

To the Graduate Council:

I am submitting herewith a thesis written by Nathan D. Miller entitled "Rapid Molecular Detection of *Salmonella* From Produce Using real-time PCR." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science & Technology.

Doris D'Souza, Major Professor

We have read this thesis
and recommend its acceptance:

P. Michael Davidson

Gina Pighetti

Accepted for the Council:

Carolyn R. Hodges

Carolyn R. Hodges, Vice Provost and
Dean of the Graduate School

(Original signatures are on file with official student records.)

**Rapid Molecular Detection of *Salmonella* From Produce
Using real-time PCR**

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Nathan D. Miller
May 2009

Copyright © 2006 by Nathan D. Miller
All rights reserved.

ACKNOWLEDGEMENTS

I would like to thank my major professor/advisor Dr. Doris D'Souza for her help and giving me the opportunity to complete my M.S. degree. She has been a great advisor that has helped me adjust to the ups and downs of graduate life. With her continual challenges to go beyond what was expected has provided me with a much appreciated educational experience. Not only is she my major professor but a friend that looks out for me and guided me through the ups and downs of my graduate career. For that, I am greatly appreciative to have worked under her for these last two years. I would also like to thank Faith Critzer and Ray Trejo for their encouragement and friendship. They both are a remarkable friend that has always been there to guide me and for that I am truly grateful.

I would like to express my appreciation to my committee members, Dr. P. Michael Davidson and Dr. Gina Pighetti for their input and challenges. To the Food Science Department, thanks for all the great times and laughs that we have shared. There has been too many to just name one. The faculty, staff, and students are among the best showing great enthusiasm and openness to everyone. This is just one of the many great qualities about this department that makes it one of the best.

Last but certainly not least, I would like to thank my family for their endless support and prayer. Without the solid support from ya'll, it would have been a much harder road to travel. I am grateful for the sacrifices they were made in order to help me achieve where I am today.

ABSTRACT

Recent outbreaks of *Salmonella* linked to fresh produce emphasize the need for rapid and sensitive assays to help control outbreaks. Reverse-transcriptase PCR (RT-PCR) detects the presence of mRNA (shorter half-life than DNA), with greater potential of detecting viable cells. Rapid real-time methods using fluorescent dyes and probes simultaneously detect and confirm the presence of target nucleic acid, eliminating the need for gel electrophoresis. The objective of this research was to rapidly detect *Salmonella* Typhimurium from spiked lettuce, tomatoes, and peppers using real-time RT-PCR. Washed and ultraviolet light treated lettuce (~25gram), tomato (~100g), and peppers (~130g) samples were inoculated with high (10^8 to 10^6 CFU) and low (10^3 to 10^1 CFU) *Salmonella* Typhimurium overnight cultures. Samples were then rinsed or hand massaged with 225 ml 0.05 M glycine-saline buffer containing 0.05% Tween and 3% beef extract. Un-inoculated washed produce and sterile buffer were used as negative controls; with *S. Typhimurium* as a positive control. RNA was extracted from each sample using the Qiagen RNeasy Mini Kit. RT-PCR was carried out using a SYBR Green I RT-PCR kit with previously described *Salmonella* specific *invA* gene primers and an internal amplification control (IAC) to eliminate false negatives. Reaction conditions were RT at 50 degree Celsius/40min; PCR at 95 degree Celsius /45s, 58 degree Celsius /45s, 72 degree Celsius /45s for 40 cycles followed by melt temperature(T_m) analysis in a BioRad iCycler to determine specific *invA* product ($\sim T_m$ = 87.5 degree Celsius) and IAC (T_m =82 degree Celsius). To improve detection sensitivity of low inocula, spiked lettuce,

tomatoes, and peppers were pre-enriched in buffered peptone water for 6 hours at 37 degree Celsius, followed by RNA extraction and RT-PCR detection. Each experiment was repeated twice. Real-time RT-PCR after 6-h pre-enrichment, Qiagen RNA extraction and the SYBR Green I kit gave *Salmonella* detection up to 10^3 CFU/25g from lettuce, 10^4 CFU/~130g from tomatoes, and 10^4 CFU/25g from peppers. Without enrichment, detection limits were 10^6 CFU/25g for lettuce, 10^7 CFU/25g for tomatoes, and 10^7 CFU/25g for peppers. Sensitive and rapid detection of *Salmonella* from spiked lettuce, tomatoes, and peppers could still be obtained within one day (~ 2 working shifts).

TABLE OF CONTENTS

Chapter	Page
CHAPTER I.....	1
Literature Review	1
Traditional Methods Used to Detect <i>Salmonella</i>	5
Novel Technology Used to Detect <i>Salmonella</i>	13
Factors Affecting the Polymerase Chain Reaction (PCR) Within the Food Matrix	22
Actions to Increase the Sensitivity and Specificity of PCR and the Recovery of Bacteria from the Food Matrix.....	27
References	36
CHAPTER II.....	48
Real-time reverse-transcriptase PCR for <i>Salmonella</i> Typhimurium detection from Lettuce and Tomatoes	48
Abstract	49
Introduction	51
Materials and Methods:.....	56
Growth of <i>Salmonella enterica</i> serovar Typhimurium	56
Preparation of Lettuce and Tomatoes.....	56
Extraction from Inoculated Lettuce and Tomatoes.....	57
Enumeration of Bacteria	57
Isolation of Total RNA from Bacteria	58
DNase I Treatment.....	59
Nanodrop Quantification of RNA.....	59
Real-Time Reverse-Transcriptase Polymerase Chain Reaction (rt-RT-PCR) Procedure	59
Preparation of the Internal Amplification Control (IAC).....	60
Results	61
Bacterial Cell Recovery from Lettuce and Tomatoes.....	61
RNA Extraction Yields from Lettuce and Tomatoes.....	62
rt-PCR Detection and Specificity	62
DNase I Treatment	63
Discussion.....	63
List of References.....	69
Appendix	73
CHAPTER III.....	89
Real-time reverse-transcriptase PCR for <i>salmonella typhimurium</i> detection for jalapeño and Serrano peppers	89
Abstract	90
Introduction	91

Materials and Methods:	94
Growth of <i>Salmonella enterica</i> serovar Typhimurium	94
Preparation of Food Source	94
Extraction from Inoculated Food Source	95
Enumeration of the Bacteria	95
Isolation of Total RNA from Bacteria	96
DNase I Treatment	97
Nanodrop Quantification of RNA	97
Real-Time Reverse-Transcriptase Polymerase Chain Reaction (rt-RT-PCR) Procedure	97
Preparation of the Internal Amplification Control (IAC)	98
Results	99
Bacterial Cell Recovery from Peppers	99
RNA Extraction Yields from Peppers	100
rt-PCR Detection and Specificity	100
DNase I Treatment	101
Discussion	101
List of References	108
Appendix	112
Vita	122

LIST OF TABLES

Table	Page
Table 1: <i>Salmonella</i> foodborne outbreaks occurring over the past several years.....	44
Table 2: Recent Molecular Methods used for the detection of <i>Salmonella</i> in foods.....	46
Table 3: Recovery of <i>Salmonella</i> Typhimurium from lettuce by plating on XLT4 agar and nanodrop readings of RNA extracts without enrichment	74
Table 4: Recovery of <i>S. Typhimurium</i> without enrichment from peppers by plating on XLT4 agar and nanodrop readings of RNA extracts	113

LIST OF FIGURES

Figure	Page
Figure 1: Detection limits of <i>Salmonella</i> showing cycle threshold values (Ct) from spiked Lettuce by rt-PCR without enrichment. Positive Control (1), Log 8 (2), Log 7 (3), Log 6 (4) CFU/25g, and Negative Control water (5)	75
Figure 2: Melt temperature curve depicting specific <i>Salmonella</i> amplification products at ~85°C with detection limits of 106 without enrichment form Lettuce. The T _m of the invA product occurs ~85°C and the T _m of the IAC occurs at 82°C.	76
Figure 3: Agarose gel electrophoresis of rt-PCR amplified products (347bp) from lettuce spiked with <i>Salmonella</i> Typhimurium without enrichment. Lane (M): 100 bp ladder marker. Lane (1-8): products from lettuce spiked with log 8 to log 1 of <i>S. typhimurium</i> . Lane (9): positive control of RNA extracted from <i>S. Typhimurium</i> . Lane (10): negative control (water)	77
Figure 4: Detection limits of <i>Salmonella</i> showing cycle threshold values (Ct) from spiked Lettuce by rt-PCR with enrichment. Positive Control (1), Log 5 (2), Log 4 (3), and Negative Control water (4) showing detection limits of 104 CFU/25g for lettuce... ..	78
Figure 5: Melt temperature curves depicting specific <i>Salmonella</i> amplification products at ~85°C with detection limits of log 4 CFU/25g with enrichment from lettuce. . The T _m of the invA product occurs ~85°C and the T _m of the IAC occurs at 82°C.....	79
Figure 6: Agarose gel electrophoresis of rt-RT-PCR amplified products (347) from lettuce spiked with <i>Salmonella</i> Typhimurium after enrichment. Lane (M): 100 bp ladder marker. Lane (1-5): products from lettuce spiked with log 8 to log 4 CFU of <i>S. Typhimurium</i> . Lane (6): positive control of RNA extracted from <i>S. Typhimurium</i> . Lane Lane (7): negative control (water).....	80
Figure 7: Detection limits of <i>Salmonella</i> showing cycle threshold values (Ct) from spiked tomatoes by rt-PCR without enrichment. Positive Control (1), Log 8 (2), Log 7 (3), and Negative Control water (5) showing a detection limit of 10 ⁷ CFU/ml for tomatoes.	81
Figure 8: Melt temperature curve depicting specific <i>Salmonella</i> amplification products at ~85°C with detection limits of log 7 CFU/25g without enrichment from Tomatoes. The T _m of the invA product occurs ~85°C and the T _m of the IAC occurs at 82°C....	82
Figure 9: Agarose gel electrophoresis of rt-PCR amplified products (347bp) from Tomatoes spiked with <i>Salmonella</i> Typhimurium without enrichment. Lane (M): 100 bp ladder marker. Tomato control no inoculation (1), Lane (2-4): products from Tomatoes spiked with log log 8 to log 4 CFU of <i>S. Typhimurium</i> . Lane (6): positive control of RNA extracted from <i>S. Typhimurium</i> . Lane (7): negative control (water)83	83
Figure 10: Detection limits of <i>Salmonella</i> showing cycle threshold values (Ct) from spiked Tomatoes by rt-PCR with enrichment. Positive Control (1), Log 6 (2), Log 5 (3), Log 4 (4), and Negative Control water (5)	84

- Figure 11: Melt temperature curve depicting specific *Salmonella* amplification products at ~85°C with detection limits of 10⁷ without enrichment form Tomatoes. . The T_m of the invA product occurs ~85°C and the T_m of the IAC occurs at 82°C..... 85
- Figure 12: Agarose gel electrophoresis of rt-PCR amplified products (347bp) from Tomatoes spiked with *Salmonella* Typhimurium without enrichment. Lane (M): 100 bp ladder marker. Lane (1-8): products from Tomatoes spiked with 10⁸ to 10¹ CFU of *S. Typhimurium*. Lane (9): positive control of RNA extracted from *S. Typhimurium* pure culture. Lane (10): negative control (water) 86
- Figure 13: Agarose gel electrophoresis of PCR products using RNA treated with DNase I. Lane (M): 100 bp ladder marker. Lane (1-7): products from *Salmonella* Typhimurium. Lane (8-11): products from Spiked Lettuce (10⁷, 10⁶, 10⁵). Lane (12-15): products from Spiked Tomatoes (10⁷, 10⁶, 10⁵). Lane (16): negative control (water)..... 87
- Figure 14: Agarose gel electrophoresis of reverse-transcriptase PCR treated with DNase I. Lane (M): 100 bp ladder marker. Lane (1-7): products from *Salmonella* Typhimurium. Lane (8-11): products from Lettuce. Lane (12-15): products from Tomatoes. Lane (16): positive control of *S. Typhimurium* from RNA extraction. Lane (17): negative control (water)..... 88
- Figure 15: Detection limits of *Salmonella* showing cycle threshold values (Ct) from spiked Jalapeno peppers by rt-PCR without enrichment. Positive Control (1), Log10⁸ (2), Log10⁷ (3), Pepper control no inoculation (4), and Negative Control water (5) 114
- Figure 16: Melt temperature curve depicting specific *Salmonella* amplification products at ~85°C with detection limits of 10⁷ without enrichment form Jalapeno peppers. 115
- Figure 17: Agarose gel electrophoresis of rt-PCR amplified products (347bp) from Jalapeno peppers spiked with *Salmonella* Typhimurium without enrichment. Lane (M): 100 bp ladder marker. Lane (1-6): products from Jalapeno peppers spiked with 10⁸ to 10³ CFU/ml of *S. Typhimurium*. Lane (7): positive control of *S. Typhimurium* from RNA extraction. Lane (8): negative control (water) 116
- Figure 18: Detection limits of *Salmonella* showing cycle threshold values (Ct) from spiked Jalapeño peppers by rt-PCR with enrichment. Positive Control (1), 10⁶ - 10⁴ CFU (2-4), Negative Control water (5), and 10³ CFU (6) 117
- Figure 19: Melt temperature curves depicting specific *Salmonella* amplification products at ~85°C with detection limits of 10⁴ with enrichment from Jalapeno peppers.... 118
- Figure 20: Agarose gel electrophoresis of rt-RT-PCR amplified products (347 bp) from Jalapeno peppers spiked with *Salmonella* Typhimurium after enrichment. Lane (M): 100 bp ladder marker. Lane (1-9): products from Jalapeno peppers spiked with 10⁸ to 10⁰ CFU/ml of *S. Typhimurium*. Lane (12): positive control of RNA extracted from *S. Typhimurium*. Lane (13): negative control (water)..... 119
- Figure 21: Agarose gel electrophoresis of PCR products (347 bp) using RNA treated with DNase I from Jalapeno peppers spiked with *Salmonella* Typhimurium. Lane (M): 100 bp ladder marker. Lane (1-5): products from Jalapeno peppers spiked with 10⁷

to 10 ³ CFU/ml of <i>S. Typhimurium</i> . Lane (6): positive control of RNA extracted from <i>S. Typhimurium</i> . Lane (7): negative control (water).....	120
Figure 22: Agarose gel electrophoresis of rt-PCR products (347 bp) using RNA treated with DNase I from Jalapeno peppers spiked with <i>Salmonella Typhimurium</i> . Lane (M): 100 bp ladder marker. Lane (1-5): products from Jalapeno peppers spiked with 10 ⁷ to 10 ³ CFU/ml of <i>S.typhimurium</i> . Lane (6): positive control of RNA extracted from <i>S. typhimurium</i> . Lane (7): negative control (water).....	121

CHAPTER I

Literature Review

According to the Centers for Disease Control and Prevention (CDC), in the United States there are an estimated 76 million foodborne related illnesses each year. Many of these cases go unreported due to symptoms being mild and only lasting a few days (CDC, 2008a). Of the foodborne related illnesses, about 325,000 people are hospitalized and about 5,000 of the related illnesses result in death each year. Over the past several years *Salmonella* enterica serovars have been increasingly gaining more attention in the news media. There has been an estimated 40,000 cases of Salmonellosis each year in the U.S. alone (CDC, 2008a). These cases of Salmonellosis can be attributed to the 2,000 different *Salmonella* serovars that have been discovered.

Salmonellosis is an infectious disease caused by the ingestion of *Salmonella* that belongs to the “Enterobacteriaceae” family. *Salmonella* is typically a rod-shaped, motile, Gram-negative bacteria. *Salmonella* gallinarum and pullorum are two pathogenic strains believed to be non-motile (Meerburg and Kijlstra, 2007). *Salmonella* is a current common problem for the food industry. Infection with *Salmonella* can cause gastroenteritis, inflammation of the intestinal tract, nausea, fever, vomiting, and diarrhea (Stone and others 1994). Many of these symptoms occur within 12-72 hours of infection and last about 4-7 days (CDC, 2008a). *Salmonella* infection starts in the small intestine by invading the enterocytes. Once it has invaded the enterocytes, it causes villus blunting and the loss of the absorptive lining in the small intestine. The problem

continues with polymorphonuclear leucocytes, a white blood cell that looks like it has more than one nuclei, moving to the mucosa infected area and causing the onset of watery diarrhea. The problem can further continue if the bacteria moves past the mucosa cells to the underlying tissue and into the blood stream where it can be distributed throughout the body fairly quickly (Meerburg and Kijlstra, 2007). The elderly, infants, and people with suppressed immune systems are at high risk of infection and need to seek treatment with antibiotics (CDC, 2008a).

Salmonella outbreaks in the past have mainly been associated with pork, poultry meat, eggs and egg products (Klerks and others 2007). In today's market, many of the consumers are buying more ready-to-eat foods and fresh produce with an increasing trend of vegetarian food consumption. With the popularity of this vegetarian movement / trend on the rise, understanding the source of produce contamination is greatly significant. In recent years, it has been noted that manure and irrigation water can contribute to the spread of human pathogens on crops growing in the fields (Klerks and others 2007). This practice can greatly contribute to the spread of salmonellosis from farm to table and to the human population (Klerks and others 2007). Being able to understand more about areas of contamination: during harvesting, processing, and retail markets, can greatly help reduce the spread of *Salmonella* (Bhagwat, 2003).

Understanding the transmission process of *Salmonella* is crucial in order to detect and curb outbreaks. *Salmonella* is mainly transmitted through the fecal oral route. Contamination of produce can occur by various processes. For example, when

one considers fresh produce on the farm, there are many different routes of contamination such as use of contaminated water which can come from many different sources. As an example, a farmer may irrigate his fields with waste water from the dairy in order to use the nutrients from the accumulated manure at the barn. Although this practice may improve the quality of the produce, it is necessary because of environmental laws on removing of waste. The down side of this practice is this practice is a highly specific contributor to the spread of *Salmonella* (Klerks and others 2007). Another point of contamination is the use of organic compost for fertilizer (Yun and others 2007). This type of fertilizer is usually plowed in before the produce has been planted. If the organic compost was applied before the produce has grown, the bacterial organisms still present can contaminate the produce during a rain storm just by the simple action of the soil bouncing on the leaves. Also, during harvest time if a worker drops the fresh cut produce on the ground, it could also be a source of contamination not only for that particular produce but for the rest of the harvest as well. On moving away from the farm and to the processing plant, many other potential sources of contamination exist.

From the process aspect, a processing plant could be a high risk establishment for the cross contamination of produce with *Salmonella*, but it can be the point of least contamination because of a company's ability to control the environment of the product. Several points of contamination include poor personal hygiene of workers, contaminated equipment, improper storage temperatures and unsafe sources of food

(Sivapalasingam and others 2004). Poor personal hygiene could be attributed to not washing hands after using the restroom or even touching the floor or contact surfaces of the processing plant. Many processing plants understand the importance of controlling bacteria and have their employees step in a dish of sanitizing liquid before entering the processing rooms, while washing hands or using gloves should be a practice done every time one begins working again. Contaminated equipment can be the source of different types of bacteria. Equipment used in the processing plants could have been contaminated from the produce being processed. This would cause cross contamination to the products that are processed after the point of contamination if not washed / decontaminated properly. Also if the equipment is not cleaned properly, it could attract different insects and rodents to the equipment. Rats or mice have been known to cause the spread of *Salmonella* through the dropping of fecal pellets (Meerburg and Kijlstra, 2007). Retail markets could also deal with the same aspects of contamination as a processing plant does. Therefore, they should use proper procedures to help ensure that contamination from *Salmonella* or any other bacteria does not occur. In spite of all the control strategies in place, outbreaks of *Salmonella* do occur. In order to determine source of contamination, rapid and sensitive detection methods are needed to allow for timely product recall.

