistant pathogens was designed in two sets. The first set included five primer-pairs for the amplification of bla$_{KPC}$, bla$_{NDM}$, bla$_{CTX}$, bla$_{CMY}$, bla$_{VIM}$ genes with IAC (Table 5.1). The second set targeted bla$_{IMP}$, bla$_{OXA}$, bla$_{SHV}$, bla$_{TEM}$, bla$_{ACC}$ antibiotic resistant genes and IAC (Table 5.2). Real-time PCR assay was performed in 15 µL reaction volume with 2× MeltDoctor™ HRM master mix (Applied Biosystems, Foster City, CA, USA). A touchdown PCR amplification protocol was used on LightCycler® 96 real-time PCR platform (Roche Diagnostics Corporation, Indianapolis, USA). The PCR program for the amplification consisted for following step: 10 min of initial denaturation step at 95 °C followed by 40 cycles of denaturation at 95 °C for 10 s, initial annealing at 65 °C for 40 s (gradual temperature decrement of 0.2 °C per cycle, final annealing temperature 60 °C), extension and data collection at 72°C for 10 sec. Melt Cure was performed at 0.04 °C/s (25 reading/ °C).

Table 5.1 Primer set for the first multiplex assay.

Oligo	Oligo sequence	Oligo conc.	Product size (bp)	Amplicon T_m (°C)
KPC-688-F	GTCGGAGACAAAACCGGAAC	0.26 μM	51	77.8
KPC-738-R	ATAGTCATTTGCCGTGCCATA	0.26 μM		
NDM-310-F	TGGATCAAGCAGGAGATCAA	0.26 μM	250	88.4
NDM-559-R	GGCCGGGGTAAAATACCTT	0.26 μM		
CTX-583-F	AATCTGACGCTGGGTAAAG	0.16 μM	140	85.5
CTX-722-R	CCGCTGCCGGTTTTATC	0.16 μM		
CMY-55-F	GTTCAGGAGAAAACGCTCCA	0.2 μM	87	82.2
CMY-141-R	CCAGCCTAATCCCTGGTACA	0.2 μM		
VIM-215-F	TCATTGTCCGTGATGGTGAT	0.26 μM	51	74.4
VIM-265-R	ACCCCACGCTGTATCAATCC	0.26 μM		
IAC-NDM-pp	catataTGGATCAAGCAGGAGATCAAAT ATATATATTATCAAAATAAGACTAAT AAAGAAGGTATTTTACCCCGGCCcatac	10 fg/15 μl	74	69

Table 5.2 Primer set for the second multiplex assay.

Oligo	Oligo sequence	Oligo conc.	Product size (bp)	Amplicon T_m (°C)
IMP-311-F	TAGAGTGGCTTAATTCTCRATC	0.16 μM	75	77.8
IMP-577-R	CTTCTAWATTTGCGTCACCC	0.16 μM		
OXA48-63-F	AGCAAAGGAATGGCAAGAAA	0.23 μM	65	73.6
OXA48-127-R	CGCCCTGTGATTTATGTTCA	0.23 μM		
SHV-309-F	GGTCAGCGAAAAACAYCTTG	0.4 μM	195	88.2
SHV-503-R	GCCTCATTCAGTTCCGTTTC	0.4 μM		
TEM-94-F	GATACGGGAGGGCTTACCAT	0.2 μM	146	85.0
TEM-239-R	GGATGGAGGCGGATAAAGTT	0.2 μM		
ACC-238-F	GAGCAAATTCGGCAGAGAAA	0.2 μM	115	81.6
ACC-352-R	CAAGATGCAACAGGCTCTGA	0.2 μM		
IAC-SHV-pp	catataGGTCAGCGAAAAACAYCTTGAT ATATATATTATCAAAATAAGACTAAT AAAGGAAACGGAACTGAATGAGGCcatac	10 fg/15 μl	75	70

5.2.6 Validation of the assay

The assay for the detection of ESBL resistant pathogens developed in this study was validated using DNA samples obtained from four laboratories: Robert Koch Institute (Berlin, Germany), Calgary Laboratory Services (Calgary, AB, Canada), Institute of Microbiology, University of Lausanne and University Hospital Center (Lausanne, Switzerland), and University of Missouri Hospital (MO, USA). A total of 38 strains that have been previously characterized for ESBL and carbapenem resistance genetic markers were analyzed using the multiplex assay developed in our study.

5.2.7 *In silico* analysis of primer pairs used for multiplex assay

The diversity of the family of enzymes conferring antibiotic resistance is very high. In addition, each family of enzymes comprises of hundreds of allelic variants of the specific gene. Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was performed for each of the primer-pairs used in this study to find allelic variants that possessed similar primer binding sites.

5.3 RESULTS

5.3.1 Multiplex real-time PCR

Two multiplex real-time PCR reactions were developed for the identification of 10 genes, coding for ESBLs and carbapenemase enzymes. The first set targeted bla_{CTX}, bla_{CMY}, bla_{VIM}, bla_{KPC}, bla_{NDM} and IAC (Figure 5.1). The IAC molecule (IAC-NDM-pp) in this reaction was amplified using primer pairs, NDM-310-F and NDM-559-R, generating a separate melting peak of 69 °C. Because two targets (bla_{NDM} and IAC) were

simultaneously amplified using one primer pair, the IAC molecule was added at a very low concentration (10 pg/15 µL reaction) in order to allow preferential amplification of the target DNA (instead of IAC). As a result, the melt peak formed by the IAC amplicon was much smaller than the other peaks of the multiplex assay (Figure 5.1A). However, in the absence of the NDM target or in the non-template control (NTC), a bigger IAC melt peak was observed (Figure 5.1B). The second multiplex reaction targeted bla$_{IMP}$, bla$_{OXA}$, bla$_{SHV}$, bla$_{TEM}$, bla$_{ACC}$ and IAC (Figure 5.2A). In this multiplex reaction, the IAC molecule (IAC-SHV-pp) was co-amplified by SHV-309-F and SHV-503-R primer-pairs and the IAC amplicon generated a separate melt peak of 70 °C (Figure 5.2B).

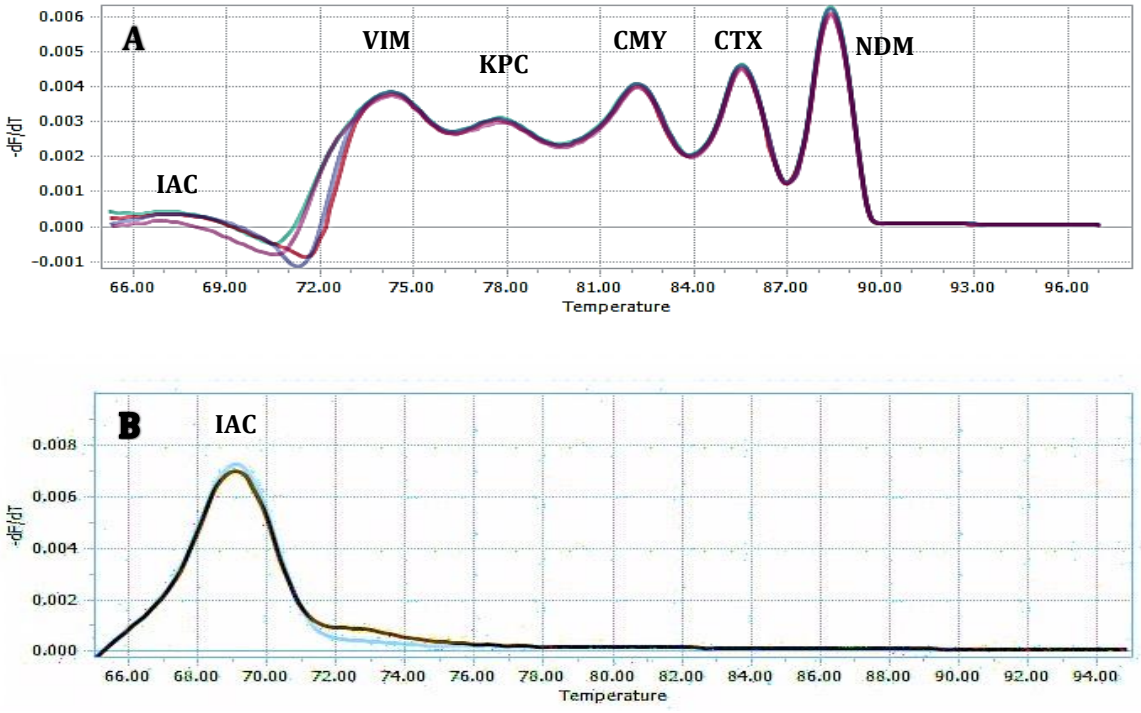

Figure 5.1 Multiplex assay for the detection of bla$_{VIM}$, bla$_{KPC}$, bla$_{CMY}$, bla$_{CTX}$ and bla$_{NDM}$ with IAC (A) bla$_{VIM}$, bla$_{KPC}$, bla$_{CMY}$, bla$_{CTX}$ and bla$_{NDM}$ with IAC. (B) Non-template control sample showing the IAC melt peak.

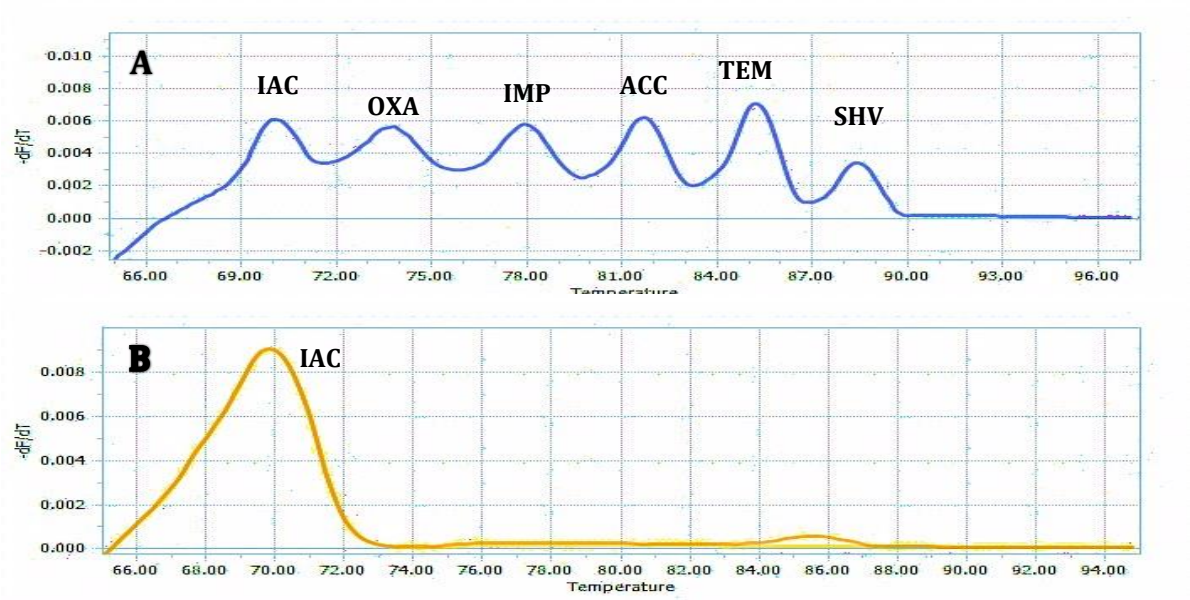

Figure 5.2: Multiplex assay for the detection bla$_{OXA}$, bla$_{IMP}$, bla$_{ACC}$, bla$_{TEM}$ and bla$_{SHV}$ with IAC (A) bla$_{OXA}$, bla$_{IMP}$, bla$_{ACC}$, bla$_{TEM}$ and bla$_{SHV}$ with IAC. (B) Non-template control sample showing the IAC melt peak.

5.3.2 Validation of the assay

The result of our multiplex PCR assay mostly concurred with earlier reports (Eller and others 2013; Pfeifer and others 2011; Pfeifer and others 2012; Yong and others 2009; Peirano and others 2011a; Peirano and others 2011b), but in addition to the previously reported antibiotic resistant (ABR) determinants (+), our multiplex assay was also able to detect the presence of a few other ABR genes (+) (Table 5.3). *Salmonella enterica* Newport 209/10 and *Escherichia coli* 2/10, were previously reported as positive for bla$_{CTX}$ and bla$_{OXA}$ respectively. However, *Salmonella enterica* Newport 209/10 and *Escherichia coli* 2/10 tested negative for bla$_{CTX}$ and bla$_{OXA}$ genes, respectively, using our multiplex assay. This might be due to the high allelic diversity within each antibiotic hydrolyzing enzyme family.

