# SIMULTANEOUS QUANTITATION OF *ESCHERICHIA COLI* O157:H7, *SALMONELLA* AND *SHIGELLA* IN GROUND BEEF BY MULTIPLEX REAL-TIME PCR AND IMMUNOMAGNETIC SEPARATION

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

**SIMULTANEOUS QUANTITATION OF *ESCHERICHIA COLI* O157:H7, *SALMONELLA* AND *SHIGELLA* IN GROUND BEEF BY MULTIPLEX REAL-TIME PCR AND IMMUNOMAGNETIC SEPARATION**

Presented by Luxin Wang

A candidate for the degree of Master of Science

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

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## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

**Table** **Page**

**SIMULTANEOUS QUANTITATION OF *ESCHERICHIA COLI* O157:H7, *SALMONELLA* AND *SHIGELLA* IN GROUND BEEF BY MULTIPLEX REAL-TIME PCR AND IMMUNOMAGNETIC SEPARATION**

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## ABSTRACT

The objectives of this study were to establish a real-time multiplex polymerase chain reaction (PCR) for simultaneous quantitation of *Escherichia coli* O157:H7, *Salmonella* and *Shigella* that have been implicated in a number of foodborne disease outbreaks. Genomic DNA for the real-time PCR was extracted by the boiling method. Three sets of primers and corresponding TaqMan® probes were designed to target these three pathogens. Multiplex real-time PCR was carried out with TaqMan® Universal PCR Master Mix in an ABI Prism 7700 Sequence Detection System. Final standard curves were calculated by plotting the threshold cycle (*Ct*) value against $\log_{10}$ CFU/ml by linear regression to analyze the results for each pathogen. With optimized conditions, the quantitative detection ranges of the real-time multiplex PCR for pure cultures were $10^2$ to $10^9$ CFU/ml for *E. coli* O157:H7, $10^3$ to $10^9$ CFU/ml for *Salmonella* and $10^1$ to $10^8$ CFU/ml for *Shigella*. When this established multiplex real-time PCR system was applied to ground beef samples, the lowest detection concentration of three pathogens were increased to $10^5$ CFU/g for *E. coli* O157:H7, $10^3$ CFU/g for *Salmonell*a and $10^4$ CFU/g for *Shigella*. Immunomagnetic separation was then used to isolate *E. coli* O157:H7 and *Salmonella* from the beef samples. The lowest detection concentrations of

three pathogens were reduced to $10^3$ CFU/g. TaqMan® real-time PCR, combined with IMS has the potential to be a faster and more reliable method for rapid quantitation of *E. coli* O157:H7, *Salmonella* and *Shigella* in food, which will take 3 h for the whole process.

# CHAPTER 1

# INTRODUCTION

Foodborne diseases remain a persistent challenge to the public health. Foodborne disease outbreaks, which cause approximately 76 million illnesses and 5,000 deaths every year (Mead et al., 1999), have more than doubled in the United States since 1987 (Tauxe et al., 1997). These outbreaks also cause a serious economic loss for both the consumer and the industry. Most reported foodborne disease outbreaks from the consumption of contaminated produce were caused by pathogens, such as *Escherichia coli* O157:H7, *Salmonella* and *Shigella*. Among these outbreaks, *E. coli* O157:H7 caused about 73,500 cases of infections every year, resulting in syndromes like hemorrhagic colitis and non-bloody diarrhea (Buchanan et al., 1997). Members of the genus *Salmonella* are responsible for a large number of food poisoning, approximately 1.4 million according to estimates (Mead et al., 1999). Virulent *Shigella* spices, including *Shigella dysenteriae*, *Shigella boydii* and *Shigella sonnei*, can cause severe illnesses like hemolytic uremic syndrome with a very low infection dose (10 CFU) (Kothary et al., 2001).

Human pathogen contamination of raw meat products is caused by a wide array of pre-harvest, harvest, and post-harvest processes (Li et al., 2005). During slaughter, pathogenic bacteria may contaminate a carcass and be distributed by cut meat materials. Although thorough cooking kills pathogens, cooked meat may become re-contaminated by food handlers during processing or from the environment.

Standard conventional detection methods for *E. coli* O157:H7, *Salmonella* and *Shigella* are described in the *FDA Bacteriological Analytical Manual*, such as culturing the organisms in selective agar and identifying isolates according to their biochemical or immunological characteristics. These protocols, however, are time consuming and labor intensive.

The polymerase chain reaction (PCR) is a rapid technique with high specificity and sensitivity for the identification of target organisms. Multiplex PCR is a technology that can amplify more than two gene sequences in the same reaction. As *E. coli* O157:H7, *Salmonella* and *Shigella* have the potential to contaminate the same products, it is useful to detect them simultaneously. The application of multiplex PCR for the detection of *E. coli* O157:H7, *Salmonella* and *Shigella* have been done, with a detection limit of 1.25 CFU/g after a 24-h enrichment (Li et al., 2002b). However, quantitative detection of target genes is not feasible in multiplex PCR assays because amplified products can only be visualized in agarose gels after the completion of PCR, and the strength of the target band cannot be measured correctly.

Recently, real-time PCR assays were developed for the detection and quantification of pathogen-specific gene products with the use of Taqman™ probes and molecular beacons (Lyons et al., 2000, Nogva et al., 2000). TaqMan™ probes and molecular beacons are oligonucleotides labeled with a reporter and a quencher dye at the 5' and 3' end respectively. With the amplification of target sequences, probes that attach to the sequences will break, and the reporter dye that is suppressed by the quencher dye, will give out fluorescence. The intensity of the fluorescence will

increase with the process of amplification. The measurement of incremental fluorescence increases for each PCR cycle will provide an accurate estimate of the number of specific pathogenic cells in the contaminated food samples. Multiplex real-time PCR has been used for detection of different bacterial species from meat samples, realizing the quantitation and identification of target strains at the same time.

Even though the concentrations of most foodborne pathogens in food are typically low, the infectious doses of some of these pathogens are quite low. The minimum infection dose of *E. coli* O157:H7 and *Shigella* are approximately 10 CFU, while that of *Salmonella* is around $10^6$ CFU (Kothary et al., 2001). Enrichment is usually done to increase the concentration of target pathogens in order to increase the detection sensitivity. However, it becomes impossible, after enrichment, to determine the actual initial concentration of the pathogens present in a food sample.

Immunomagnetic separation (IMS) is a technique that has been as an alterative to selective enrichment broths to remove target microbial cells from biological samples. It has been effectively combined with several biological methods, such as PCR and real-time PCR.

The objective of this research was to develop a rapid, specific and sensitive detection method by combining the IMS technique with real-time PCR assay which has the potential to be used for quantitation and identification of *E. coli* O157:H7, *Salmonella* and *Shigella* present in ground beef.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Microbial contamination of food

In recent years, the importance of food safety has increased dramatically because of the growing number of reported illnesses associated with foodborne pathogens. The Council for Agricultural Science and Technology estimated in its 1994 report, *Foodborne Pathogens: Risks and Consequences*, that as many as 9,000 deaths and 6.5 to 33 million illnesses in the United States each year are food-related. The lost caused by foodborne illness is immense, with an estimated 9,000 deaths and up to $3 billion in health care expenditures and lost productivity (Lundberg et al., 1997; Buzby et al., 1999). Illnesses due to the consumption of foods including animal products are increasing due to changes in food production, food processing methods, globalization of food supply, new packaging technologies, and changing eating habits (Manchester et al., 1995; Notermans et al., 1997). Other promoting factors include microbial adaptation, such as antibiotic resistance, transmission methods (various improperly cooked meats), and both human demographics (AIDS), and human behavior (increased food consumption at fast food restaurants).

## 2.2 Economic loss caused by food contamination

Although the American food supply is one of the safest in the world, significant annual economic losses (approximately 33 million cases of foodborne illnesses; over 9,000 deaths; and an estimated loss of between 5.6 and 9.4 billion

dollars) are reported due to consumption of contaminated food (FDA, 1997; Buzby et al., 1999). For example, the annual economic loss of outbreaks due to *Escherichia coli* O157:H7 alone is estimated at 216 to 580 million dollars (Clarke et al., 1994). This cost includes losses at the consumer (sickness and decreased productivity), producer (condemnation of contaminated products), and national (gross national product) levels (Bean et al., 1990).

## 2.3 Microbial contamination of beef

Microbial contamination represents a major cause of foodborne diseases. Different foods may contain various types of pathogenic bacteria, fungi and viruses, because of their different compounds and processing procedures. Among different foods, it has been established that farm animals are the primary reservoirs of many serious foodborne pathogens in the U.S., due to improper handlings in pre-harvest, harvest and post-harvest processes.

### 2.3.1 Origin of microflora during processing steps and control measures

Microorganisms present on a muscle food product represent an accumulation of organisms picked up at different stages in the production process. A proportion of these organisms are associated with the live animal, coming from either the environment or the feed. Additional organisms may have been introduced during transport of the live animal, perhaps as a result of co-mingling with other animals. Such organisms present on the animal at the time of harvesting or slaughtering represent what maybe called the “primary microflora” of the muscle food product.

During harvesting or slaughtering procedures, the primary microflora may be transferred from the exterior surface of the animal or from the intestinal tract onto the muscle surface.

After harvesting or slaughtering, additional microorganisms that contaminate the muscle food represent a sort of "secondary microflora". In many cases, secondary flora originates in the processing facility, either from the environment and equipment, or contamination by human operatives, or improper product storage. Even with optimal processing conditions, some microbial contamination will occur. Subsequent to leaving the production facility, a product may be further contaminated during distribution and while in the consumer's possession.

Because it is not possible to totally eliminate contamination and subsequent deterioration of muscle foods, great care must be taken during the storage and preparation of muscle foods. All harvesting, slaughtering, handling, storage and food preparation practices should be designed to minimize contamination and prevent or retard microbial growth. Generally, if muscle foods are handled so as to minimize adverse microbial changes, non-microbial deterioration will also be minimized.

#### 2.3.1.1 Source and control of microorganisms

At the production level, the first source of microorganisms is the animals and their carriage of pathogens. The feed supply of captive-reared animals (including those grown under aquaculture) can also be a significant source of pathogens. Water is another source that will contaminate a meat product. The environment and pests are other factors that could contaminate animals (Kinsman, et al, 1994).

The source and control of microorganisms during harvesting and slaughtering also need to be considered. Equipment used in the harvesting and slaughtering, and hide removal processes of cattle contributes heavily to the bacterial load on superficial carcass tissues. Rinsing carcasses with chlorinated water and organic acids will reduce microbial contamination, but only when used in conjunction with good manufacturing practices. Tanks in which the beef are placed can be a major venue for cross contamination as well (Kinsman et al. 1994).

## 2.4 Pathogenic bacteria in contaminated food

Although various causes of foodborne illness, pathogenic bacteria remain the predominant agents, Food-related bacteria account for approximately 5.2 million illness, 37,000 hospitalizations, and 1,300 deaths per year in the U.S. (Mead et al., 1999). According to the U.S. Department of Agricultural/Economic Research Service (USDA/ERS) (1996), six bacterial pathogens present in food products, including *Salmonella*, *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium perfringens*, cost 2.9 to 6.7 billion dollars in human illness annually in this country. Various meat products have been associated with outbreaks caused by *E. coli* O157:H7, *Salmonella* and *Shigella* (CDC, 2002).

### 2.4.1 *Escherichia coli* O157:H7

It has become evident that *E. coli* can cause a variety of diseases in humans and animals (AGA, 1995). *E. coli* O157:H7 is an *E. coli* serotype of most interest, because of its association with human foodborne illnesses (Doyle, 1991; Buchanan et

al., 1997). It was first recognized as a human pathogen in 1982 after two major outbreaks of hemorrhagic colitis in Oregon and Michigan (Riley et al., 1983). Although *E. coli* O157:H7 is not host-specific (Armstrong et al., 1996; Faith et al., 1996; Besser et al., 1997; Dargatz et al., 1997; Kudva et al., 1997a; Hancock et al., 1998a), it is more prevalent in ruminants in general and in cattle in particular (Riemann et al., 1998). Many outbreaks of foodborne illnesses have been traced to consumption of beef products such as undercooked ground beef (Riley et al., 1983; Belongia et al., 1991; Tarr, 1993; Orr et al., 1994; Abdul-Raouf et al., 1996)

Extensive research with beef or dairy cattle showed that the presence of *E. coli* O157:H7 in their feces. In most foodborne illness outbreaks, the cause was attributed to consumption of undercooked beef (Cieslak et al., 1997) or processed raw beef (Reis et al., 1980) which had been contaminated with ruminant feces (Brown et al., 1997). Infection often leads to bloody diarrhea, and occasionally to kidney failure. Person-to-person contact in families and child care centers is also an important mode of transmission.

Beef products have been implicated in *E. coli* O157:H7 illnesses since 1982 (Riley et al., 1983), many studies (Hancock et al., 1994; Chapman et al., 1997; Hancock et al., 1997) have focused on the detection and isolation of the pathogen in beef cattle on pasture or in the feedlot.

#### 2.4.1.1 Characteristics of *Escherichia coli* O157:H7

The species, *E. coli*, is a rod-shaped, gram-negative, facultatively anaerobic, non-spore forming bacterium. It belongs to the Enterobacteriaceae family, and can

ferment lactose, producing acid and gas. In the IMVIC (indole, methyl red, Voges-Proskauer and citrate) tests, it yields a typical + + - - results. Serotypes of *E. coli* are determined on the basis of somatic (O), flagellar (H) and capsule (K) antigens (Feng, 2001). While most *E. coli* strains are harmless, some pathogenic strains have been recognized as enteropathogenic, enterotoxigenic, enterohemorrhagic and enteroinvasive. Enterohemorrhagic *E. coli* (EHEC) is the most virulent among all pathogenic *E. coli* strains. It represents the bacterium producing *Shiga*-like toxin (SLT), which causes hemorrhagic colitis (severe bloody diarrhea) that may progress to life-threatening hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) in humans. The known serotypes of EHEC include O157:H7, O111:H8, O103:H2, O26:H11, and their non-motile variants (Feng, 2001). Stressful conditions, such as storage under refrigeration temperatures, acid tolerance of *E. coli* O157:H7 will be enhanced. This trait might enable the pathogen to survive in the low pH of the gastric environment and cause disease at a low infectious dose in humans (Buchanan and Doyle, 1997).