Lettuce, tomatoes, peppers, and cantaloupes have received much scrutiny in the past several years due to outbreaks linked to *Salmonella* contamination. *Salmonella's* ubiquitous nature should not be taken lightly (Mercanoglu and Griffiths, 2005) with the

most common source of contamination being the fecal-oral route of infection (CDC, 2008b). Lettuce is a cool-season crop that has a temperature growth range from 45° to 75°F (MacGillivray, 1953). With *Salmonella*'s mesophilic growth range, these temperatures (20° - 40°C) (Talaro and Talaro, 1999b) and nutrients from the plant create an environment optimal for *Salmonella* to multiply (D'Aoust and others 2001). Methods to help sensitively, specificity, and rapidly detect the source of pathogen entry in the plant/produce are in high demand.

Traditional Methods Used to Detect Salmonella

Traditional cultural methods for the detection of *Salmonella* and other food-borne pathogens are laborious and time consuming. As the demand for large quantities of fresh produce by consumers increase, assurance of product safety for consumption with timely delivery of fresh produce is crucial. Traditional detection methods usually consist of a several step process: pre-enrichment, selective enrichment, propagation / growth, presumptive identification, identification, and characterization (Serotyping) in order to determine the presence or absence of target bacteria (Feng, 2007).

Pre-enrichment process is one of the key elements in helping injured target bacteria recover and reach a level of optimal growth. The use of a nonselective media like Tryptic Soy Broth (TSB) provides the organism with the nutrients needed in order to divide (Seo and others 2006). A selective enrichment process is used to inhibit bacteria other than the target bacteria. This process provides less competition aiding the target bacteria to selectively grow (Difco[™], 2003). Triple sugar iron agar (TSI) is used in the

presumptive identification of *Salmonella* (Seo and others 2006) to determine if carbohydrate fermentation is taking place and if hydrogen sulfide is being produced (Difco™, 2003). It requires a 24 hr incubation period between each nonselective and selective step in order for the cells to grow and obtain results. The entire process takes a total of about 5 days for preliminary results.

According to the Bacteriological Analytical Manual (BAM), updated by FDA, there are over 50 different types of media and reagents that are approved to be used for testing of *Salmonella*. The media can be broken down into two different groups, enrichment and differentiation. Some of the enrichment media are Lactose broth, Selenite cystine (SC) broth, Tetrathionate (TT) broth, Rappaport-Vassiliadis (RV) broth, Trypticase Soy Broth (TSB), Nutrient broth, Brain heart infusion agar, and Universal pre-enrichment broth (Andrews and Hammack, 2007). Some of the differentiation medias are Xylose lysine desoxycholate (XLD) agar, Hektoen enteric (HE) agar, Bismuth sulfite (BS) agar, Triple sugar iron (TSI) agar, Malonate broth, Lysine iron agar (LIA), MacConkey agar, and Xylose lactose tergitol 4 (XLT4) (Andrews and Hammack, 2007). All of these media have unique and specific characteristics to aid in the determination of the target bacterium.

The properties of each general enrichment media are unique in their own way. Lactose broth is used as a pre-enrichment step in order to study coliform organisms and to determine lactose fermentation of any type of bacteria. The available nutrients within the media are lactose, peptone, and beef extract. The bacteria can ferment the

carbohydrate source lactose, thereby producing gas bubbles to provide a positive test for a coliform. The peptone and beef extract provide nutrients needed for the growth of the organism (Difco[™], 2003).

Trypticase Soy Broth is a general purpose medium that will support the growth of a wide variety of bacteria. It has the capabilities to promote the growth of fastidious and nonfastidious microorganisms. The media consists of several ingredients that allows for the growth of many different microorganisms. The amino acids needed to support the growth of microorganisms come from the casein and soybean meal provided in the media. Dextrose is added to provide a source of energy. Also sodium chloride is added to help control the osmotic equilibrium (Difco[™], 2003).

Nutrient broth is a general purpose media that is similar to Lactose broth but without the ingredient lactose. The media provides the nutrients needed to support the growth of nonfastidious organisms. The peptone provides a source of organic nitrogen and the beef extract contains carbohydrates, vitamins, organic nitrogen, and salts. This media can support many different types of microorganisms (Difco[™], 2003).

Brain heart infusion agar is also a general purpose media that has the ability to support the growth of bacteria, yeasts, and molds. The infused portions, brain and heart, and peptone provide a good source of organic nitrogen, carbon, sulfur, and vitamins. Microorganisms begin to breakdown the dextrose in order to carryout the fermentation process, while the pH is controlled by the disodium phosphate (Difco[™], 2003).

Selenite cystine is a selective enrichment that is used to separate *Salmonella* from other organisms. This media is used to promote the growth of *Salmonella* when sanitary conditions are not the best (Difco[™], 2003). The advancement of *Salmonella* is due to the selenite in the media which inhibits fecal streptococci and coliforms for the first 8-12 hours of incubation (Leifson, 1936). Cystine is added in order to further promote the growth of *Salmonella* (North and Bartram, 1953).

Tetrathionate broth is also another type of selective enrichment media. This media supports the growth of *Salmonella* that are present in small numbers that are in constant competition with the intestinal microflora of the host organism. With the addition of iodine-iodide solution, it allows for *Salmonella* to be isolated from conditions that are not deemed sanitary (Difco[™], 2003).

Rappaport-Vassiliadis broth is a selectively enriching media used for *Salmonella* that are present in sewage-polluted water, feces, dairy products, and meat. This media is used in conjunction with a pre-enrichment media in order to isolate *Salmonella* colonies. With the addition of malachite green, other intestinal microflora will be inhibited of growth. Also a slight disadvantage is that if the osmotic pressure, a low pH, or both occur in conjunction with malachite green, it cause *S. Typhimurium* and *S. Choleraesuis* to be inhibited as well (Difco[™], 2003).

Universal pre-enrichment broth helps aid in the recovery of sublethally injured cells from *Salmonella* and *Listeria*. The media does not contain any type of antibiotic that could potentially inhibit the growth of sublethal cells. Peptone in the media provides

a source of carbon and nitrogen. Sodium and potassium provide a great buffering capacity in order to keep the pH stable so as not to inhibit any sublethally injured cells that could be vulnerable to low pHs. The metabolism of the organisms is increased by the addition of sodium pyruvate, while dextrose provides an energy source (Difco[™], 2003).

Differential media allows the differentiation of organisms grown together on the same media to improve identification of the target species. Xylose lysine desoxycholate agar is a differentiation media, that works through a set of different ingredients that the target organism needs to survive and helps in the identification process. The ability of this media to differentiate *Salmonella* is due to the presence of sodium thiosulfate and ferric ammonium citrate. When broken down, this causes the colonies to produce hydrogen sulfide therefore turning the colonies black. Also this media has a pH indicator phenol red which turns yellow under acidic condition. As the bacteria begin to break down the lysine, it begins to produce an acid therefore causing the phenol red to turn yellow indicating that acid is produced (Difco[™], 2003).

Xylose lactose tergitol is a differentiating media used to differentiate between enteric pathogens (Difco[™], 2003). The peptone is the source of nitrogen within the media. Vitamins enter the media through the addition of yeast extract. The ingredient ferric ammonium citrate gives the media the ability to differentiate by turning the colonies black upon breakdown of this ingredient. The pH indicator in this media is

phenol red with a color change from red to yellow and yellow meaning acidic conditions (Difco™, 2003).

Hektoen enteric agar is a differentiation and selective agar. The media contains bile salts that inhibit gram positive bacteria. Like the XLD media described above, HE also has sodium thiosulfate and ferric ammonium citrate. When broken down, it causes the formation of hydrogen sulfide therefore turning the colonies black. A pH indicator, a combination of acid fuchsin and bromthymol blue, is also incorporated into the media with a change in color going from light blue to yellow. The yellow color indicating the production of an acid (Difco™, 2003).

Bismuth sulfite agar is a selective media that inhibits gram-positive bacteria and coliform organisms (Difco™, 2003). This media being extremely selective, is primarily used in testing bacteria present in fecal and urinary matter (Cope and Kasper, 1937). The ingredients within the medium that provide nutrients for the growth of the bacteria are beef extract and peptone, which supplies the nitrogen and vitamins, while the sugar dextrose provides a rich energy source for the target organism to grow. The inhibiting agents that creates an environment not suitable for gram-positive and coliforms are bismuth sulfite indicator and brilliant green. There is also a differentiating ingredient, ferrous sulfate, that determines if the organism is producing hydrogen sulfide (Difco™, 2003).

Triple sugar iron agar is a differentiating agar made up of three sugars dextrose, lactose, and sucrose. The third sugar sucrose (Hajna, 1945) is added to help improve the

detection of sucrose-fermenting bacteria in addition to lactose and dextrose fermenters. This media also contains a pH indicator phenol red which turns yellow in the presence of an acid and under basic conditions will remain red. The ingredient ferrous ammonium sulfate is an indicator for hydrogen sulfide production and turns the colonies black upon breaking down iron. If the media contains a red layer around the top of the media and a yellow layer at the bottom of the media then it is a dextrose fermenter. If the color of the media changes to completely yellow, it is fermenting all three sugars. With the breakdown of the ferrous ammonium sulfate, the color of the medium turns black indicating the production of hydrogen sulfide (Difco[™], 2003).

Malonate Broth is a differentiating media used in the food and dairy industry (Difco[™], 2003). Ammonium sulfate alone, within this media, provides the only source of nitrogen for the target bacteria to use. Also there is only one source of carbon and it is contributed by the ingredient sodium malonate. This media is unique because it allows for the growth of the *Enterobacter* group while inhibiting the growth of the *Escherichia* group. This differentiating phenomenon is caused by the sodium malonate which does not support the growth of the *Escherichia* group. A pH indicator, bromothymol blue, is also incorporated into the media to show the color change from green to blue as the media becomes more basic (Difco[™], 2003).

Lysine iron agar is a differentiation media that was produced for the detection of *Salmonella* because of the production of lysine decarboxylase which forms large amounts of hydrogen sulfide (Edwards and Fife, 1961). The sugar, dextrose, is the

carbohydrate source that the target organism ferments in order to maintain life processes. The addition of the bromocresol purple indicates a pH change in the media. A change at a pH of 5.2 creates a yellow color indicating an acidic pH. When the media changes at 6.8 or above, it creates a purple color indicating a basic pH. Ferric ammonium citrate and sodium thiosulfate are both added to the media to indicate when hydrogen sulfide is produced therefore causing the colonies to turn black (Difco[™], 2003).

MacConkey agar has a selective and differential property which helps to expose the presence of coliforms and enteric pathogenic organisms. With a low bile salt concentration of about 2.9%, the media is only somewhat selective against gram-positive bacteria. The ingredient crystal violet is also added to the media to aid in the inhibition of gram-positive bacteria. The lactose fermentation shows a color change because of the addition of the neutral red indicator. Since *Salmonella* does not break down lactose, the indication of the presence of the bacteria is identified by the colorless colonies present on the plate (Difco[™], 2003).

Traditional methods for detection can take several days to give results about the target bacteria. This process can take 4 days just to get the presumptive test results. These four days consist of pre-enrichment step, selective enrichment, and differential/selective agars for a presumptive identification (Feng, 2007). A further 3 days will be used in order to confirm the identity of the target isolates (Mercanoglu and Griffiths, 2005). These three days consist of biochemical testing and serotyping. With a

total of at least 7 days to give a positive identification, this could adversely affect the processor whose produce is being held for safety concerns.

Novel Technology Used to Detect *Salmonella*

Recently in the past several years, there has been great improvement in the detection of *Salmonella*. Novel technologies that amplifies the nucleic acid of cells have shown great improvement in sensitivity and specificity of targeting a particular type of bacterial DNA. With this improved technology, faster detection and containment of outbreaks can greatly reduce the chance of great economic loss and protect public health. Some of the improved technology consist of polymerase chain reaction (PCR), PCR-enzyme-linked immunosorbent assay (PCR-ELISA), and real-time PCR and reverse transcriptase PCR (rt-RT-PCR) (Perelle and others 2004). Each one of the improved methods has greatly reduced the time for positive detection of the target bacteria by skipping the pre-enrichment, non-selective, and selective steps in the traditional method of detection. While these improved technologies have made detection faster, they also have draw backs, because of the inhibitors within the food matrix and low detection sensitivity.

PCR technology has made great strides with advances in the speed of recovery of the target bacteria for determining its presence in the food system. This system works in an ingenious way that allows manipulation of the replication of deoxyribonucleic acid (DNA). A short piece of DNA or primer, which is a sequence of DNA about 16-30 bp long complementary to target DNA, binds to the target DNA sequence and the enzyme Taq

polymerase used in a thermocycler replicates the DNA or increases the number of DNA copies (Feng, 2001). This process acts just like a photocopy machine. Taq polymerase, the enzyme found in *Thermus aquaticus*, was first found and isolated from the hot springs or thermal reservoirs in Yellow Stone National Park. The finding of this bacterium, created much advancement in the PCR process because of the stability of the enzyme at high temperatures. This stability was a remarkable improvement because the enzyme was not destroyed by the high temperature when the thermocycler underwent the denaturation process of DNA at 95°C (Fairchild and others 2006). This enzyme eased the process of having to add more enzymes after each cycle of annealing and extension.

The PCR procedure consists of a three step process carried out in a thermocycler. These include denaturing of the DNA, annealing of the primers, and extending or copying of the DNA (Fairchild and others 2006). The denaturing of the DNA is carried out at a temperature around 94°C for about 15 seconds. Annealing of the primers is the next step carried out at a temperature that is specific to the target primer sequence for about 15-30 seconds (Stone and others 1994). The annealing temperature is dependent on the G/C ration which should be about 45 to 55% of the primer. The last step is the extension of DNA process which is heated to about 72°C (Stone and others 1994). These three steps conclude one complete cycle. In order to achieve several thousand copies of the target DNA, this process is done over and over about 30 or 40 cycle times. It is clear that the advent of the thermocycler and thermostable Taq polymerase made this

process go much faster because a scientist did not have to keep manually adding enzyme to the process and moving the samples from one water bath to another (Fairchild and others 2006). This entire process takes only about 2-3 hours.

This three step process using a thermocycler, only replicates the DNA but does not provide a method of detection in order to determine if the target product is present. Gel electrophoresis is a method of detection used. The DNA fragments that were amplified during the thermocycler step are loaded onto an agarose gel. The agarose gel has a dye, ethidium bromide, added to it that binds to DNA and fluoresces under UV light. Ethidium bromide is an excellent detection dye because it binds to all double stranded DNA. For detection of an amplified product, one is required to have some previous knowledge of where the forward and reverse primers are going to bind on the template strand and how many base pairs the primers are apart. So if we break this down as an example, with the forward primer binding at the 200 base pair on the template strand and the reverse primer binding to the 400 base pair on the template strand, the difference between the two primers is 200 base pairs. This would be the size of one's DNA fragment that will be illuminated by the ethidium bromide under ultraviolet light when run out on the agarose gel. A standard or control DNA marker is also used in order to determine the size of the target DNA that is present (Fairchild and others 2006). An electrical current is used in order to move the DNA fragments from the negative to the positive end through the agarose gel therefore causing the sizes to separate based upon the larger sizes closer to the top and the smaller fragment towards the bottom (Fairchild

and others 2006). Ultraviolet light is used to visualize the DNA. The issues with agarose gel electrophoresis include cross-contamination due to opening of reaction tubes and increased detection time and cost.

Primer design and accurate optimization is a must for the PCR amplification process to be effective. The PCR process can also be designed to carry out a multiplex process meaning it can amplify two or more target sequences in the same reaction. Multiplexing has its advantages because it has the ability to save time and labor. However, primer design needs careful attention as the target products should vary in size and be easily distinguished by gel electrophoresis or other novel detection systems. Also when multiplexing, each bacterial DNA is competing for the use of reagents within the mix therefore decreasing efficiency. This technology is not without its drawbacks. One of the drawbacks is each set of primers need to be close to the same annealing temperatures. Also if the target sequences are close in size, separating them on an agarose gel will be difficult because the target products are close that they cannot be distinguished between the two sequences (Fairchild and others 2006). Although this technology can amplify a single gene a million-fold in 2 hours, it has lower detection limits than its monoplex PCR counterpart. (Feng, 2001).

In the reverse transcriptase PCR, an additional initial step where mRNA is transformed into cDNA by the reverse transcriptase enzyme is used followed by regular PCR steps. The advantage of using mRNA from the target bacteria is that mRNA has a shorter half life than DNA and therefore has greater potential of detecting primarily

viable cells that could cause infection (Maurer, 2006). After this first reverse transcriptase step has taken place, it then undergoes the PCR steps that have been previously described where the denaturation, annealing, and extension of the DNA takes place. Real time PCR is used to produce “real time” results without the need for the days of labor intensive culture work and eliminate agarose gel electrophoresis. This method uses a fluorescence measurement in order to determine the amount of DNA present. In order for the fluorescence to be detected, a camera measures the amount of light given off by the double stranded DNA (dsDNA) at the end of the extension step bound to a fluorescence dye (Fairchild and others 2006). Real-time chemistries include intercalating dyes such as SYBR Green I, hydrolyzing (TaqMan) probes and hybridizing probes (Molecular Beacons). Intercalating dyes like SYBR Green I binds to all DNA and so a melt temperature (T_m) curve specific for the target DNA is needed to be run at the end of the PCR process. The T_m aids in further confirmation that the target product is really present. Binding to all double stranded DNA, results in low specificity. Target products will have specific T_m 's that will confirm the specific product and not just primers. When using fluorescent dyes such as SYBR green I, primers need to be specific for the target bacteria and hence primer design is a crucial step (Feng, 2007) and also should not form primer dimers. Primers should be approximately 20 bases long.

SYBR Green I dye is the easiest of the real-time PCR methods and is the cheapest. This dye binds to all double stranded DNA which allows for it to be universal in detecting different types of bacterial DNA or any other type of dsDNA (Feng, 2007). A

disadvantage to SYBR Green I is that it will bind to all double stranded DNA even if it is not the target sequence (Maurer, 2006). Bhagwat (2004) produced a study on mixed-salad, which contained 80% lettuce, 10% cabbage, and 10% carrots. They found that since SYBR Green I bound to all double stranded DNA a melt temperature was need to determine the presence of the target after the PCR process has taken place (Bhagwat, 2004). The T_m refers to the point when half the DNA is double stranded and half is single stranded (De Medici and others 2003). The T_m is dependent on the GC content, length of the sequence, and the sequence itself (Ririe and others 1997). The T_m of the product is specific to that product therefore gel electrophoresis is no longer needed (Feng, 2007).

Molecular beacons are hybridizing probes consisting of short single-stranded DNA that binds to the complementary target DNA strand (Fairchild and others 2006). These short single-stranded DNA or probes are made up of a specific hairpin loop oligonucleotide that bind to the target with a reporter at the 3' end, a quencher at the 5' end, and a stem that is complementary to each other and not the target DNA. When this loop binds to the DNA, it is stretched out, this causes the reporter and quencher to move farther apart so the fluorescence can now be given off and detected (Feng, 2007). An advantage to molecular beacons is its hybridization capabilities to be very specific and sensitive to the target DNA and RNA in which the probe is targeting amongst thousands of other sequences (Snyder and Champness, 2007a). Disadvantages include tricky and challenging design of primer and increased cost. This method has been used

by Liming and Bhagwat (2004) against food products such as alfalfa sprouts, cantaloupe, mixed salad, and cilantro for the detection of *Salmonella* with a detection limit of 1-4 CFU (Liming and Bhagwat, 2004).