Table 5.3 Antibiotic resistance gene profiling using our multiplex assay.

Organism	**CTX**	**CMY**	**NDM**	**KPC**	**VIM**	**OXA-48 like**	**IMP**	**ACC**	**TEM**	**SHV**
Salmonella enterica Infantis 50/07									+	
Salmonella enterica Typhimurium 58/07										+
Salmonella enterica Infantis 49/07	+								+	
Salmonella enterica Newport 209/10	ND									
Salmonella enterica Kentucky 184/10	+									
Salmonella enterica Paratyphi 77/08		+							+	
Salmonella enterica Bareilly 277/10								+		
Klebsiella pneumoniae 93/08				+					+	+
Klebsiella pneumoniae 229/09	+					+			+	+
Klebsiella pneumoniae 93/10	+	+	+						+	+
Escherichia coli 2/10	+		+			ND			+	
Enterobacter cloacae 146/09					+					+
Pseudomonas aeruginosa 82/10							+			
Escherichia coli 85.01	+								+	
Escherichia coli MHO1	+		+						+	
Klebsiella pneumoniae KPCG02				+					+	+
Escherichia coli 14-115-002523									+	
Escherichia coli 14-139-00319	+									
Escherichia coli 14-125-002399	+									+
Escherichia coli 229-1384	+								+	

+ = Antibiotic resistant marker reported by previous reports.
+ = Other antibiotic resistant genetic marker found positive using our multiplex-assay
ND = Antibiotic resistant genes reported by previous researcher but not detected using our multiplex assay

In another comparative study, DNA samples obtained from the Institute of Microbiology, University of Lausanne and University Hospital Center (Lausanne, Switzerland) was evaluated using our multiplex assay. In a previous study, these isolates were evaluated using MALDI-TOF (Vogne and others 2014) for the presence of antibiotic resistance. The multiplex assay developed in our study, worked equally well for a wide range of pathogenic bacteria that included *Enterobacter cloacae, E. coli, Klebsiella pneumoniae, Morganella morganii, Pseudomonas aeruginosa, Providencia stuartii, Serratia marcescens* and *Salmonella.* With the exception of *Serratia marcescens* 8057, the antibiotic resistant profile of all other isolates were either in agreement with data obtained from MALDI-TOF or our assay generated much more superior results detecting a greater number of antibiotic resistant genes for the same strain (Table 5.4).

Table 5.4 Antibiotic resistant profiling using multiplex real-time PCR assay and its comparison with previous reports.

Strain	MALDI TOF	Our real-time PCR Multiplex Assay
Enterobacter cloacae 8121	VIM	VIM
Serratia marcescens 5965	OXA-48	OXA-48
Serratia marcescens 8057	KPC	--
Morganella morganii 7572	NDM	NDM
Providencia stuartii 8117	VIM	VIM, TEM
Providencia stuartii 8118	VIM	VIM, TEM
Pseudomonas aeruginosa 7622	VIM	VIM
Pseudomonas aeruginosa 6487	VIM	VIM
Pseudomonas aeruginosa 7072	VIM	VIM
Klebsiella pneumoniae 7677	VIM	VIM, SHV
Klebsiella pneumoniae 7678	VIM	VIM, SHV
Klebsiella pneumoniae 7877	NDM	NDM, CTX, TEM, SHV
Klebsiella pneumoniae 7932	NDM	NDM, CTX, SHV
Klebsiella pneumoniae 8052	KPC	KPC, TEM, SHV
Klebsiella pneumoniae 8083	KPC	KPC, TEM, SHV
Klebsiella pneumoniae 8161	KPC	KPC, TEM, SHV
Escherichia coli 7469	OXA-48	OXA-48, TEM
Enterobacter cloacae 8088	OXA-48	OXA-48

5.3.3 *In silico* analysis of primer pair used for multiplex assay

Conserved primer pairs were designed for the multiplex real-time PCR assays to amplify the dominant as well as other less commonly or geographically restricted allelic variants. *In silico* analysis (Primer-BLAST) was performed in order to check the number of allelic variants that can be targeted using conserved primer-pairs used in our study. The primer-BLAST results shows that, in addition to dominant allelic variants, our primer pairs were capable of amplifying a vast number of allelic variants that are commonly found in the Enterobacteriaceae (Table 5.5).

5.4 DISCUSSION

In this study, a multiplex real-time PCR assay for the detection of ESBL and carbapenem resistance genes was developed. Because the diversity of genes expressing resistance towards ESBL and the carbapenem group of antibiotics is very high, only 10 of the most common genes (bla$_{KPC}$, bla$_{NDM}$, bla$_{CTX}$, bla$_{CMY}$, bla$_{VIM}$, bla$_{IMP}$, bla$_{OXA}$, bla$_{SHV}$, bla$_{TEM}$, bla$_{ACC}$) were targeted for development of this multiplex real-time PCR assay. According to a recent review (Lupo and others 2013), *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{CMY}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{OXA-48}}$, 16S rRNA methylases genes and mutations in *gyr*A and *par*C are the most common antibiotic resistance genes in the Enterobacteriaceae family and these genes should be targeted for the development of non-phenotypic tests to detect and characterize antibiotic resistance mechanisms. The same review also emphasized the importance of bla$_{VIM}$ and bla$_{IMP}$ for the detection of carbapenem resistance. The 16S rRNA methylases gene is responsible for conferring high-level aminoglycoside resistance, whereas mutations in *gyr*A, *gyr*B and *par*C genes provide resistance towards

quinolone (Doi and other 2004; Tankovic and other 1996). Consequently, 16S rRNA methylases, *gyr*A, *gyr*B and *par*C were not included in our study.

The assay developed in the study detected all the genetic determinants that have been previously reported for the sample. However, for some of the samples this multiplex PCR assay detected the presence of more antibiotic resistance genes. Because conserved regions of the antibiotic resistance genes were targeted for primer designing, that may have facilitated the detection of a higher of number allelic variants. A large number of samples were found to be positive for KPC, CMY, CTX-M genes using this multiplex real-time PCR assay, which is conflicting with previous studies. DNA sequencing of the KPC, CMY and CTX-M PCR amplicons must be performed for the validation of the assay. The diversity of allelic variants of each antibiotic resistance genes targeted in this study is high. However, there are a few allelic variants that are dominant in the enteric pathogens (e.g. KPC-1, KPC-2, NDM-1, CTX-M-15, CMY-2, VIM-1, VIM-2, IMP-1, IMP-2, OXA-48, SHV-12, TEM-1, ACC-1). In addition to these dominant allelic variants, PCR primers designed in this study were able to detect the presence of other less frequently found variants (Table 5.5). *Salmonella enterica* Newport 209/10 possess the CTX-M-8 enzyme, which cannot be amplified by the primers used in this study, hence it was not detected. Similarly, *E. coli* 2/10 codes for OXA-1 and OXA-2 enzymes, which have a very different sequence from OXA-48, thus their detection is not possible with OXA-48-like primers.

Table 5.5 Allelic variants with the same primer-binding site

Gene	Primer sequence	Allelic variants
KPC	GTCGGAGACAAAACCGGAAC ATAGTCATTTGCCGTGCCATA	**blaKPC-1, blaKPC-2** , blaKPC-3, blaKPC-4, blaKPC-5, blaKPC-6, blaKPC-7, blaKPC-8, blaKPC-9, blaKPC-11, blaKPC-13, blaKPC-15, blaKPC-16, blaKPC-17, blaKPC-19
NDM	TGGATCAAGCAGGAGATCAA GGCCGGGGTAAAATACCTT	**blaNDM-1**, blaNDM-3, blaNDM-4, blaNDM-5, blaNDM-6, blaNDM-7, blaNDM-8, blaNDM-9, blaNDM-10, blaNDM-12
CTX	AATCTGACGCTGGGTAAAG CCGCTGCCGGTTTTATC	blaCTX-M-1, blaCTX-M-2, blaCTX-M-3, blaCTX-M-4, blaCTX-M-5, blaCTX-M-12, **blaCTX-M-15**, blaCTX-M-22, blaCTX-M-29, blaCTX-M-32, blaCTX-M-28, blaCTX-M-30, blaCTX-M-31, blaCTX-M-34, blaCTX-M-35, blaCTX-M-36, blaCTX-M-37, blaCTX-M-44, blaCTX-M-54, blaCTX-M-55, blaCTX-M-56, blaCTX-M-57, blaCTX-M-58, blaCTX-M-59, blaCTX-M-61, blaCTX-M-66, blaCTX-M-68, blaCTX-M-69, blaCTX-M-71, blaCTX-M-79, blaCTX-M-82, blaCTX-M-89, blaCTX-M-101, blaCTX-M-103,blaCTX-M-108, blaCTX-M-109, blaCTX-M-114, blaCTX-M-116, blaCTX-M-117, blaCTX-M-131, blaCTX-M-132, blaCTX-M-136, blaCTX-M-137, blaCTX-M-141, blaCTX-M-142, blaCTX-M-144
CMY	GTTCAGGAGAAAACGCTCCA CCAGCCTAATCCCTGGTACA	**blaCMY-2**, blaCMY-4, blaCMY-6, blaCMY-7, blaCMY-12, blaCMY-14, blaCMY-15, blaCMY-16, blaCMY-18, blaCMY-21, blaCMY-22, blaCMY-23, blaCMY-25, blaCMY-26, blaCMY-27, blaCMY-28, blaCMY-29, blaCMY-30, blaCMY-31, blaCMY-32, blaCMY-33, blaCMY-37, blaCMY-38,blaCMY-39, blaCMY-40, blaCMY-41, blaCMY-42, blaCMY-43, blaCMY-45, blaCMY-46, blaCMY-47, blaCMY-48, blaCMY-49, blaCMY-50, blaCMY-51, blaCMY-53, blaCMY-54, blaCMY-55, blaCMY-56, blaCMY-57, blaCMY-58, blaCMY-59, blaCMY-60, blaCMY-61, blaCMY-62, blaCMY-63, blaCMY-64, blaCMY-65, blaCMY-66, blaCMY-67, blaCMY-68, blaCMY-69, blaCMY-71, blaCMY-72, blaCMY-73, blaCMY-75, blaCMY-77, blaCMY-78, blaCMY-79, blaCMY-80, blaCMY-81, blaCMY-84, blaCMY-85, blaCMY-