#### 2.4.1.2 Disease and virulence

The infectious dose of *E. coli* O157:H7 is as low as approximately 10 colony forming units (CFUs) (Kothary and Babu, 2001). Initial symptoms of the infection include non-bloody diarrhea, "crampy" abdominal pain, and short-lived fever. The major illness caused by the pathogen is Hemorrhagic colitis, which is characterized by bloody diarrhea, moderate dehydration and acute abdominal cramps. HUS occurs in all ages but often in children under 10. The associated complications are pallor, intravascular

destruction of red blood cells, depressed platelet counts, lack of urine formation and acute renal failure. While the mortality rate of HUS is 3-5%, many survivors suffer permanent disabilities, such as renal insufficiency and neurological deficits. On the other hand, TTP commonly occurs in adults. The disease is similar to HUS except that it causes less renal damage but affects the central nervous system significantly (Buchanan et al., 1997; Feng, 2001).

The illness-causing Shiga-like toxin (SLT) produced by *E. coli* O157:H7 is named due to its similarity to the Shiga toxin produced by *Shigella dysenteriae*. SLT is a 70 KDal protein composed of a single A subunit and five B subunits. The B subunit confers tissue specificity, enabling the toxin to adhere to a specific glycolipid receptor on endothelial cell surfaces. The A subunit is then delivered to the host cell where it binds with 28S ribosome, inhibits protein synthesis and kills kidney cells which ultimately leads to HUS (Buchanan et al., 1997; IFT, 2000). In addition to the SLT, other virulent factors of *E. coli* O157:H7 include a chromosomal *eaeA* gene that encodes for an outer membrane protein called intimin which is associated with attachment (Dean-Nystrom et al., 1998) as well as an approximately 60-MDa plasmid that encodes enterohemolysin (Makino et al., 1998).

### 2.4.2 *Salmonella*

*Salmonella* is one of the leading causes of foodborne illness throughout the world. Estimates indicate that *Salmonella* accounts for approximately 1.4 million illnesses, 17,000 hospitalizations and 600 deaths per year in the U.S (Mead et al.,

1999), resulting in a cost of around 4.0 billion dollars annually in medical expenses and productivity losses (Todd, 1989).

A few large-scale salmonellosis outbreaks were recorded in the U.S. during the last century. One occurred in 1974 on the Navajo Indian reservation when approximately 11,000 individuals attended a barbecue. The infections, caused by *S.* Newport-contaminated and improperly handled potato salad, resulted in 3,400 persons becoming ill (Horwittz et al., 1977). In 1985, a massive outbreak of salmonellosis occurred in six states, resulting in 16,284 confirmed cases and subsequently seven deaths. So far, the largest outbreak of foodborne salmonellosis in the U.S. happened in 1994. This outbreak involved at least 41 states and more than 224,000 persons. Zhao et al. (2001) investigated the presence of multiplex foodborne pathogens in retail chicken, turkey, pork and beef from the greater Washington D.C. area between June 1999 and July 2000 (Zhao et al., 2001). In a total of 825 samples, *Salmonella* was isolated from 1.9% of 210 beef samples. A more comprehensive study, carried out by the USDA Food Safety and Inspection Service (FSIS) showed that *Salmonella* outbreaks involved 98,204 raw meat and poultry products in the U.S for the period 1998 to 2000 (Rose et al., 2002). This study detected *Salmonella* on 29.2% of 3,481 samples, 15.7% of 735 ground chicken samples, and 10.7% of 24,452 broiler samples.

Due to the contamination of *Salmonella* in foods, a number of recalls have been issued by the food industry. In March 2002, Double D Meat Company Inc. in Louisiana recalled approximately 14,100 pounds of fresh, ready-to-eat pork sausage that may have been contaminated with *Salmonella* (USDA/FSIS, 2002). More recently, a notable recall occurred in October, 2003. M. D. Chavez/Old Santa Fe Trail

in New Mexico firm voluntarily recalled approximately 22,000 pounds of beef jerky because of *Salmonella* contamination (USDA FSIS, 2003).

#### 2.4.2.1 Characteristics of *Salmonella*

*Salmonella* is a rod-shaped, facultative anaerobic, non-spore forming, Gram-negative bacterium. As a member of the family Enterobacteriaceae, it was named after D.E. Salmon, a bacteriologist who had identified *Salmonella choleraesuis* in 1885 (Tauxe, 1991). The genus includes 2449 serovars under two species, *Salmonella enterica* and *Salmonella bongori*. Based on DNA-DNA hybridization and multilocus enzyme electrophoretic characterization, *S. enterica* is further divided into the six subspecies, *enterica*, *salamae*, *arizonae*, *diarizonae* and *indica*. The serotypes commonly isolated from humans, agricultural products and foods, belong to the subspecies *enterica* (D' Aoust, 2001). *Salmonella* can grow in temperatures from 2$^{o}$C to 47$^{o}$C (with the optimum being from 25$^{o}$C to 43$^{o}$C). Most of them can ferment glucose and certain other monosaccharides but generally cannot ferment lactose, sucrose or salicin. It can grow in the low pH environment of the human stomach which may explain the high infectious dose of this pathogen. However, *Salmonella* can be killed under high salt concentrations (Jay, 2000).

The incidence of antibiotic resistance among *Salmonella* strains continues to increase, leading to the emergence of highly virulent serotypes, such as *S.* Typhimurium DT104 in Europe and North American. *S.* Typhimurium DT 104 is resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline, and can be tolerant against other medically important antibiotics. This has raised a

great public health concern as these antibiotics are usually utilized as treatments for suspected extraintestinal *Salmonella* infections and serious gastroenteritis in humans (IFT, 2000).

#### 2.4.2.2. Disease and virulence

The clinical syndromes linked to human salmonellosis include enteric fever, enterocolitis, and invasive systematic disease. Enteric fever is caused by *Salmonella* Typhi and *Salmonella* Paratyphi A, B or C. The main symptoms consist of watery diarrhea, prolonged and spiking fever, nausea and abdominal cramps. The serious typhoidal disease was predominant in the U.S. from the later 1800s to 1949 (Tietjen et al., 1995). However, it currently seldom occurs, with approximately 824 illnesses, 618 hospitalizations, and 3 deaths per year, mainly as a result of international travel (Mead et al., 1999). In comparison, the incidence of outbreaks due to nontyphoidal *Salmonella* (*S*. Enteritidis, *S*. Typhimurium and others) continues to increase since the middle of the last century. Estimates indicate that nontyphoidal *Salmonella* accounts for 1.4 million illnesses, 16,430 hospitalizations, and 582 deaths annually in the U.S. (Mead et al., 1999). The symptoms of enterocolitis include severe abdominal pain, diarrhea, vomiting and fever. All serotypes of *Salmonella* are potentially pathogenic to humans. The mortality rate of human salmonellosis depends on the age of the subject, with an average of 4.1%. Special populations like newborns, the elderly, and patients with immune deficiencies are particularly prone to the infection (D'Aoust, 2001). In general, the infectious dose of *Salmonella* is approximately $10^5$ CFUs. However, outbreaks may occur from consumption of relatively low numbers of *Salmonella* cells.

It has been estimated that more than 200 virulence factors contribute to the pathogenic properties of *Salmonella*. The virulence determinants are encoded by *Salmonella* pathogenicity islands or plasmid (IFT, 2001). During the initial stage of salmonellosis, at least six different adhesions are involved in the intestinal colonization of the pathogen (IFT, 2001). SPI-1 is responsible for the invasion of *Salmonella* into the gastrointestinal epithelium. The proteins secreted by the bacteria are delivered to the host cells and lead to cytoskeletal rearrangement, membrane ruffing, and bacterial uptake macropinocytosis. Once inside, the engulfed bacteria remain to inhabit and proliferate in the membrane-bound vacuoles. An SPI-2-encoded type III secretion system might account for the survival of *Salmonella* in the intracellular compartment by avoiding lysosomal fusion, a host defense mechanism that usually targets invasive microbes (Hansen-Wester et al., 2001; IFT, 2001). In addition, toxins play a notable role in the illness. Most serotypes of *S*. enterica can produce a thermolabile polypeptide enterotoxin that causes diarrhea. Cytotoxins located in the bacterial outer membrane might be involved in spreading the *Salmonella* into deeper host tissues via inhibition of protein synthesis and lysis of host cells. An endotoxin, also located in the bacterial outer membrane, is associated with leukocyte-dependent inflammatory response (D'Aoust, 2001). The prevalence of *Salmonella* in raw meat and poultry products in the U. S. from 1998 through 2000 are shown in Table 2.1.

### 2.4.3 *Shigella*

*Shigella* is a major etiological agent of diarrheal disease in tropical developing countries, where it has been estimated to account for at least 500,000

deaths annually in young children (Smith, 1987). Although it is not as notorious as *E. coli* O157:H7 and *Salmonella* in the U.S., *Shigella* causes approximately 448,240 illnesses, 6,231 hospitalizations, and 70 deaths each year (Mead et al., 1999). It has been involved in a number of foodborne outbreaks. In 1985, a large outbreak of

**TABLE 2.1.** Prevalence of *Salmonella* in raw meat and poultry products in the United States from 1998 to 2000 (Rose et al., 2002).

| Product | Number of Samples | % Positive |
|---|---|---|
| Broilers | 24,452 | 10.7 |
| Market hogs | 9,733 | 5.4 |
| Cows/bulls | 4,102 | 2.2 |
| Steers/heifers | 2,114 | 0.4 |
| Ground beef | 53,587 | 3.4 |
| Ground chicken | 735 | 15.7 |
| Ground turkey | 3,481 | 29.2 |

shigellosis occurred in Midland-Odessa, Texas, with over 5,000 individuals stricken ill. The source of the infections was chopped, bagged lettuce that had been contaminated with *Shigella sonnei* (CDC, 1986). In 1988, another outbreak of *S. sonnei* in Michigan was associated with uncooked tofu. In this case, a total of 3,175 individuals were infected by the pathogen while attending a music festival. *Shigella* is quite different

from *E. coli* O157:H7 and *Salmonella* in its habitat (Table 2.2). The pathogen is present only in humans and only infects humans and certain primates, but is not usually carried by animals (Acheson, 2001). Direct or indirect human fecal contamination is the main source of *Shigella* responsible for the outbreaks. Vehicles of transmission can be either common-source water supplies or foods. In 1999, a survey of imported produce for multiple pathogens showed that 9 of 1,003 tested samples were tainted by *Shigella*

**TABLE 2.2.** Comparisons in selected physiological properties of *Escherichia*, *Salmonella* and *Shigella*.

| Genus | Glucose | Motility | $H_2S$ | Iodole | Citrate | Mol% (G+C) |
|---|---|---|---|---|---|---|
| *Escherichia* | acid and gas | +[a] | _ | +[b] | _ | 48-52 |
| *Salmonella* | acid and gas | +[a] | + | _ | + | 50-53 |
| *Shigella* | acid | _ | _ | _ | _ | 49-53 |

Adapted from Jay (2000)

[a]Usually

[b]Type 1 strains.

(FDA/CFSAN, 2001). While the contamination rate of *Shigella* seems low, the associated foods remain highly risky in that they are subjected to hand processing and preparation, receive limited heat treatment, or sometimes served raw (Wu et al., 2000).

Sandridge Food Corp. recalled 7,348 pounds of various salad products and surfside pasta because the products were manufactured using celery that may have been contaminated with *Shigella* (FDA, 1999).

#### 2.4.3.1 Characteristics of *Shigella*

Like *Escherichia* and *Salmonella*, *Shigella* belongs in the family *Enterobacteriaceae* that is characterized as gram-negative, facultatively anaerobic, non-sporeforming straight bacilli. A brief comparison of the three genera is shown in Table 2.2 (Jay, 2000). *Shigella* is nonmotile, oxidase negative, catalase positive, methyl red positive, Voges-Proskauer and Simmons citrate negative, and lysine decarboxylase and arginine dihydrolase negative. It produces acid but not gas from glucose and other carbohydrates, does not hydrolyze urea, utilize malonate, grow on KCN agar, and does not produce $H_2S$ (Holt et al., 1994). Genetically, it is more closely related to *Escherichia* than to *Salmonella*. *Shigella* can grow at a range of temperature from $10^oC$ to $48^oC$. The optimal pH for growth is from 6 to 8 (Jay, 2000).

#### 2.4.3.2 Disease and virulence

The genus *Shigella* consists of four species: *S. sonnei*, *S. flexneri*, *S. boydii*, and *S. dysenteriae*. Geographic distribution, clinical manifestations, and outcome following infection of *Shigella* are different among the different species. Those differences are shown in Table 2.3. While all species cause diseases in humans, only the first three are generally regarded as foodborne pathogens. Shigellosis in developed countries is usually linked to *S. sonnei* or *S. flexneri*. U.S. shigellosis surveillance in

1999 indicated that *S. sonnei* caused 73% of the total cases, followed by *S. flexneri* (20.1%), *S. boydii* (1.6%) and *S. dysenteriae* (0.5%). Species identification was not available for 4.8% of shigellosis cases. A seasonal distribution of shigellosis cases has been observed, with a peak incidence of cases occurring during the summer and fall months (CDC, 2001).

The infectious dose of *Shigella* is as low as 10 CFU (Kothary and Babu, 2001). This pathogen is usually transmitted from person to person by the fecal-oral route (FDA/CFSAN, 2003c). Table 1.3 lists the geographic distribution, clinical manifestations, and disease

**TABLE 2.3.** Geographic distribution, clinical manifestation, and outcome following infection with various *Shigella* species.

| *Shigella* Species | Geographic distribution | Clinical manifestation | Outcome |
|---|---|---|---|
| *S. sonnei* | U.S | watery diarrhea | self-limiting |
| *S. boydii* | developed countries, found in Indian subcontinent | some dysentery | antibiotic therapy |
| *S. flexneri* | many part of the world, even in developed countries | usually dysentery | antibiotic therapy |
| *S. dysenteriae* | mainly in developing countries | dysentery and major complications | antibiotic therapy |

Adapted from Acheson (2001).

outcome of various *Shigella* species. *Shigella* usually colonizes the intestinal tract and adheres to epithelial cells, penetrating the mucosal surface (Acheson, 2001). Most of the virulence factors are encoded on chromosome and plasmids. Initial symptoms of shigellosis, which usually develops within 12-48 h after exposure to the pathogen, include fever, aches, fatigue and loss of appetite. Watery diarrhea might occur, which can even degenerate into bloody stools or dysentery, which is due to the transient multiplication of the organisms as they pass trough the jejunum. Other major features are severe abdominal cramps, tenesmus and anorexia. Sometimes, Shiga toxin binds to galabiose and inhibits mammalian protein synthesis, leading to hemolytic uremic syndrome, a severe complication that can cause kidney failure and death (Acheson, 2001).