The fluorescence resonance energy transfer (FRET) is another hybridizing probe system that has two probes. There is a probe that binds to the 3' end that has the donor dye and a probe at the 5' end that has the acceptor dye. When these two probes bind to the target DNA and are close enough by a distance of only one base pair, the donor dye excites the acceptor dye and causes it to fluoresce (Fairchild and others 2006). FRET has been used in nonfat dry milk in order to detect *Listeria monocitogenes* and was able to detect 10^3 CFU (Koo and Jaykus, 2003).

TaqMan based PCR is a hydrolyzing probe that takes advantage of the *Taq* DNA polymerase activity during the extension step (Chen and others 1997). During the extension step in the PCR process, *Taq* DNA polymerase hydrolyzes the probe. As the 3'-reporter is cleaved from the rest of the probe it begins to fluoresce. This works because the 3'-reporter is no longer next to the 5'-quencher. When the reporter and quencher are close to each other, the quencher will absorb the excited state of the reporter. As amplification increases, the amount of reporter separated increases and so therefore the amount of fluorescence is proportional to the amplified DNA (Nogva and Lillehaug, 1999).

Some disadvantages to this new technology include incorporation of these new concepts in a food matrix. There are many things that can go wrong causing the PCR

procedure to be inhibited. Also while dealing with a food matrix, inhibitors such as lipids, proteins, enzymes, chemical additives, fiber, other bacteria, and pH changes which might cause false negatives (Lee and Fairchild, 2006) that prevent enzymes and reagents from amplifying the target product. These are just some of the disadvantages that scientists face when working with new technology in a food matrix.

Almost all of those serovars contain a gene known as the invasive A gene (*invA* gene). The *invA* gene is presently located on the chromosome and is specific to *Salmonella* (Cheng and others 2008). Invasive A gene is needed for *Salmonella* to target the epithelial cells (Galan and Curtiss, 1989). The invasive A gene is located on the chromosome and is part of a 40-kb pair region known as *Salmonella* pathogenicity island (SPIs) (Amavisit and others 2003). Of the 2,000 different serovars, Typhimurium, Choleraesuis, Dublin, Enteritidis, and Gallinarum-Pullorum, all contain a virulence plasmid that have the *spvC* gene (Chiu and Ou, 1996). Targeting the plasmid for detection would not be wise because each plasmid may code for a different trait and can be distributed among the population unevenly (Snyder and Champness, 2007b) and plasmid loss (curing) can occur over time, due to chemical treatment, environment challenges and during conditions of stress. Hence detecting an unstable population of genes borne on plasmids is not optimal. Ideally targeted genes for detection by PCR and molecular detection methods should be chromosomally present, be conserved and constitutively expressed.

Over the years, there have been several types of antibody-based assays that have been developed. Some of the assays are latex agglutination, enzyme-linked immunosorbent assay (ELISA), with methods such as immunomagnetic separation to help concentrate the product used for downstream detection. These assays continue to be used because of their simplicity and specificity in the detection of food borne pathogens (Feng, 2007). When using the latex agglutination method, it takes antibody-coated colored latex beads to bind to the target bacteria. If the target bacterium is present, then the antibody will bind to the antigen to form a precipitate. This precipitate will be known because of the visible clumping that will be produced. Since this type of assay is not as sensitive as some others, an enrichment process may need to be done when using this test for food analysis. This is because the assay requires about 10^7 CFU or more for the reaction to give good results (Feng, 2001).

The enzyme-linked immunosorbent assay is currently known as the sandwich assay. This is because it uses an antibody-coated matrix to grab the bacteria from the food matrix while a second antibody conjugate binds to the target bacteria causing an antibody-antigen-antibody conjugate to form (Feng, 2001). Once this sandwich has formed, a substrate is added to the product in order for it to produce a color for detection visually to the naked eye or by a spectrophotometer (Feng, 2007). ELISA is much more sensitive to detecting the target bacteria than latex agglutination because ELISA only needs between 10^4 and 10^5 CFU/ml in order to detect the target bacteria

(Feng, 2001). With a lower detection limit, ELISA is a much more sensitive test compared to latex agglutination.

Immunomagnetic separation (IMS) is another assay that uses the antibody principle to concentrate target bacteria. Antibodies attach to magnetic beads and attach itself to the target bacteria. A magnetic tool is put into solution attracting the beads to the tool and is separated with concentrated target bacteria (Feng, 2007). However, some drawbacks include reduced efficiency as for example, when IMS was added to ground beef in order to recover 'Escherichia Coli 0157:H7' it did not bind even 50% of the target cells (Feng, 2007).

Factors Affecting the Polymerase Chain Reaction (PCR) Within the Food

Matrix

The food matrix is a harsh working environment for the PCR process. This process requires a clean environment almost completely free of any inhibiting substrates. Many of the inhibiting substrates are naturally present within the food itself. Some of these substances in food that inhibit / prevent the synthesis of DNA are: lipids, proteins, enzymes, chemical additives, fiber, other bacteria, and pH changes (Lee and Fairchild, 2006). According to Lee and Fairchild, a general way to look at inhibitors is as substrates that bind to or degrade part of the reaction reagents that will prevent the DNA from being replicated. Since many of these inhibiting substances are also present in the enrichment broths and other media used during the process, one needs to take careful attention to be sure and wash the cells and reduce inhibitory food matrix

substances before moving onto the PCR process (Lee and Fairchild, 2006). Also good aseptic techniques need to be practiced in order to prevent cross-contamination, so results of false positives and false negatives are not obtained. This can easily be overcome by keeping the work station clean using UV irradiation, spray with sodium hypochlorite and ethanol, and / or enzymatic methods (Wilson, 1997).

The challenges that one faces with the inhibitors can be reduced usually by taking some simple measures. One measure might be trying to understand how the organism's DNA and RNA is extracted from the cell (Lee and Fairchild, 2006). When following the protocol like Qiagen, the steps for extraction along with the simple wash step can help give a much purer sample of RNA or DNA. A common commercial extraction kit such as Qiagen RNeasy kit, aims at purifying RNA to help reduce the amount of inhibitors present by breaking them down. According to the RNeasy Mini Handbook, the first wash step is to add RLT, a proprietary buffer, which is the RNeasy Lysis Buffer (Qiagen, 2001) containing guanidine thiocyanate. This compound is responsible for protecting RNA from degradation by RNases and aiding in cell lysis and removes PCR inhibitors such as proteins by denaturing. The protein becomes denatured because guanidine thiocyanate causes it to randomly coil in another shape (Sambrook and Russell, 2001). Ethanol is the next wash step in the RNeasy Mini Handbook (Qiagen, 2001). This organic solvent aids in the removal of proteins and lipids while causing the DNA to precipitate out (Sambrook and Russell, 2001). In order to make sure the procedure works properly, the entire chromosomal DNA needs to be removed from the

solution. This is done only when RNA is the target because some applications are sensitive to small amount of DNA. For this part of the procedure RW1 is added to solution. RW1 breaks down DNA therefore providing a much purer RNA sample (Qiagen, 2001). Also DNases that selectively degrade DNA and not RNA can be added. The last buffer to be added is RPE. With the RPE wash, it allows for further purification of the RNA (Qiagen, 2001). With these simple wash steps supplied by Qiagen, extraction of RNA is easy and yields a much purer RNA sample. This procedure thus helps to alleviate some of the false positive and negatives that might arise during the PCR process due to protein and lipid contamination.

Another RNA extraction method available commercially is the TRIzol™ reagent. It produces a lower quality of mRNA because of the organic carry over when compared to the Qiagen method. Much of the TRIzol™ method requires the addition of organic solvents such as chloroform and ethanol. Many times the solvents do not evaporate off in the allotted time given during the procedure. One part of the procedure requires the addition of chloroform to the product. After it has incubated at room temperature with the chloroform, the upper aqueous phase needs to be removed and added to isopropanol. Some of the bottom precipitate could come with the supernatant and continue through the rest of the process. Also during the ethanol removal, it requires a 5 to 10 minute air dry period. This is sometimes not long enough for all the ethanol to be removed. Many of the reagents used during the TRIzol™ method if carried out can later hinder the PCR process. Hence while procedures are needed to improve yields and

quality of nucleic acid, caution must be used to prevent addition of external inhibitory substance during the DNA/RNA extraction process.

Another type of problem that PCR runs into is false positives and negatives from the reagents themselves. Most of these problems are simple and can be avoided using good lab practices like cleaning as a prevent measure. The reagent, such as the enzyme needed for the PCR procedure, should be kept at the appropriate storage temperature (frozen) when it is not in use and kept on ice while it is waiting to be used during a reaction. False negative will thus be avoided due to reaction failure. Another way to prevent the cross-contamination of the reagent stock solution is to use different barrier tips each time one goes to the stock reagent, use disposable gloves while changing them out in between mixing of the reagents and the PCR process, and cleaning of the lab work bench and area with bleach or overnight ultraviolet illumination before and after the use of the equipment (Maurer, 2006). Other things that can cause cross-contamination and inhibit the PCR process include the equipment / laboratory items and the air itself within the lab. Many things like pollen moving into the room as scientist come and go, powder from the gloves, plasticware that has not been cleaned and sterilized properly, and cellulose (Wilson, 1997). One suggestion for this problem is to have different lab areas or two different labs altogether in order to prevent cross-contamination between the RNA / DNA sample preparation and PCR preparation step (Maurer, 2006). When opening the microcentrifuge tubes the DNA can become aerosolized therefore causing our hands, pipettes, and lab bench area to be quickly contaminated with DNA. So also

separating the area where loading of the agarose gel takes place from the RNA/DNA preparation would be a good practice as well, in order to prevent contamination of the stock reagents (Maurer, 2006). As technology further advances with PCR, it begins to make the process much faster, by real-time methods, and reducing false positive and negative results in a one-step enclosed tube format.

The new technology begins to put many of the media plating steps that could pose potential contamination points at rest. These points of contamination can no longer cause problems because they have become all-inclusive meaning that these steps have been combined and therefore do not cause cross-contamination with other products or reagents within the lab. Some of this new technology is the advancement of PCR. Some of the features of real-time PCR are quite impressive while making the process much faster and preventing contamination of other product. Amplified product tubes need not be opened and gels do not need to be run. RT-PCR allows for what is called a T_m or melt temperature to be taken in order to determine if the target product is actually there. With this type of process, the DNA does not become aerosolized during the loading of the agarose gel meaning that one point of contamination is now controlled for. Not only have there been technological advances with equipment but there have been advances in the reagents as well.

As scientists learn more and more each day about the different chemical processes that it takes to sustain life, they begin to learn how to manipulate them and are able to begin to reproduce these actions within a lab setting. The need for more

rapid detection tests have grown over the past several years, therefore creating a call for a process that could validate and determine if the novel technology is working properly. These validation steps somewhat come out of necessity because false negative and positive results are always a possibility that could take place. A step that is now needed for any PCR reaction/assay is an Internal Amplification Control (IAC). This control procedure takes short pieces of RNA or DNA complementary to target primers and adds them to the PCR mix that are amplified by the same target primers in the mix but yield a different size product. This provides assurance that the PCR mix along with the reagents within the mix are working properly. If the IAC is amplified, it means that there is no false negative and all reagents and equipment are working properly. This method allows one to carry out further dilutions of the product in order to get a concentration that is consistent with each use and serial-dilution end-point PCR or real-time quantitative PCR can be obtained. This procedure is a simple way to provide a validation step that can be used to prove that no false negatives have taken place. Along with correcting for false negatives, procedures for sensitivity and specificity are also being carried out to further increase detection limits.

Actions to Increase the Sensitivity and Specificity of PCR and the Recovery of Bacteria from the Food Matrix

Sensitivity and specificity are both key aspects of PCR and the recovery of bacteria. They provide the users with an idea of how well the product or method will perform. First, when trying to understand sensitivity and specificity one needs to look at

the definitions. Sensitivity is the ability to detect very low numbers of the target product which results in a positive test and is truly positive (Peplow and others 1999). Specificity is the ability to receive a negative/positive result when in fact the target sample is negative/positive without cross-reacting with non-target organisms (Peplow and others 1999). With these two definitions a foundation of understanding the detection limits of PCR and its reaction reagents is attained.

The PCR mix is an important part of the whole PCR procedure. The reagents that make up the mix help to create an environment that will promote the enumeration of the bacterial DNA. With the use of a PCR kit provided by the manufacturer, it allows for one to do a PCR run at a faster rate. PCR kits can be supplied by several different companies such as Invitrogen™, Qiagen, etc. Invitrogen™ provides a PCR kit called SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit. This PCR kit has some of the necessary reagents, except the primer sets, needed to run the PCR process. The primer sets are unique to ones target bacterium. The first reagent / component is SuperScript™ III RT/Platinum® *Taq* Mix. This reagent has all the dNTPs needed to run the reaction along with RNaseOUT™, which is a ribonuclease inhibitor (Invitrogen, 2005). The ribonuclease inhibitor helps to prevent RNA breakdown by enzymes that may be present within the reaction therefore making the PCR process much more sensitive and specific to the target RNA. Another reagent within the PCR mix is 2X SYBR® Green Reaction Mix (Invitrogen, 2005). SYBR® Green is a nonspecific DNA-binding dye that binds to all double stranded DNA (Lee and Fairchild, 2006). This dye does not increase

the sensitivity or specificity of the PCR mix, but is a key player in the ability of RT-PCR to provide the data based on fluorescence. 50-mM Magnesium Sulfate is another reagent that is provided in the kit (Invitrogen, 2005). Magnesium Sulfate is added to the mixture because it is a cofactor that aids *Taq* polymerase in the replication of the nucleic acid. Too much or too little magnesium sulfate will cause the specificity and sensitivity of the reaction to decrease. Many compounds such as calcium ions can interfere with the magnesium sulfate therefore inhibiting its ability to work properly with the *Taq* polymerase (Wilson, 1997). The reagent, 20X Bovine Serum Albumin, is also part of the invitrogen™ kit. Bovine Serum Albumin (BSA) helps to make the PCR mix more specific and sensitive by blocking nonspecific binding (Sambrook and Russell, 2001). With this type of commercial kit available, “it makes the world of PCR easy for routine use in one simple step.” Target primers must be specific to the target bacteria and don’t react with non-target organisms. The genes that are chromosomally located and continually expressed are preferred. Primer annealing temperature should be optimized and PCR buffer and reaction condition optimized against cross reactivity.

Primer design is important for sensitivity and specificity to the target product. The starting place for most researchers is PUBMED and the DNA database search called GenBank (Fairchild and others 2006). When using GenBank database search, a BLAST search is done to see if the target sequence of the gene matches any other gene sequences presently known. Once determined that no closely related strains or serovars exist, a proper primer size of ~18 bp is preferred and should coincide with the gene

search done previously (Fairchild and others 2006). Creating a small primer sequences runs a risk of finding other sequences within the genome. The G-C content should be approximately 50% to help control the melt temperature. Created primers should not form hairpin loops, primer dimers (Fairchild and others 2006), and avoid long runs of the same nucleotide. The forward and reverse primers for *invA* gene used in the present study, (**F: 5'CACGCTCTTTCGTCTGGCA3'**; **R2: 5'TACGGTTCCTTTGACGGTGCGA3'**), underwent a cross-reactivity study. This cross-reactivity study consisted of 38 different foodborne pathogens. Of the foodborne pathogens tested, only *Salmonella* species presented a positive result <30 Ct value. The negative control, water, had a Ct value of 30 therefore the foodborne pathogens tested had a Ct value ≥ 30 (D'Souza and others 2007).

In actuality this process is quite complicated. *Taq* polymerase is an enzyme that joins deoxynucleotides together to form long chains of DNA (Snyder and Champness, 2007a). Once the DNA sequence has been denatured or unwound, a primer binds to the template strand and is a signal / starting point for DNA polymerase (Talaro and Talaro, 1999a). Now that the primer has bound to the template strand, DNA polymerase will attach the appropriate nucleotide to match the template strand. The enzyme moves along the template strand in a 3'-to-5' direction while creating a new strand in a 5'-to-3' direction (Snyder and Champness, 2007a). All reactions are carried out in automated PCR machines such as BioRad.

Other than PCR and real-time PCR, novel nucleic acid amplification methods are also being pursued for food application. These include Nucleic Acid Sequence-Based Amplification (NASBA) and Loop-mediated isothermal amplification (LAMP). NASBA is an amplification technique that amplifies only RNA and not DNA under isothermal conditions. This process is carried out in a single tube under 41°C conditions in a water bath. This process only needs one temperature and does not need expensive PCR machines (Gracias and McKillip, 2007). This method was first described by Guatelli and others (1990) showing that the sample started with RNA which then formed cDNA. The cDNA, RNA / DNA hybrid, then underwent RNase H treatment which removed the RNA and the binding of the primer to the single stranded DNA would finish out the DNA strand. This new DNA strand would have a T₇ promoter region that would allow for reverse transcription to take place for continues replication of the strand (Guatelli and others 1990). NASBA has been applied to several different foodborne pathogens including *Salmonella* Enteritidis in poultry products with a detection limit <10 cfu/10g (Cook and others 2002) and in peppers with a detection limit of 10¹ cfu (D'Souza and Jaykus, 2003), *Campylobacter jejuni* in chicken skin with a detection limit of 10⁵ cfu/10g (Uyttendaele and others 1997), *Salmonella* Enteritidis and Typhimurium from a pure overnight culture (Simpkins and others 2000), and *Listeria monocytogenes* in Gouda cheese and Ice cream with a detection limit of 0.5 cfu/g and 0.2 cfu/g (Blais and others 1997).

The LAMP assay is also an isothermal assay that does not require a denatured DNA template and can be combined with reverse transcription to amplify RNA sequences (Nagamine and others 2002). This technique is designed to use 4-6 different primers which have the ability to identify different regions on the target gene. A constant temperature between 60-65°C is maintained for the procedure which is then completed in 1 hour (Okamura and others 2008). In order to get the LAMP assay started two inner primers bind to the target DNA to initiate complementary strand synthesis. Then the outer primers bind to initiate strand displacement on the ends of the DNA strand to allow for a loop structure to form. This loop then serves as a template for the backwards inner primer to initiate DNA synthesis. The loop quickly forms a stem-loop which serves as the starting material for the second stage in the LAMP assay (Okamura and others 2008). This cycle continues with each new stem-loop that forms. Chicken meat samples is an example of where this procedure has been applied (Yamazaki and others 2009). Also LAMP has been applied to several different foodborne pathogens including *Salmonella enterica* in chickens with a detection limit of 10^1 cfu/g (Okamura and others 2008) and in a pure overnight culture (Wang and others 2008), *Campylobacter jejuni and coli* from chicken meat (Yamazaki and others 2009), and *Clostridium botulinum* type B in fish with a detection limit of 10 cfu in vegetative cells (Sakuma and others 2009).