		86, blaCMY-87, blaCMY-90, blaCMY-96, blaCMY-97, blaCMY-99, blaCMY-102, blaCMY-104, blaCMY-105, blaCMY-108, blaCMY-110, blaCMY-111
VIM	TCATTGTCCGTGATGGTGAT CACCCCACGCTGTATCAATC	**blaVIM-1, blaVIM-2**, blaVIM-4, blaVIM-5, blaVIM-19, blaVIM-23, blaVIM-24, blaVIM-25, blaVIM-27, blaVIM-31, blaVIM-33, blaVIM-34, blaVIM-35, blaVIM-39, blaVIM-40
IMP	TAGAGTGGCTTAATTCTCRATC CTTCTAWATTTGCGTCACCC	**blaIMP-1, blaIMP-2,** blaIMP-4, blaIMP-6, blaIMP-8, blaIMP-10, blaIMP-13, blaIMP-19, blaIMP-24, blaIMP-26, blaIMP-27, blaIMP-32, blaIMP-34, blaIMP-38
OXA	AGCAAAGGAATGGCAAGAAA CGCCCTGTGATTTATGTTCA	**blaOXA-48,** blaOXA-162, blaOXA-163, blaOXA-181, blaOXA-232, blaOXA-244, blaOXA-247, blaOXA-370
SHV	GGTCAGCGAAAAACAYCTTG GCCTCATTCAGTTCCGTTTC	blaSHV-1, blaSHV-2, blaSHV-5, blaSHV-7, blaSHV-8, blaSHV-11, **blaSHV-12**, blaSHV-13, blaSHV-14, blaSHV-18, blaSHV-24, blaSHV-25, blaSHV-26, blaSHV-27, blaSHV-28, blaSHV-29, blaSHV-30, blaSHV-31, blaSHV-33, blaSHV-36, blaSHV-37, blaSHV-38, blaSHV-40, blaSHV-41, blaSHV-42, blaSHV-44, blaSHV-45, blaSHV-46, blaSHV-49, blaSHV-50, blaSHV-51, blaSHV-52, blaSHV-53, blaSHV-55, blaSHV-56, blaSHV-59, blaSHV-60, blaSHV-61, blaSHV-62, blaSHV-63, blaSHV-64, blaSHV-65, blaSHV-66, blaSHV-67, blaSHV-69, blaSHV-71, blaSHV-72, blaSHV-73, blaSHV-74, blaSHV-76, blaSHV-77, blaSHV-78, blaSHV-79, blaSHV-80, blaSHV-82, blaSHV-83, blaSHV-85, blaSHV-86, blaSHV-92, blaSHV-93, blaSHV-94, blaSHV-95, blaSHV-98, blaSHV-99, blaSHV-101, blaSHV-102, blaSHV-103, blaSHV-104, blaSHV-105, blaSHV-106, blaSHV-107, blaSHV-108, blaSHV-109, blaSHV-110, blaSHV-119, blaSHV-120, blaSHV-121, blaSHV-122, blaSHV-123, blaSHV-124, blaSHV-125, blaSHV-126, blaSHV-128, blaSHV-129, blaSHV-132, blaSHV-133, blaSHV-134, blaSHV-135, blaSHV-137, blaSHV-140, blaSHV-141, blaSHV-142, blaSHV-143, blaSHV-144, blaSHV-145, blaSHV-147, blaSHV-148, blaSHV-149, blaSHV-150, blaSHV-152, blaSHV-153, blaSHV-154,

		blaSHV-155, blaSHV-156, blaSHV-157, blaSHV-158, blaSHV-159, blaSHV-160, blaSHV-161, blaSHV-162, blaSHV-163, blaSHV-164, blaSHV-165, blaSHV-167, blaSHV-168, blaSHV-172, blaSHV-173, blaSHV-178, blaSHV-179, blaSHV-183,
TEM	GATACGGGAGGGCTTACCAT GGATGGAGGCGGATAAAGTT	**blaTEM-1,** blaTEM-2, blaTEM-15, blaTEM-17, blaTEM-20, blaTEM-21, blaTEM-22, blaTEM-24, blaTEM-34, blaTEM-40, blaTEM-43, blaTEM-52, blaTEM-53, blaTEM-54, blaTEM-63, blaTEM-70, blaTEM-71, blaTEM-76, blaTEM-78, blaTEM-79, blaTEM-81, blaTEM-82, blaTEM-83, blaTEM-84, blaTEM-88, blaTEM-89, blaTEM-90, blaTEM-95, blaTEM-106, blaTEM-107, blaTEM-109, blaTEM-112, blaTEM-113, blaTEM-114, blaTEM-115, blaTEM-116, blaTEM-120, blaTEM-121, blaTEM-123, blaTEM-124, blaTEM-125, blaTEM-126, blaTEM-131, blaTEM-132, blaTEM-134, blaTEM-135, blaTEM-136, blaTEM-137, blaTEM-142, blaTEM-143, blaTEM-144, blaTEM-149, blaTEM-150, blaTEM-153, blaTEM-154, blaTEM-155, blaTEM-158, blaTEM-159, blaTEM-160, blaTEM-167, blaTEM-169, blaTEM-184, blaTEM-185, blaTEM-195, blaTEM-197, blaTEM-201, blaTEM-207, blaTEM-209, blaTEM-212, blaTEM-216, blaTEM-217
ACC	GAGCAAATTCGGCAGAGAAA CAAGATGCAACAGGCTCTGA	**blaACC-1,** blaACC-4, blaACC-5

Monteiro and other (2012) reported a real-time PCR melt curve assay for the detection of carbapenemase genes. The assay targeted the most common types of serine-β-lactamase and metallo-β-lactamase genes: *bla*$_{KPC}$ type, *bla*$_{IMP}$ type, *bla*$_{GES}$ type, *bla*$_{VIM}$ type, *bla*$_{OXA-48}$ and *bla*$_{NDM-1}$. The assay generated some melt peaks that were very close to one another (*bla*$_{IMP}$ type -T_m 80.1 °C, *bla*$_{OXA-48}$ - T_m 81.6 °C, *bla*$_{VIM}$ type - T_m 90.3 °C and *bla*$_{KPC}$ type - T_m 91.6 °C). Additionally, the assay also lacked an IAC,

which is an essential component of all diagnostic assays. A similar SYBR® Green dye-based multiplex real-time PCR melt curve assay for the detection of carbapenemases resistance was reported (Hofko and others 2014). The assay's panel 1 amplified IMP-1, IMP-2, GES, KPC, VIM-2, and 16S rRNA as an IAC, whereas panel 2 targeted the OXA-23-like, VIM-1, OXA-48- like, and NDM genes. The assay not only generated overlapping peaks (IMP-1 and IMP-2, GES and VIM-2, OXA-23-like and OXA-48- like, VIM-1 and NDM), which can be barely separated if bacterial samples possess both the genes. Another drawback of the assay was that panel 2 of the assay lacked an IAC. At present, there are two commercial real-time PCR kits available for the detection of ESBL and carbapenem resistant pathogens. Check-Direct CPE Kit (Check-Points Health B.V., Wageningen, Netherlands) targets four genes (e.g. KPC, OXA-48, VIM, NDM) whereas Check-MDR ESBL kit (Check-Points Health B.V., Wageningen, Netherlands) targets three genes (e.g. CTX-M, TEM, SHV). The ESBL and carbapenem detection assay developed in our study targets a much higher number of genes (10 genes) that is much higher than the combined number of targets of the two kits. Further, our assay does not use expensive dual-labeled fluorescent probes or require a high end real-time PCR machine like ABI 7500, CFX96TM, LightCycler® 480 which are required by these commercial kits. Our assay has a modest requirement of oligonucleotides, real-time PCR master mix with a high resolution melting dye and a basic real-time PCR machine making it one of the most commercially viable methods. Irrespective of the above-mentioned facts, the assay developed in this study does have some limitations. The assay does not target CTX-M-14, which is the most common enzyme of the CTX family and it is not designed to detect other rare variants of the CTX-M-8 and CTX-M-25 groups.

5.5 CONCLUSIONS

In this study, a multiplex real-time melt curve PCR assay was developed that can be used to detect the presence of 10 genes that confer resistance towards the ESBL and carbapenem groups of antibiotics. This assay can be used by regulatory agencies (USDA and CDC) for conducting surveillance of antibiotic resistant foodborne pathogens.

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Chapter 6 Multiplex real-time PCR assays for the Detection of Eight Shiga Toxin-Producing *Escherichia coli* Using Melting Curve Analysis

ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) are pathogenic strains of *E. coli* that cause bloody diarrhea. Seven STEC serogroups, O157, O26, O45, O103, O111, O121 and O145 are responsible for more than 71% of total infections caused by this group of pathogen. Thus, in addition to *E. coli* O157, the USDA-FSIS declared additional six STEC serogroups as adulterants. Two sets of multiplex melt curve real-time PCR assays with an internal amplification control were standardized for the detection of STEC serogroups. The first multiplex assay targeted *E. coli* O145, *E. coli* O121, *E. coli* O104, *E. coli* O157, *E. coli* O26, *E. coli* O45, *E. coli* O103 and *E. coli* O111; while the second set detected *E. coli* O145, *E. coli* O121, *E. coli* O157, *E. coli* O26, *E. coli* O45, *E. coli* O103, *E. coli* O111, stx_1 and stx_2, and *Salmonella*. The applicability of the assays was tested using 11 different meat and produce samples. For food samples spiked with a cocktail of four STEC serogroups with a combined count of 10 CFU/25 g food, all targets of the multiplex assays were detected after an enrichment period of 6 h. The assays also worked efficiently when 325 g of food sample was spiked with 10 CFU of STEC. The assays developed in this study can be used for the detection of eight STEC serogroups and can be completed in less than 11 h.

6.1 INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are a group of *E. coli* strains with the ability to produce shiga toxin via the expression of the stx_1 and stx_2 genes. STECs are broadly divided into *E. coli* serotype O157 and non-O157 serogroups. Non-O157 serogroups in the last decade has emerged as a major food-borne pathogen of concern worldwide (Smith and others 2014). In Canada, they were involved in 63% of the total STEC infections (Thompson and others 2005), in Denmark, 74% of cases are related to non-O157 (Nielsen and others 2006), and data from Germany showed the highest incidence rate (82%) (Werber and others 2008), whereas the infection rate in Netherland was reported to be 80% (Van Duynhoven and others 2008). STEC infections lead to a variety of illnesses with varying severity, including diarrhea, hemorrhagic colitis, hemolytic uremic syndrome (HUS), and death. Hence in the year 2000, STEC strains causing human illness were included as notifiable pathogens to the Nationally Notifiable Diseases Surveillance System (Gould and others 2013). Till date, a large number of STEC serogroups have been identified, but not all serogroups are pathogenic to humans. The frequency of infections caused by a few serogroups is much higher than by other STEC serogroups. Among various non-O157 STEC serogroups, six serogroups are most commonly reported: O26 (26%), O103 (22%), O111 (19%), O121 (6%), O45 (5%), and O145 (4%) (Gould and others 2013). According to the Centers for Disease Control and Prevention (CDC), in the last five years (2010 to 2014), STECs led to 14 foodborne outbreaks in the United States (US). Hale and others (2012), reported that around 231,157

cases of infections are caused by this group of pathogens in the U.S. annually. Out of these, *E. coli* O157 led to 40.3% of infections, whereas non-O157 serogroups caused the remaining 59.7% cases. In another study that analyzed data collected from 2000 to 2010 by USDA Food Safety and Inspection Service (FSIS), a total of 7694 STEC-related cases was reported, out of which 5688 (73.9%) were linked to the O157 serogroup, while as of 2006, 35.2% cases were caused by non-O157 STECs. Hence, in light of these new data and also due to increasing incidences of non-O157 STECs, the USDA in 2012 declared these six non-O157 STEC serogroups, also known as the big six non-O157 (O26, O103, O111, O121, O45, and O145) as adulterants (zero tolerance policy) in non-intact raw beef products (USDA 2011).

STEC serogroup O104 has been associated with sporadic cases of food-borne outbreaks related to milk and sprouts. In 2011, *E. coli* O104:H4 led to a major multi-country outbreak in Europe, which was linked to the consumption of fenugreek sprouts (Baranzoni and others 2014). In the recent past, consumption of fresh produce and sprouts has increased and along with it, the foodborne outbreaks associated with them. Detection of STECs in sprouts is a challenging task as the product has a high background microflora, the pathogen is internalized and the presence of other coliforms can interfere with their detection (Weagant and Bound 2001).

STECs are zoonotic pathogens, beef cattle and ruminants act as common reservoirs of STECs, including O157 and non-O157 serogroups (Kaspar and others 2010). These pathogens are found in the intestinal tract and animal feces, hence they make their way into food products of animal origin during the slaughtering process. Beef products contaminated with animal feces have been associated with STEC infections in human

(Smith and Fratamico 2005). These pathogens have been also reported to contaminate milk, cheese and other dairy products (Kaspar and others 2010).

Immunological methods, such as immunomagnetic separation (IMS) and latex agglutination, are rapid and easy to perform for the detection of STECs. However, manufacturing these kits requires high quality antibodies and reagents. Further, commercially available differential agar media used for the isolation of non-O157 suffer from their inability to differentiate STECs from non-pathogenic *E. coli* in mixed bacterial samples (Smith and others 2014). Molecular methods, such as TaqMan™ real-time PCR are specific as well as sensitive methods for the detection of STECs, but these methods have a high running cost. Thus, the objective of this study was to develop a low-cost and equally sensitive multiplex real-time PCR assay for simultaneously detecting eight serogroups of STEC, *viz* O145, O121, O104, O157, O26, O45, O103, and O111.

6.2 MATERIALS AND METHODS

6.2.1 Bacterial strains

Non-O157 STEC reference strains set was procured from the STEC Center at Michigan State University (Michigan, USA) (Table 6.1). *E coli* O157:H7 and *Salmonella* strains were obtained from the University of Missouri Food Microbiology Lab culture collection. Cultures were grown at 37 °C in Tryptic Soy broth (TSB) (Difco Labs., MD, USA). The cultures were maintained at -50 °C in TSB (Difco Labs., MD, USA) supplemented with 30% glycerol.