## 2.5 Detection of foodborne bacterial pathogens

Identification of pathogenic bacteria in foods is very important in ensuring the safety of food supplies as well as for confirming food-related outbreaks. However, microbial identification is a challenge. First of all, high sensitivities are required for preliminary enrichment and subsequent isolation steps that separate the microorganisms from the food. Secondly, high specificities are needed for microbial identification. The identification step can effectively separate the target pathogens from the background microflora.

### 2.5.1 Conventional pathogen detection methods

Traditionally, pathogen detection is based on cultural enrichment and isolation in non-selective and selective media. Usually, the procedure involves cultivation, isolation, screening and confirmation. Cultivation is a step that consists of pre-enrichment and selective enrichment. Pre-enrichment is aimed at restoring the injured target microorganisms to a stable physiological condition in a non-selective environment (Bailey et al., 1991), in the presence of proper growing factors, including intrinsic factors, such as pH, moisture, and extrinsic factors, like temperature and oxygen availability (Sperber et al., 2001). Usually, compounds in an enrichment broth will facilitate the growth of target pathogens and suppress the non-target microflora. Other factors, like temperature, oxygen concentrations must also been taken into account because all these factors can help the growth of specific microorganisms.

In the isolation step, enriched cultures from the enrichment step are streaked on a selective agar medium. Usually, the target microorganisms will give a unique color or shape. Based on the appearance of the colonies, suspects will be picked and used for further analyses. Subsequent confirmation steps include biochemical tests which are based on whether or not the bacterial isolates can utilize a specific substrate during growth (Kalamaki et al., 1997). Color changes or gas production are usually observed for identification.

In recent years, commercial diagnostic kits have been developed for many biochemical or chemical tests, which not only save time, but also materials, labor and space (Kalamaki et al., 1997). Examples of popular commercial biochemical testing kits in the market are MICRO-ID® (REMEL, Lenexa, KS), BBL® Enterotube ™ II

(Becton Dickinson, Sparks, MD), and API® 20E System (bioMerieux, Hazelwood, MO). Presumptive positive isolates, identified by the biochemical tests, are confirmed by serotyping, based on the reactions against somatic (O) and /or flagella (H) antigens.

#### 2.5.1.1 Bacteriological Analytical Manual (BAM)

The FDA Bacteriological Analytical Manual (BAM) (FDA, 8th Ed, 1995) is a widely used microbiological manual in a food pathogen detection lab. Figures 2.1, 2.2 and 2.3 summarize the general BAM protocols for detection of *E. coli* O157:H7, *Salmonella* and *Shigella* from beef samples.

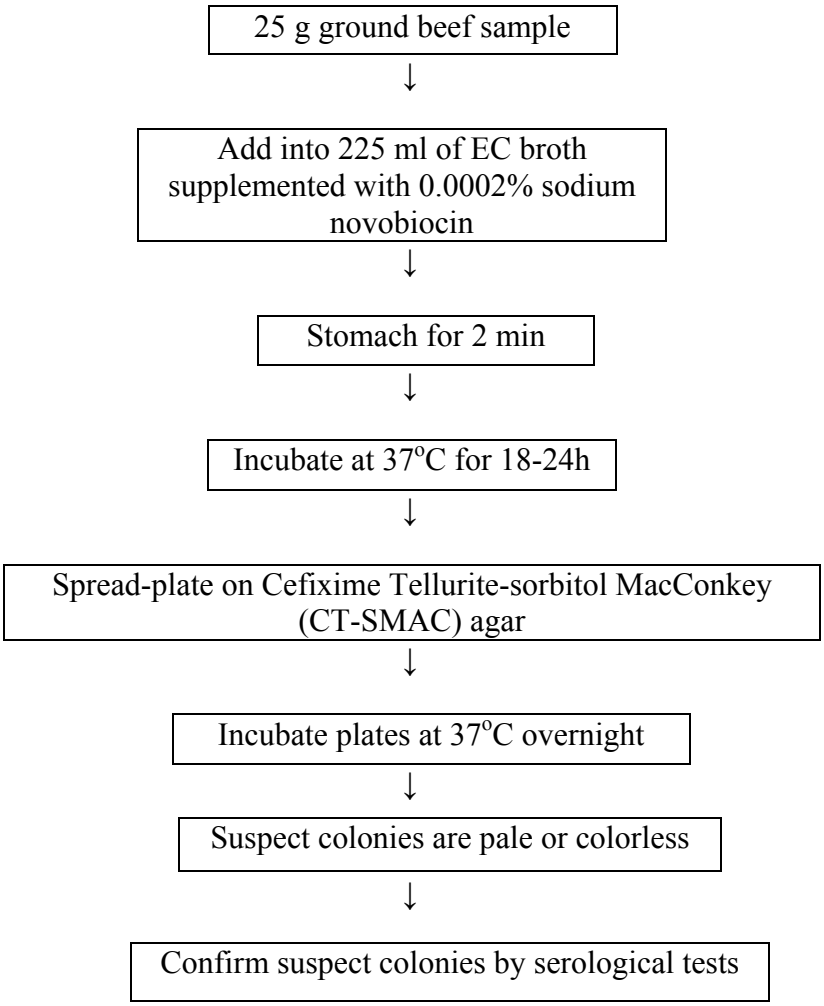


**FIGURE 2.1.** BAM procedure for isolation of *E. coli* O157:H7 from foods **(**FDA, 1995).

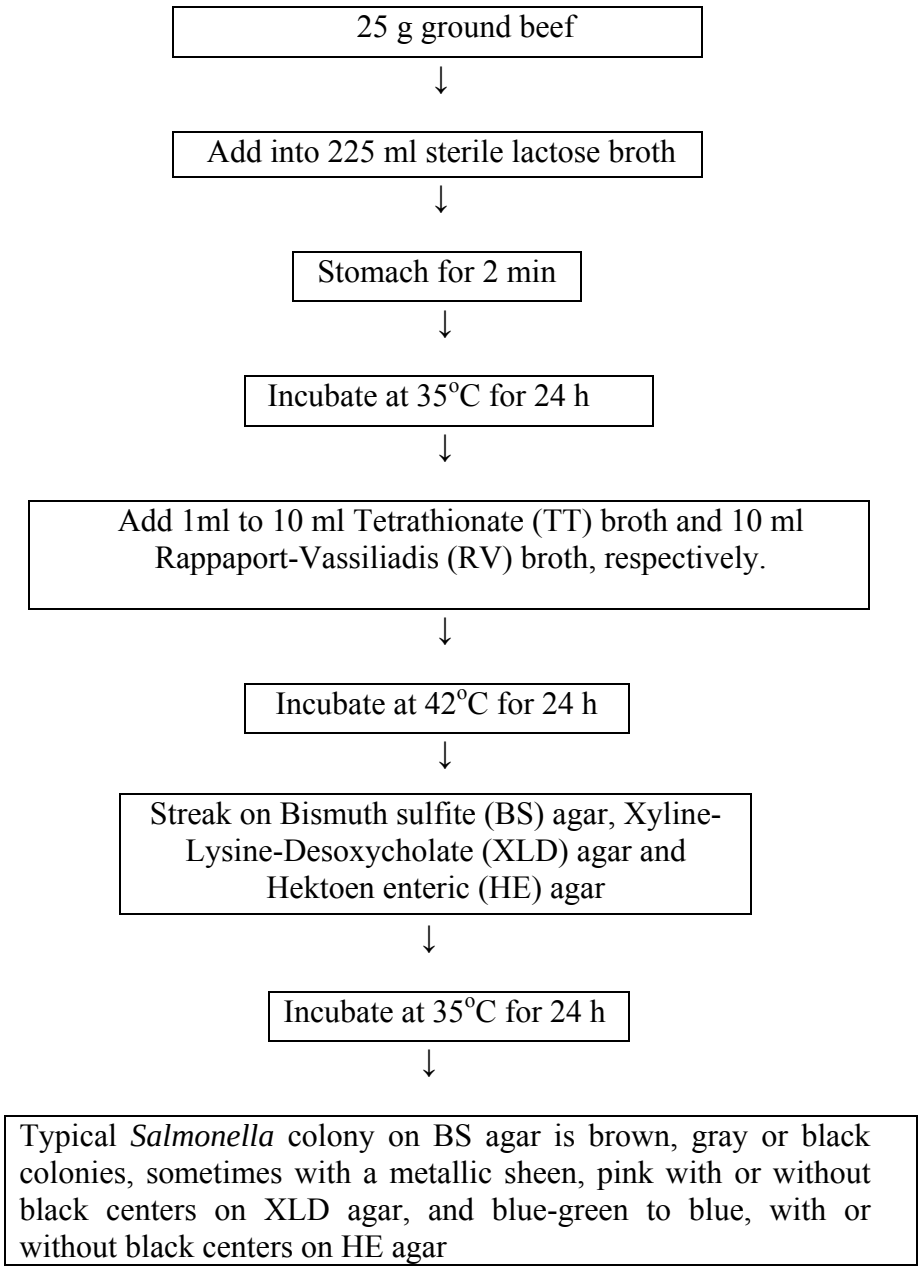


**FIGURE 2.2.** BAM procedure for isolation of *Salmonella* from foods (FDA, 1995).

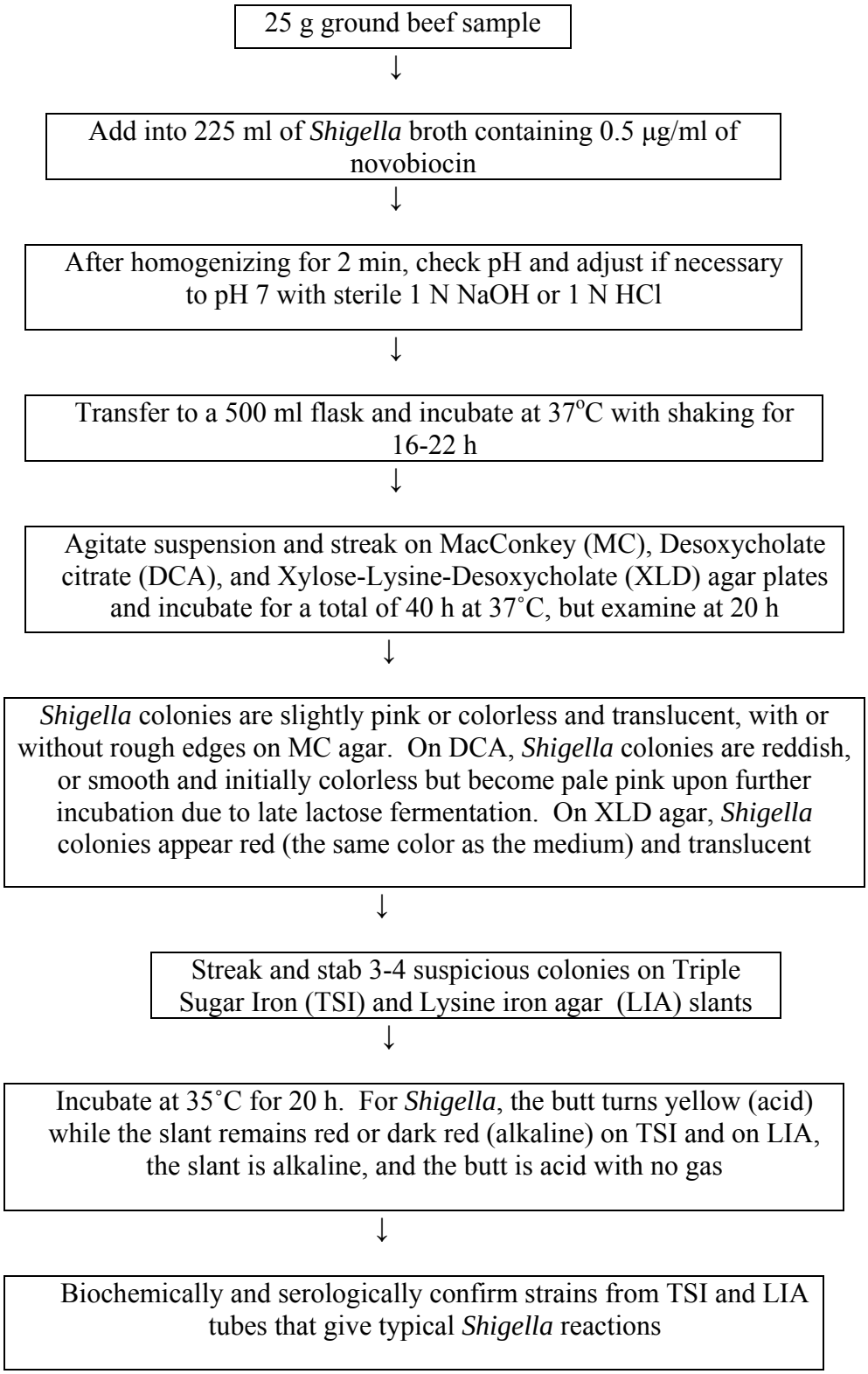


**FIGURE 2.3.** BAM procedure for isolation of *Shigella* from foods **(**FDA, 1995).

### 2.5.2 Rapid pathogen detection methods

In the food industry, early identification of bacterial pathogens is required from screening incoming raw materials and ingredients, monitoring processing equipment and the environment, to ensuring the safety and quality of finished products. Below, various rapid methods for food pathogen detection are described.

#### 2.5.2.1 Immunological methods

Immunological assays are based on the antigenic characteristics of target microorganisms. The antibody employed can be either polyclonal or monoclonal. The former type of antibody binds to a wide range of antigenic determinants while the latter significantly improves the specificity of immunological techniques by reacting with a unique epitope. With the labeling of antibody and antigen-antibody complexes, this assay can be in radioactive, fluorescent, agglutination, immobilization, precipitation or enzymatic format.

##### 2.5.2.1.1 ELISA

The enzyme-linked immunosorbent assay (ELISA) represents the most common format of immunological methods. ELISA is performed with a solid-phase matrix, such as a microwell plate, coated with antibodies. While a sample containing specific bacterial antigens is applied to the matrix, the coated antibodies are added to the well to form fastened antibody-antigen-antibody (sandwich) complexes. Following rinsing of unbound enzyme-conjugated antibodies, the enzyme remaining in the well is

measured by a colorimetric reaction and optical instrumentation (Entis et al., 2001). Currently, a number of commercial kits are available for ELISA detection of foodborne pathogens and some of them have been approved as standard methods by the Association of Official Analytical Chemists (AOAC) International.

#### 2.5.2.1.2 Commercial ELISA kit

EHEC-TEK (Organon-Teknika, Durham, NC) is based on a murine monoclonal antibody (MAb), 4E8C12, specifically directed against the antigen unique to *E. coli* O157:H7 and *E. coli* O26:H11 (Padhye et al., 1991). Nevertheless, Johnson et al. (1995) disclosed that the target antigens of MAb 4E8C12 were also present in other *E. coli* serotypes, such as O22:H8, O46:H38, O88:H49, O91:H21, O111:H11, O111:NM, and O118:H16.