While much attention has focused on detection, research needs to be focused on bacterial recovery from the food as well to improve detection sensitivity. This

process can be confusing because representative samples from a large quantity of product are needed in order to increase chances of detection of the target bacterium. According to the Compendium of Methods for the Microbiological Examination of Foods, preenrichment is recommended to recover ones targets cells from the food matrix (Flowers and others 1992). This pre-enrichment step is needed in order to bring pathogenic bacteria counts up to a detectable level. Since many food products also have nonpathogenic species that can outcompete pathogens, a series of selective enrichment media are used in order to confirm pathogen presence in the food product. A series of step-wise media would start with nonselective media, moving to selective enrichment media, selective isolation media, and followed by a biochemical test in order to confirm the strain of bacteria (Flowers and others 1992). The relative sample size can depend on the amount of product that a company is producing. This means that they may want to sample every lot that has been produced that day. There are three different categories of sampling described by the Compendium of Methods for Microbiological Examination of Foods (Flowers and others 1992). Category I takes 60 samples of 500g of food to be sampled. Category II takes 29 samples of 250g of food to be sampled. Category III takes 13 samples of 125g of food to be sampled. A study that was done by Burnett and others (2001), showed that the sample size used for lettuce was 50g with 50 ml of peptone water, to rinse the cells off the lettuce, while shaking in a stomach bag. Also for the same study Burnett and others (2001), 20g sample of lettuce in buffered phosphate buffer was blended for recovery of *Salmonella* with a detection limit of 10^7 CFU (Burnett

and Beuchat, 2001). In another study done by Himathongkham and others (2007), a one hundred and fifty gram sample of lettuce was used and placed in a stomach bag to be analyzed for the detection of *Escherichia coli* O157:H7 which produced a detection limit of 0.07 CFU/g mixed (Himathongkham and others 2007). As one can imagine, sampling of products can be quite large at times when dealing with products of high volume being produced each day. Also multiple samples need to be processed. Recent research is focused on sampling methods and sampling schemes to ensure absence of contamination in the food processing environment.

In Table 1 (All tables are in the Appendix), it shows many of the outbreaks that have occurred in the last few years. Much of the problem has been trying to recover and detect the bacteria. Detection limits are dependent on the amount of target bacteria that has been recovered. Molecular detection techniques like PCR or RT-PCR have been used and applied to different foods Table 2. When the target pathogenic bacteria are not detected and eliminated, it can create an outbreak of food poisoning. This in turn can injure lives and cost the food industry millions of dollars. Further research on developing better rapid and sensitive detection methods, can help keep the consumer safe. Tracking of outbreaks to the contamination source is very complicated. Gold standard methods such as Pulse Field Gel Electrophoresis (PFGE) are used for this process by the CDC and state and public health labs.

Recent outbreaks of *Salmonella* linked to fresh produce emphasize the need for rapid detection methods to help control outbreaks. Reverse-transcriptase-PCR (rt-PCR)

detects the presence of mRNA (shorter half-life than DNA), with greater potential of detecting viable pathogens. Use of real-time PCR eliminates the need for gel electrophoresis which enhances the speed of detection. To improve the speed of detection and assay sensitivity, the objectives of this research were to apply real-time rt-PCR to detect *Salmonella* from spiked lettuce, tomatoes, and peppers with low and high inoculum levels. Recovery of bacteria was also analyzed using various buffers to remove/elute bacteria from the food matrix. New rapid detection methods will allow the food industry to initiate timely recalls, protect public health, prevent outbreaks, and save the industry millions of dollars by limiting the number of recalls by preventing release of contaminated product.

References

- Amavisit P, Lightfoot D, Browning GF & Markham PF. 2003. Variation between pathogenic serovars within *Salmonella* pathogenicity islands. *J. Bacteriol.* 185(12):3624-3635.
- Andrews WH & Hammack T. 2007. *Salmonella*. Bacteriological Analytical Manual *Online*, 8th Edition, Revision A, 1998. Chapter 5.
- Bhagwat AA. 2003. Simultaneous detection of *Escherichia coli* O157 : H7, *Listeria monocytogenes* and *Salmonella* strains by real-time PCR. *International Journal of Food Microbiology* 84(2):217-224.
- Bhagwat AA. 2004. Rapid detection of *Salmonella* from vegetable rinse-water using real-time PCR. *Food Microbiology* 21(1):73-78.
- Blais BW, Turner G, Sooknanan R & Malek LT. 1997. A nucleic acid sequence-based amplification system for detection of *Listeria monocytogenes* hlyA sequences. *Applied and Environmental Microbiology* 63(1):310-313.
- Bowen A, Fry A, Richards G & Beuchat L. 2006. Infections associated with cantaloupe consumption: a public health concern. *Epidemiology and Infection* 134(4):675-685.
- Burnett AB & Beuchat LR. 2001. Comparison of sample preparation methods for recovering *Salmonella* from raw fruits, vegetables, and herbs. *Journal of Food Protection* 64(10):1459-1465.
- CDC. 2008a. Foodborne Illness. Centers for Disease Control and Prevention.
- CDC. 2008b. Salmonellosis. Centers for Disease Control and Prevention.
- CDC. 2009. *Salmonella* Outbreak Investigations. Centers for Disease Control and Prevention. Atlanta, GA.
- Chen S, Yee A, Griffiths M, Larkin C, Yamashiro CT, Behari R, PaszkoKolva C, Rahn K & DeGrandis SA. 1997. The evaluation of a fluorogenic polymerase chain reaction assay for the detection of *Salmonella* species in food commodities. *International Journal of Food Microbiology* 35(3):239-250.
- Cheng CM, Lin W, Van KT, Phan L, Tran NN & Farmer D. 2008. Rapid Detection of *Salmonella* in Foods Using Real-Time PCR. *Journal of Food Protection* 71(12):2436-2441.
- Chiang YC, Fan CM, Liao WW, Lin CK & Tsen HY. 2007. Real-time PCR detection of *Staphylococcus aureus* in milk and meat using new primers designed from the heat shock protein gene htrA sequence. *Journal of Food Protection* 70(12):2855-2859.
- Chiu CH & Ou JT. 1996. Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, invA and spvC, by an enrichment broth culture-multiplex PCR combination assay. *Journal of Clinical Microbiology* 34(10):2619-2622.
- Cook N, Ellison J, Kurdziel AS, Simpkins S & Hays JP. 2002. A nucleic acid sequence-based amplification method to detect *Salmonella enterica* serotype enteritidis strain PT4 in liquid whole egg. *Journal of Food Protection* 65(7):1177-1178.

- Cope EJ & Kasper JA. 1937. Cultural Methods for the Detection of Typhoid Carriers. *American Journal of Public Health* 28:1065 - 1068.
- D'Aoust J-Y, Maurer J & Bailey JS. 2001. *Salmonella* Species. In: Doyle, M. P., Beuchat, L. R. & Montville, T. J., editors. *Food Microbiology: Fundamentals and Frontiers*, 2nd Ed. Washington, D.C.: ASM Press. p. 141 - 178.
- D'Souza DH, Critzer FJ & Golden DA. 2007. Reverse-Transcriptase-PCR for the Rapid Detection of *Salmonella* Using *invA* Primers. *International Association for Food Protection* 2007. Lake Buena Vista, Florida: International Association for Food Protection p. 181.
- D'Souza DH & Jaykus LA. 2003. Nucleic acid sequence based amplification for the rapid and sensitive detection of *Salmonella enterica* from foods. *Journal of Applied Microbiology* 95(6):1343-1350.
- De Medici D, Croci L, Delibato E, Di Pasquale S, Filetici E & Toti L. 2003. Evaluation of DNA extraction methods for use in combination with SYBR Green I real-time PCR to detect *Salmonella enterica* serotype enteritidis in poultry. *Applied and Environmental Microbiology* 69(6):3456-3461.
- Difco™. 2003. Difco™ & DDL™ Manual. Sparks, Maryland: Becton, Dickinson and Company.
- Edwards PR & Fife MA. 1961. Lysine-Iron Agar in the Detection of Arizona Cultures. *Applied Microbiology* 9:478 - 480.
- Fairchild A, Lee MD & Maurer JJ. 2006. PCR Basics. In: Maurer, J., editor). *PCR Methods in Foods*. New York, NY: Springer Science+ Business Media, Inc. p. 1 - 25.
- Feng P. 2001. Development and Impact of Rapid Methods for Detection of Foodborne Pathogens. In: Doyle, M. P., Beuchat, L. R. & Montville, T. J., editors. *Food Microbiology: Fundamentals and Frontiers*, 2nd Ed. Washington D.C.: ASM Press. p. 775 - 796.
- Feng P. 2007. Rapid Methods for the Detection of Foodborne Pathogens: Current and Next-Generation Technologies. In: Doyle, M. P. & Beuchat, L. R., editors. *Food Microbiology: Fundamentals and Frontiers*, 3rd Ed. Washington, D.C.: ASM Press. p. 911 - 934.
- Flowers RS, D'Aoust J-Y, Andrews WH & Bailey JS. 1992. *Salmonella*. In: Vanderzant, C. & Splittstoesser, D. F., editors. *Compendium of methods for the Microbiological Examination of Foods*. Washington, D.C.: American Public health Association.
- Galan JE & Curtiss R. 1989. CLONING AND MOLECULAR CHARACTERIZATION OF GENES WHOSE PRODUCTS ALLOW *SALMONELLA-TYPHIMURIUM* TO PENETRATE TISSUE-CULTURE CELLS. *Proceedings of the National Academy of Sciences of the United States of America* 86(16):6383-6387.
- Gracias KS & McKillip JL. 2007. Nucleic acid sequence-based amplification (NASBA) in molecular bacteriology: A procedural guide. *Journal of Rapid Methods and Automation in Microbiology* 15(3):295-309.
- Greene SK, Daly ER, Talbot EA, Demma LJ, Holzbauer S, Patel NJ, Hill TA, Walderhaug MO, Hoekstra RM, Lynch MF & Painter JA. 2008. Recurrent multistate outbreak

- of Salmonella Newport associated with tomatoes from contaminated fields, 2005. *Epidemiology and Infection* 136(2):157-165.
- Guatelli JC, Whitfield KM, Kwoh DY, Barringer KJ, Richman DD & Gingeras TR. 1990. Isothermal, *in vitro* amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. *Proceedings of the National Academy of Sciences of the United States of America* 87:1874-1878.
- Guo X, Chen J, Beuchat LR & Brackett RE. 2000. PCR detection of Salmonella enterica serotype Montevideo in and on raw tomatoes using primers derived from *hlyA*. *Applied and Environmental Microbiology* 66(12):5248-5252.
- Hajna AA. 1945. Triple-Sugar Iron Agar Medium for the Identification of the Intestinal Group of Bacteria. *Journal of Bacteriol* 49:514 - 515.
- Himathongkham S, Dodd ML, Yee JK, Lau DK, Bryant RG, Badoiu AS, Lau HK, Guthertz LS, Crawford-Miksza L & Soliman MA. 2007. Recirculating Immunomagnetic separation and optimal enrichment conditions for enhanced detection and recovery of low levels of Escherichia coli O157 : H7 from fresh leafy produce and surface water. *Journal of Food Protection* 70(12):2717-2724.
- Invitrogen. 2005. SuperScriptTM III Platinum^R SYBR^R Green one-Step qRT-PCR Kit. Carlsbad, Ca: invitrogen.
- Johnston LM, Elhanafi D, Drake M & Jaykus LA. 2005. A simple method for the direct detection of Salmonella and Escherichia coli O157 : H7 from raw alfalfa sprouts and spent irrigation water using PCR. *Journal of Food Protection* 68(11):2256-2263.
- Klerks MM, Franz E, van Gent-Pelzer M, Zijlstra C & van Bruggen AHC. 2007. Differential interaction of Salmonella enterica serovars with lettuce cultivars and plant-microbe factors influencing the colonization efficiency. *Isme Journal* 1(7):620-631.
- Koo K & Jaykus LA. 2003. Detection of Listeria monocytogenes from a model food by fluorescence resonance energy transfer-based PCR with an asymmetric fluorogenic probe set. *Applied and Environmental Microbiology* 69(2):1082-1088.
- Lee MD & Fairchild A. 2006. Sample Preparation for PCR. In: Maurer, J., editor). *PCR Methods in Foods*. New York, NY: Springer Science+ Business Media, Inc. p. 41 - 50.
- Leifson E. 1936. New selenite enrichment media for the isolation of typhoid and paratyphoid (salmonella) bacilli. *American Journal of Hygiene* 24(2):423-432.
- Li Y & Mustapha A. 2004. Simultaneous detection of Escherichia coli O157 : H7, Salmonella, and Shigella in apple cider and produce by a multiplex PCR. *Journal of Food Protection* 67(1):27-33.
- Liming SH & Bhagwat AA. 2004. Application of a molecular beacon - real-time PCR technology to detect Salmonella species contaminating fruits and vegetables. *International Journal of Food Microbiology* 95(2):177-187.
- MacGillivray JH. 1953. *Vegetable Production*. New York: The Blakiston Company, Inc.

- Maurer JJ. 2006. The Mythology of PCR: A Warning to the Wise. In: Maurer, J., editor). PCR Methods in Foods. New York, NY: Springer Science+ Business Media, Inc. p. 27 - 40.
- Meerburg BG & Kijlstra A. 2007. Role of rodents in transmission of Salmonella and Campylobacter. *Journal of the Science of Food and Agriculture* 87(15):2774-2781.
- Mercanoglu B & Griffiths MW. 2005. Combination of Immunomagnetic separation with real-time PCR for rapid detection of salmonella in milk, ground beef, and alfalfa sprouts. *Journal of Food Protection* 68(3):557-561.
- Mohle-Boetani JC, Farrar J, Bradley P, Barak JD, Miller M, Mandrell R, Mead P, Keene WE, Cummings K, Abbott S, Werner SB & Invest T. 2009. Salmonella infections associated with mung bean sprouts: epidemiological and environmental investigations. *Epidemiology and Infection* 137(3):357-366.
- Nagamine K, Hase T & Notomi T. 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes* 16(3):223-229.
- Nogva HK & Lillehaug D. 1999. Detection and quantification of Salmonella in pure cultures using 5'-nuclease polymerase chain reaction. *International Journal of Food Microbiology* 51(2-3):191-196.
- North WR & Bartram MT. 1953. THE EFFICIENCY OF SELENITE BROTH OF DIFFERENT COMPOSITIONS IN THE ISOLATION OF SALMONELLA. *Applied Microbiology* 1(3):130-134.
- Okamura M, Ohba Y, Kikuchi S, Suzuki A, Tachizaki H, Takehara K, Ikedo M, Kojima T & Nakamura M. 2008. Loop-mediated isothermal amplification for the rapid, sensitive, and specific detection of the O9 group of Salmonella in chickens. *Veterinary Microbiology* 132(1-2):197-204.
- Peplow MO, Correa-Prisant M, Stebbins ME, Jones F & Davies P. 1999. Sensitivity, specificity, and predictive values of three Salmonella rapid detection kits using fresh and frozen poultry environmental samples versus those of standard plating. *Applied and Environmental Microbiology* 65(3):1055-1060.
- Perelle S, Dilasser F, Malorny B, Grout J, Hoorfar J & Fach P. 2004. Comparison of PCR-ELISA and LightCycler real-time PCR assays for detecting Salmonella spp. in milk and meat samples. *Molecular and Cellular Probes* 18(6):409-420.
- Qiagen. 2001. RNeasy Mini Protocol for Isolation of Total RNA from Bacteria. RNease^R Mini Handbook. Valencia, Ca: Qiagen. p. 56 - 60.
- Ririe KM, Rasmussen RP & Wittwer CT. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical Biochemistry* 245(2):154-160.
- Sakuma T, Kurosaki Y, Fujinami Y, Takizawa T & Yasuda J. 2009. Rapid and simple detection of Clostridium botulinum types A and B by loop-mediated isothermal amplification. *Journal of Applied Microbiology* 106(4):1252-1259.

- Sambrook J & Russell DW. 2001. *Molecular Cloning A Laboratory Manual*, 3rd Ed., 3 ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Saroj SD, Shashidhar R, Karani M & Bandekar JR. 2008. Rapid, sensitive, and validated method for detection of *Salmonella* in food by an enrichment broth culture - Nested PCR combination assay. *Molecular and Cellular Probes* 22(3):201-206.
- Seo KH, Valentin-Bon IE & Brackett RE. 2006. Detection and enumeration of *Salmonella* Enteritidis in homemade ice cream associated with an outbreak: Comparison of conventional and real-time PCR methods. *Journal of Food Protection* 69(3):639-643.
- Shearer AEH, Strapp CM & Joerger RD. 2001. Evaluation of a polymerase chain reaction-based system for detection of *Salmonella enteritidis*, *Escherichia coli* O157 : H7, *Listeria* spp., and *Listeria monocytogenes* on fresh fruits and vegetables. *Journal of Food Protection* 64(6):788-795.
- Simpkins SA, Chan AB, Hays J, Popping B & Cook N. 2000. An RNA transcription-based amplification technique (NASBA) for the detection of viable *Salmonella enterica*. *Letters in Applied Microbiology* 30(1):75-79.
- Sivapalasingam S, Barrett E, Kimura A, Van Duyn S, De Witt W, Ying M, Frisch A, Phan Q, Gould E, Shillam P, Reddy V, Cooper T, Hoekstra M, Higgins C, Sanders JP, Tauxe RV & Slutsker L. 2003. A multistate outbreak of *Salmonella enterica* serotype Newport infection linked to mango consumption: Impact of water-dip disinfection technology. *Clinical Infectious Diseases* 37(12):1585-1590.
- Sivapalasingam S, Friedman CR, Cohen L & Tauxe RV. 2004. Fresh produce: A growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *Journal of Food Protection* 67(10):2342-2353.
- Snyder L & Champness W. 2007a. The Bacterial Chromosome: DNA Structure, Replication, and Segregation. *Molecular Genetics of Bacteria* 3rd Ed. Washington, D.C.: ASM Press. p. 13 - 70.
- Snyder L & Champness W. 2007b. Plasmids. *Molecular Genetics of Bacteria* 3rd ed. Washington, DC: ASM Press. p. 197 - 242.
- Stone GG, Oberst RD, Hays MP, McVey S & Chengappa MM. 1994. DETECTION OF *SALMONELLA* SEROVARS FROM CLINICAL-SAMPLES BY ENRICHMENT BROTH CULTIVATION PCR PROCEDURE. *Journal of Clinical Microbiology* 32(7):1742-1749.
- Szabo EA & Mackey BM. 1999. Detection of *Salmonella enteritidis* by reverse transcription-polymerase chain reaction (PCR). *International Journal of Food Microbiology* 51(2-3):113-122.
- Talaro DP & Talaro A. 1999a. *Microbial Genetics. Foundations in Microbiology* 3rd ed. Boston: McGraw-Hill Companies, Inc. p. 256 - 295.
- Talaro KP & Talaro A. 1999b. *Elements of Microbial Nutrition, Ecology, and Growth. Foundations in Microbiology*, 3rd ed. Boston: McGraw-Hill Companies, Inc. p. 109 - 220.