Table 6.1 STEC and *Salmonella* strains used in this study

	Serotype	Strain		Serotype	Strain
1.	*E. coli* O26:H11	DEC10B	**22.**	*E. coli* O145:H[28]	4865/96
2.	*E. coli* O26:H11	97-3250	**23.**	*E. coli* O145:H NM	GS G5578620
3.	*E. coli* O26:H	MT#10	**24.**	*E. coli* O145:H NT	IH 16
4.	*E. coli* O26:H N	TB352A	**25.**	*E. coli* O104:H	ECOR 228
5.	*E. coli* O45:H2	M103-19	**26.**	*E. coli* O104:H	TW04909
6.	*E. coli* O45:H2	MI01-88	**27.**	*E. coli* O104:H	TW04911
7.	*E. coli* O45:H2	MI05-14	**28.**	*E. coli* O104:H	TW01435
8.	*E. coli* O45:H NM	DA-21	**29.**	*E. coli* O157:H7	505B
9.	*E. coli* O103:H2	MT#80	**30.**	*E. coli* O157:H7	3178-85
10.	*E. coli* O103:H6	TB154A	**31.**	*E. coli* O157:H7	43894
11.	*E. coli* O103:H25	8419	**32.**	*E. coli* O157:H7	C7927
12.	*E. coli* O103:H N	PT91-24	**33.**	*E. coli* O157:H7	MF 1847
13.	*E. coli* O111:H2	RD8	**34.**	*S.* Typhimurium	13311
14.	*E. coli* O111:H8	3215-99	**35.**	*S.* Typhimurium	14028
15.	*E. coli* O111:H11	0201 9611	**36.**	*S.* Enteritidis	13076
16.	*E. coli* O111:H NM	3007-85	**37.**	*S.* Typhimurium	LJH 666
17.	*E. coli* O121:H19	3377-85	**38.**	*S.* Newport	692
18.	*E. coli* O121:H19	MT#2	**39.**	*S.* Agona	LJH 1132
19.	*E. coli* O121:H	MT#18	**40.**	*S.* Agona	LJH 1122
20.	*E. coli* O121:H[19]	DA-5	**41.**	*S.* Typhimurium	LJH 788
21.	*E. coli* O145:H NT	D177	**42.**	*S.* Enteritidis	F3-1
			43.	*S.* Enteritidis	G5-02

6.2.2 Bacterial DNA extraction

Genomic DNA from all bacterial isolates used in the study and from enriched food samples was isolated using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The concentrations and purity of the obtained DNA samples were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

6.2.3 Primer design

Serotype-specific primer-pairs for the identification of six STEC serogroups were designed based on the *wzx* gene which encodes a flippase, whereas serotype-specific primer pairs for amplification of *E. coli* O104 was designed based on the glycosyl transferase gene sequence using the Primer3 software (Untergasser and others 2012). The *uid*A gene primer designed by (Cebula and others 1995; Wang and others 2007) was used for detection of *E. coli* O157:H7. In order to test for the presence of STEC *stx*$_1$ and *stx*$_2$, virulence genes, two pairs of conserved primers were designed for the detection seven *stx* subtypes. The *stx*$_1$ primer pair amplified *stx*$_{1a}$, *stx*$_{1c}$ and *stx*$_{1d}$ subtypes, whereas the *stx*$_2$ primers targeted *stx*$_{2a}$, *stx*$_{2c}$ *stx*$_{2d}$, and *stx*$_{2e}$ subtypes. For the detection of *Salmonella*, PCR primers based on the *Salmonella* invasin (*inv*A) virulence gene were designed (Table 6.2, 6.3, 6.4 and 6.5). The oligonucleotides were commercially synthesized (IDT, Coraville, IA, USA). The primers were designed such as to keep the melting temperature (T_m) of the PCR amplicons between 67 °C and 87 °C. The specificity of the designed PCR primers was tested using the NCBI/Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The T_m of all amplicons was estimated using the BioEdit software (Hall 1999).

Table 6.2 Primer set for the detection of eight Shiga toin producing *E. coli*.

Oligo name	Oligo sequence	Product size (bp)	Oligo conc.	Amplicon T_m (°C)
O145-F-602	ACTGGGATTGGACGTGGATA	137	0.20 µM	77.54 ± 0.05
O145-R-738	TCCTCCCAAAACTTCTAGGC		0.20 µM	
O121-F-716	TGGCTAGTGGCATTCTGATG	150	0.20 µM	75.49 ± 0.06
O121-R-865	ATGCGCTTACTCCCAAGATG		0.20 µM	
O104-F-491	CCGTAATTGAAAAGCTTGGTG	81	0.20 µM	71.45 ± 0.11
O104-R-571	CGGCTGCAAGTATCCTAAGC		0.20 µM	
O157:H7-F	TTGACCCACACTTTGCCGTAA	226	0.30 µM	83.32 ± 0.07
PT-2	GCGAAAACTGTGGAATTGGG		0.30 µM	
IAC-104121145157	5'-TGGCTAGTGGCATTCTGATGCATGGTGGC ATGGGGATTTTTTGCTGCAAGTGGGCTGTCC AGACAGTTCATAGTTGGTTTGGCCATATCTG TCGCATTACGAGAAACTTTCATCGTTGGTTT AACATGGCATCTTGGGAGTAAGCGCAT-3'	151	100 fg/10 µl	80.14 ±0.10

Table 6.3 Primer set for the detection of eight Shiga toxin producing *E. coli*.

Oligo name	Oligo sequence	Product size (bp)	Oligo conc.	Amplicon T_m (°C)
O26-F- 562	TCTGGCGTGCTATCGCTTAT	72	0.40 µM	67.6 ± 0.27
O26-R- 633	TTCCGCCCATTGAATTTTAG		0.40 µM	
O45-F- 305	GTCTGGCTGCAGGGACTTT	160	0.07 µM	73.4 ± 0.11
O45-R- 464	AGACGAGCCTGGCTTTGATA		0.07 µM	
O103-F- 752	TAGAGGATGCCGGATATTGG	169	0.07 µM	77.09 ± 0.10
O103-R- 920	GCGAGCGGTACAACAATACA		0.07 µM	
O111-F-287	AAGGCGAGGCAACACATTAT	85	0.20 µM	81.34 ± 0.13
O111-R-371	CGATGTTGATCATCTGGGAGA		0.20 µM	
IAC-2611110345	5'-AAGGCGAGGCAACACATTATTGACCC TGCGCTCTACCCGATAGCTGAGGCGGAC TGCAGGCTGGTGGTAGCACTCAGCGCAGCGGGAT GGCATCGCCACCCGCACCGGTCACCTCGACCCGA GACGCGCTCGATCTCCCAGATGATCAACATCG-3'	155	100 fg/10 µl	87.35 ± 0.13

Table 6.4 Primer set for the detection of seven Shiga toxin producing *E. coli* and *Salmonella.*

Oligo name	Oligo Sequence	Product Size (bp)	Oligo conc.	Amplicon Tm (°C)
O145-F-602	ACTGGGATTGGACGTGGATA	137	0.20 μM	77.54 ± 0.05
O145-R-738	TCCTCCCAAAACTTCTAGGC		0.20 μM	
O121-F-716	TGGCTAGTGGCATTCTGATG	150	0.30 μM	75.49 ± 0.06
O121-R-865	ATGCGCTTACTCCCAAGATG		0.30 μM	
O157:H7-F	TTGACCCACACTTTGCCGTAA	226	0.40 μM	83.32 ± 0.07
O157:H7-R	GCGAAAACTGTGGAATTGGG		0.40 μM	
Stx_2-F	TACCACTCTGCAACGTGTCG	164	0.12 μM	81.5 ± 0.2
Stx_2-R	AGGCTTCTGCTGTGACAGTG		0.12 μM	
Stx_1-F	ATCGCTTTRCTGATTTTTCA	56	0.40 μM	73.8 ± 0.2
Stx_1-R	CAATGTAACCGCWSTTGTACC		0.40 μM	
IAC-O157-set-121pp	CATATATGGCTAGTGGCATTCTGATGATA TATATATTATCAAAATAAGACTAATAAAG CATCTTGGGAGTAAGCGCATCATAC	72	30 fg/10 μl	70.56 ± 0.05

Table 6.5 Primer set for the detection of seven Shiga toxin producing *E. coli* and *Salmonella*

Oligo name	Oligo Sequence	Product Size (bp)	Oligo conc.	Amplicon Tm (°C)
O26-F- 999	AAGCGCGTTCATCCCTTTAT	83	0.30 µM	72.2 ± 0.07
O26-R- 1081	ACAATCCAACCGAACCAAAC		0.30 µM	
O45-F- 317	GGACTTTCGTTGCGTTGTG	142	0.10 µM	80.9± 0.1
O45-R- 458	GCCTGGCTTTGATACCATGT		0.10 µM	
O103-F- 752	TAGAGGATGCCGGATATTGG	169	0.25 µM	77.8 ± 0.09
O103-R- 920	GCGAGCGGTACAACAATACA		0.25 µM	
O111-F-287	AAGGCGAGGCAACACATTAT	72	0.20 µM	75.1± 0.1
O111-R- 385	GCCAAAGGTATTCACGATGTT		0.20 µM	
***Salmonella*-F**	CGGTGGGTTTTGTTGTCTTC	237	0.10 µM	83.3± 0.05
***Salmonella*-R**	TCATCGCACCGTCAAAGGA		0.10 µM	
IAC- Sal-set-103pp	CATATATAGAGGATGCCGGATATTGGATA TATATATTATCAAAATAAGACTAATAAAG TGTATTGTTGTACCGCTCGCCATAC	73	10 fg/10 µl	69.7 ± 0.05

6.2.4 Development of real-time PCR melt curve assay

All primer pairs were initially screened for their specificity using DNA samples from standard cultures. Final primer sets for the multiplex assay were selected based on PCR product melting temperature, reaction efficiency and product size. The multiplex assay was designed in four sets: (A) *E. coli* O145, *E. coli* O121, *E. coli* O104, *E. coli* O157 and IAC (Table 6.2); (B) second set targeted *E. coli* O26, *E. coli* O45, *E. coli* O103, *E. coli* O111 and IAC (Table 6.3); (C) *E. coli* O26, *E. coli* O45, *E. coli* O103, *E. coli* O111, *Salmonella* and IAC (Table 6.4); and (D) *E. coli* O145, *E. coli* O121, *E. coli* O157, stx_1, stx_2 and IAC (Table 6.5).

6.2.5 IAC design

A synthetic single stranded DNA sequence was designed that could be amplified using one of the primer pairs of the multiplex assay (generating a separate melt curve peak). IAC-104121145157, IAC-2611110345, IAC-O157-set-121pp and IAC- Sal-set-103 pp was added to the multiplex assay at a concentration of 100 fg, 10 fg, 500 fg and 500 fg per 10 µL reaction, respectively.

6.2.6 Real-time PCR

Real-time PCR assay was performed using 2× MeltDoctor HRM Master Mix (Applied Biosystems, Foster City, CA, USA). The multiplex assay was standardized on a StepOnePlus® real-time PCR (Applied Biosystems, Foster City, CA, USA) and also tested on a LightCycler® 96 real-time PCR (Roche Diagnostics Corp., Indianapolis, USA). The StepOnePlus® real-time PCR (Applied Biosystems, Foster City, CA, USA) was additionally calibrated for the MeltDoctor™ dye, whereas the fluorescence data on

LightCycler® 96 real-time PCR (Roche Diagnostics Corp., Indianapolis, USA) was collected in the FAM channel. PCR was performed with a 10µL reaction volume in duplicate with primer concentrations mentioned in Tables 6.2, 6.3, 6.4 and 6.5. A two-step amplification protocol included an initial denaturation at 94 °C for 10 min; 40 cycles of 94 °C for 30 s, 60 °C for 45 s; and a melt curve step at the end of the PCR (from 60 °C to 95 °C, with gradual temperature increments of 0.1 °C/s). A melt curve plot was prepared by plotting the negative derivative of fluorescence (–Rn) versus temperature. Mean T_m values for each product was calculated by averaging the T_m values.