For *Salmonella*, the AOAC International has approved the following kits as presumptive alternatives to conventional methods: *Salmonella*-TEK (Organon-Teknika), TECRA (TECRA), Q-TROL (Dynatech Laboratories, Inc., Chantilly, VA), VIDAS (BioMerieux, France), and LOCATE (Rhone-Poulenc, Scotland) (Andrews et al., 2001).

As for the detection of *Shigella*, no ELISA kit is currently available in the market. However, immunological assays based on latex agglutination, such as Bactigen (Wampole Labs, Cranbury, NJ) and Wellcolex (Laboratoire Wellcome, France), have been commercially established and applied for serological confirmation of *Shigella* isolates (Bouvet and Jeanjean, 1992; Metzler et al., 1988).

ELISA is quite efficient because of its rapidity, simplicity, suitability for simultaneous analysis of a larger number of samples, and potentiality for automation. Nevertheless, the detection limit of ELISA for bacterial pathogens is generally about $10^5$ to $10^6$ cells, which is far higher than the typical concentration of pathogens in food samples. Thus, a pre-enrichment step is required for the usage of a commercial ELISA kit.

### 2.5.3 Genetic methods

Techniques relying on the fact that specific characteristics (phenotypes) of a bacterium are ultimately determined by genetic information conveyed in the sequence of its DNA, have been increasingly exploited. Below, the polymerase chain reaction (PCR), multiplex PCR and real-time PCR will be discussed.

#### 2.5.3.1 Polymerase chain reaction

The polymerase chain reaction was invented by Kary Mullis of Perkin ElmerCetus Corporation in 1983 (Mullis et al., 1986). This simple but elegant technique is based on *in vitro* amplification of selected nucleic acid sequences. It is carried out in repetitive cycles, each of which consists of three steps: denaturation, annealing and extension (Figure 2.4). In the denaturation step, the heating temperature is higher than 90$^o$C which separates double-stranded DNA into single strands. During annealing, the temperature is lowered, thus enabling two specific primers to hybridize to their complementary sequences in the single strands of target DNA. A temperature of 72$^o$C is used for the extension step. Then, thermostable DNA polymerase catalyzes

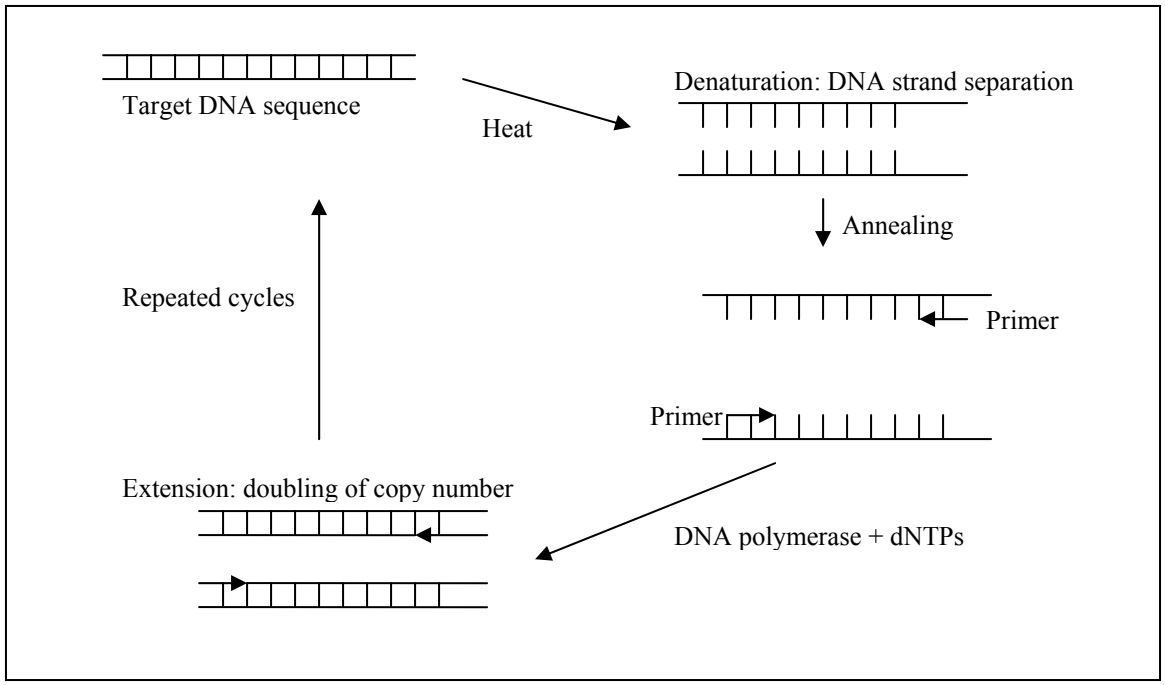


**FIGURE 2.4.** Steps of the polymerase chain reaction (Adapted from Entis et al., 2001).

continuous additions of nucleotides to the 3' ends of two annealed primers and extends two synthesized DNA strands. Cycles are repeated with double newly generated DNA as the template. Gel electrophoresis is usually performed to verify the specifically amplified products based on their sizes (Entis et al., 2001).

### 2.5.3.2 Multiplex polymerase chain reaction

Conventional PCR assays only provide for the detection of a single pathogen. In a real food contamination scenario, various pathogenic bacteria might contaminate similar types of foods, thus necessitating screening for multiple suspect pathogens at the same time. Simultaneous PCR detection, which can amplify more than one target sequence in one reaction system, would significantly reduce the

analysis time, labor and cost required in conventional systems because several organisms are analyzed at the same time. Thus, multiplex PCR realizes a faster and sensitive diagnosis of bacterial contamination in environmental, food and clinical samples.

The principle behind the multiplex PCR is the same as that of single or conventional PCR. Genomic DNA from different target pathogens is extracted and mixed in one reaction system in the presence of specific primers targeting each pathogen. In this mixed system, concentrations of individual components of the reaction mixture have to be adjusted and optimized accordingly in order to ensure an equal and stable amplification for all target pathogens.

Concurrent detection of a range of foodborne pathogens can be achieved via multiplex PCR. This technique has been well established for rapid identification of multiplex virulence factors in *E. coli* O157:H7 (Fratamico et al., 1995; 2000). Also, multiplex PCR can be used for species and serotype differentiation, and bacterial pathogens belonging to different genera. Unlike PCR assays amplifying single DNA sequences, the multiplex PCR requires a high accuracy in selecting the appropriate primer sets that are compatible with one another as well as in seeking reaction conditions that would facilitate similar efficiencies for amplification of different target genes.

#### 2.5.3.3 Real-time polymerase chain reaction

Conventionally, the analysis of PCR products is a separate step performed after the completion of a PCR. Usually, gel electrophoresis is used to assess the size

and purity of the PCR products. In a recent study, the detection limit of multiplex PCR for *E. coli* O157:H7, *Salmonella* and *Shigella* were 0.2 $\log_{10}$ CFU/g after 24-h enrichment (Li, et al., 2005). Thus, the entire process from obtaining the sample to concluding the result took 30 h. If analysis could be performed during cycling, no extra analysis steps would be required and the time to obtain results would be faster. Analyzing PCR products during amplification is known as "real-time" PCR.

#### 2.5.3.3.1 Difference between conventional PCR and real-time PCR

Real-time PCR suggests a "kinetic" rather than an "equilibrium" paradigm for PCR (Wittwer et al., 1999). Conventional PCR is usually considered as a repetitive process where three reactions occur at three temperatures three times during each cycle. In contrast, the kinetic paradigm emphasizes temperature transitions (Figure 2.5). Denaturation and annealing times are often reduced to "zero" and the temperature may always be changing. Denaturation, annealing and extension occur at different rates, and, depending on the temperature, multiple reactions may occur simultaneously. The kinetic paradigm is more correct, theoretically and practically. Sample temperatures do not change instantaneously but occur as smooth transitions. Most protocols, however, do not use zero second denaturation times (Wittwer et al., 1991, 1994).

The easiest way to monitor PCR during amplification is with the use of fluorescence technology. Many applications require only a double strand-specific dye such as SYBR Green I (Morrison et al., 1998, Bay et al., 1999). Using a generic dye eliminates the cost and problems associated with probe synthesis. However, certain

applications require greater sequence specificity, and a variety of fluorescently-labeled oligonucleotide probes can be used to monitor the progress of PCR (Pritham et al., 1998). These include exonuclease ("TaqMan®") probes and hybridization probes.

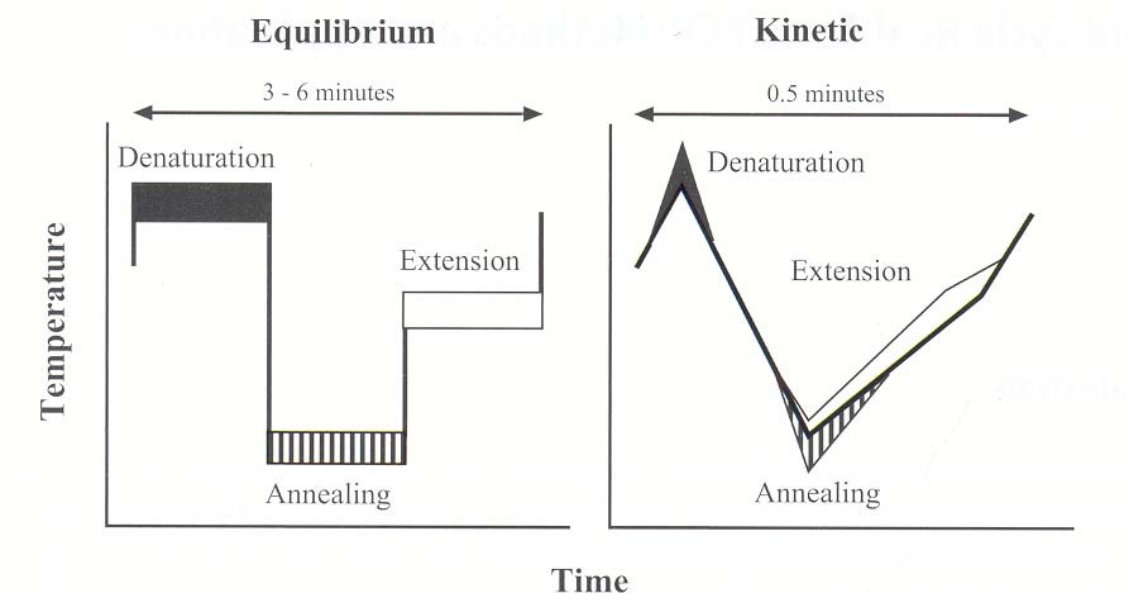


**FIGURE 2.5.** Comparison of equilibrium and kinetic paradigms of PCR (adapted from Wittwer, 2001).

TaqMan® probes are used to detect specific sequences in PCR products by employing the 5'-->3' exonuclease activity of *Taq* DNA polymerase. The TaqMan® probe (20-30 bp) is disabled from extension at the 3' end, and consists of a site-specific sequence labeled with a fluorescent reporter dye and a fluorescent quencher dye. During PCR, the TaqMan® probe hybridizes to its complementary single strand DNA sequence within the PCR target. When amplification occurs, the TaqMan® probe is degraded due to the 5'-->3' exonuclease activity of *Taq* DNA polymerase, thereby

separating the quencher from the reporter during extension. Due to the release of the quenching effect on the reporter, the fluorescence intensity of the reporter dye increases. During the entire amplification process, this light emission increases exponentially, with the final level being measured spectrophotometrically after termination of the PCR. Because increase in the fluorescence intensity of the reporter dye is only achieved when probe hybridization and amplification of the target sequence have occurred, the TaqMan® assay offers a sensitive method to determine the presence or absence of specific sequences. Figure 2.6 below shows the process of giving out fluorescence by TaqMan probes.

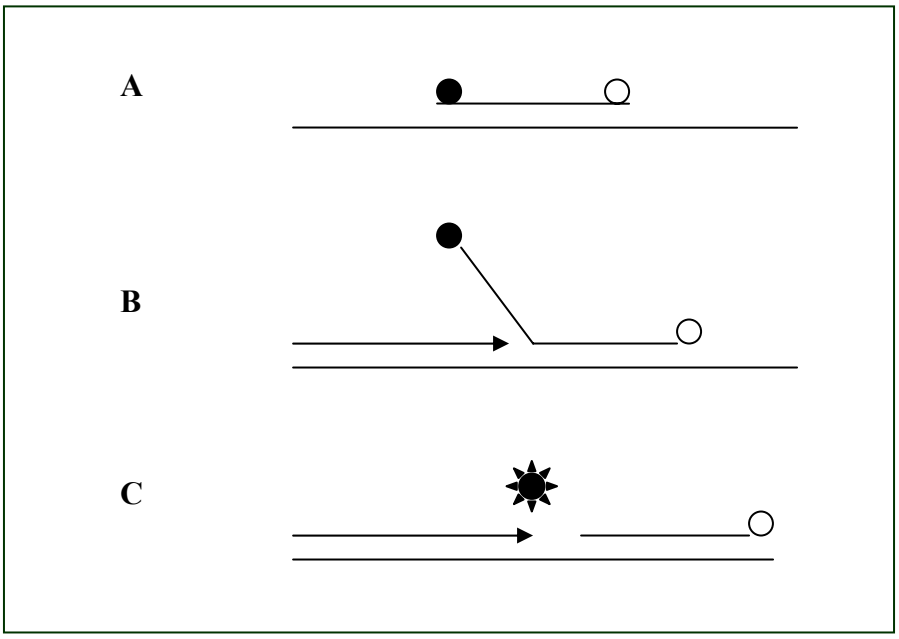


**FIGURE 2.6.** Basic principle for TaqMan ™ probe (adapted from Franklin, 2002).

Quantitation can be realized by real-time PCR. A PCR reaction profile can be thought of as having three segments: an early background phase, an exponential growth phase (or log phase) and a plateau. The background phase lasts until the signal from the PCR product is greater than the background signal of the system. The exponential growth phase begins when sufficient product has accumulated to be detected above the background, and ends when the reaction efficiency falls as the reaction enters the plateau phase. During the "log" phase the amplification course is described by the equation:

$$T_n = T_o (E)^n \quad (1)$$

where $T_n$ is the amount of target sequence at cycle n, $T_o$ is the initial amount of target, and E is the efficiency of amplification. The maximum efficiency possible in PCR is 2 because every PCR product is replicated in every cycle. Typical real-time PCR curves monitored on a light cycler are shown in Figure 2.7. The concentration is different from left to right, whereby the left has the highest concentration. The cycle where each reaction first rises above background is dependant on the amount of target present at the beginning of the reaction. The higher the initial concentration, the sooner the signal from reporter dye suppress the background dye. The *Ct* value is the point where the fluorescence given out by the reporter dye is stronger than that of the background dye. The *Ct* value is decided by setting up a threshold line on a standard curve made by plotting the *Ct* value with the initial DNA concentration. The threshold line is a factor that influences the standard curve and this relationship is shown on Figure 2.8.