- Uyttendaele M, Bastiaansen A & Debevere J. 1997. Evaluation of the NASBA(R) nucleic acid amplification system for assessment of the viability of *Campylobacter jejuni*. *International Journal of Food Microbiology* 37(1):13-20.
- Wang H & Slavik MF. 2005. A multiplex polymerase chain reaction assay for rapid detection of *Escherichia coli* O157 : H7, *Listeria monocytogenes*, *Salmonella* Typhimurium and *Campylobacter jejuni* in artificially contaminated food samples. *Journal of Rapid Methods and Automation in Microbiology* 13(3):213-223.
- Wang L, Shi L, Alam MJ, Geng Y & Li L. 2008. Specific and rapid detection of foodborne *Salmonella* by loop-mediated isothermal amplification method. *Food Research International* 41(1):69-74.
- Wilson IG. 1997. Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology* 63(10):3741-3751.
- Yamazaki W, Taguchi M, Kawai T, Kawatsu K, Sakata J, Inoue K & Misawa N. 2009. Comparison of Loop-Mediated Isothermal Amplification Assay and Conventional Culture Methods for Detection of *Campylobacter jejuni* and *Campylobacter coli* in Naturally Contaminated Chicken Meat Samples. *Applied and Environmental Microbiology* 75(6):1597-1603.
- Yuk HG, Warren BR & Schneider KR. 2006. Preliminary evaluation of flow-through immunocapture followed by real-time PCR for the detection of *Salmonella* serovars on tomato surfaces within 8 hours. *Journal of Food Protection* 69(9):2253-2257.
- Yun HJ, Lim SY, Song HP, Kim BK, Chung BY & Kim DH. 2007. Reduction of pathogenic bacteria in organic compost using gamma irradiation. *Radiation Physics and Chemistry* 76(11-12):1843-1846.

Appendix

Table 1: *Salmonella* foodborne outbreaks occurring over the past several years

<u><i>Year</i></u>	<u><i>Serotype</i></u>	<u><i>Food Product</i></u>	<u><i>Location</i></u>	<u><i>Reference</i></u>
1974	Typhimurium	Apple Juice	New Jersey	(Sivapalasingam and others 2004)
1979	Oranienburg	Watermelon	Illinois	(Sivapalasingam and others 2004)
1985	Berta	Apple Juice	Pennsylvania	(Sivapalasingam and others 2004)
1989	Chester	Cantaloupe	Multiple States	(Sivapalasingam and others 2004)
1990	Anatum	Alfalfa	Washington	(Sivapalasingam and others 2004)
1991	Javiana	Watermelon	Michigan	(Sivapalasingam and others 2004)
1991	Poona	Cantaloupe	Multiple States	(Sivapalasingam and others 2004)
1993	Javiana	Watermelon	Wisconsin	(Sivapalasingam and others 2004)
1993	Heidelberg	Lettuce	Minnesota	(Sivapalasingam and others 2004)
1994	Thompson	Lettuce	Minnesota	(Sivapalasingam and others 2004)
1994	Braenderup	Lettuce	New York	(Sivapalasingam and others 2004)
1995	Hartford	Orange Juice	Florida	(Sivapalasingam and others 2004)
1995	Stanley	Alfalfa	Multiple States	(Sivapalasingam and others 2004)
1995	Newport	Alfalfa	Oregon, Canada	(Sivapalasingam and others 2004)
1996	Stanley	Alfalfa	Virginia	(Sivapalasingam and others 2004)
1996	Montevideo	Alfalfa	California	(Sivapalasingam and others 2004)
1997	Saphra	Cantaloupe	California	(Sivapalasingam and others 2004)
1997	Infantis/Anatum	Alfalfa	Missouri, Kansas	(Sivapalasingam and others 2004)
1997	Senftenberg	Alfalfa, Clover	California, Nevada	(Sivapalasingam and others 2004)

Table 1, Continued.

<u><i>Year</i></u>	<u><i>Serotype</i></u>	<u><i>Food Product</i></u>	<u><i>Location</i></u>	<u><i>Reference</i></u>
1999	Newport	Mangoes	Multiple States	(Sivapalasingam and others 2003)
2000-2002	Poona	Cantaloupe	Multiple States	(Bowen and others 2006)
2000-2002	Enteritidis	Mung Beans Sprouts	US, Canada, Netherlands	(Mohle-Boetani and others 2009)
2005	Newport	Tomato	Multiple States	(Greene and others 2008)
2006	Typhimurium	Tomato	Multiple States	(CDC, 2009)
2006	Tennessee	Peanut Butter	Multiple States	(CDC, 2009)
2007	Wandsworth	Veggie Booty	Multiple States	(CDC, 2009)
2007	Schwarzengrund	Pet Food	Multiple States	(CDC, 2009)
2007	I 4,[5], 12:i:-	Pot Pies	Multiple States	(CDC, 2009)
2008	Litchfield	Cantaloupe	Multiple States	(CDC, 2009)
2008	Agona	Puffed Rice Cereals	Multiple States	(CDC, 2009)
2008	Saintpaul	Jalapeno & Serrano	Multiple States	(CDC, 2009)
2008-09	Typhimurium	Peanut Butter	Multiple States	(CDC, 2009)

Table 2: Recent Molecular Methods used for the detection of *Salmonella* in foods

<u>Food Product</u>	<u>Molecular Technique</u>	<u>Detection Sensitivity</u>	<u>Incubation Period (hr)</u>	<u>Reference</u>
Lettuce	PCR	10 ⁴ cfu	1	(Wang and Slavik, 2005)
Tomato	PCR	10 ⁴ cfu	9	(Guo and others 2000)
Alfalfa Sprouts	RT-PCR	~4 cfu	18	(Liming and Bhagwat, 2004)
Cantaloupe	RT-PCR	~4 cfu	18	(Liming and Bhagwat, 2004)
Mixed Salad	RT-PCR	~4 cfu	18	(Liming and Bhagwat, 2004)
Cilantro	RT-PCR	~4 cfu	18	(Liming and Bhagwat, 2004)
Poultry	RT-PCR	<10 ³ cfu	24	(De Medici and others 2003)
Minced Beef	RT-PCR	10 cfu	16	(Szabo and Mackey, 1999)
Egg	RT-PCR	10 cfu	16	(Szabo and Mackey, 1999)
Milk	RT-PCR	1 cfu	10	(Chiang and others 2007)
Mixed Salad	PCR	1 cfu	20	(Bhagwat, 2003)
Tomato	PCR	1 cfu	24	(Shearer and others 2001)
Alfalfa Sprouts	PCR	10 ² cfu	0	(Johnston and others 2005)
Apple Cider	PCR	0.8 cfu	24	(Li and Mustapha, 2004)
Cantaloupe	PCR	0.8 cfu	24	(Li and Mustapha, 2004)
Lettuce	PCR	0.8 cfu	24	(Li and Mustapha, 2004)
Tomato	PCR	0.8 cfu	24	(Li and Mustapha, 2004)

Table 2. Continued.

<u><i>Food Product</i></u>	<u><i>Molecular Technique</i></u>	<u><i>Detection Sensitivity</i></u>	<u><i>Incubation Period (hr)</i></u>	<u><i>Reference</i></u>
Watermelon	PCR	0.8 cfu	24	(Li and Mustapha, 2004)
Alfalfa Sprouts	PCR	80 cfu	24	(Li and Mustapha, 2004)
Tomatoes	FTI-PCR	10 ⁰ cfu	4	(Yuk and others 2006)
Chicken Wash	PCR	10 ⁴ cfu	1	(Wang and Slavik, 2005)
Watermelon	PCR	10 ⁴ cfu	1	(Wang and Slavik, 2005)
Broccoli	PCR	10 ⁴ cfu	1	(Wang and Slavik, 2005)
Mushrooms	PCR	10 ⁴ cfu	1	(Wang and Slavik, 2005)
Ground Beef	PCR	10 ⁴ cfu	1	(Wang and Slavik, 2005)
Yogurt	PCR	10 ⁴ cfu	1	(Wang and Slavik, 2005)
Cottage Cheese	PCR	10 ⁴ cfu	1	(Wang and Slavik, 2005)
Carrot	Nested-PCR	4 cfu	6	(Saroj and others 2008)
Cucumber	Nested-PCR	4 cfu	6	(Saroj and others 2008)
Poultry Meat	Nested-PCR	4 cfu	6	(Saroj and others 2008)
Sprouts	Nested-PCR	4 cfu	6	(Saroj and others 2008)

CHAPTER II

Real-time reverse-transcriptase PCR for *Salmonella* Typhimurium detection from Lettuce and Tomatoes

PCR Detection of *S. Typhimurium* from Produce

Real-time reverse-transcriptase PCR for *Salmonella* Typhimurium detection from Lettuce and Tomatoes

Nathan D. Miller, Doris H. D'Souza and P.M. Davidson

University of Tennessee, 2605 River Drive Room 100 FSPB, Knoxville, TN 37996

Key words: *Salmonella* Typhimurium, Real-time PCR, Lettuce and Tomatoes

Doris H. D'Souza. Tel: (865)974-2753; Fax: (865)974-7332
E-mail: ddsouza@utk.edu

Abstract

Recent outbreaks of *Salmonella* linked to fresh produce emphasize the need for rapid detection methods to help control outbreaks. Real-time reverse-transcriptase-PCR (RT-PCR) detects the presence of mRNA (shorter half-life than DNA), with greater potential of detecting viable pathogens and eliminates the need for gel electrophoresis. The objectives of this research were to apply RT-PCR to detect *Salmonella* from spiked lettuce and tomatoes. Twenty-five grams of lettuce and ~130 gram of tomato were inoculated with 10^8 to 10^1 CFU overnight culture of *Salmonella* Typhimurium. Outer leaves of iceberg lettuce were removed and the inner leaves, as well as tomatoes, were thoroughly rinsed with water and dried under ultraviolet light for 10 minutes. After testing of various buffers, the produce was either rinsed or hand massaged, for optimal recovery, in 0.05M glycine saline-buffer (0.05% Tween, 3% beef extract) that gave optimal recovery. For low inocula levels, short pre-enrichment of 6 hours in peptone buffer was needed. Serial dilutions were plated on Xylose Lactose Tergitol 4 (XLT4) agar and portions used for RNA extraction using the Qiagen RNeasy® Mini Kit. SYBR Green one step RT-PCR kit with *invA* gene primers and an internal amplification control was used for detection. Reaction conditions were 50°C/40 minutes followed by 94°C/45s, 58°C/45s, 72°C/45s for a total of 45 cycles followed by melt temperature analysis. RT-PCR detected up to 10^4 CFU/25g of lettuce and $\sim 10^3$ CFU/ ~ 25 g of tomatoes after pre-enrichment. Without pre-enrichment, detection for lettuce was 10^6 CFU/25g and tomato detection was $\sim 10^6$ CFU/ ~ 25 g. These results showed that RT-PCR can be used to

identify contaminated produce in about 10-12 hours when compared to other reports that require 18 – 24 hour enrichment.

Introduction

Salmonella is one of the major foodborne bacteria that is ubiquitously present in the environment (Mercanoglu and Griffiths, 2005). *Salmonella* is a facultative anaerobe that belongs to the *Enterobacteriaceae* family. Under normal conditions, *Salmonella* is a mesophile, however the bacterium can survive and grow at 54°C when pre-exposed to thermal stress (D'Aoust and Maurer, 2007). Even though there has been an increase in education about the bacteria, its ability to continually cause problems in the food system is a difficulty faced everyday (Mercanoglu and Griffiths, 2005). It is imperative to find improved and rapid methods to detect *Salmonella* which is an area of constant research.

Traditional cultural methods, such as the use of selective and differential microbiological media, can be used to effectively detect and identify different bacteria. However, these methods are much slower than what is needed to rapidly detect the presence of potential pathogens in foods. For *Salmonella*, traditional methods can take 4 days to show preliminary results regarding the presence of *Salmonella* and another 3 days to confirm the serotype of the bacteria (Mercanoglu and Griffiths, 2005). This could take a total of 4 – 7 days to identify the bacteria. While researchers have devised much faster and more accurate molecular detection methods for foodborne pathogens (Feng, 2007), all methods have the same problems in that the food matrix is a complex system. It has many inhibitors that can cause molecular methods, such as the polymerase chain reaction (PCR) process, to fail and give false results (Maurer, 2006).

The food matrix is a difficult working environment for the PCR process. This process requires an environment almost completely devoid of substances or conditions that inhibit or prevent the replication of DNA. Inhibitors are substrates that bind or degrade part of the reaction reagent that will prevent replication of DNA. In food such inhibitors and condition include lipids, proteins, enzymes, chemical additives, fiber, and pH changes (Lee and Fairchild, 2006). Many of these inhibiting substances are also present in the enrichment broths and other media used during the recovery of bacteria from food samples. Therefore, washing steps used for the cells before moving into the PCR process are critical (Lee and Fairchild, 2006). Prevention of cross-contamination is another important aspect so as to avoid false positives and false negatives. This can easily be overcome by keeping the work station clean using UV irradiation, sodium hypochlorite, photochemical, and/or enzymatic methods (Wilson, 1997).

Specificity of a PCR process can be increased by targeting specific virulence genes of a particular microorganism that is not present in other species. One such gene specific to *Salmonella* is the *invA* gene. All invasive strains of *Salmonella* have the *invA* gene present (Perera and Murray, 2008), while this gene has been absent from other closely related organisms like *Escherichia coli* (Baumler and others 1998) and other foodborne pathogens. The *invA* gene allows *Salmonella* to target epithelial cells of the intestinal wall (Rahn and others 1992). The chromosomal location of the *invA* gene also makes it a good target for use in PCR assays. By targeting the *invA* gene, positive results by PCR indicate the presence of *Salmonella* that it is in fact an invasive species.

In recent years, the use of molecular technologies such as polymerase chain reaction (PCR) and real-time polymerase chain reaction (RT-PCR) has enabled rapid detection of foodborne pathogens. PCR uses gel electrophoresis to visualize the presence or absence of the target product, while real-time PCR uses Fluorescence probes or fluorescent dyes where melt temperature of the target DNA helps visualize the presence or absence of the target product. Reverse-transcriptase PCR (rt-PCR) can detect viable bacteria by targeting its mRNA which has a shorter half-life than DNA (Maurer, 2006). This allows detection of live organisms introduced or recent contamination rather than organisms that have been killed due to implemented control measures to eliminate bacteria from the food product or environment. Since rapid technology is available, detecting target bacteria at a faster rate can help the “processor” recall fresh produce from the market more quickly than in the past due to bacterial safety issues.

As the demand for fresh produce such as tomatoes and lettuce increases, supplying safe products remain a challenge. Over the past 20 years, sale and consumption of tomatoes and tomato products have almost increased 30 percent (Plummer, 2000). This is about 93 pounds of tomatoes consumed per person in 1998 (Plummer, 2000). Much of the tomato products consist of sauces, ketchup, pastes, salsa, and juice. These tomato products make up about 81 percent of the total 93 pounds consumed. This rise could be attributed to an increase in public awareness of the health benefits as tomato rich diets have been linked to reducing the risk of some cancers and

heart diseases (Plummer, 2000). According to the Economic Research Service of the USDA, a study examined the future growth of the fruits and vegetable markets (Lin, 2004). It is suggested that better education about health benefits along with dietary knowledge will increase tomato consumption. The percentage of tomatoes will likely increase 1.3% within this time period (Lin, 2004). With continual consumption increase, product safety will also need to increase.

The demand for lettuce is also on the rise as shown in a study by the United States Department of Agriculture (USDA) as reported in the *Vegetables and Specialties Situation and Outlook Yearbook* (Kaufman and others 2000). The report involved a ten year study from 1987 to 1997. In 1987 potatoes, lettuce, and tomatoes led the charts with 162.4 pounds being consumed per person where as in 1997, 185.6 pounds were consumed with potatoes, lettuce, and onions being the leading vegetables consumed per person (Kaufman and others 2000) . Convenience is of driving factors of the fresh produce. Many packaged salads and fresh-cut vegetables show a continual increase in their shelf space in retail markets. Along with rising incomes, consumers are able to purchase a broader variety of produce in order to reach the new flavors normally tasted at restaurants in their own homes (Kaufman and others 2000).

Produce safety has been a concern for a long time. Looking back to the mid 90's, lettuce was implicated in several outbreaks. The serovars associated with the lettuce outbreaks were *Salmonella* Heidelberg in 1993, *Salmonella* Thompson and also *Salmonella* Braenderup in 1994 (Sivapalasingam and others 2004). Serovars that were

associated with tomato outbreaks were *Salmonella* Typhimurium in 2006 (CDC, 2009) and *Salmonella* Javiana in 1990 (Sivapalasingam and others 2004) to name only a few of the several associated with produce in the past.

Previous studies have shown that detection of *Salmonella* from produce using RT-PCR or PCR is able to detect lower limits of 10^3 CFU from chicken wash and 10^4 CFU from fresh cut lettuce (Wang and Slavik, 2005), ≤ 10 CFU from minced beef and whole eggs (Szabo and Mackey, 1999), and $< 10^3$ CFU from poultry samples (De Medici and others 2003). Much of these lower limits of detection were aided by a pre-enrichment media process of no more than 24 hours. For example, incubating poultry meat for 24 hours allowed a detection limit of 10^3 CFU/ml (Kanki and others 2009) using PCR. A detection limit of 10^3 CFU/g was demonstrated for buffalo meat trimmings using an 18 hour incubation period (Biswas and others 2008). Finally using RT-PCR, a detection limit of less than 5 CFU/25g was shown for alfalfa sprouts, cantaloupe, mixed salad and cilantro when incubated for 24 hours (Liming and Bhagwat, 2004).

The purpose of this study was to determine if a method could be developed for detection of *Salmonella* from lettuce and tomatoes within one day using real-time PCR with a SYBR Green I dye that is relatively cheaper than using fluorescent probes. High (10^8 to 10^6 CFU/ml) and low (10^2 to 10^0 CFU/ml) inocula were used and, for the latter, short enrichment times were applied. Previously described *invA* primers were used that targeted the chromosomally located *invA* gene specific for *Salmonella* species.

Materials and Methods:

Growth of *Salmonella enterica* serovar Typhimurium

Salmonella enterica serovar Typhimurium (DT104 2486; UT Cultural Collection) was grown in 10 ml of Trypticase Soy Broth (TSB; Difco, Becton Dickinson and company Sparks MD) at 35°C for 24 h. The cultures were transferred two consecutive times at 24 h intervals before use. A ten-fold serial dilution of an overnight culture in 9 ml of peptone buffer (Oxoid LTD; Basingstoke Hampshire, England) per tube, was plated on Trypticase Soy Agar (TSA; Difco, Accumedia Lansing, Michigan) and incubated to determine counts.

Preparation of Lettuce and Tomatoes

The fresh produce, lettuce and tomatoes, used in this study were purchased at a local grocery store. Before inoculation, the produce was washed for 1 minute using tap-water and placed in an open sterile Petri plate in a hood to dry under ultraviolet light for 10 minutes at room temperature. Iceburg lettuce (25g) and Roma tomatoes (120-150g) were individually inoculated with 0.1 ml *Salmonella enterica* serovar Typhimurium high inoculums (10^8 to 10^6) and low inoculums (10^5 to 10^0) and allowed to dry for 10 min at room temperature. Washed and dried un-inoculated produce was used as controls and each experiment was replicated at least twice.

Extraction from Inoculated Lettuce and Tomatoes

Bacteria were eluted from the produce by washing with 0.05 M glycine-0.14N/saline buffer (pH 9.0) (glycine, Fisher Scientific, Fair Lawn, New Jersey), buffer with 0.05% Tween-20 (Promega; Madison, WI) and buffer with 0.1% Tween-20 and 3% beef extract to compare extraction efficiency and aid in the removal of bacterial cells. Fifty-ml of each buffer was added to the lettuce and tomatoes in a sterile stomach bag. The tomatoes were hand rubbed for 1 minute and the lettuce was 'stomached' at 230 RPM for 30 seconds (Stomacher® 400 Seward; England). Fifty-ml samples of eluted buffer were centrifuged at 8000 x g for 10 min at 4°C (Sorvall Legend 23 R; Thermo Electron Corporation; Asheville, NC). The supernatant was decanted and the cell pellet was resuspended in 1 ml of peptone buffer before proceeding with plate counts and the isolation of RNA (Oxoid LTD; Basingstoke Hampshire, England).