6.2.7 Limit of detection of the real-time PCR assay

To determine the limit of detection of the developed multiplex assay, DNA from standard cultures of STEC strains was isolated using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA). Two equal mixture DNA samples was prepared: mixture 1 comprised of *E. coli* O145, *E. coli* O121, *E. coli* O104 and *E. coli* O157; and mixture 2 comprised of *E. coli* O26, *E. coli* O45, *E. coli* O103, *E. coli* O111 DNA sample. These DNA mixtures were then serially diluted. One microliter of each serially diluted DNA, in triplicate, was used to determine the limit of detection of the multiplex assay.

To further validate the limit of detection of the assay, overnight cultures of STECs were serially diluted in 9 ml peptone water (1.0 g/L) and enumerated using Tryptic Soy Agar (TSA) (Difco Labs., MD, USA). DNA was isolated from 1 mL of each dilution, using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) and 2 □L of the obtained DNA sample was used for performing real-time PCR in a singleplex format. The obtained real-time PCR results were then correlated with

bacterial count (CFU/mL) to estimate the limit of detection of the assay in singleplex format.

6.2.8 Comparison of enrichment media

For the selection of suitable enrichment media for the enrichment of STECs in food samples, the applicability of three enrichment media: brain heart infusion broth (BHI) (Difco Labs., MD, USA), TSB (Difco Labs., MD, USA) and buffered peptone water (BPW) (Remel, Lenexa, KS, USA) was tested and compared. All trials for the selection of suitable enrichment media were performed using ground beef with the highest fat content (73% lean/27% fat), which were inoculated with a cocktail of four STECs at a rate of 10 CFU (combined count of four STEC strains) per 25 g of ground beef. The effect of adding vancomycin cefixime cefsulodin (VCC) selective supplement (Sigma-Aldrich, United States) on the enrichment time was also tested using the three enrichment media. Because the addition of the VCC supplement slowed the growth of the STECs during enrichment, the effect of each antibiotic constituent of the VCC supplement (vancomycin - 8.0 mg/L [Sigma-Aldrich], cefixime - 0.05 mg/L [Fluka, Sigma-Aldrich], and cefsulodin - 10.0 mg/L [Sigma-Aldrich]) was further investigated using BPW (Remel) as the enrichment media.

6.2.9 Preparation of artificially spiked food samples

Ground beef of different fat contents (73% lean/27% fat, 80% lean/20% fat, 85% lean/15% fat and 93% lean/7% fat), beef stew meat, ground chicken, ground turkey (85% Lean/15% Fat), apple cider, alfalfa sprouts, spinach, shredded iceberg lettuce, and shredded romaine lettuce were purchased from a local supermarket. Beef trims (80%

lean/20% fat) were obtained from the University of Missouri (Columbia, MO, USA) Meat Lab. To study the effect of natural microflora of each food sample on the multiplex PCR assay, aerobic plate counts of each food sample were determined using the pour plate method. The inoculum for artificially contaminating food samples was prepared by growing STEC strains in TSB overnight at 37 °C. The bacterial cultures were serially diluted in 9 mL peptone water (1.0 g/L), enumerated using TSA (Difco Labs.) and the inocula was stored in the refrigerator. Based on the obtained count, calculated amount was used to inoculate the food samples and simultaneously the inoculum was also plated using TSA. This approach helped to achieve close to accurate inoculation levels. The enrichment process for the spiked food samples was performed in sterile filter stomacher bags (Fisherbrand, Houston, Texas, USA). Twenty-five grams of food sample were inoculated with either of the two STEC cocktails at a rate of 10 CFU (combined count of four STEC strains). Cocktail 1 comprised of *E. coli* O157:H7, *E. coli* O145, *E. coli* O121 and *E. coli* O104; whereas cocktail 2 comprised of *E. coli* O26, *E. coli* O45, *E. coli* O103 and *E. coli* O111. Cefixime and cefsulodin antibiotics were not added to the enrichment medium used in this study, which are used to deter the growth of *Pseudomonas and Proteus* during enrichment process. To verify the robustness of the assays food samples were additionally spiked with 10^4 CFU of *Pseudomonas aeruginosa* and 10^4 CFU of *Proteus mirabilis* per 25 g of food sample. The inoculated food samples were allowed to attach to the food matrix for 15 min at room temperature. After attachment, spiked food samples were diluted with 225 mL pre-warmed (42 °C) BPW (Remel, Lenexa, KS, USA) containing vancomycin (8 mg/L) (Sigma-Aldrich, St. Louis, USA). A negative process control was also included in the study as described by Malorny and others

(2007). A negative process control consisted of sterilized enrichment broth, with 25 g of food sample. The spiked food samples were stomached (Seward, London, UK) for 2 min after which it was incubated at 42 ± 1 °C, without shaking for 6 h. Each food sample for the two multiplex assays for the detection of eight STEC was processed in duplicates. After enrichment, DNA was isolated from 2 mL of enriched broth. Samples were centrifuged at 0.1 ×*g* for 1 min to separate suspended food particles from the media, DNA was isolated using PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) and 1.5 µL of the obtained DNA samples were used for performing real-time PCR.

6.2.10 Spiked food sample according to USDA recommendation

As according to USDA recommendations, 325 g of ground beef (85% lean/15% fat) and beef trim were measured. Each was inoculated with all eight strains of STECs at a concentration of 10 CFU/325 g (combined inoculum concentration of 80 CFU/325 g food sample). After inoculation, the cells were allowed to attach for 15 min at room temperature and the samples were diluted with 975 mL of pre-warmed (42 °C) BPW (Remel, Lenexa, KS, USA) containing vancomycin (8 mg/L) (Sigma-Aldrich, St. Louis, USA). Samples were stomached (Seward, London, UK) for 2 min and incubated for 8 h at 42 °C ± 1 °C without shaking. Two milliliters of enrichment broth were collected at 6 h and 8 h. DNA from the enriched broth samples was isolated as described above and diluted to (1:1) with nuclease free water. Two microliters of the obtained DNA sample were used for performing real-time PCR in both singleplex and multiplex formats. Additionally, the second set of multiplex reaction targeting seven STECs, *stx*$_1$, *stx*$_2$ and *Salmonella* food samples were similarly spiked and enriched as mentioned above.

6.3 RESULTS

6.3.1 Real-time PCR

The grouping of STEC serogroups in the multiplex assay was based on the frequency of occurrence of these serogroups. Among the non-O157 STEC serogroups, *E. coli* O26, *E. coli* O103, *E. coli* O111, and *E. coli* O45 are found at a much higher frequency than other serogroups (USDA, 2012). The first attempt to standardize the multiplex assay was performed without an IAC. This approach helped us to locate the free region on the melt curve that can be targeted for designing a reaction-specific IAC molecule. The melt curve showed a big gap between *E. coli* O 145 and *E. coli* O157 peaks for multiplex 1 (Figure 6.1A), while the region after the *E. coli* O45 peak was also found to be free in multiplex reaction 2 (Figure 6.1B). Two reaction-specific single stranded DNA molecules (151bp and 155bp) were designed to target the vacant regions on the melt curve. These molecules acted as the IAC for each multiplex reaction. The amount of IAC for both multiplex PCR assays was separately optimized for each of the multiplex real-time reactions.

Both multiplex assays amplified all targets and generated five peaks without any overlaps (Figure 6.2). The assay was developed using MeltDoctor™ HRM Master Mixes, which being a high resolution saturating dye unlike SYBR® Green, it equally intercalated to all five amplicons of the multiplex and generated a melt curve whereby all five peaks were clearly resolved (Figure 6.2). The average melting temperatures (T_m) of each amplicon generated in the multiplex reaction and IAC are mentioned in Table 6.2, 6.3, 6.4 and 6.5.

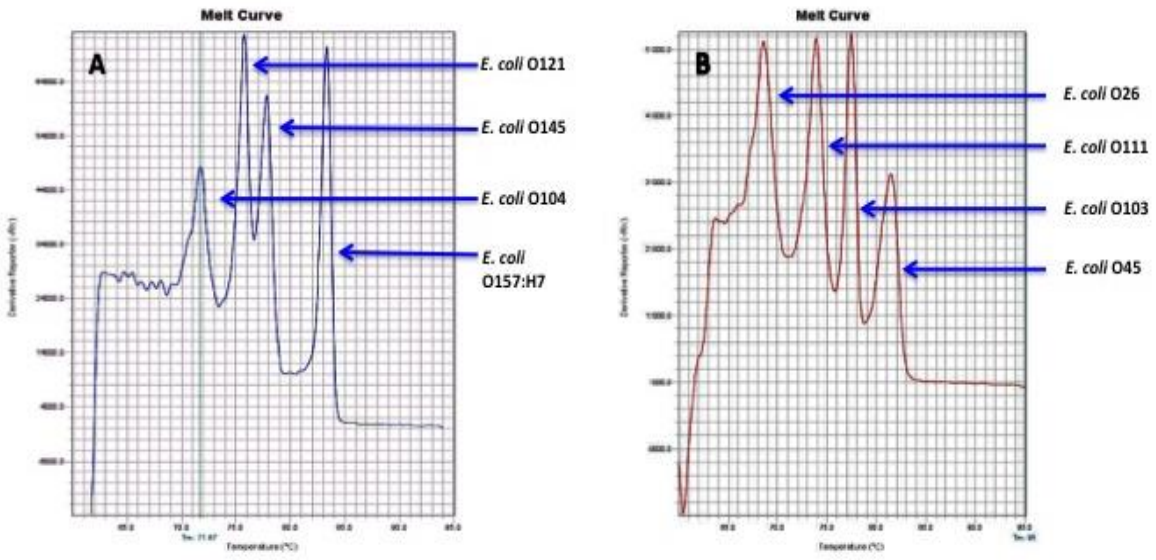

Figure 6.1 Multiplex real-time PCR melt curve assay without IAC: A) *E. coli* O104, O121, O145, and O157. B) *E. coli* O26, *E. coli* O45, *E. coli* O103 and *E. coli* O111.

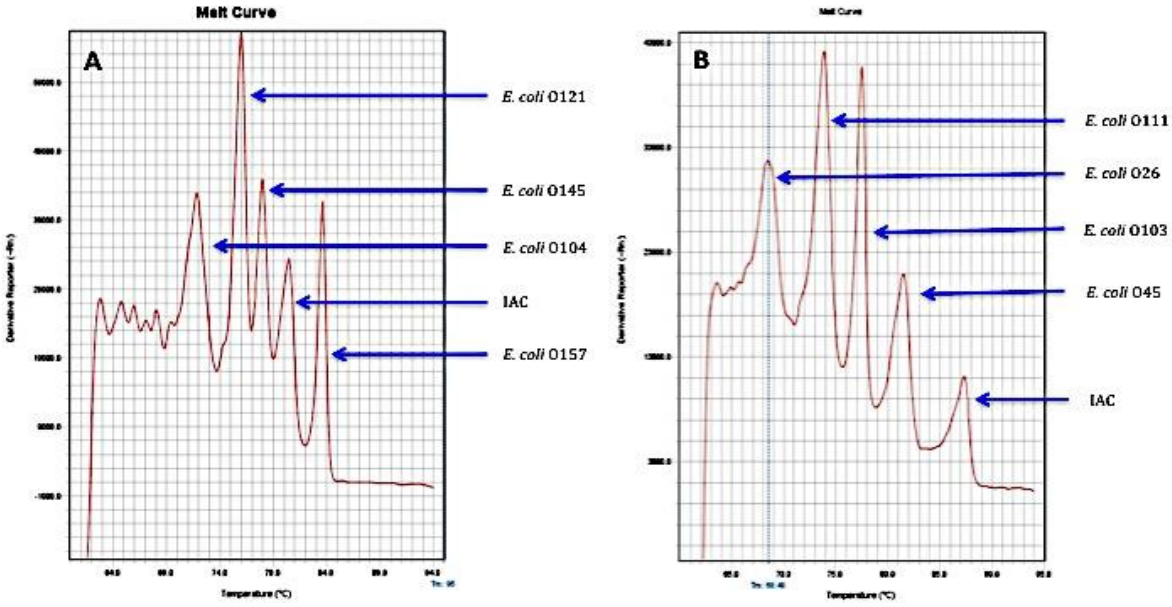

Figure 6.2 Multiplex real-time PCR melt curve assay with: A) *E. coli* O145, *E. coli* O121, *E. coli* O104, *E. coli* O157 and IAC. B) *E. coli* O26, *E. coli* O45, *E. coli* O103, *E. coli* O111 and IAC.