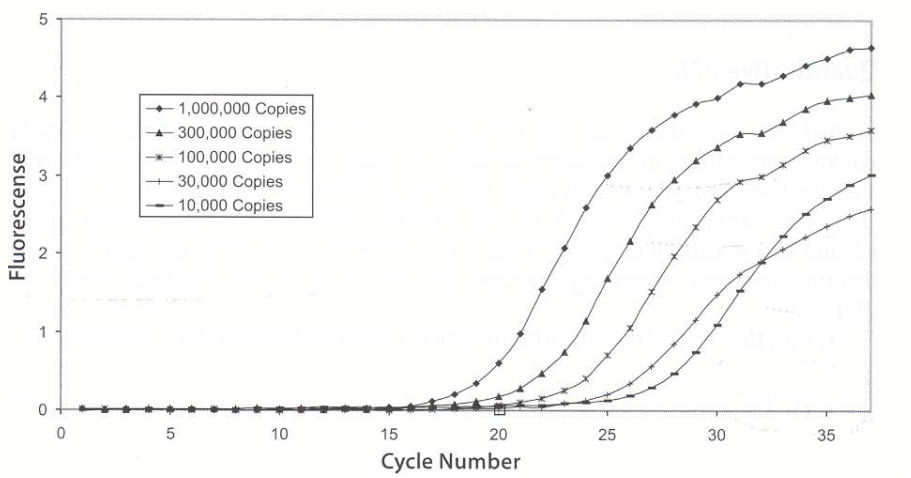


**FIGURE 2.7.** Typical real-time PCR result for different dilutions (adapted from Rasmussen, 2001).

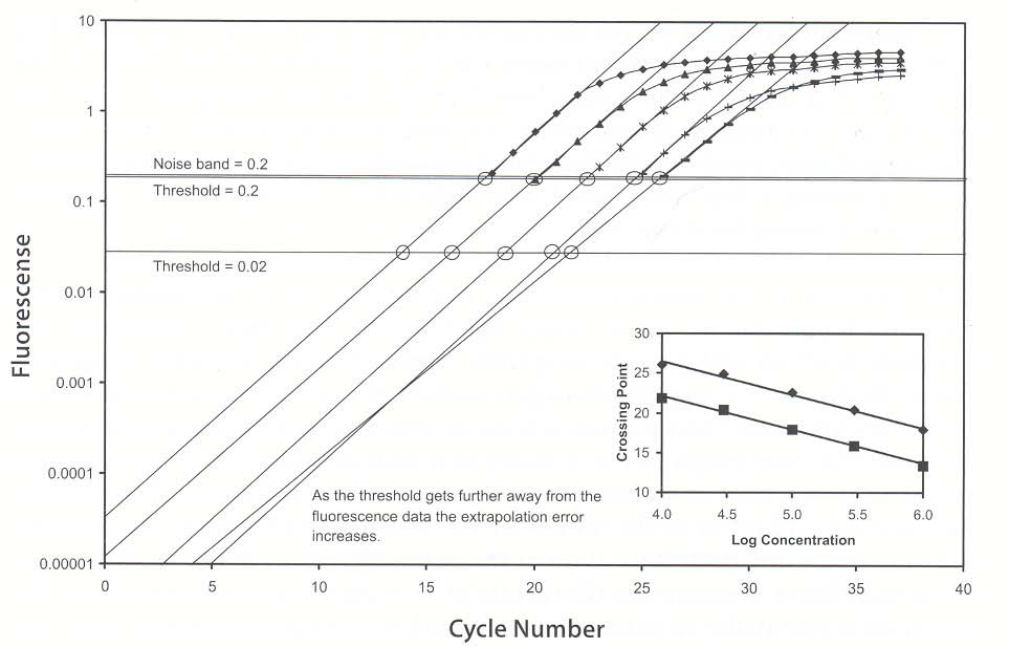


**FIGURE 2.8.** Threshold line influences the standard curve (adapted from Rasmussen, 2001).

#### 2.5.3.3.2 Application of real-time PCR

Recently, real-time PCR assays were developed for the detection and quantification of pathogen-specific gene products with the use of molecular beacons (Lyons et al., 2000, Nogva et al., 2000). In 2000, Nogva et al. applied Taqman™ probes to quantitatively detect *Listeria monocytogenes* in pure cultures, water, skim milk and unpasteurized whole milk. TaqMan™ probes and molecular beacons are oligonucleotides labeled with a reporter and quencher dyes at the 5' and 3' end, respectively. With the amplification of a target sequence, probes that attach to the sequences break, and the reporter dye, suppressed by the quencher dye, will give out fluorescence. The intensity of the fluorescence will increase with the process of amplification. The measurement of incremental fluorescence increases for each PCR cycle provides an accurate estimate of the number of cells of a pathogen in a contaminated food sample. Multiplex real-time PCR has been used to detect different species from meat samples, realizing the quantitation and identification of target strains at the same time (Sharma, 2002).

### 2.5.3.4 Immunomagnetic separation

Immunomagnetic separation (IMS) is a technique that can efficiently isolate cells from bodily fluids or cultured cells. It can also be used as a quantification method of pathogens in food, blood or feces (Engstrand, et al., 1995). In this technique, antibodies coating paramagnetic beads will bind to antigens present on the surface of cells, thus, capturing the cells and facilitating the concentration of these

bead-attached cells. The concentration process is created by a magnet placed on the side of the test tube bringing the beads to it.

This technology has been successfully used in combination with other techniques. It has also been effectively combined with several biological methods, such as PCR and real-time PCR, in food pathogen detection (Fluit et al., 1993; Mercanoglu and Griffiths, 2005). In 1996, Blanco et al. applied the immunomagnetic separation method to the detect enterohemorrhagic *E. coli* O157:H7 in minced beef. The principle and procedure of IMS are shown in Figure 2.9. The first step of IMS is to add the medium containing the food suspension and antibody-coated Dynabeads® to tubes. The tube is then placed in a rotator for mixing the beads and target microorganisms together. Next, the broth is removed and the beads that are attached to the tube wall are washed with washing buffer. The beads are collected after three washes and can then be used for the next step, such as DNA extraction.

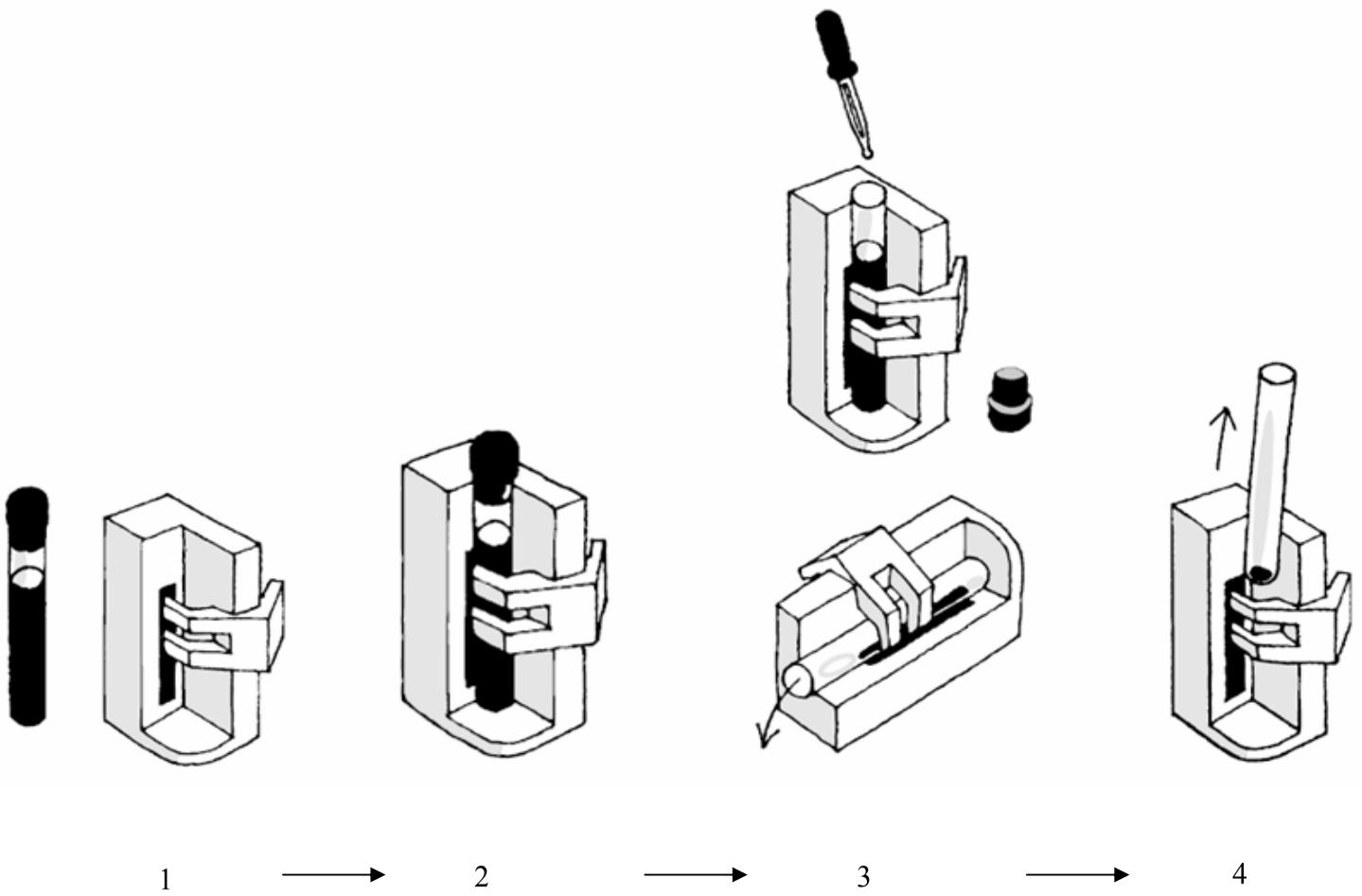


**FIGURE 2.9.** Immunomagnetic separation (adapted from Dynal manual, Dynal, Lake Success, N.Y.).

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Bacterial strains

Seventy bacterial strains from the American Type Culture Collection (ATCC, Rockville, MD) or the culture collection of the Food Microbiology Laboratory in the Food Science Program, University of Missouri, Columbia, were used in this study. All of the strains were used for testing the selectivity of DNA primers used in this study (Table 3.1). All strains, except *Bacillus*, *Clostridium* and *Lactobacillus*, were cultivated aerobically overnight at 37$^{o}$C in tryptic soy broth (Difco Labs., BD Diagnostic Systems, Sparks, Md.) supplemented with 0.5% yeast extract (TSBYE) (Difco Labs.). *Bacillus stearothermophilus* was aerobically grown in TSBYE at 55$^{o}$C. The clostridia were anaerobically propagated in TSBYE in a vacuum incubator at 37°C. *L. plantarum* was anaerobically cultivated in MRS broth (Difco Labs.) in a vacuum incubator at 37°C. The target strains, *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Shigella flexneri* were grown overnight at 37$^{o}$C in TSBYE, to a final concentration of $10^{9}$ CFU/ml.

**TABLE 3.1.** Bacterial strains used.

| Strain | Number tested | Source |
|---|---|---|
| *Escherichia coli* | | |
| K12 C5 | 1 | UMC[a] |
| K12 23716 | 2 | ATCC[b] |
| O55:H$^-$ 12014 | 1 | ATCC |
| O103:H8 23982 | 1 | ATCC |
| O111:NM 43887 | 1 | ATCC |
| O142:H6 23985 | 1 | ATCC |
| O157:H7 3178-95 | 2 | ATCC |
| O157:NM 700376 | 1 | ATCC |
| *Salmonella* | | |
| Arizonae | 1 | BioControl[c] |
| Bareilly | 1 | |
| Berta | 1 | ATCC |
| Binza | 1 | ATCC |
| Cerro | 1 | UMC |
| Derby | 1 | UMC |
| Drypool | 1 | ATCC |
| Enteritidis | 2 | UMC |
| Heidelberg | 1 | UMC |
| Infantis | 1 | UMC |
| Oranienburg | 1 | UMC |
| Pullorum | 1 | BioControl |
| Rubislaw | 1 | BioControl |
| Senftenburg | 2 | UMC |
| Tennessee | 1 | ATCC |

a

| | | |
|---|---|---|
| Typhimurium 14028 | 1 | ATCC |
| *Shigella boydii* 35965 | 1 | ATCC |
| *S. dysenteriae* 29028 | 1 | ATCC |
| *S. flexneri* 12022 | 1 | ATCC |
| *S. sonnei* 9290, 25931 | 2 | ATCC |
| *Aeromonas hydrophila* 7965 | 1 | ATCC |
| *Alcaligenes faecalis* 8750 | 1 | ATCC |
| *A. viscolactis* | 1 | UMC |
| *Bacillus cereus* | 1 | UMC |
| *B. coagulans* | 1 | UMC |
| *B. megaterium* | 1 | UMC |
| *B. sphaericus* | 1 | ATCC |
| *B. stearothermophilus* | 1 | UMC |
| *Citrobacter diversus* | 1 | UMC |
| *C. freundii* | 1 | UMC |
| *Clostridium butyricum* | 1 | UMC |
| *C. nigrificans* | 1 | UMC |
| *C. perfringens* | 1 | UMC |
| *Enterobacter aerogenes* 13048 | 1 | ATCC |
| *E. faecalis* | 1 | UMC |
| *Flavobacterium devorans* | 1 | UMC |
| *Klebsiella pneumoniae* | 1 | UMC |
| *Lactobacillus plantarum* | 1 | UMC |
| *Listeria ivanovii* | 1 | UMC |
| *L. monocytogenes* | 4 | UMC |
| *Micrococcus luteus* | 1 | UMC |
| *M. varians* | 1 | UMC |
| *Pediococcus acidilactici* | 1 | UMC |
| *Proteus vulgaris* | 1 | UMC |

| | | |
|---|---|---|
| *P. mirabilis* 55100 | 1 | Remel |
| *Providencia rettgeri* | 1 | UMC |
| *Pseudomonas aeruginosa* | 1 | UMC |
| *P. fluorescens* | 1 | UMC |
| *P. fragi* | 1 | UMC |
| *Serratia marcescens* | 1 | UMC |
| *Staphylococcus aureus* | 1 | UMC |
| *S. epidermidis* | 1 | UMC |
| *Yersinia enterocolitica* 35669 | 1 | ATCC |

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[a]University of Missouri-Columbia, Food Microbiology Lab Culture Collection

[b]American Type Culture Collection, Rockville, MD

[c]BioControl Systems, Inc., Bellevue, WA

[d]Micro-ID, Remel, Lenexa, KS

### 3.2 Design of primers and fluorogenic probes

Designing of primers and probes were conducted by using the software ABI Primer Express® (Applied Biosystems, Foster City, CA). The *uid*A gene of *E. coli* O157:H7 was selected as a target sequence for designing an *E. coli* O157:H7-specific primer, because a highly conserved single pair mutation is present in this gene. The *uid*A gene encodes ß-glucuronidase in *E. coli*, although O157:H7 isolates do not exhibit glucuronidase activity. The gene has been applied in a mismatch amplification mutation assay (MAMA) format to specifically identify *E. coli* O157:H7 and its toxigenic and non-motile variant, *E. coli* O157: $H^-$. The amplicon for *Salmonella* used

in this study is part of a sequence common in all *Salmonella* strains. The primers designed according to this sequence have been demonstrated to be specific for 144 of 146 *Salmonella* strains (116 of 118 serovars) (Aabo et al., 1993). The *ipa*H gene, a multicopy invasion plasmid antigen gene from *Shigella flexneri*, was used to design the *Shigella*-specific primer. Detailed information of each sequence is included in Table 3.2. DNA primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa).