Enumeration of Bacteria

For low inocula levels, short-enrichment times were needed. The lettuce was stomached and the tomatoes were hand rubbed in 50 ml of sterile peptone water (Oxoid) and incubated for 6 h at 35°C. Following incubation a 50 ml sample was removed and centrifuged at 8000 x g for 10 min at 4°C (Sorvall). The supernatant was decanted and the cell pellet was resuspended in 1 ml of peptone buffer (Oxoid). Serial dilutions of enriched and un-enriched samples were plated on Xylose Lactose Tergitol 4 (XLT4) agar and incubated at 37°C for 24 to 48 h to enumerate typical black colonies for *Salmonella* detection.

Isolation of Total RNA from Bacteria

RNA in samples was first stabilized by adding 1.2 ml of RNAlater Stabilization Reagent (Qiagen) to 0.6 ml of each sample. The combination of stabilization reagent and bacteria culture/extract was incubated at room temperature for 10 min and vortexed several times. The cells were centrifuged (Eppendorf 5417C; New York, NY) for 10 min at 8000 x g at 4°C and the pellet was stored at -80°C or used directly for extraction. The RNeasy Mini Purification Kit (Qiagen; Valencia, CA) was used to extract RNA from *Salmonella* Typhimurium, pure culture, inoculated and enriched, and un-enriched lettuce and tomato samples, un-inoculated lettuce and tomato negative controls, and un-inoculated sterile water.

One hundred µl of Tris-EDTA (TE; 10 mM Tris-Cl, 1 mM EDTA, pH 8.0; Promega; Madison, WI) containing lysozyme (3 mg/ml; Sigma-Aldrich; St. Louis, MO) was added to each pellet, vortexed, and incubated at room temperature for 10 minutes with mixing. RLT buffer (350 µl) was added followed by 250 µl of Absolute-200 proof ethanol (Aaper Alcohol; Shelbyville, KY) and transferred to the RNeasy mini column. The column was centrifuged at 8000 x g for 15 sec and the collection tube was discarded. RW1 buffer (700 µl) was added to the column and centrifuged for 15 sec at 8000 x g to wash the column. RPE buffer (500 µl) was added and centrifuged at 8000 x g for 15 sec and repeated once. The RNA was then eluted off the column with 40 µl of RNase-free water and centrifuging at 8000 x g for 1 min. The collection tubes containing RNA were capped and stored at -80°C until further analyses.

DNase I Treatment

DNase I treatment was carried out using the TURBO DNA-free™ Kit (Ambion®; Applied Biosystems; Foster City, CA). The procedure consisted of 5 µl of 10x TURBO DNase Buffer, 1 µl of rDNase I DNA-free™, and 34 µl of Nuclease-free Water. The reagents then were mixed with 10 µl of RNA and incubated at 37°C for 30 min. Then 5 µl of DNase Inactivation Reagent was added and incubated for 2 minutes. The sample then centrifuged at 10,000xg for 2 min. The supernatant was removed and placed in a new tube.

Nanodrop Quantification of RNA

After extraction of the RNA, a quick method to quantify the RNA was done. Quantification was done using a NanoDrop® Spectrophotometer (NanoDrop® ND-1000 Spectrophotometer; Thermo Scientific; Wilmington, DE). The concentration of the nucleic acid was confirmed using UV-spectrophotometry at 260 nm while, the purity was determined using the ratio 260/280 for proteins and 260/230 for organic contamination. The two ratios needed to be above 1.85 to provide a good sample of RNA with little to no protein or organic carryover or contamination.

Real-Time Reverse-Transcriptase Polymerase Chain Reaction (rt-RT-PCR) Procedure

The SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen; Carlsbad, California) was used in the rt-RT-PCR step. Twenty-five µl reactions contained 5 µl RNA extracts in RNase-DNase free water, Superscript III (SSIII) one-step RT-PCR kit

reagents (Invitrogen, Carlsbad, CA, USA) with SYBR Green I, 0.02 μ M of each *invA* primer (**F: 5' CACGCTCTTTCGTCTGGCA3'**; **R2: 5' TACGGTTCCTTTGACGGTGCGA3'**) and bovine serum albumin (BSA) with a final concentration of 1.6 μ l and an internal amplification control (IAC) (see below) obtained from Sigma-GenoSys (St. Louis, MO, USA). The thermocycler conditions were carried out as follows, RT at 50°C/30min, denaturation at 95°C/5min, followed by 40 cycles at 95°C/45s, 58°C/45s, 72°C/45s, and final extension at 72°C/7min in a Bio-Rad iCycler (Hercules, CA, USA). After amplification, melt temperature analyses from 50°C to 95°C with 0.5°C increments, was carried out. The software provided by iCycler was used to obtain the threshold cycle (C_T) values and melt temperatures (T_m). Melt temperature at ~85°C for the *Salmonella* specific *invA* product was expected. Products were separated on 2% agarose gels by electrophoresis (in 1X Tris-acetic acid-EDTA (TAE) buffer; Fisher Scientific, Pittsburgh, PA, USA), stained with ethidium bromide, visualized under ultraviolet light, and photographed using the Gel Doc System/Station (Bio-Rad). A DNA marker, with a molecular weight of 100 bp (Promega), was also run on the agarose gel to determine the size of the amplified target RT-PCR product.

Preparation of the Internal Amplification Control (IAC)

The Internal Amplification Control was added to the reaction mix, for each sample, in order to eliminate the concern of false negatives due to PCR inhibitors, enzyme inactivation or instrument malfunction. If the IAC did not produce a T_m and there was no product T_m , it was indicative of reaction failure. The Beacon Designer

Software (BioRad) was used to design the *stx 1* primer set from the shiga toxin region of *E. coli* O157:H7 DNA to yield a product of 109 bps. The forward and reverse *invA* primer was coupled to the *stx1* forward and reverse primers respectively to amplify a 182 bp product. A T₇ promoter was added to the forward primer and RNA was amplified using the MEGAscript T7 Transcription Kit (Ambion). The concentration of the amplified RNA product was diluted in order to reach an optimal concentration of 1.9 fg/μl for the PCR procedure.

Results

Bacterial Cell Recovery from Lettuce and Tomatoes

Extracting the target *Salmonella* from the lettuce proved to be much easier than removal of the target from the outer surface of the tomato. Our recovery of *Salmonella* Typhimurium cells, as determined by plating from the surface of the lettuce, was about 10³ CFU/ml from the initial inocula of 10⁶ CFU/ml and from the surface of the tomato was about 10² CFU from the initial inocula of 10⁷ CFU/ml using the optimal buffer containing Tween-20 and beef extract. The addition of 0.05% Tween-20 and 3% Beef Extract to the extraction buffer increased the recovery of target *Salmonella* cells and was found to be optimal. The recovery increased from 10¹ CFU using Glycine-saline buffer to 10³ CFU using the optimal buffer, Glycine-saline with 0.05% Tween-20 and 3% Beef Extract. Tween-20 acted as a surfactant to help lift the cells off the tomato skin and

into solution to improve recovery and detection. Table 3 (All tables and figures are in the Appendix) shows the CFU counts recovered using various extraction buffers.

RNA Extraction Yields from Lettuce and Tomatoes

RNA extraction from un-enriched lettuce spiked with 10^6 CFU *Salmonella* using the Qiagen RNeasy Mini Purification Kit yielded 55.42 ± 41.61 ng/ μ l nucleic acid and un-enriched tomatoes spiked with 10^7 CFU *Salmonella* yielded 2.83 ± 2.41 ng/ μ l respectively. After an enrichment process of 6 h for lettuce and tomato spiked with 10^4 CFU *Salmonella*, RNA extraction yielded 15.67 ± 16.72 ng/ μ l of nucleic acid for lettuce and 46.32 ± 4.34 ng/ μ l of nucleic acid for tomatoes.

rt-PCR Detection and Specificity

Using real-time RT-PCR, *Salmonella* could be detected up to 10^6 CFU/25g of lettuce spiked with high inocula *Salmonella* without enrichment (Figure 1). For low inocula levels, a 6 hour enrichment was necessary (Figure 4). After a 6 h enrichment process, the detection limit increased by two logs to 10^4 CFU/25g of lettuce. For tomatoes, *Salmonella* was detected at 10^7 CFU/ $\sim$ 25g without enrichment (Figure 7). For low inocula levels, a 6 hour enrichment was necessary for detection that gave a 10^4 CFU/ $\sim$ 25g detection limit (Figure 10) This shows that enrichment will in fact aid in better detection of the target bacteria at lower levels with results in 1 day. To determine specificity of the amplified products, Tm curves showed peaks $\sim 85^\circ\text{C}$ as expected (Figure 2 & 5) for lettuce. The IAC showed peaks at 82°C as expected showing that false

negatives were not obtained. Products were confirmed by gel electrophoresis. The T_m curves for tomatoes (Figure 8 & 11) confirmed that the target product was in fact present at the detected level. Gel electrophoresis revealed the 347 bp target product as expected, with similar detection sensitivity at RT-PCR C_t values of 10^4 CFU (Figure 9 & 12). Negative controls did not show any T_m 's at 85°C or any product on the gel. Negative controls consisted of water, TSB, peptone, or uninoculated lettuce (Figure 3 & 6).

DNase I Treatment

DNase I treatment was performed in order to determine if amplification is from RNA or carryover DNA. When the treatment was applied to our study, the ability to detect the targets RNA from lettuce and tomato was reduced. A one log reduction in the detection limit for lettuce and tomato when applied to rt-PCR was obtained to give 10^7 CFU/25g in un-enriched lettuce and 10^8 CFU/~25g in un-enriched tomatoes. Also, PCR on the DNase I treated extracts did not yield any positive PCR products as expected, showing the absence of any DNA carryover (Figure 13 & 14).

Discussion

Salmonella was recovered from lettuce and tomato through an extraction buffer or a pre-enrichment process in peptone water. After testing combinations of glycine-saline buffers, an optimal buffer was found to be glycine-saline buffer solution containing 3% beef extract and 0.05% Tween-20. Optimization of the extraction buffer is

shown in Table 3. Previous research has suggested that attachment of bacterial cells to the surface of produce is due to the roughness or hydrophobic properties of the produce surface (Iturriaga and others 2003). This study used a buffer solution that contained a surfactant to aid in the removal of the *Salmonella* cells from the surfaces of lettuce and tomatoes. . Our recovery of *Salmonella* Typhimurium cells, as determined by plating from the surface of the lettuce, was about 10^3 CFU/ml from the initial inocula of 10^6 CFU/ml and from the surface of the tomato was about 10^2 CFU from the initial inocula of 10^7 CFU/ml using the optimal buffer containing Tween-20 and beef extract. Although this percentage is low, other factors may have played a role in the low recovery percentage. According to a study done by Shi and others (2007), the different serovars of *Salmonella* may play a role in how the bacteria may attach to the surface (Shi and others 2007). Another factor that can affect recovery is cell viability. The death or injury of the cell can be attributed to the cells drying time on the produce surface (Lang and others 2004). Traditional methods have been used to increase cell numbers by applying an enrichment process which can take several days.

The major advantage of real-time-PCR is the speed of detection with results achieved within 10 - 12 h. Results can be obtained within one day as compared to traditional culturing methods which can take up to 4-7 days (Feng, 2007). An advantage of using reverse-transcriptase-PCR that targets mRNA is that it potentially allows for the detection of viable cells within the food product. As mRNA has a shorter half life than DNA it has greater potential of detecting primarily viable cells or at the very least recent

contamination that could cause infection (Maurer, 2006). Several other studies used a 24 hour or less enrichment process in order to detect *Salmonella* using rt-PCR or PCR (De Medici and others 2003; Szabo and Mackey, 1999; Wang and Slavik, 2005). The study done by De Medici et. al. (2003) had a detection limit of $< 10^3$ on poultry samples which was incubated for 24 h (De Medici and others 2003). Another study by Szabo and Mackey (1999) had a detection limit of 10^7 CFU on minced beef with a 4 h enrichment and ≤ 10 CFU on minced beef with a 16 h enrichment (Szabo and Mackey, 1999). Also in the same study whole eggs were studied and found to have a detection limit of 10^7 CFU with a 4 h enrichment and ≤ 10 CFU with a 16 h enrichment (Szabo and Mackey, 1999). A study done by Wang and Slavik (2005) had a detection limit of 10^4 CFU on fresh-cut lettuce and chicken wash which had only a 1 h incubation period at room temperature (Wang and Slavik, 2005). A 10 h pre-enrichment step was used by Chiang et. al. (2007) in order to obtain a detection limit of 1 CFU in milk and meat samples (Chiang and others 2007). With our study we have been using a 6 h enrichment process to detect the lower inoculum levels and have been able to detect up to a 10^4 CFU/25g or 400 CFU/g on lettuce and tomatoes. While this method shows similar detection limits to other reports, it is still much faster because of shorter enrichment times and also cheaper as it uses SYBR Green I dye instead of fluorescent probes.

With simultaneous amplification and detection using fluorescence in real-time PCR, this allows for product specificity to be confirmed by melt temperature (T_m) rather than using gel electrophoresis. A melt temperature is when 50% of the amplified DNA is

double stranded and 50% is single stranded (De Medici and others 2003). The T_m determines if the specific target amplicon is present or not. Since the T_m is part of the rt-PCR process this allows for much faster results, less labor intensive, and less likely for the product to become contaminated.

A validation step, using an internal amplification control (IAC), was applied in order to confirm the results of the experiment. The IAC was added to identify potential false-negatives that may have been caused by PCR inhibitors (Moreira and others 2009). PCR inhibitors could be lipids, proteins, enzymes, chemical additives, fiber, other bacteria, and pH (Lee and Fairchild, 2006). In our study the size of the IAC was smaller than the target, even though it has been recommended that the IAC be larger (Hoorfar and others 2004). This was done to compensate for the high T_m of the target product and increase the ability to differentiate between the two different products T_m 's. The target and IAC were both amplified using the same primer sets that use only one set of PCR reaction conditions (Hoorfar and others 2004). If the IAC is amplified without target product, it shows that the results were not a false negative. Thus a robust assay was developed that detected *Salmonella* from lettuce at 10^6 CFU/25g and at 10^7 CFU/25g without enrichment and 10^4 CFU/25g and 10^4 CFU/25g with enrichment for lettuce and tomatoes respectively. While this method cannot conclusively detect viable cells only, *Salmonella* can be detected from produce at 10^4 CFU/25g from lettuce and tomatoes within 10-12 hours.

A DNase I treatment was performed in order to eliminate genomic DNA that may have still present (Werbrouck and others 2007). When this treatment was applied to our study, the ability to detect the targets RNA from lettuce and tomatoes was reduced by one log. Even though this procedure was done, it has been expressed by Harper and others (2003) that all genomic DNA may be completely removed by DNase I and some DNA can be carried over (Harper and others 2003). It could also be possible that reduced detection after DNase I treatment was due to the DNase I not being 100% free of contamination by RNases. This could result in the DNase I treatment degrading also some of the RNA present (Werbrouck and others 2007), therefore leading to lower yields. The loss of RNA from the reaction could lead one to believe that the produce is free of the target pathogen. Therefore the results must be interpreted with caution. Therefore PCR was conducted on the RNA not treated with DNase I. The lower yield of RT-PCR after DNase I treatment showed that precautions must be taken while extracting RNA to prevent carry-over DNA that could lead to extraneous results. However it is also crucial to ensure that DNase I used does not have RNases present that could destroy the total RNA as well decrease yield while keeping RNA pure (Figure 13 & 14).

This method rapidly detected *Salmonella* faster than traditional plating. With continued research on improving detection sensitivity, bacterial recovery, and RNA yields, this method has the potential to ultimately save the food industry millions of dollars as well as costly recalls, and protect public health. This method has allowed for

us to obtain results with detection sensitivities of 10^4 CFU/25g lettuce and 10^4 CFU/ 25g tomatoes within a 10-12 h period rather than the 4-7 day traditional plating technique. However, further research should focus on improving recovery of the bacteria from the produce surface that could aid in improving detection sensitivity.

List of References

- Baumler AJ, Tsois RM, Ficht TA & Adams LG. 1998. Evolution of host adaptation in *Salmonella* enterica. *Infection and Immunity* 66(10):4579-4587.
- Biswas AK, Kondaiah N, Bheilegaonkar KN, Anjaneyulu ASR, Mendiratta SK, Jana C, Singh H & Kumar RR. 2008. Microbial profiles of frozen trimmings and silver sides prepared at Indian buffalo meat packing plants. *Meat Science* 80(2):418-422.
- CDC. 2009. *Salmonella* Outbreak Investigations. Centers for Disease Control and Prevention. Atlanta, GA.
- Chiang YC, Fan CM, Liao WW, Lin CK & Tsen HY. 2007. Real-time PCR detection of *Staphylococcus aureus* in milk and meat using new primers designed from the heat shock protein gene htrA sequence. *Journal of Food Protection* 70(12):2855-2859.
- D'Aoust J-Y & Maurer J. 2007. *Salmonella* Species. In: Doyle, M. P. & Beuchat, L. R., editors. *Food Microbiology: Fundamentals and Frontiers*, 3rd Ed. 3rd Ed. ed. Washington, D.C.: ASM Press. p. 187 - 236.
- De Medici D, Croci L, Delibato E, Di Pasquale S, Filetici E & Toti L. 2003. Evaluation of DNA extraction methods for use in combination with SYBR Green I real-time PCR to detect *Salmonella* enterica serotype enteritidis in poultry. *Applied and Environmental Microbiology* 69(6):3456-3461.
- Feng P. 2007. Rapid Methods for the Detection of Foodborne Pathogens: Current and Next-Generation Technologies. In: Doyle, M. P. & Beuchat, L. R., editors. *Food Microbiology: Fundamentals and Frontiers*, 3rd Ed. Washington, D.C.: ASM Press. p. 911 - 934.
- Harper LV, Hilton AC & Jones AF. 2003. RT-PCR for the pseudogene-free amplification of the glyceraldehyde-3-phosphate dehydrogenase gene (gapd). *Molecular and Cellular Probes* 17(5):261-265.
- Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M & Fach P. 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. *Journal of Clinical Microbiology* 42(5):1863-1868.
- Iturriaga MH, Escartin EF, Beuchat LR & Martinez-Peniche R. 2003. Effect of inoculum size, relative humidity, storage temperature, and ripening stage on the attachment of *Salmonella* Montevideo to tomatoes and tomatillos. *Journal of Food Protection* 66(10):1756-1761.
- Kanki M, Sakata J, Taguchi M, Kumeda Y, Ishibashi M, Kawai T, Kawatsu K, Yamasaki W, Inoue K & Miyahara M. 2009. Effect of sample preparation and bacterial concentration on *Salmonella* enterica detection in poultry meat using culture methods and PCR assaying of preenrichment broths. *Food Microbiology* 26(1):1-3.
- Kaufman PR, Handy CR, McLaughlin EW, Park K & Green GM. 2000. Understanding the Dynamics of Produce Markets. Agriculture Information Bulletin Number 758. Economic Research Service/USDA.
- Lang MM, Harris LJ & Beuchat LR. 2004. Evaluation of inoculation method and inoculum drying time for their effects on survival and efficiency of recovery of *Escherichia*