The presence of Shiga toxin genes (stx_1, stx_2) in a STEC strain is a prerequisite for the strain to cause HUS complications following infection. *Salmonella* is the second most frequently reported cause of foodborne outbreak and illness in the United States. Therefore, additional primer-pairs were added to our multiplex reactions for the detection of seven *stx* gene subtypes and *Salmonella*. In order to accommodate new targets (stx_1, stx_2 and *Salmonella*) in the melt curve plot, a few of the old primer pairs were replaced (*E. coli* O26, *E. coli* O45, *E. coli* O111) and new IAC molecules were also designed. The improved multiplex assay generated all six melt curve peak without any overlap (Figure 6.3).

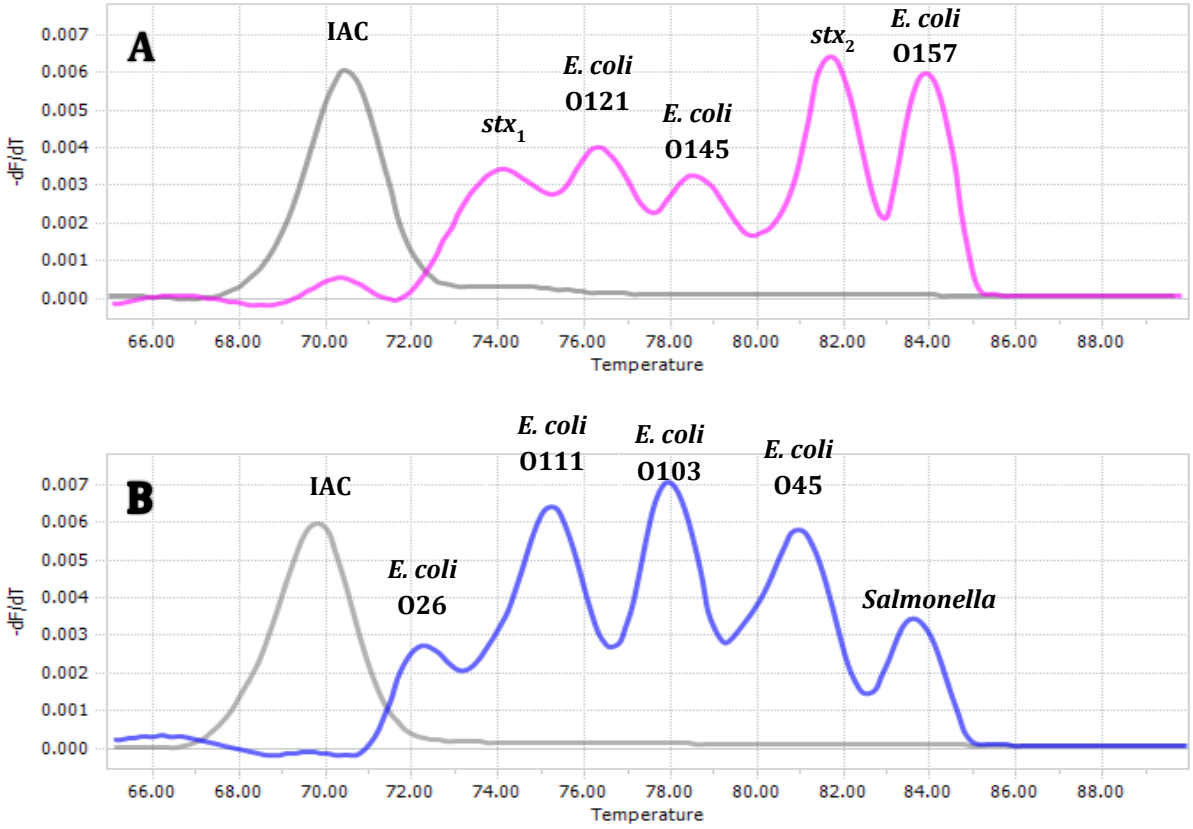

Figure 6.3 Multiplex real-time PCR assay for the detection of STEC and *Salmonella*: A) stx_1, *E. coli* O121, *E. coli* O145, stx_2, *E. coli* O157 and IAC. B) *E. coli* O26, *E. coli* O111, *E. coli* O103, *E. coli* O45, *Salmonella* and IAC.

The new multiplex reactions obtained after incorporation of additional primer pairs were able to detect: *stx*$_1$ (*stx*$_{1a}$, *stx*$_{1c}$ and *stx*$_{1d}$ subtypes), *stx*$_2$ (*stx*$_{2a}$, *stx*$_{2c}$, *stx*$_{2d}$, and *stx*$_{2e}$ subtypes), *E. coli* O121, *E. coli* O145, *E. coli* O157, *E. coli* O26, *E. coli* O111, *E. coli* O103, *E. coli* O45 and *Salmonella*. Reaction-specific IAC molecules were designed and their concentrations were separately optimized for each multiplex reaction (Figure 6.3).

6.3.2 Limit of detection of the assay

The two multiplex assays developed in this study, when tested using equal mixtures of eight STEC DNA samples, was found to be working efficiently over a broad DNA concentration range. Multiplex 1 targeting *E. coli* O145, *E. coli* O121, *E. coli* O104, *E. coli* O157 and IAC generated positive results for all targets from13.5 ng to 135 pg per reaction, whereas multiplex 2 targeting *E. coli* O26, *E. coli* O45, *E. coli* O103, *E. coli* O111 and IAC detected all targets in a DNA concentration range of 10.3 ng to 103 pg per reaction (Figure 6.4).

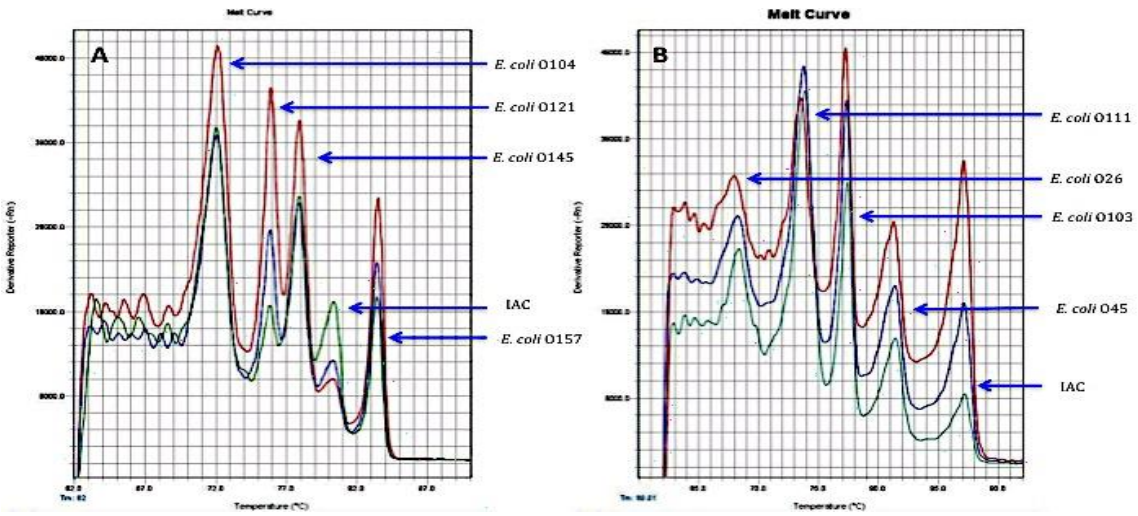

Figure 6.4 Sensitivity of multiplex real-time PCR at different DNA concentration: A) Multiplex 1 targeting *E. coli* O145, *E. coli* O121, *E. coli* O104 and *E. coli* O157 with IAC B) Multiplex 2 targeting *E. coli* O26, *E. coli* O45, *E. coli* O103 and *E. coli* 111 and IAC.

The limit of detection for each primer set, using DNA isolated from decimally diluted pure broth culture, in singleplex format was found to be in between 1.4×10^2 to 4.3×10^2 CFU/mL. The corresponding Ct values obtained for each primer pair are shown in the Table 6.6.

Table 6.6 Limit of detection of each primer in singleplex.

Bacteria	Count (CFU/ml)	Ct
***E. coli* O26**	4.3×10^2, 1.6×10^2	33.8, 34.8
***E. coli* O 45**	2.6×10^2, 2.2×10^2	34.5, 34.5
***E. coli* O103**	3.0×10^2, 1.9×10^2	34.9, 35.6
***E. coli* O111**	1.4×10^2, 1.4×10^2	35.4, 35.2
***E. coli* O121**	1.8×10^2, 1.6×10^2	34.8, 34.2
***E. coli* O145**	1.7×10^2, 1.5×10^2	34.6, 34.8
***E. coli* O104**	3.3×10^2, 2.7×10^2	36.7, 34.0
***E. coli* O157**	1.5×10^2, 1.5×10^2	36.0, 35.2

6.3.3 Comparison of enrichment media

The applicability of BHI, TSB and BPW broths was tested for the enrichment of STECs in food samples. With ground beef (73% lean/27% fat), artificially contaminated with a cocktail of four STECs at a rate of 10 CFU/25 g, all targets of the multiplex assays were detected after an enrichment period of 6 h. The addition of VCC supplement to all three enrichment media slowed the growth of STEC during the incubation. When the three antibiotics that are constituents of VCC supplement (vancomycin, cefixime,

cefsulodin) were individually tested for their inhibitory effect on enrichment time, vancomycin was the only antibiotic that had no inhibitory effect on the growth of STEC serogroups. Hence, BPW with vancomycin (8 mg/L) was selected to be the only antibiotic added to the BPW enrichment medium for further studies.

6.3.4 Artificially spiked food samples

Standard aerobic plate count of food samples used in this study were as follows: ground beef (96% Lean/4% Fat) - 5.9×10^4 CFU/g, ground beef (85% Lean/15% Fat) - 2.3×10^3 CFU/g, beef trims (80% Lean/20% Fat) - 2.0×10^3 CFU/g, ground beef (73% Lean/27% Fat) - 2.0×10^5 CFU/g, beef stew meat - 1.2×10^4 CFU/g, ground chicken - 9.1×10^8 CFU/g, ground turkey (85% Lean/15% Fat) - 1.1×10^7 CFU/g, apple cider - < 10 CFU/g, alfalfa sprouts - 1.3×10^9 CFU/g. In all food samples inoculated with a cocktail of four STECs, (10 CFU/25 g food: combined count of four STEC strains), all targets of the multiplex assay were detected following a 6-h enrichment period. The IAC in each multiplex reaction formed a separate melt peak. The IAC molecule generated a very small peak in the multiplex real-time PCR assay in presence of *E. coli* O121 or *E. coli* O103 target DNA. But, the size of IAC peaks was comparable to other peaks in absence of *E. coli* O121 or *E. coli* O 103 DNA or negative control samples. The addition of 10^4 CFU of *P. aeruginosa* and *P. mirabilis* to the 25 g of artificially contaminated food sample showed no negative effect on the assay. All targets of the multiplex assay were detected even in the presence of a high level of *P. aeruginosa* and *P. mirabilis.* The assay performed robustly with food samples of different fat contents, phenolic compounds and microbial load.

6.3.5 Spiked food sample according to USDA recommendation

As according to the USDA recommendations, 325 g of ground beef (85% lean/15% fat) was artificially inoculated with all eight strains of STECs at a concentration of 10 CFU/325 g of each strain. All targets of the multiplex assay were detected after an enrichment period of 8 h (Figure 6.5).