TaqMan® probes were designed according to the target genes of the three pathogens by the same software (ABI, Inc.). Each probe was synthesized and labeled with a reporter dye and a quencher dye Carboxytetramethylrhodamine (TAMRA) by ABI (ABI, Inc.). For the *E. coli* probe, the 5' end was labeled with VIC, and 3' end was labeled with TAMRA. *Salmonella* probe was labeled with Carboxyfluorescein (FAM) on the 5' end, and TAMRA on the 3'end. The *Shigella* probe was labeled with 5'-Tetrachloro-Fluorescein (TET) on the 5' end and TAMRA on the 3' end.

VIC, FAM and TET are three different fluorescent dyes that can generate different signals. The signal map of the three dyes is showed in Figure 3.1. DNA sequences, target genes, amplicon sizes, three pairs of primers and the probes used in this study are shown in Table 3.3.

**TABLE 3.2.** Amplicons for *E. coli* O157:H7, *Salmonella* and *Shigella*.

| Strain | Sequence of amplicon | Size |
|---|---|---|
| *E. coli* O157:H7 | ttgacccacactttgccgtaatgagtgaccgcatcgaaacgcagcacgatacgctggc ctgcccaacctttcggtataaagacttcgcgctgataccagacgttgcccacataattac gaatatctgcatcggcgaactgatcgttaaaactgcctggcacagcaattgcccggcttt cttgtaacgcgctttcccaccaacgctgctcaattccacagttttcgcg | 227 bp |
| *Salmonella* | gcgactatcaggttaccgtggacactatcgaacataaaacgaagccagttctgacgct gtggtctgccttgcctgaagcggtagccagcgaggtgaaaacgacaaaggggagtct ggcgcagaagttaggttgtcgatgagaagcgctatacggcgcgtagaaagataatgga gaaaccctgccaagggtcttgatttgctacagagtgatgcaatctccctttttttagtgttac catcgtcatgccggacgaaaatagc | 261 bp |
| *Shigella* | cttgaccgcctttccgataccgtctctgcacgcaatacctccggattccgtgaacaggtc gctgcatggctggaaaaactcagtgcctctgcggagcttcgacagcagtctttcgct | 117 bp |

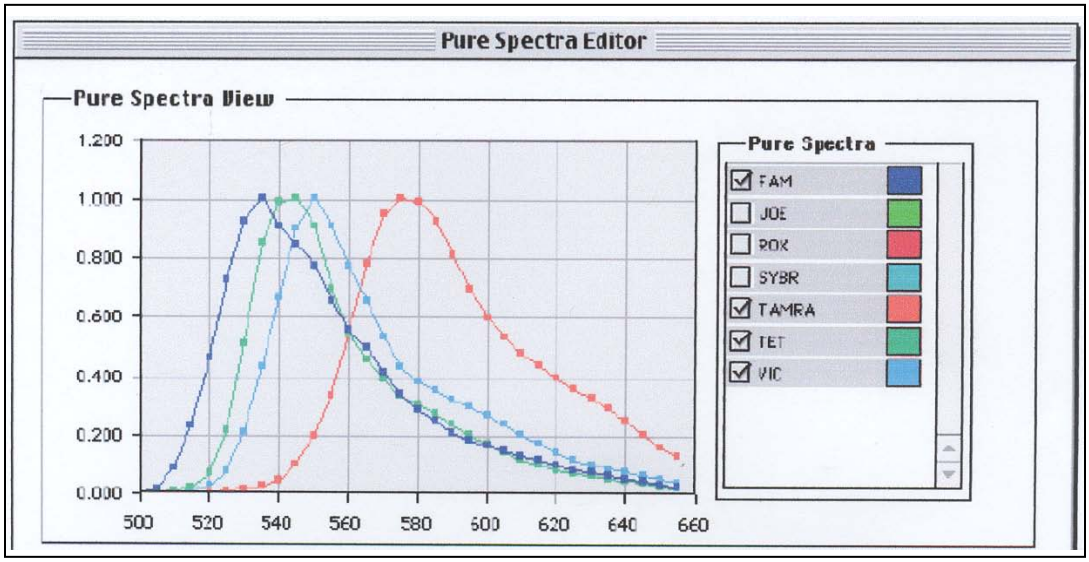



**FIGURE 3.1.** The signal map of TAMRA, TET, VIC and FAM.

**TABLE 3.3**. Oligonucleotide primers and fluorescence probes used in the multiplex real-time PCR.

| Primer/Probe | Sequence (5' to 3') | Target gene | Amplicon size |
|---|---|---|---|
| *E. coli*-1 | TTGACCCACACTTTGCCGTAA | *uidA* | 227 bp |
| *E. coli*-2 | GCGAAAACTGTGGAATTGGG | | |
| *E. coli-p* | 5'- VIC- TGACCGCATCGAAAC GCAGCT-TAMRA-3' | | |
| *Sal*-1 | GCTATTTTCGTCCGGCATGA | NA[a] | 117 bp |
| *Sal*-2 | GCGACTATCAGGTTACCGTGGA | | |
| *Salmonella-p* | 5'- FAM- TAGCCAGCGAGGTGA AAACGACAAAGG-TAMRA-3' | | |
| *Shig*-1 | CTTGACCGCCTTTCCGATA | *ipaH* | 261 bp |
| *Shig*-2 | AGCGAAAGACTGCTGTCGAAG | | |
| *Shigella-p* | 5'-TET- AACAGGTCGCTGCATG GCTGGAA-TAMRA-3' | | |

[a]Not available.

### 3.3 DNA extraction from pure cultures

To determine the specificity of the primers designed by the software, 100 µl each of fresh overnight-grown *E. coli* O157:H7, *Salmonella* and *Shigella* cultures were transferred to fresh 5 ml TSBY broth, and incubated for 24 h. Sterile peptone water (9 ml) was used to make serial dilutions of each bacterial strain. The method of Pitcher et al. (1989) was used to recover DNA from 1.5 ml of each dilution, and from a mixture

of all three pathogens (0.5 ml *E. coli* O157:H7, 0.5 ml *Salmonella* and 0.5 ml *Shigella* from each dilution).

### 3.4 Specificity of primers

The PCR was used to determine the specificity of the primers. Each 50 µl reaction mixture contained 1× PCR buffer (2 mM Tris-HCl [pH 8.0], 10 mM KCl, 0.01 mM EDTA, 0.1 mM DTT, 5% glycerol, 0.05% Tween® 20 and 0.05% Nonidet® -P40), 200 µM each of deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, three pair of primers, 1.25 U of Taq DNA polymerase and 10 µl of DNA solution. With the reaction temperature of 94°C for 4 min and an additional 35 cycles at a melting temperature of 94°C for 1 min, annealing temperature of 62°C for 1 min, and extension temperature of 72°C for 1 min, the temperature of samples were maintained at 72°C for 7 min to complete the synthesis of all strands after the final cycle. Sterile distilled water and genomic DNA of *Salmonella* Typhimurium, *E. coli*O157:H7 and *Shigella* were included in each PCR to act as a negative and positive control, respectively.

After the amplification, 10 µl of each sample were electrophoresed through a 1.8% horizontal agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). The gel was stained in 300 ml of TAE buffer containing 0.5 µg/ml ethidium bromide for 20 min, and DNA bands were visualized with a Model 310 UV transilluminator (Fotodyne Inc., New Berlin, WI) and photographed. PCR Markers™ (Promega, Madison, WI, USA) were included in each gel to determine the size of the PCR amplified products.

### 3.5 Optimization of the multiplex real-time PCR

Real-time PCR was carried out in a 96-well ABI Prism® 7700 Sequence Detection System (ABI). In order to obtain the best amplification from real-time PCR, each 50 µl reaction mixture contained 25.0 µl of TaqMan™ Universal PCR Master Mix (2×), with a final concentration of 1×, 5.0 µl forward and reverse primers, with the final concentration of 50 to 900 nM, 5.0 µl TaqMan™ probe, with the final concentration of 250 nM, and sterile distilled water (ABI, Inc.).

In order to detect all three pathogens in the same reaction system, the 5.0 µl forward and reverse primers were divided into three equal parts, 1.5 µl for *E. coli* primers, 1.5 µl for *Salmonella* primers, and 1.5 µl for *Shigella* primers. The volume of each of the respective probes for the three pathogens was adjusted to 1 µl. Because the amplification efficiency of each primer was affected in this multiplex real-time PCR system, the optimal concentrations of each primer were determined by increasing the concentration of *E. coli* and *Salmonella* primers accordingly.

Multiplex real-time PCR was carried out in a 96-well ABI Prism® 7700 Sequence Detection System. Ten microliters of DNA from *E. coli* O157:H7, *S.* Typhimurium and *S. flexneri* were applied for the establishment of the multiplex PCR protocol. A reaction volume of 50 µl of PCR mixture contained 25 µl of TaqMan™ Universal PCR Master Mix (PE Biosystems, Brachburg, NJ), primers (*E. coli*-1, *E. coli*-2, *Sal*-1, *Sal*-2, *Shig*-1, *Shig*-2), probes (*E. coli-p, Salmonella-p, Shigella-p*), 10 µl of DNA mix from the three pathogens, and distilled water. The multiplex real-time PCR was performed through 45 cycles of 95$^{o}$C for 15 sec and 62$^{o}$C for 1 min, following preheat steps at 50$^{o}$C for 2 min and 95$^{o}$C for 10 min. Final standard curves

were calculated by plotting *Ct*, which is the point at which fluorescence exceeded an arbitrary threshold signal, versus $\log_{10}$ of the CFU/ml.

### 3.6 Preparation of artificially contaminated ground beef

Fresh ground beef was purchased from a local grocery store and determined to be free of *E. coli* O157:H7, *Salmonella* and *Shigella* by standard methods (FDA BAM, 8th Ed., 1995). To determine the numbers of each initial inoculum, ten-fold serial dilutions of each organism were prepared in 0.1% (w/v) sterile peptone water (Difco Labs.). Each dilution was pour-plated in plate count agar (Difco Labs) and incubated overnight at 37°C for 24 h, after which time, colonies were enumerated. Preparation of artificially contaminated ground beef was performed according to the method established by Soumet et al. (1994; 1997), with minor modifications. Ground beef (25 g) was transferred to a sterile Whirl-Pak® sample bag (Nasco, Fort Atkinson, WI) and inoculated with 2.5 ml of each bacterial strain to achieve $10^o$ to $10^8$ CFU/g. An uninoculated control was prepared by seeding the ground beef with 2.5 ml of sterile peptone water.

### 3.7 DNA extraction from beef samples

Bacterial DNA from overnight grown pure cultures was extracted according to the method of Pitcher et al. (1989). For DNA extraction from inoculated beef samples, sterile peptone water (225 ml) was added and the suspensions of artificially contaminated samples were stomached (Stomacher 400, Seward, UK) for 2 min. Beef suspensions (1.5 ml) were transferred to a microcentrifuge tube. After centrifuging at

low speed to separate out debris, 20 µl of PrepMan$^{TM}$ ultra sample preparation reagent (ABI) was added to resuspend the pellets, and the boiling method was used to extract the DNA (Li, et al., 2002a).

### 3.8 Enrichment for the lower pathogen concentration

25 g of beef samples, artificially contaminated with lower pathogen concentrations, were added to 225 ml of TSBY broth, and incubated in a shaker incubator. Samples were taken out at 8 h, 10 h and 24 h. PrepMan$^{TM}$ ultra sample preparation reagent (ABI) was used to extract genomic DNA. Real-time PCR was performed to determine the effects of long time enrichment methods.

### 3.9 Immunomagnetic capture of pathogens

Anti-*Salmonella* and anti-*E. coli* O157:H7 Dynabeads® (Dynal, Lake Success, N.Y.) were used for isolation of *Salmonella* and *E. coli* O157:H7 from the beef samples by immunomagnetic separation (IMS). Twenty microliters of anti-*Salmonella* beads were incubated with 1 ml of samples at room temperature. After rotating on a Labquake® Rotisserie (Barnstead International, Dubuque, IA) for 10 min, 20 µl of anti-*E. coli* O157:H7 beads were added to the same sample, followed by an additional 10 min of rotation. The bead-bacteria complexes were subsequently placed in a magnetic particle concentrator (Dynal MPC-1, Dynal Biotech ASA, Oslo, Norway) for separating the magnetic beads from the beef suspensions. One milliliter of washing buffer (phosphate-buffered saline, pH 7.4) was added to wash the beads,

and the separation steps were repeated. The beads were washed twice before using for DNA extraction.

### 3.10 DNA extraction for bacteria caught by IMS beads

For immunomagnetic-captured bacteria from beef suspensions, beads captured by the magnet were resuspended in 20 μl of PrepMan$^{TM}$ ultra sample preparation reagent (PE, Foster City, CA) following the method described by Li et al., 2002.

# CHAPTER 4

# RESULTS

## 4.1 Specificity of the primers

The specificity of the primers designed by the ABI software was tested by PCR. Based on the 70 strains tested, the results showed that the primers were specific for only the target three pathogens. The detail information of all the strains used and the results are shown in Table 4.1.