- coli O157 : H7, *Salmonella*., and *Listeria monocytogenes* inoculated on the surface of tomatoes. *Journal of Food Protection* 67(4):732-741.
- Lee MD & Fairchild A. 2006. Sample Preparation for PCR. In: Maurer, J., editor). *PCR Methods in Foods*. New York, NY: Springer Science+ Business Media, Inc. p. 41 - 50.
- Liming SH & Bhagwat AA. 2004. Application of a molecular beacon - real-time PCR technology to detect *Salmonella* species contaminating fruits and vegetables. *International Journal of Food Microbiology* 95(2):177-187.
- Lin B-H. 2004. Fruit and Vegetable Consumption Looking Ahead to 2020. *Agriculture Information Bulletin* 792-7. Economic Research Service/USDA.
- Maurer JJ. 2006. The Mythology of PCR: A Warning to the Wise. In: Maurer, J., editor). *PCR Methods in Foods*. New York, NY: Springer Science+ Business Media, Inc. p. 27 - 40.
- Mercanoglu B & Griffiths MW. 2005. Combination of Immunomagnetic separation with real-time PCR for rapid detection of salmonella in milk, ground beef, and alfalfa sprouts. *Journal of Food Protection* 68(3):557-561.
- Moreira AN, Conceicao FR, Conceicao RDS, Dias CN, Carvalhal JB, Dellagostin OA & Aleixo JAG. 2009. IMS USING IN-HOUSE MONOCLONAL ANTIBODY-COATED MAGNETIC BEADS ASSOCIATED TO PCR ASSAY FOR DETECTION OF SALMONELLA TYPHIMURIUM IN RAW MEATS. *Journal of Food Safety* 29(1):59-72.
- Perera K & Murray A. 2008. Development of a PCR assay for the identification of *Salmonella* enterica serovar Brandenburg. *Journal of Medical Microbiology* 57(10):1223-1227.
- Plummer C. 2000. Cutbacks Ahead for Processing Tomato Acreage. *Agriculture Outlook magazine*. Economic Research Service/USDA.
- Rahn K, Degrandis SA, Clarke RC, McEwen SA, Galan JE, Ginocchio C, Curtiss R & Gyles CL. 1992. AMPLIFICATION OF AN INVA GENE SEQUENCE OF SALMONELLA-TYPHIMURIUM BY POLYMERASE CHAIN-REACTION AS A SPECIFIC METHOD OF DETECTION OF SALMONELLA. *Molecular and Cellular Probes* 6(4):271-279.
- Shi X, Namvar A, Kostrzynska M, Hora R & Warriner K. 2007. Persistence and growth of different *Salmonella* serovars on pre- and postharvest tomatoes. *Journal of Food Protection* 70(12):2725-2731.
- Sivapalasingam S, Friedman CR, Cohen L & Tauxe RV. 2004. Fresh produce: A growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *Journal of Food Protection* 67(10):2342-2353.
- Szabo EA & Mackey BM. 1999. Detection of *Salmonella* enteritidis by reverse transcription-polymerase chain reaction (PCR). *International Journal of Food Microbiology* 51(2-3):113-122.
- Wang H & Slavik MF. 2005. A multiplex polymerase chain reaction assay for rapid detection of *Escherichia coli* O157 : H7, *Listeria monocytogenes*, *Salmonella* Typhimurium and *Campylobacter jejuni* in artificially contaminated food

- samples. *Journal of Rapid Methods and Automation in Microbiology* 13(3):213-223.
- Werbrouck H, Botteldoorn N, Uyttendaele M, Herman L & Van Coillie E. 2007. Quantification of gene expression of *Listeria monocytogenes* by real-time reverse transcription PCR: Optimization, evaluation and pitfalls. *Journal of Microbiological Methods* 69(2):306-314.
- Wilson IG. 1997. Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology* 63(10):3741-3751.

Appendix

Table 3: Recovery of *Salmonella* Typhimurium from lettuce by plating on XLT4 agar and nanodrop readings of RNA extracts without enrichment

Buffer	Inoculum Size	Cells Recovered by Plating on XLT4	RNA Extracted ng/ μ l
Glycine-Saline	10^6	$9.0 \times 10^1 \pm 9.8$	16.83 ± 0.49
Glycine-Saline w/ 0.05% Tween [®] 20	10^6	$1.0 \times 10^1 \pm 0$	9.95 ± 8.22
Glycine-Saline w/ 0.1% Tween [®] 20	10^6	$2.45 \times 10^2 \pm 12$	17.35 ± 1.08
Glycine-Saline w/ .05% Tween- 20 & 3% Beef Extract	10^6	$2.52 \times 10^3 \pm 3.7$	23.13 ± 1.37

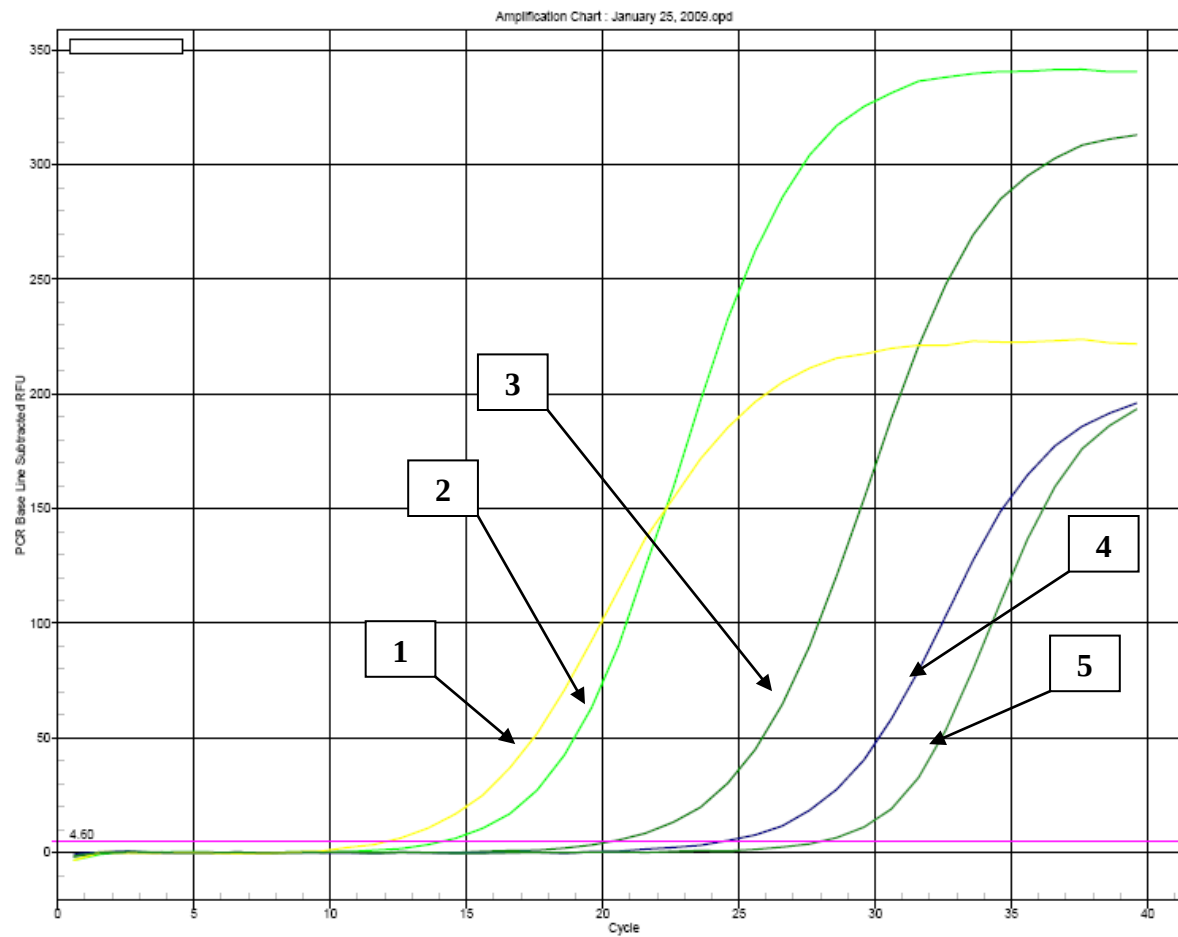


Figure 1: Detection limits of *Salmonella* showing cycle threshold values (Ct) from spiked Lettuce by rt-PCR without enrichment. Positive Control (1), Log 8 (2), Log 7 (3), Log 6 (4) CFU/25g, and Negative Control water (5)

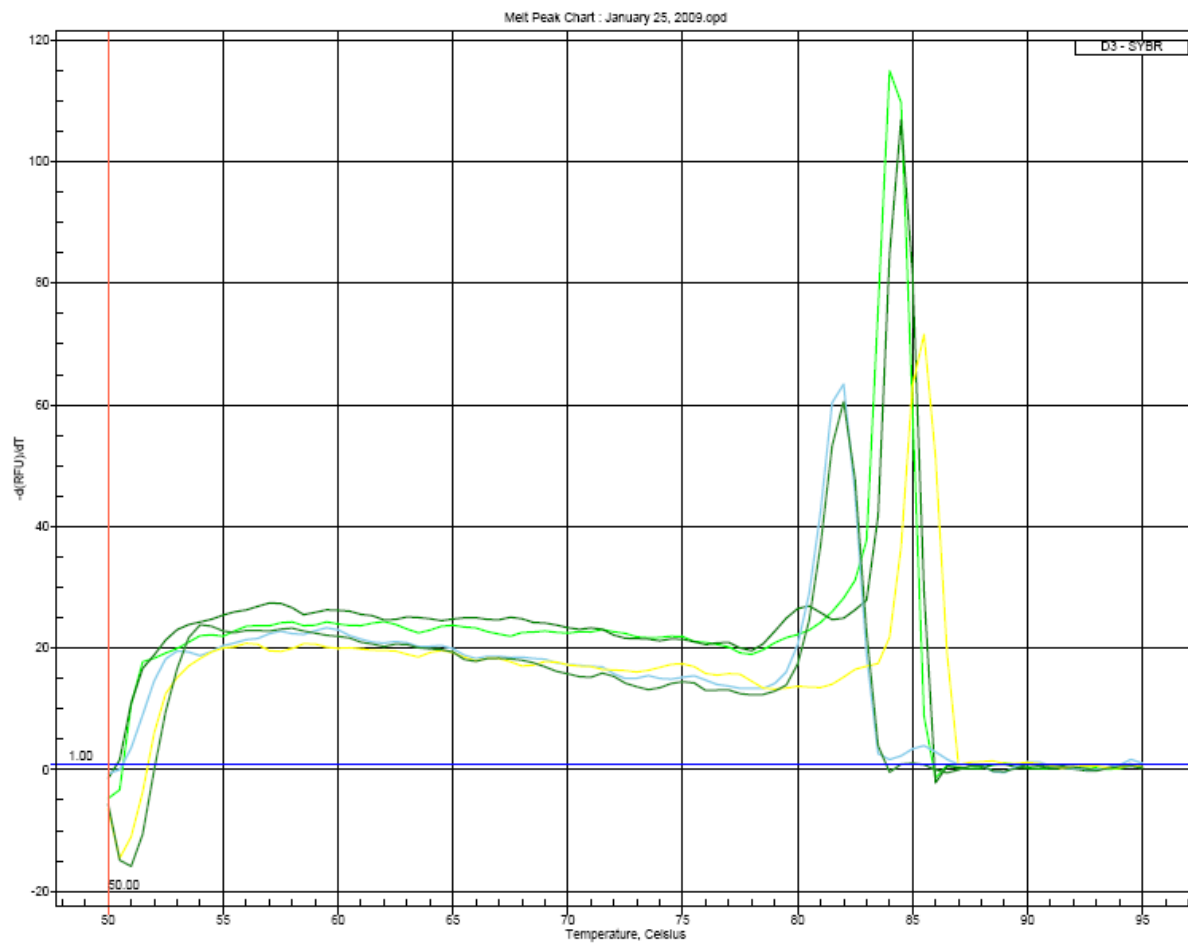


Figure 2: Melt temperature curve depicting specific *Salmonella* amplification products at ~85°C with detection limits of 106 without enrichment from Lettuce. The T_m of the *invA* product occurs ~85°C and the T_m of the IAC occurs at 82°C.

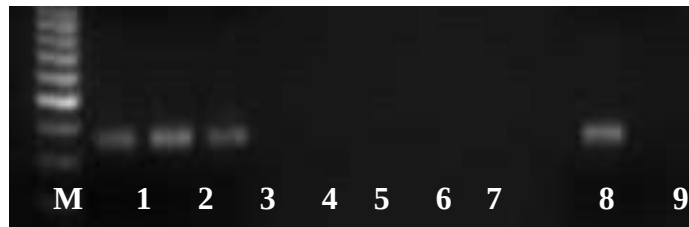


Figure 3: Agarose gel electrophoresis of rt-PCR amplified products (347bp) from lettuce spiked with *Salmonella* Typhimurium without enrichment. Lane (M): 100 bp ladder marker. Lane (1-8): products from lettuce spiked with log 8 to log 1 of *S. typhimurium*. Lane (9): positive control of RNA extracted from *S. Typhimurium*. Lane (10): negative control (water)

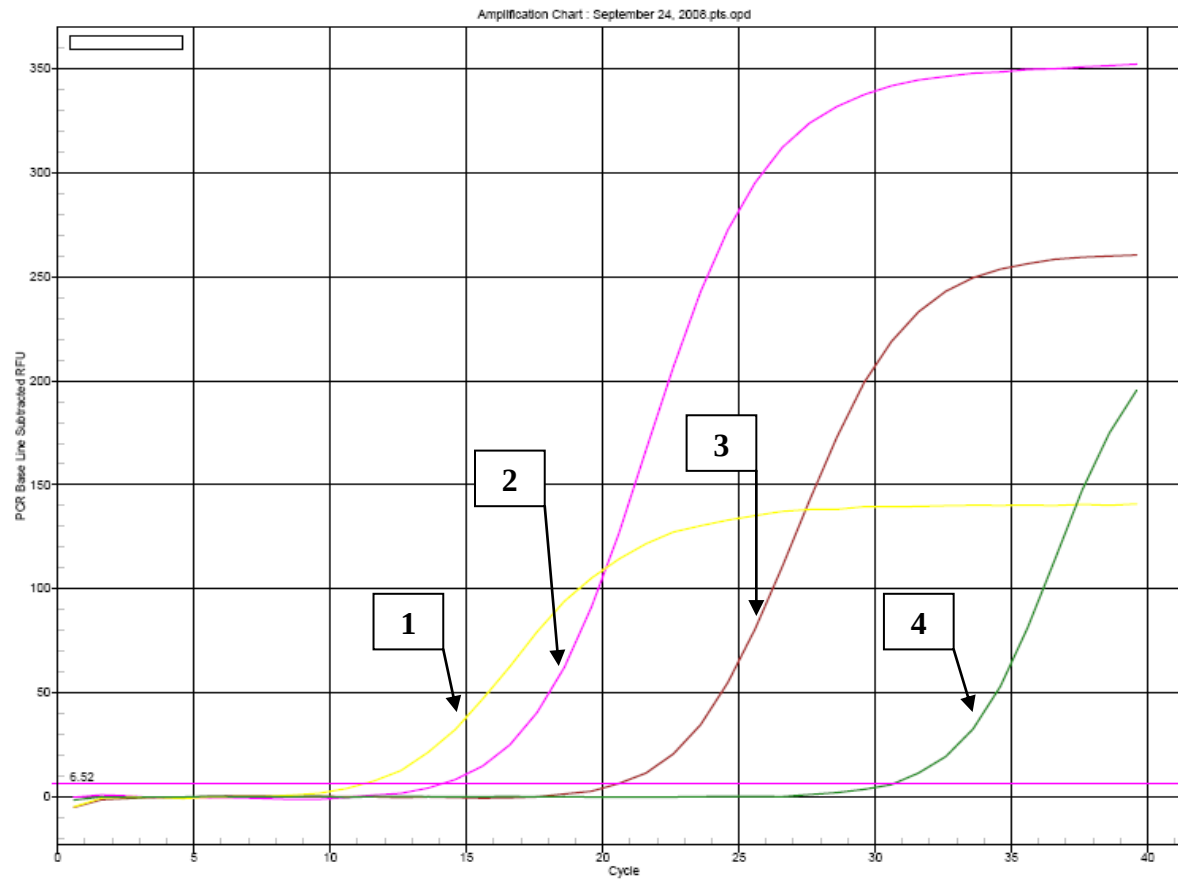


Figure 4: Detection limits of Salmonella showing cycle threshold values (Ct) from spiked Lettuce by rt-PCR with enrichment. Positive Control (1), Log 5 (2), Log 4 (3), and Negative Control water (4) showing detection limits of 104 CFU/25g for lettuce.

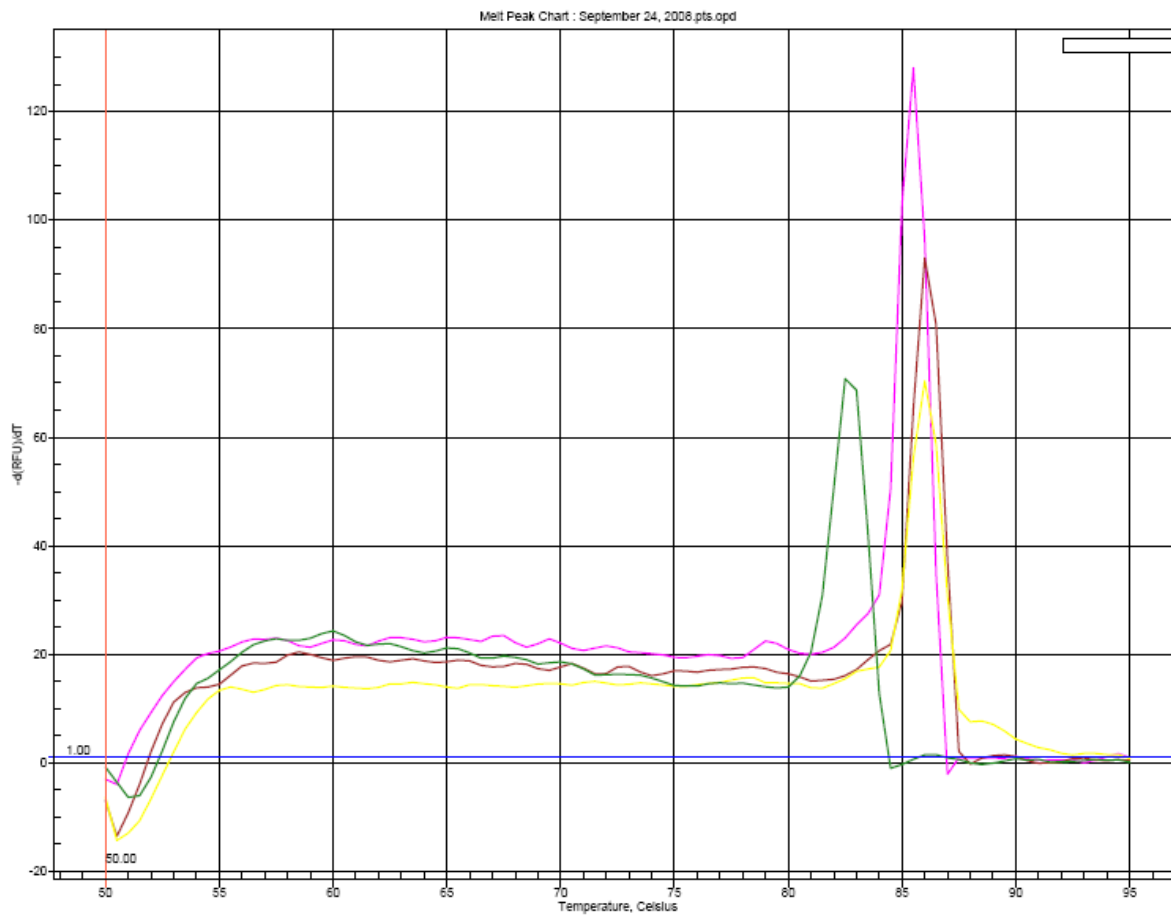


Figure 5: Melt temperature curves depicting specific *Salmonella* amplification products at ~85°C with detection limits of log 4 CFU/25g with enrichment from lettuce. . The T_m of the *invA* product occurs ~85°C and the T_m of the IAC occurs at 82°C.