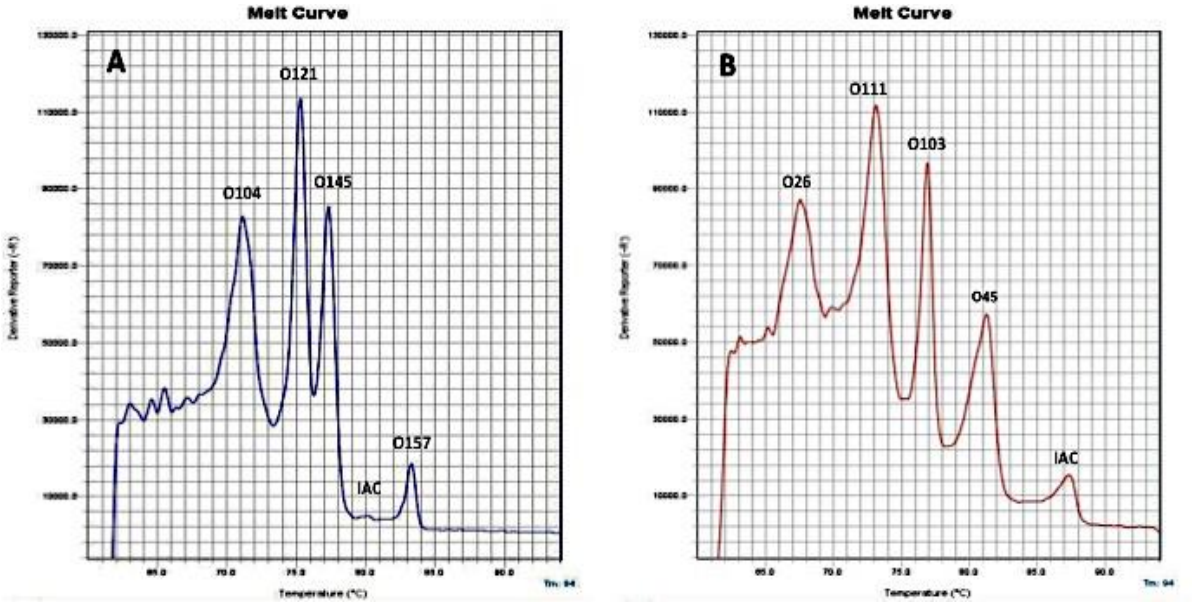

Figure 6.5 Melt curve real-time PCR using 325 g ground beef spiked with 8 STEC serogroups after 6 h of enrichment.

The DNA samples obtained after the enrichment process was used to perform real-time PCR in uniplex and multiplex format both. The obtained Ct value of each target is mentioned in Table 6.7. Almost all targets of the multiplex real-time PCR assay were detected following 6 h enrichment period, but the Ct value obtained from singleplex reaction was high. These Ct values were lowered after 8 h enrichment.

Table 6.7 Mean Ct values of each PCR primer in uniplex format, inoculated with 10 CFU/325g of ground beef.

Bacteria	**Inoculum Count**	**Ct**	
		6h	**8h**
***E. coli* O26**	10 CFU, 21 CFU	27.9, 34.2	27.5, 24.0
***E. coli* O45**	5 CFU, 19CFU	28.5, 33.4	22.3, 28.2
***E. coli* O103**	9 CFU, 15 CFU	27.8, 32.2	22.4, 20.0
***E. coli* O111**	8 CFU, 16 CFU	28.8, 32.3	23.7, 24.5
***E. coli* O121**	16 CFU, 14 CFU	27.0, 33.7	21.5, 21.9
***E. coli* O145**	11 CFU, 14 CFU	29.8, 32.7	21.3, 21.6
***E. coli* O104**	10 CFU, 12 CFU	25.6, 29.4	18.1, 21.9
***E. coli* O157**	7CFU, 21 CFU	UD, 34.6	29.8, 31.7

The multiplex reactions targeting seven STEC serogroups with *stx*$_1$, *stx*$_2$ and *Salmonella* also generated very similar results. Samples of 325 g ground beef (85% lean/ 15% fat) samples inoculated with 10-20 CFU of each pathogen could be detected following 8-h enrichment period (Figure 6.6). Addition of new primer-pairs to the multiplex real-time PCR assay and also new IAC molecules showed no adverse effect on the amplification efficiency of all primers of the multiplex assay. Hence, the enrichment time required for the detection of all the targets by multiplex assay was unaffected.

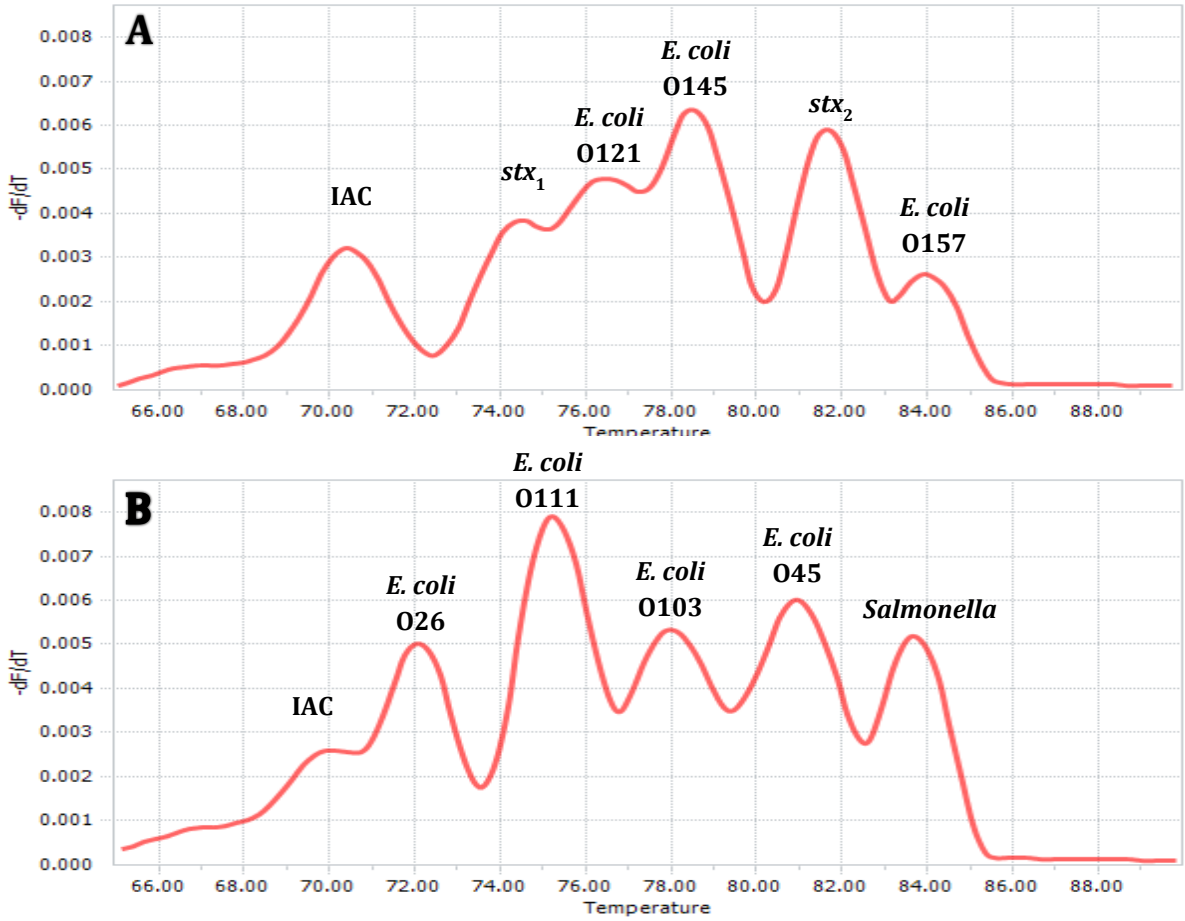

Figure 6.6 Melt curve real time PCR using 325 g ground beef spiked with seven STEC serogroups and *Salmonella* after 8 h of enrichment.

6.4 DISCUSSION

Detection of non-O157 STECs in meat and produce is a challenging task because these organisms lack typical phenotypic characteristics that differentiate them from non-pathogenic *E. coli* already present in food as part of a natural microflora. At present, various culture-based chromogenic media are available for the detection of STECs, but these methods are not stand-alone methods. They typically require an additional IMS step that confers specificity to the culture media, but in the process, increases the per-sample

cost of testing. Apart from these limitations, culture-based methods also require a much longer processing time that can vary from 18-48 h. Hence, the development of molecular methods targeting serotype-specific genetic determinants, appears to be a promising solution. O-antigen gene clusters of *E. coli* encodes the *wzx* O-antigen flippase and *wzy* O-antigen polymerase. These genes are involved in processing and translocation of the specific O units across the membrane and their polymerization into O antigen respectively (Lin and others 2011). As a result, these genes act as excellent targets for detection of non-O157 STECs at the serotype level. Multiplex assays targeting serotype-specific O-antigen cluster genes for the detection of multiple strains of STECs have been previously described (Bai and others 2012; Fratamico and others 2011; Valadez and others 2011).

A total of 115 primer pairs were designed and screened for the standardization of these multiplex assays. The primers were initially screened for their specificity using DNA isolated from a standard non-O157 STEC reference set (STEC Center, Michigan State University, MI, USA). Melting temperature (T_m) was calculated for all the primers generating specific amplicons. Multiple primers were found working for the multiplex assay. The final primer pair for the assay was selected on the basis of the following criteria: primers with higher amplification efficiency, greater T_m difference with neighboring peaks, and smaller amplicon size.

IAC is the most important component of a diagnostic assay because food matrices can harbor inhibitory components that can interfere with PCR amplification and lead to false negative results. In this study, a single-stranded synthetic oligonucleotide sequence was added to the real-time PCR reaction. The IAC oligonucleotides were amplified by

one of the primer pairs already present in the multiplex reaction mixture. IAC-104121145157 was amplified by O121-F-716 and O121-R-865, whereas IAC-2611110345 was amplified using O111-F-287 and O111-R-371 (Tables 6.2 and 6.3). For the other two multiplex reactions, IAC-O157-set-121pp was amplified using O121-F-716 and O121-R-865 while IAC- Sal-set-103pp used O103-F- 752, O103-R- 920 primer pairs for amplification (Tables 6.4 and 6.5). This approach helped in reducing the total number of primer-pairs used for the multiplex assays. The IAC oligonucleotides were added to the PCR reaction at a very low concentration. The lower concentration of IAC oligonucleotide allowed preferential amplification of STEC genomic DNA, and in the process, the size of the IAC peak generated in the multiplex reaction was smaller when compared with other peaks. However, in the case of the negative control, the size of the IAC was peak was bigger as all primers was available for its amplification. Furthermore, to the best of our knowledge, this is the first reported melt curve real time PCR with an IAC. All targets of each multiplex assay developed in this study were very clearly resolved and the obtained melt curve peaks showed no overlap with each other. The superior resolution of all melt curve peaks in the assay can be associated with the use of the high resolution melting (HRM) dye, SYTO9. Unlike commonly used intercalating dyes like SYBR® Green, SYTO9 binds to all amplicons generated in the PCR reaction in high concentrations without inhibiting the reaction, therefore generating melt curves of higher resolution (Monis and others 2005; Singh and Mustapha 2014).

Novobiocin is the most commonly recommended antibiotic used for the enrichment of *E. coli* O157:H7. But various reports in recent past suggested its inhibitory and significantly lower recovery rate of non-O157 STEC cells (Kanki and others 2011; Baylis

2008). Fratamico and others (2011) reported that mTSB containing novobiocin (8 mg/L) slowed the growth of STEC O111. VCC supplement is also recommended for the selective enrichment of *E. coli* O157:H7. VCC supplement comprises of three antibiotics: cefixime suppresses the growth of *Proteus* spp., cefsulodin is inhibitory towards *Pseudomonas* spp., whereas vancomycin deters the growth of Gram-positive bacteria. Because cefixime and cefsulodin were excluded from the enrichment broth used in this study, the assay was challenged with the addition of 10^4 CFU of *P. aeruginosa* and 10^4 CFU of *P. mirabilis* per 25 g of food sample to ensure that these organisms do not interfere with the assay. The presence of both organisms and a high microflora of food samples showed no negative effect on the performance of the assay. This might be due to the fact that enrichment was performed at 42 °C, which may have conferred additional selectivity to the enrichment process by discouraging the growth of psychotropic bacteria present in the food samples.