The melting temperature ($T_m$) values for the three primer pairs were different. Ensuring a proper melting temperature for the PCR and real-time PCR is important because a proper $T_m$ value can maintain the stability of the primers, and thus, make the amplification specific enough for the target sequence. Different $T_m$ values were tested in this study, with 62$^{o}$C being the most optimal because the primers for *Escherichia coli* O157:H7, *Salmonella* and *Shigella* had the most specific amplification efficiency at this $T_m$. Figure 4.1 shows the results of the PCR using a $T_m$ of 62$^{o}$C. Lanes 2-4 in the gel show the amplification results from *E. coli* O157:H7 (227 bp), *Salmonella* (261 bp) and *Shigella* (117 bp), respectively, when each strain was tested individually in conventional PCR. Lanes 5-7 show bands corresponding to each respective strain when tested in combination in a multiplex PCR.

**TABLE 4.1**. Specificity of the multiplex real-time PCR primers for different bacterial strains.

| Strain | Number tested | Amplicon size (bp) | | |
|---|---|---|---|---|
| | | 227 | 261 | 117 |
| ***Escherichia coli*** | | | | |
| C5 | 1 | - | - | - |
| K12 | 2 | - | - | - |
| O55:H$^-$ | 1 | - | - | - |
| O103:H8 | 1 | - | - | - |
| O111:NM | 1 | - | - | - |
| O142:H6 | 1 | - | - | - |
| O157:H7 | 2 | + | - | - |
| O157:NM | 1 | + | - | - |
| ***Salmonella* serotypes** | | | | |
| Arizonae | 1 | - | + | - |
| Bareilly | 1 | - | + | - |
| Berta | 1 | - | + | - |
| Binza | 1 | - | + | - |
| Cerro | 1 | - | + | - |
| Derby | 1 | - | + | - |
| Drypool | 1 | - | + | - |
| Enteritidis | 2 | - | + | - |
| Heidelberg | 1 | - | + | - |

| | | | | |
|---|---|---|---|---|
| Infantis | 1 | - | + | - |
| Oranienburg | 1 | - | + | - |
| Pullorum | 1 | - | + | - |
| Rubislaw | 1 | - | + | - |
| Senftenburg | 2 | - | + | - |
| Tennessee | 1 | - | + | - |
| Typhimurium | 1 | - | + | - |
| ***Shigella*** | | | | |
| *S. boydii* | 1 | - | - | + |
| *S. dysenteriae* | 1 | - | - | + |
| *S. flexneri* | 1 | - | - | + |
| *S. sonnei* | 2 | - | - | + |
| **Others**[a] | 36 | - | - | - |

[a]Other tested strains included *Aeromonas hydrophila*, *Alcaligenes faecalis*, *A. viscolactis*, *Bacillus cereus*, *B. coagulans*, *B. megaterium*, *B. sphaericus*, *B. stearothermophilus*, *Citrobacter diversus*, *C. freundii*, *Clostridium butyricum*, *C. nigrificans*, *C. perfringens*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Flavobacterium devorans*, *Klebsiella pneumoniae*, *Lactobacillus plantarum*, *Listeria ivanovii*, *L. monocytogenes* (4), *Micrococcus luteus*, *M. varians*, *Pediococcus acidilactici*, *Proteus vulgaris*, *P. mirabilis*, *Providencia rettgeri*, *Pseudomonas aeruginosa*, *P. fluorescens*, *P. fragi*, *Serratia marcescens*, *Staphylococcus aureus*, *S. epidermidis* and *Yersinia enterocolitica*. Numbers shown in parentheses identified the number of strains of each bacterial species tested.

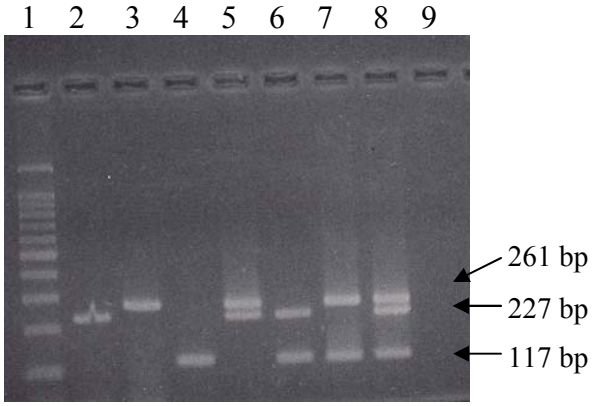



**FIGURE. 4.1**. Agarose gel electrophoresis of the multiplex PCR products from DNA of *E. coli* O157:H7, *Salmonella* and *Shigella.* 1, PCR Marker; 2, *E. coli* O157:H7; 3, *Salmonella*; 4, *Shigella*; 5, *E. coli* O157:H7 + *Salmonella*; 6, *E. coli* O157:H7 + *Shigella*; 7, *Salmonella* + *Shigella*; 8, *E. coli* O157:H7 + *Salmonella* + *Shigella*; 9, control.

### 4.2 Optimization of the multiplex real-time PCR

Different concentration of primers were tested to get the best amplification result. The concentration of primers giving the best results was 0.4 μM for *Shig*-1 and *Shig*-2, 0.8 μM for *E. coli*-1 and *E. coli*-2, 0.9 μM for *Sal*-1 and *Sal*-2.

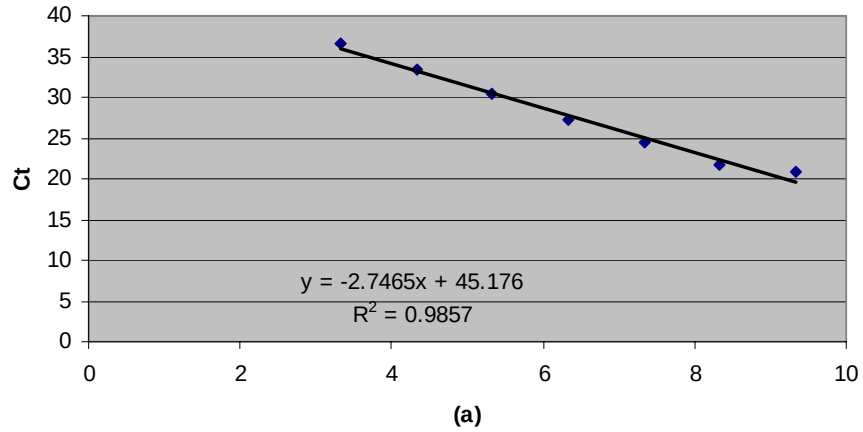
### 4.3 Real-time PCR for pure cultures

The fluorescence profile generated with the primers and probes were measured by real-time PCR on an ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The point on the curve in which the amount of

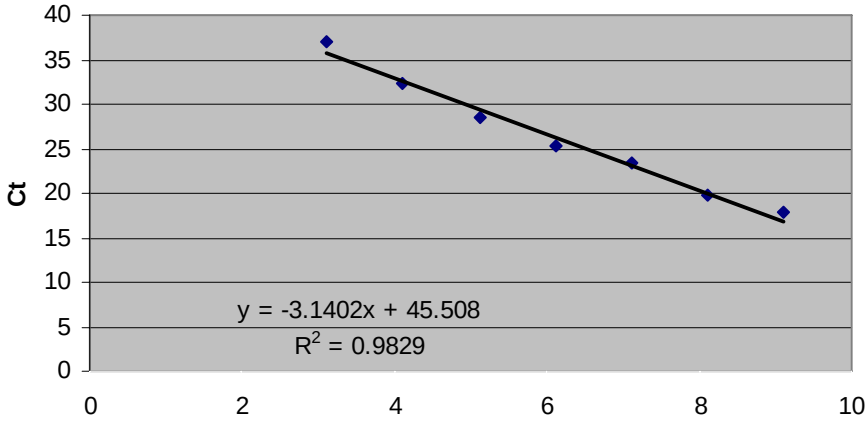
fluorescence begins to increase rapidly is termed the threshold cycle (*Ct* value). Standard curves for the pure cultures, achieved by plotting *Ct* versus $\log_{10}$ colony forming units (CFU) per ml, are shown in Figure 4.2. Quantitative detection ranges for the three pathogens with pure cultures using real-time PCR were $10^2$ to $10^9$ CFU/ml for *E. coli* O157:H7 ($R^2$ = 0.9857), $10^3$ to $10^9$ CFU/ml for *Salmonell*a (regression coefficients [$R^2$] = 0.9829), and $10^1$ to $10^8$ CFU/ml for *Shigella* ($R^2$ = 0.9986).

The sensitivity of the real-time PCR was tested when concentrations of the three pathogens were different. Overnight cultures of each strain were diluted to $10^2$ to $10^9$ CFU/ml in 0.1% peptone water. Different dilutions were mixed together, for example, $10^9$ CFU/ml of *E. coli* O157:H7 mixed with $10^2$ CFU/ml *Salmonella*, or $10^6$ CFU/ml *Salmonella* mixed with $10^2$ CFU/ml *Shigella*. DNA from the mixture of pure cultures was extracted and subjected to the real-time PCR. The results show that when the concentration of one bacterium was as high as $10^9$ CFU/ml, the *Ct* value of the other ones will increase, which meant that the lowest detection limit for the other one was higher. Detection sensitivity was not affected when the concentration of one bacterium was in a medium concentration level ($10^6$ CFU/ml) or a low level ($10^0$ CFU/ml) (Figure 4.3).

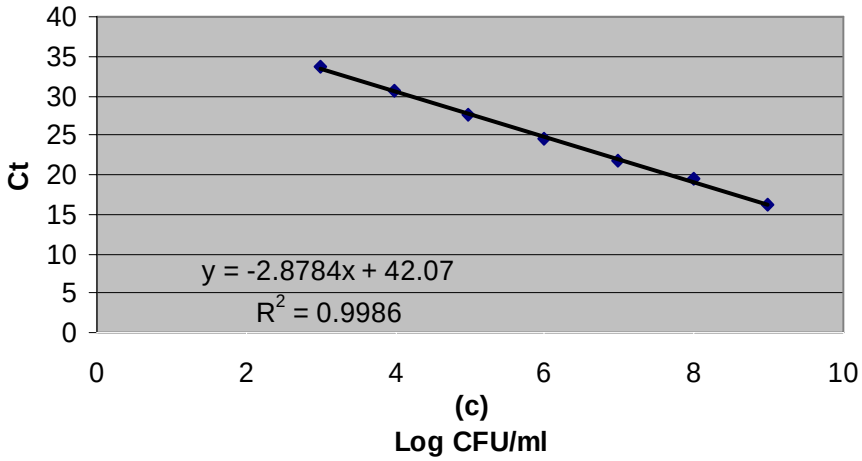
**FIGURE 4.2.** Standard curves for quantitative detection of pure cultures of *E. coli* O157:H7, *Salmonella* and *Shigella* using real-time PCR. (a) standard curve for *E. coli* O157:H7; (b) standard curve for *Salmonella*; (c) standard curve for *Shigella*. The lowest quantitative detection concentration is $10^2$ CFU/ml for *E. coli* O157:H7, $10^3$ CFU/ml for *Salmonella* and $10^1$ CFU/ml for *Shigella*.




**(a)**




**(b)**




**(c)**

**Log CFU/ml**

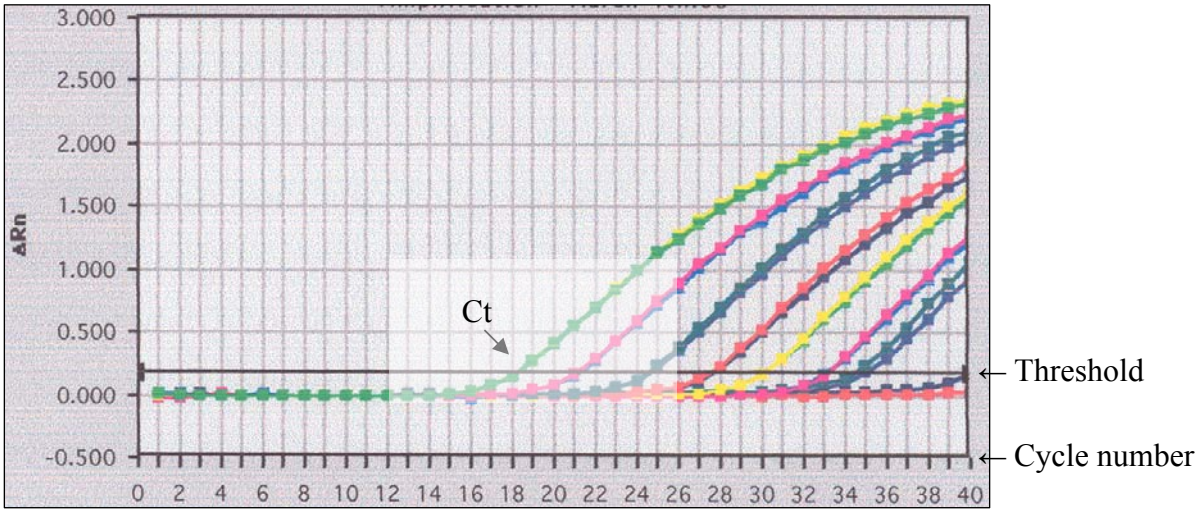



**FIGURE 4.3.** Amplification curve of *Shigella* when *E. coli* O157:H7 was $10^6$ CFU/ml. The detection range was from $10^2$ CFU/ml to $10^8$ CFU/ml. The higher the concentration, the required the cycle number for detection is less.

### 4.4 Real-time PCR for simultaneous detection of the pathogens in ground beef

When the established method was applied to ground beef that was artificially contaminated with a mixture of the three target pathogens, the detection limit was reduced to $10^5$ CFU/g for *E. coli* O157:H7, $10^3$ CFU/g for *Salmonell*a and $10^4$ CFU/g for *Shigella* (Figure 4.4).

### 4.5 Combination of immunomagnetic separation with real-time PCR

Pathogens, when present in food samples, are usually found in low concentrations. An enrichment step is necessary when lower concentrations of pathogens need to be

detected. Enrichment was applied to the lower concentrations ($10^1$ to $10^4$ CFU/g for *E. coli* O157:H7, $10^1$ to $10^2$ CFU/g for *Salmonella* and $10^1$ to $10^3$ CFU/g for *Shigella*) before performing real-time PCR. After 10 h of enrichment in TSBY broth, the lowest concentration of all three pathogens that could be detected was 10 CFU/g, but they could not be quantified. When immunomagnetic separation (IMS) was added as an additional separation step before performing the real-time PCR assay, the detection limit of *E. coli* O157:H7 and *Salmonella* increased to $10^3$ CFU/g ($R^2$ = 0.9729, 0.9023), respectively (Figure 4.5).