Lin and others (2011) described the development of a TaqMan® real-time PCR assay for the identification of nine STEC serogroups. The assay can be performed in three multiplex reactions and can be used to detect STEC serogroups O26, O45, O91, O103, O111, O113, O121, O128, and O145. Milk, apple juice, lettuce, and ground beef, samples contaminated with ≤30 CFU/25 g or mL can be detected following an enrichment period of 24 h (except for O113 in apple juice). Fratamico and others (2011) reported another TaqMan® assay, which used four multiplex reactions for the identification of six STEC serogroups, namely O26, O45, O103, O111, O121, O145, and their virulence characterization. All samples of ground beef artificially inoculated with 1–2 and 10–20 CFU/25 g gave positive results after a 24-h enrichment period. In

comparison to both the above-mentioned TaqMan® assays, our method took a lesser number of reactions and generated results with similar sensitivity in a shorter time period. Wang and others (2012) described a loop-mediated isothermal amplification (LAMP) assay for the detection of seven STEC serogroups O26, O45, O103, O111, O121, O145, and O157. Similar to our study, this group also used BPW for the enrichment of STECs. Beef samples spiked with two low STEC concentrations (1 to 2 and 10 to 20 CFU/25 g) gave positive LAMP results after an enrichment period of 6 h but the threshold time (T_t) values were high, and the T_t values became low and stable following an 8-h enrichment. Fratamico and others (2014) performed a comparative study between the DuPont BAX System (DuPont Qualicon, Wilmington, DE, USA) which is a USDA-approved method for detection of STECs and the FSIS Microbiology Laboratory Guidebook (MLG) methods for detection of STEC strains in ground beef and beef trim. The study reported limit of detection of the BAX assay in the range of 1.2×10^3 to 4.1×10^2 CFU. The limit of detection results obtained in this present study (Table 6.6) are better than when compared with the results of the BAX system. In the same study, 325 g of ground beef samples (90% lean/10% fat) were inoculated with 5-9 CFU of STEC strains and positive results were obtained for all spiked samples using both the BAX System and FSIS MLG method after an 18-h enrichment (USDA-FSIS 2014). When compared to our data in which 325 g of ground beef (85% lean/15% fat) were spiked with 5-21 CFU of STEC strains (Table 6.7), all targets were detected by the multiplex assay after an enrichment period of 6 h, the Ct values of the PCR reaction was reduced after 8 h of enrichment. One ground beef sample, which was inoculated with 7 CFU of *E. coli* O157:H7 per 325 g gave negative results after 6 h of enrichment. The assay described in the present study

generated comparable results to both the BAX system and the FSIS MLG method in a much shorter time.

Our first multiplex assay detected all eight STEC serogroups including *E. coli* O104, but at present in the United States, there is no regulation regarding the presence of *E. coli* O104 in food product. Therefore another pair of multiplex reactions was standardized in which *E. coli* O104 was omitted and three new targets (stx_1, stx_2 and *Salmonella*) were added. The *stx* gene is a diverse family of genes and consists of a total of 10 *stx* subtypes: stx_1 has three subtypes (stx_{1a}, stx_{1c}, and stx_{1d}), whereas stx_2 has seven subtypes (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, and stx_{2g}) (Feng and other 2011). The presence of stx_2 in the STEC has been correlated with STEC infection leading to HUS. The assay developed in this study detected all three stx_1 subtypes but only four stx_2 subtypes (stx_{2a}, stx_{2c} stx_{2d}, and stx_{2e}). STEC strains possessing the stx_2 gene, more specifically the stx_{2a} and stx_{2c}, are more likely to result in HUS (Friedrich and other 2002; Russo and other 2014), which is a more severe and advanced form of the disease. Friedrich and other (2002) further reported that stx_{2d} and stx_{2e} are two other commonly found stx_2 subtypes and are responsible for a milder form of the disease with a minimal risk of HUS. Out of seven stx_2 subtypes, our assay failed to detect the stx_{2b} stx_{2f} and stx_{2g} subtypes. The stx_{2f} subtype has been primarily reported from pigeons (Schmidt and others 2000), whereas the stx_{2g} has been isolated from humans, but in most of the cases, was not expressed (Prager and others 2011). Hence, it can be concluded that the assay developed in the present study can be used for the detection of all STECs that has been declared as adulterants by the USDA, the most important Shiga toxin subtypes, and *Salmonella*.

Two real-time PCR instruments (StepOnePlus® and LightCycler® 96) were used for the standardization and development of this assay. Out of these two instruments, the LightCycler® 96 real-time PCR was found to be superior, as it possessed the capability to perform melt-curve analysis at a ramp-rate of $0.04CS^{-1}$, generating melt curves with a higher resolution. The instrument also required a much shorter time to perform the melt curve analysis which helped in reducing the total time required for performing the real-time PCR. Even though the assay described in this study can detect all STEC serogroups that have been declared as an adulterants by the USDA in less than 11 h, there are still some limitations of the method. As with any PCR-based methods, the assay can only detect the presence of serotype-specific genes and does not result in bacterial colonies which would enable agencies to perform further testing, such as virulence characterization, antibiotic resistance, and genetic profiling.

6.5 CONCLUSIONS

The assay described in this study can be performed in less than 11 h, which includes sample preparation, enrichment, DNA isolation and real-time PCR, making it one of the fastest methods for detection of STECs. The assay does not use dual-labeled fluorescent probes nor antibody-based immunomagnetic separation, both of which are expensive and have shorter storage lives. Our assay has a modest requirement of oligonucleotides and real-time PCR master mix, hence making it one of the most commercially viable methods for rapid and accurate screening of STECs in food samples. In addition to food samples, the assay can also be used for screening of STECs in clinical samples of acute or bloody diarrhea cases.

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Chapter 7 CONCLUSIONS AND FUTURE STUDIES

The United States has one of the safest food supplies in the world. The real-time PCR assays developed in this study can be used for further enhancing food safety. In the first two studies, multiplex PCR assays for the detection of pathogenic as well as antibiotic resistant *Salmonella* were developed. Both the multiplex TaqMan® assay and melt-curve assay developed in this study targeted a common virulence gene (*inv*A) and antibiotic resistance determinants that are responsible for conferring resistance towards tetracycline, streptomycin, ampicillin, chloramphenicol, sulphonamide in *Salmonella*. An innovative approach was used to add an internal amplification control (IAC) in the TaqMan® assay. A single stranded (ss) 98-base long DNA sequence was uniquely designed to serve as the IAC, which has a primer-binding site for the *inv*A gene primers, but possessed a different probe binding sequence. Thus, the IAC ssDNA could be co-amplified with the same primer set (*inv*A) but detected using a different dual-labeled probe. This approach helped to reduce the number of primers used in the multiplex reaction. Another important feature of the TaqMan® assay developed in this study, was the use of a locked nucleic acid (LNA) dual-labeled probe. LNA bases have the property of binding to complementary target molecules (DNA, RNA or LNA) with higher affinity, providing higher specificity. Both the multiplex assays developed for the detection of antibiotic resistant *Salmonella* could detect 1000 genomic equivalents of *Salmonella* from serially diluted DNA samples. The detection limit for un-enriched inoculated food samples was 10^4 CFU/g, but this was improved to 10 CFU/g after a 12-h enrichment for

the TaqMan® assay, whereas a 6-h enrichment was required for the melt-curve real-time PCR assay. The two multiplex assays developed in this study can be a useful tool for epidemiologic, laboratory, and traceback investigations of tainted food samples during an outbreak caused by MDR *Salmonella*.

The extended-spectrum beta-lactam (ESBL) and carbapenem group of antibiotics are used to treat severe infections caused by members of the Family Enterobacteriaceae. The emergence of ESBL and carbapenem resistant pathogens is an important issue among zoonotic and clinical pathogens. Zoonotic pathogens often lead to human infections *via* food animals. Two multiplex real-time PCR melt-curve reactions were standardized for the detection of 10 genes that confer resistance towards ESBL and carbapenem group of antibiotics. The first reaction targeted bla_{CTX}, bla_{CMY}, bla_{VIM}, bla_{KPC}, bla_{NDM} with IAC, whereas the second reaction targeted bla_{IMP}, bla_{OXA}, bla_{SHV}, bla_{TEM}, bla_{ACC} and IAC. A similar approach as described above was used for designing the IAC. The results of the multiplex PCR assay using DNA samples obtained from previous studies either mostly paralleled the previous studies or the presence of more antibiotic resistance genes were detected using our assay. This assay can be used for rapid antibiotic resistance profiling of isolates obtained from foodborne disease outbreaks and blood or urine cultures. The assay will be valuable for detecting isolates with a low level of gene expression, which might give a negative result using traditional culture-based methods. Application of this assay in clinical settings will help clinicians come up with stewardship interventions and reduce treatment failures. The assay can also be used by regulatory agencies, such as USDA and CDC for conducting antibiotic resistant surveillance of foodborne pathogens in food, animals and the environment.

Shiga toxin producing *Escherichia coli* (STEC) contamination in food and water is one of the most serious food related concerns worldwide. Seven STEC serogroups have been declared as adulterants in the non-intact raw beef product, whereas pathogenic strains of *Salmonella* lead to 1.2 million illnesses each year in the United States. A multiplex real-time PCR assay was developed for the detection of seven STEC serogroups, STEC virulence genes (stx_1 and stx_2) and virulent *Salmonella*. The first multiplex assay targeted *E. coli* O145, *E. coli* O121, *E. coli* O104, *E. coli* O157, *E. coli* O26, *E. coli* O45, *E. coli* O103, *E. coli* O111 and IAC; while the second set detected *E. coli* O145, *E. coli* O121, *E. coli* O157, *E. coli* O26, *E. coli* O45, *E. coli* O103, *E. coli* O111, *Salmonella*, stx_1, stx_2 and an IAC. Food samples spiked with a cocktail of four STEC serogroups with a combined count of 10 CFU/25 g food, all targets of the multiplex assays could be detected after an enrichment period of 6 h, whereas 325 g of food sample spiked with 10 CFU of each STEC serotype required an enrichment period of 8 h. The assay described in this study can be performed in less than 11 h, which includes sample preparation, enrichment, DNA isolation and real-time PCR, making it one of the fastest methods for detection of STECs. A number of commercial tests are presently available, but unlike other assays, our assay does not use dual-labeled fluorescent probes nor antibody-based immunomagnetic separation, both of which are expensive and have shorter storage lives. Our assay has a modest requirement of oligonucleotides and real-time PCR master mix, making it one of the most commercially viable methods for rapid and accurate screening of STECs in food samples. In addition to food samples, the assay can also be used for screening STECs in clinical samples of acute or bloody diarrhea cases.

Future Studies

Multiplex real-time PCR melt-curve assays have become a method of choice for real-time PCR-based detection of foodborne pathogens. As a common extension to all the multiplex assays developed in this study, there is a need for development of real-time PCR tubes that are pre-loaded with lyophilized primers and other PCR reagents. These will not only simplify the real-time PCR process, but will also help to avoid contamination, save time, increase the reliability of the assay and, most importantly, make the assay easier to perform.

Recently, diagnostic companies have come up with *in vitro* diagnostic (IVD) platforms that are approved for performing nucleic acid testing in clinical laboratories, such as the BD MAX System (BD Diagnostics, Sparks, MD). These platforms can perform automated DNA extractions from a wide variety of samples, purify the obtained nucleic acids, perform real-time PCR amplification and detection of targeted pathogenic bacteria automatically. The multiplex assays for the detection of antibiotic resistant *Salmonella*, STEC, ESBL and carbapenem resistance developed in this study could be tested for their compatibility on these IVD platforms. If successful, it will lead to a reduction in total time required to perform the assays, avoid manual errors, and will also pave the way for commercialization of the assays developed in this study.

Real-time PCR instrument softwares are very well equipped for analyzing signals from multiplex TaqMan® or other types of dual-labeled probes but these softwares are not very user-friendly for melt-curve based multiplex assays. Each peak of the melt-curve plot obtained from individual reaction tubes needs to be manually analyzed and assigned. There is a need for a software tool that can analyze data from melt-curve plots,

automatically assign target name to each peak and generate a presence/absence report for each sample.

The multiplex assay for the detection of ESBL and carbapenem resistance developed in this study was validated using a limited number of DNA samples. This assay can be further tested with a larger number of clinical or zoonotic strains for the presence of antibiotic resistance genes, and the obtained results can be compared with antimicrobial susceptibility data obtained using standard microbiological methods.

VITA

Prashant Singh; born on 16th August 1982, grew up in Bokaro Steel City in the eastern part of India which is where he finished his schooling. He earned his Bachelor's Degree from Delhi University (Delhi, India), in 2004. His interest in Applied Microbiology took him to Vellore Institute of Technology (TN, India) that is where he finished his Master's Program. He worked as a Senior Research Fellow from 2006-2009 at National Bureau of Animal Genetic Resources (Haryana, India) and National Dairy Research Institute (Haryana, India) in area of dairy microbiology. He joined University of Missouri-Columbia, department of Food Science in the summer of 2010 and received a Ph.D. degree in Food Science in spring 2015. In addition to his Ph.D. he also received Food Safety and Defense Graduate Certificate, which is an inter-institutional certificate offered in cooperation with Kansas State University, Iowa State University, University of Nebraska (Lincoln) and the University of Missouri.