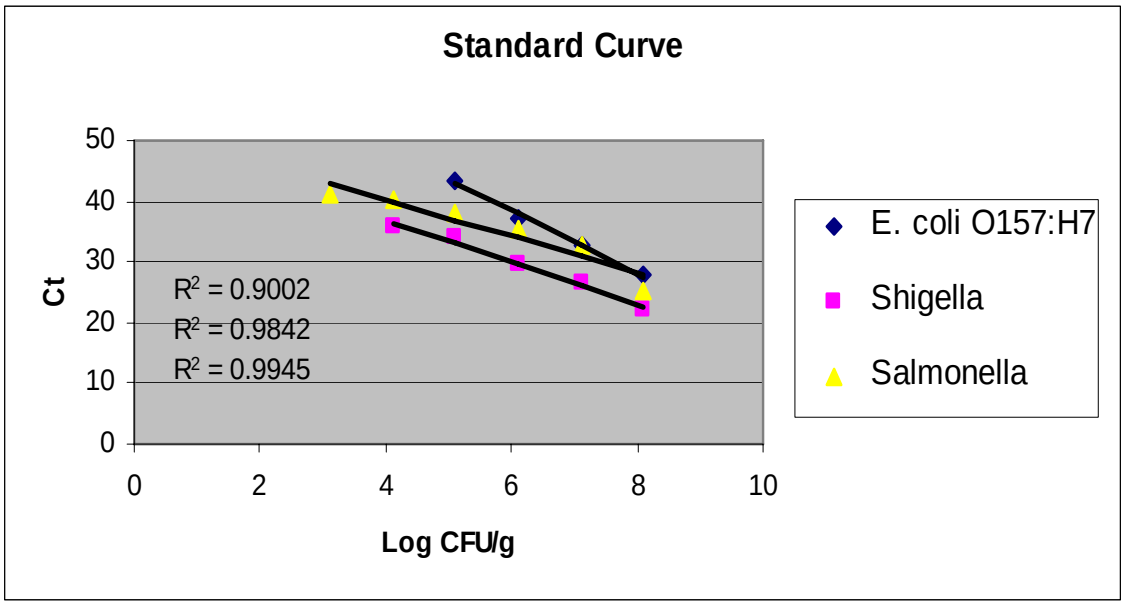



**FIGURE 4.4.** Standard curves for quantitative detection of *E. coli* O157:H7, *Salmonella* and *Shigella* in ground beef using real-time PCR. Detection limits for the three pathogens were $10^5$ CFU/g for *E. coli* O157:H7, $10^3$ CFU/g for *Salmonella*, and $10^4$ CFU/g for *Shigella*.

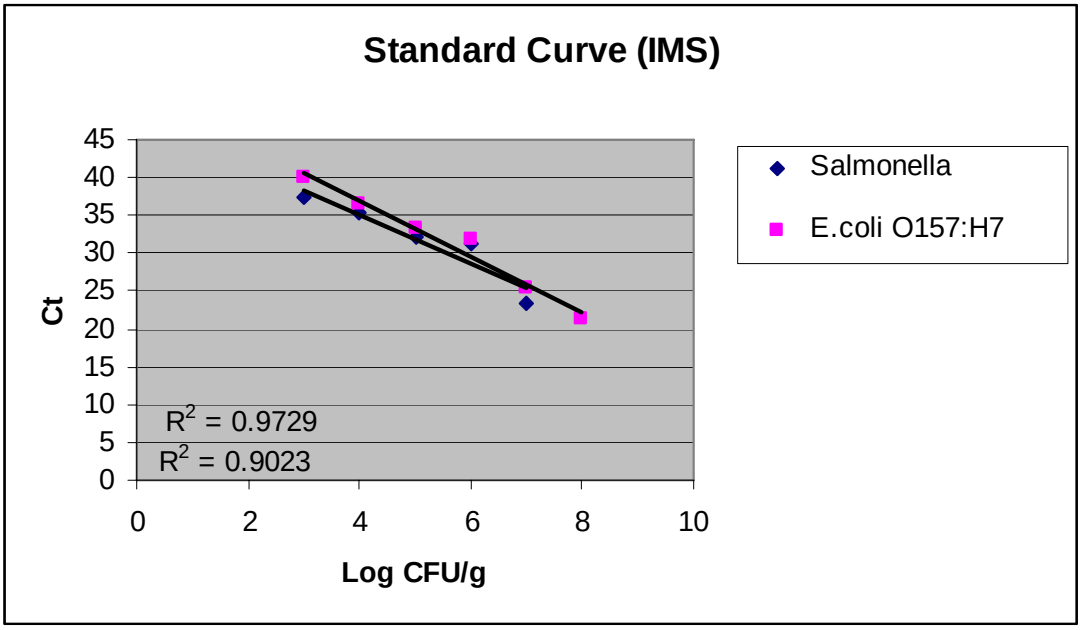



**Figure 4.5.** Standard curve for quantitative detection of *E. coli* O157:H7 and *Salmonella* in ground beef using IMS and real-time PCR. The detection limit for both of pathogens in ground beef was $10^3$ CFU/g.

### 4.6 Quantitation of *Shigella* from ground beef by real-time PCR

Ground beef artificially contaminated with only *Shigella* was used to test the application of the real-time PCR. Without the enrichment and IMS steps, a 1.5 ml suspension from different dilutions of the pathogen in ground beef was used to extract *Shigella* DNA. As shown in Figure 4.6, the detection range of *Shigella*-contaminated ground beef was from 10 CFU/g to $10^7$ CFU/g.

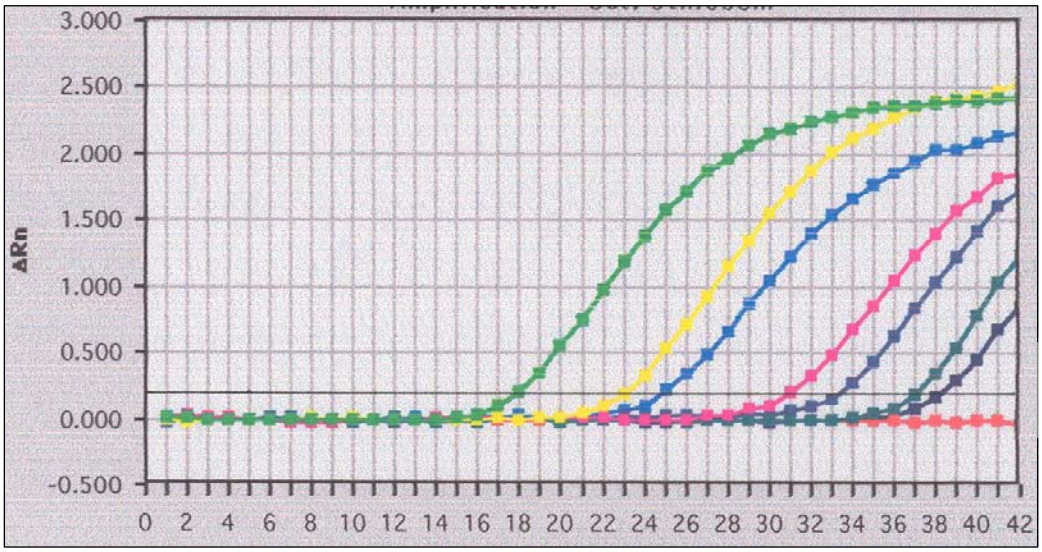



**Figure 4.6.** Amplification of *Shigella* by real-time PCR from ground beef samples. The lowest detection limit was 10 CFU/g ($R^2 = 0.9754$).

# CHAPTER 5

# DISCUSSION

Real-time PCR methods have been used for the detection, as well as quantitation, of target organisms using Taqman™ probes or molecular beacons to generate a fluorescent signal. Real-time PCR was used in this study to simultaneously detect *E. coli* O157:H7 and *Salmonella* in ground beef. Multiplex PCR is a technique that can amplify more than one target gene at the same time. Its ability to monitor several pathogens simultaneously saves time and labor as compared to conventional PCRs. Multiplex PCR has been used to detect *E. coli* O157:H7, *Salmonella* and *Shigella* from apple cider and ground beef (Li et al., 2002, 2005). In those studies, enrichment was needed when the pathogens were present in low concentrations, and quantitation could not be realized. This study, to our knowledge, is the first reported **multiplex real-time PCR** system for the simultaneous detection of *E. coli* O157:H7, *Salmonella* and *Shigella* in ground beef.

During amplification of the target gene, real-time PCR follows the same principles as conventional PCR. The specificity of the primers is very important. However, the amplification procedures of real-time PCR are quite different from those of conventional PCR assays. Therefore, new primers targeting *E. coli* O157:H7, *Salmonella Shigella* were re-designed for real-time PCR. *E.coli*-1, and *E.coli*-2 primers used in this study were designed to target a highly conserved single pair mutation in the *uid* A gene (Feng, et al., 1994). It has been used in a mismatch amplification mutation assay format to specifically identify *E. coli* O157:H7 as well as its toxigenic and non-

motile variant, *E. coli* O157: $H^-$ (Cebula, et al., 1995). The *Sal*-1 and *Sal*-2 primers were specific for all the *Salmonella* strains tested in this study. *Shi*-1 and *Shi*-2 target the *ipa*H gene of *Shigella flexneri*.

Melting temperature ($T_m$) is another factor that has to be taken into account in real-time PCR. In this system, the optimal $T_m$ for the three primer pairs were determined to be in the range of 58 to 62°C, which makes it is possible to achieve a similar efficiency for each primer set in the same system. 62°C was set as the $T_m$ for the real-time PCR in order to make the amplification more specific. The probes were also stable in this temperature since their $T_m$ were 69, 69 and 68°C, respectively for *Salmonella*, *Shigella* and *E. coli* O157:H7.

In order to achieve the best efficiency, the concentrations of each primer were adjusted. The final concentrations of the primers were determined to be 0.4 μM for *Shig*-1 and *Shig*-2, 0.8 μM for *E.coli*-1 and *E.coli*-2, and 0.9 μM for *Sal*-1 and *Sal*-2. The quality of DNA isolated from each target organism is another important factor that can greatly influence DNA amplification. PrepMan™ ultra sample preparation reagent, which was successfully applied in raw and ready-to-eat meat samples in a previous study in our laboratory (Li et al., 2002a), was used to extract bacteria DNA from the meat homogenates.

When multiplex real-time PCR was applied to pure cultures of the three pathogens, the detection efficiencies were $10^2$ to $10^9$ CFU/ml for *E. coli* O157:H7, $10^3$ to $10^9$ CFU/ml for *Salmonella* and $10^1$ to $10^8$ CFU/ml for *Shigella*, with $R^2$ of 0.9986, 0.9829 0.9857 respectively. Although the real-time PCR method performed well with mixtures of pure cultures, both the sensitivity and the efficiency decreased when this

assay was applied to ground beef (Figure 4.4 in Chapter 4). One possible reason for the decrease in sensitivity might be the presence of various food constituents, such as organic and phenolic compounds and lipids, which might influence the amplification of DNA sequences in PCR (Wilson, 1997). Another possible reason might be that the sensitivity was markedly influenced by a high level of background microflora in the ground beef or a low concentration of the target pathogens.

In order to reduce the influence of intrinsic constituents in the ground beef and the suppression of sensitivity of the real-time PCR by the background microflora, different techniques were used to increase the concentration of the target microorganisms in the ground beef. When a Tryptic Soy broth enrichment step was added before the DNA extraction step, the concentration of pathogens increased after a 10-h incubation, and they could be easily detected by the real-time PCR. This was important especially for detecting *E. coli* O157:H7 and *Shigella* whose minimum infectious dose are extremely small (approximately 10 CFU) (Kothary and Babu, 2001). However, even though enrichment did help in the detection of pathogens present in low concentrations, *quantitation* of these pathogens still could not be realized after enrichment. This is because after enrichment, the initial concentration of the pathogens present in the meat samples cannot be calculated.

Immunomagnetic separation (IMS) is a way to separate target microorganisms from the background, and it does reflect the initial concentration of each microorganism present in a food sample. In this study, IMS was coupled with real-time PCR in order to more accurately detect and quantitate the pathogens from artificially contaminated food samples. By washing the antibody-coated paramagnetic

beads used in this technique, attached target microorganisms were collected for DNA extraction. By using IMS, the detection range and $R^2$ were significantly improved for multiplex PCR detection of *E. coli* O157:H7 and *Salmonella* from ground beef. *Shigella* was not included in the assay because anti-*Shigella* paramagnetic beads were not commercial available. Thus, to quantitate *Shigella* artificially-contaminated ground beef, we used single real-time PCR (Figure 4.6 in Chapter 4). The results indicated that when low concentrations of *Shigella* need to be detected in the system, single real-time PCR will be the best way to realize this.

The entire multiplex real-time PCR detection process designed and optimized in this study took 3 h from getting the ground beef sample to calculation of the final result (including the IMS step). By this multiplex real-time PCR, all three target organisms could be detected at one time, thus reducing the amounts of reagents and cutting down on the labor and time. As a simple, rapid and efficient technique, the real-time multiplex PCR assay in this study could potentially facilitate presumptive and simultaneous monitoring of *E. coli* O157:H7, *Salmonella* and *Shigella* from ground beef and other food samples. Additionally, the effectiveness of the DNA template preparation protocols notably depended on the type of food to be tested (Grant, 2003). Therefore, alternative DNA extraction methods should be assessed with the established real-time PCR method for other food products.

# CHAPTER 6

# CONCLUSIONS AND FUTURE DIRECTIONS

The results of this study demonstrated that multiplex real-time PCR can be a potentially powerful tool to achieve simultaneous detection and quantitation of *E. coli* O157:H7, *Salmonella* and *Shigella* in ground beef. Even though the method developed in this research has not been tested with other food products, we believe that, with proper optimization, it will be applicable to other foods as well. The combination of IMS with real-time PCR allowed for a highly sensitive *simultaneous* detection and quantitation of *E. coli* O157:H7 and *Salmonella* in ground beef. However, because the multiplex PCR was not sensitive enough to detect *Shigella* as well at a low concentration and because the infectious dose of this pathogen is extremely low (10 CFU), a single real-time PCR was found to be more applicable for this organism.

Our ongoing research includes attempts at finding a way to increase the detection range and lower the influence background flora which can be used to quantitate the lowest concentration of pathogens possible in ground meats. We are also exploring further usage of IMS in the food pathogen detection field. Finally, caution has to be taken when reporting negative results from the multiplex real-time PCR for very low concentrations of pathogens. Although, real-time PCR can be used to detect the three mentioned pathogens together, enrichment is still required if identification without quantification is all that is needed.

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