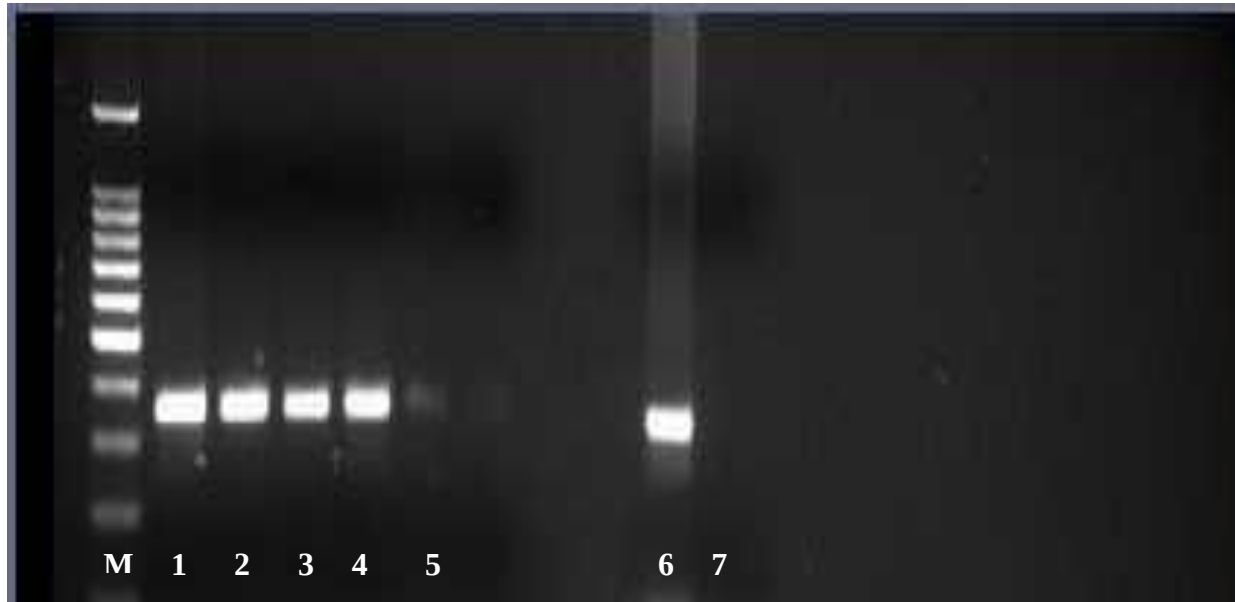


Figure 6: Agarose gel electrophoresis of rt-RT-PCR amplified products (347) from lettuce spiked with *Salmonella* Typhimurium after enrichment. Lane (M): 100 bp ladder marker. Lane (1-5): products from lettuce spiked with log 8 to log 4 CFU of *S. Typhimurium*. Lane (6): positive control of RNA extracted from *S. Typhimurium*. Lane (7): negative control (water)

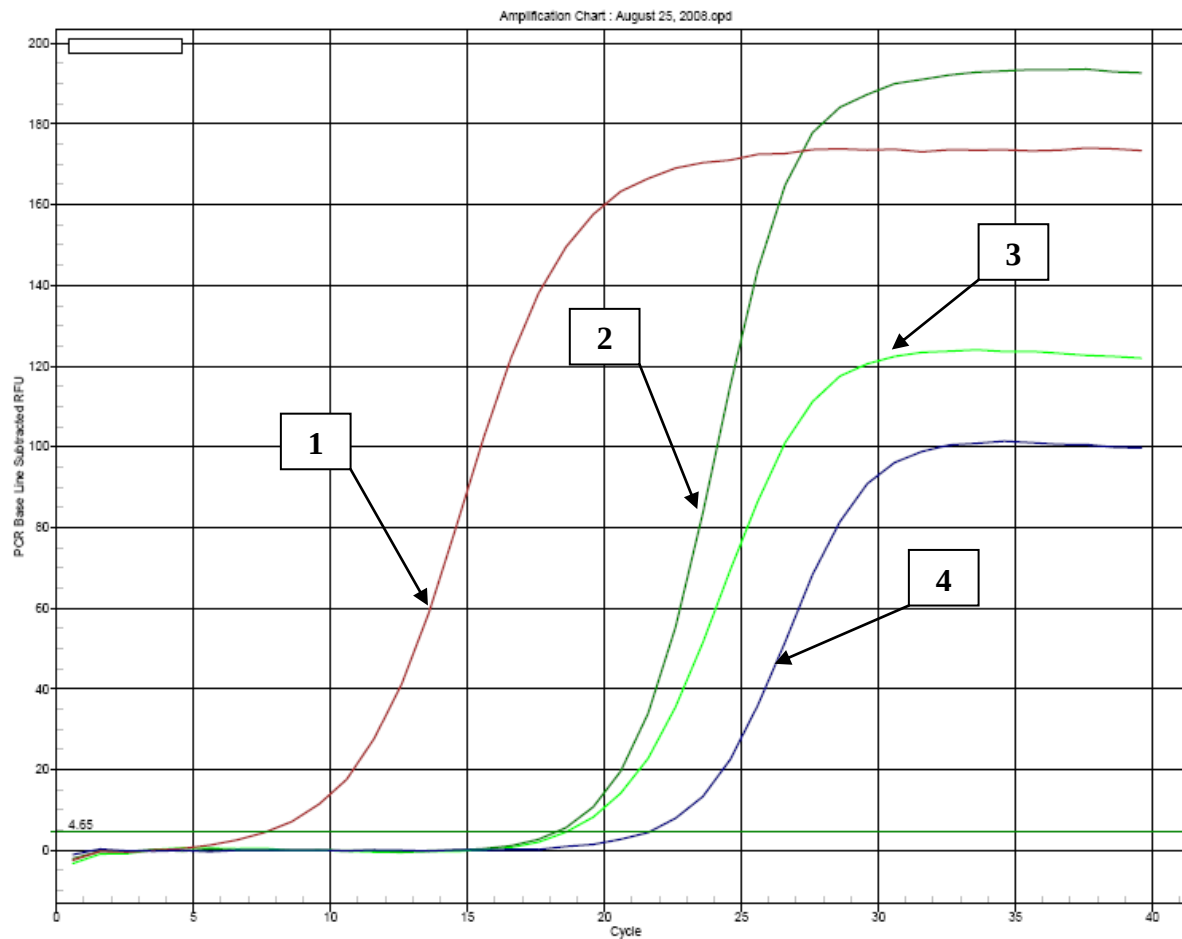


Figure 7: Detection limits of *Salmonella* showing cycle threshold values (Ct) from spiked tomatoes by rt-PCR without enrichment. Positive Control (1), Log 8 (2), Log 7 (3), and Negative Control water (5) showing a detection limit of 10^7 CFU/ml for tomatoes.

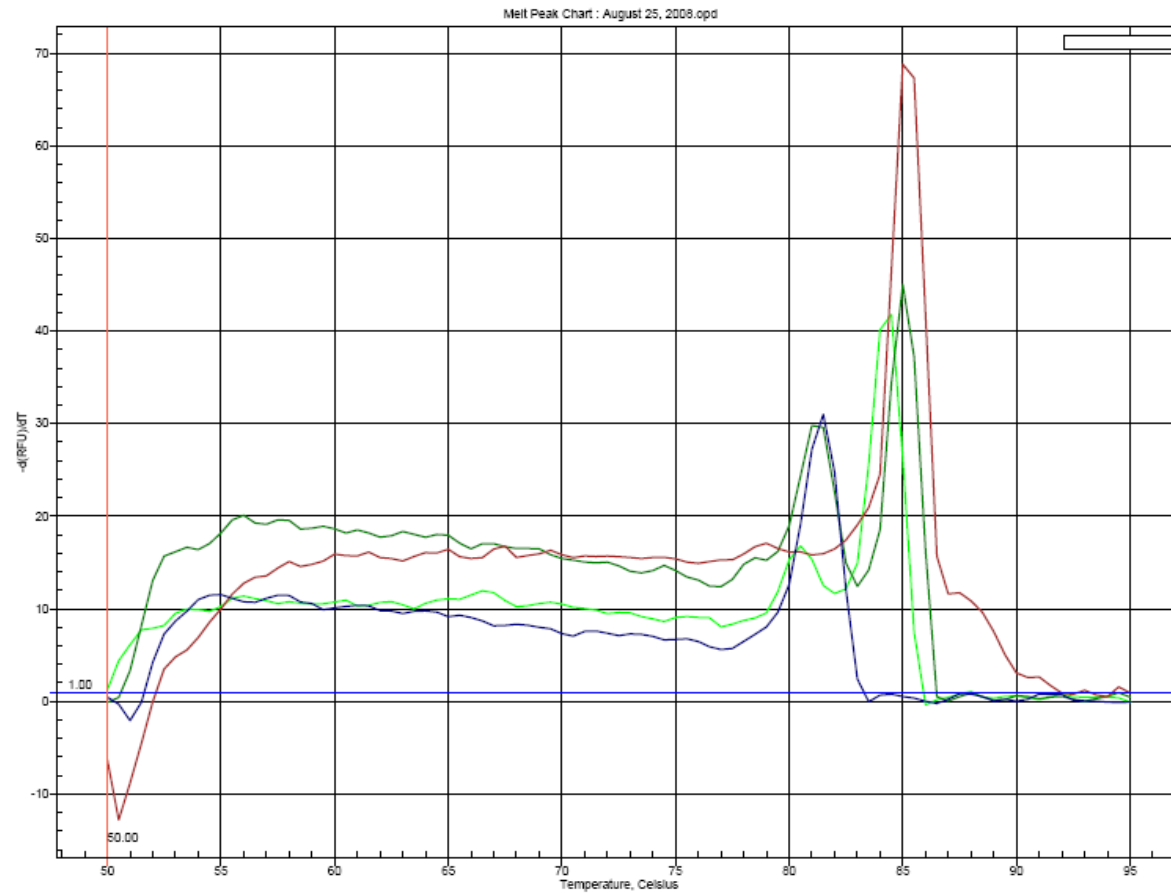


Figure 8: Melt temperature curve depicting specific *Salmonella* amplification products at ~85°C with detection limits of log 7 CFU/25g without enrichment from Tomatoes. The T_m of the invA product occurs ~85°C and the T_m of the IAC occurs at 82°C.

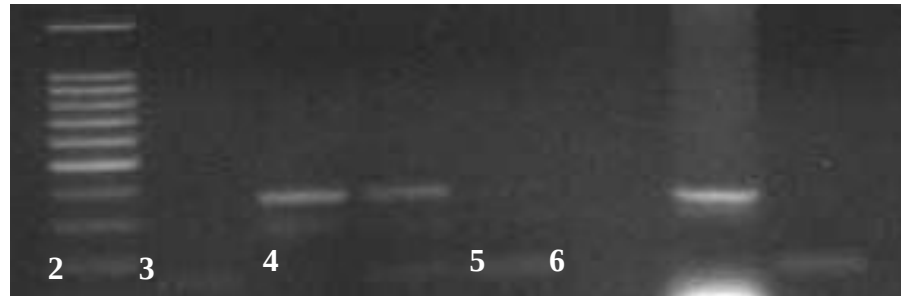


Figure 9: Agarose gel electrophoresis of rt-PCR amplified products (347bp) from Tomatoes spiked with *Salmonella* Typhimurium without enrichment. Lane (M): 100 bp ladder marker. Tomato control no inoculation (1), Lane (2-4): products from Tomatoes spiked with log log 8 to log 4 CFU of *S. Typhimurium*. Lane (6): positive control of RNA extracted from *S. Typhimurium*. Lane (7): negative control (water)

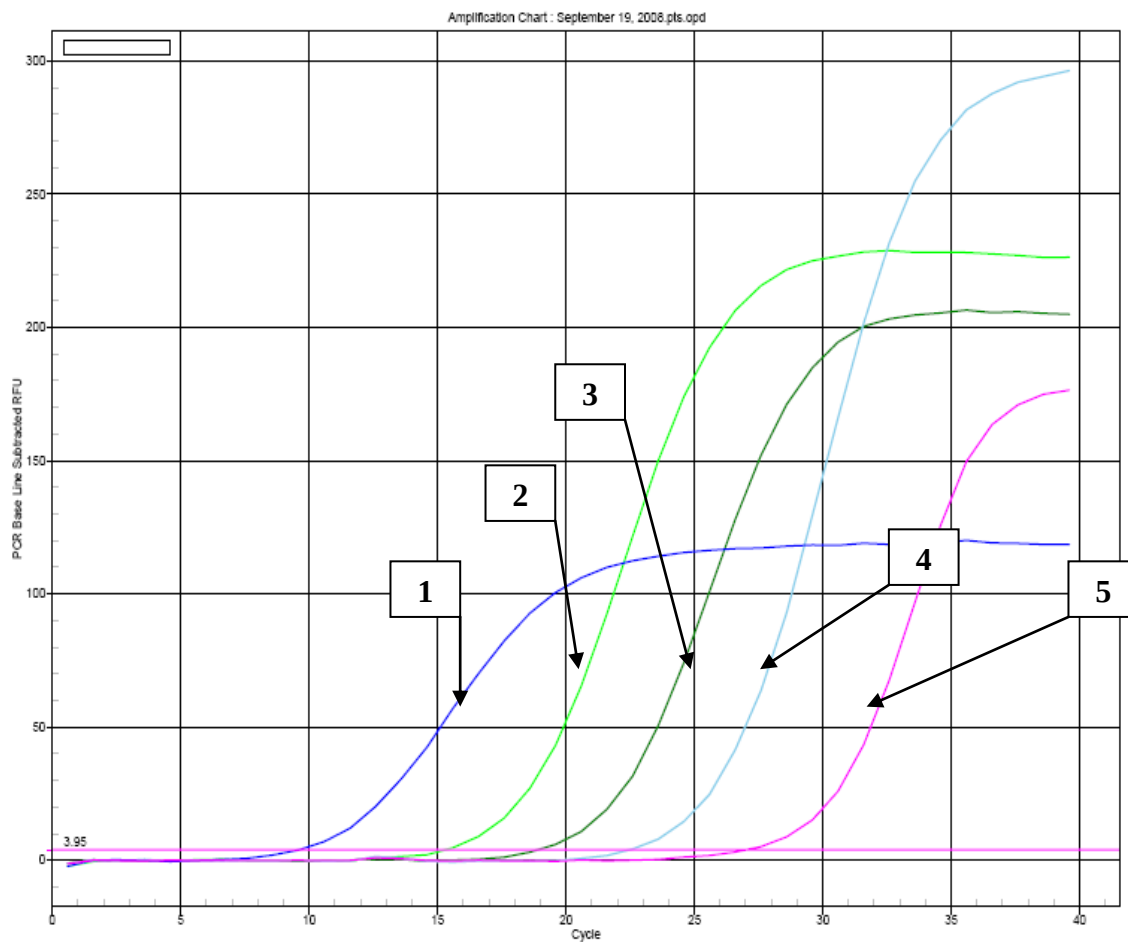


Figure 10: Detection limits of Salmonella showing cycle threshold values (Ct) from spiked Tomatoes by rt-PCR with enrichment. Positive Control (1), Log 6 (2), Log 5 (3), Log 4 (4), and Negative Control water (5)

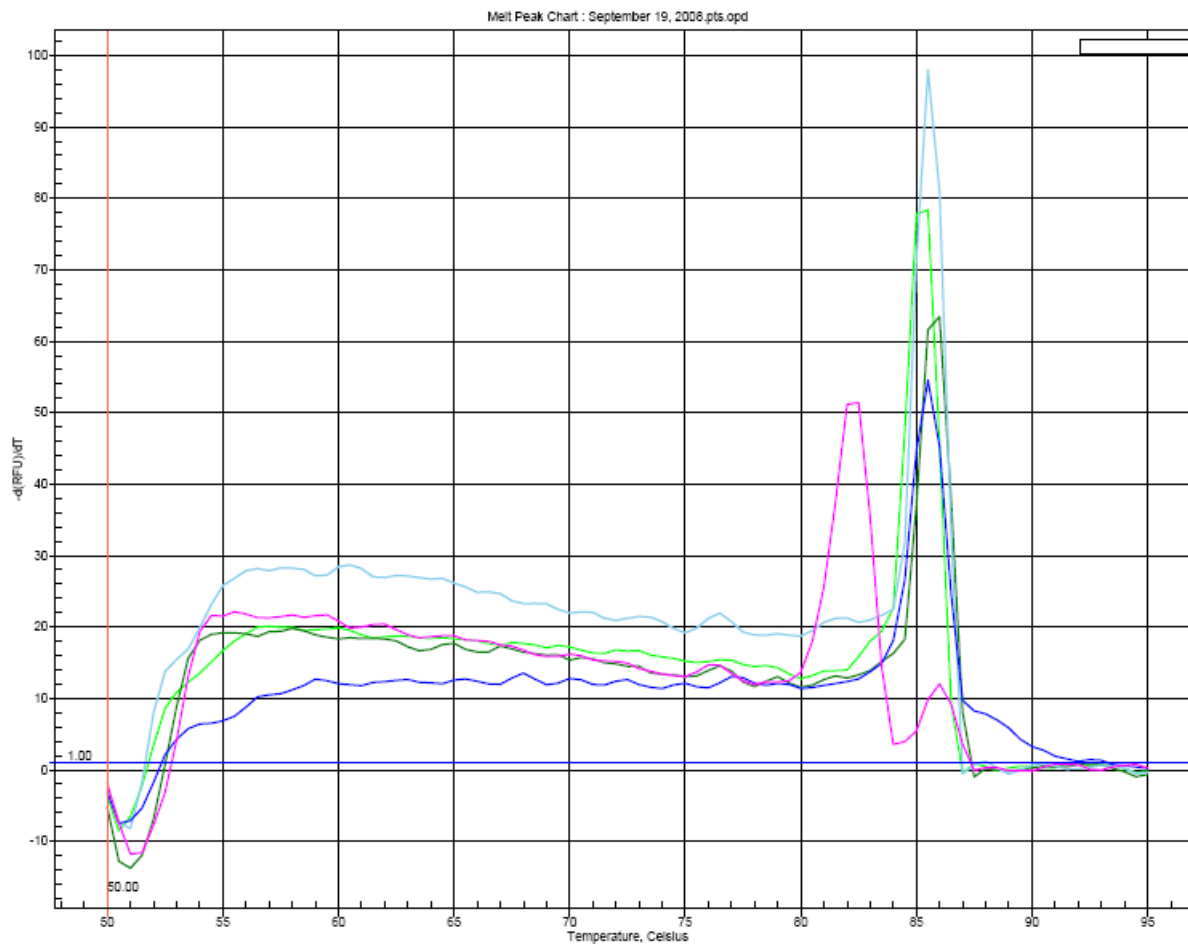


Figure 11: Melt temperature curve depicting specific *Salmonella* amplification products at ~85°C with detection limits of 10^7 without enrichment from Tomatoes. . The T_m of the *invA* product occurs ~85°C and the T_m of the IAC occurs at 82°C.

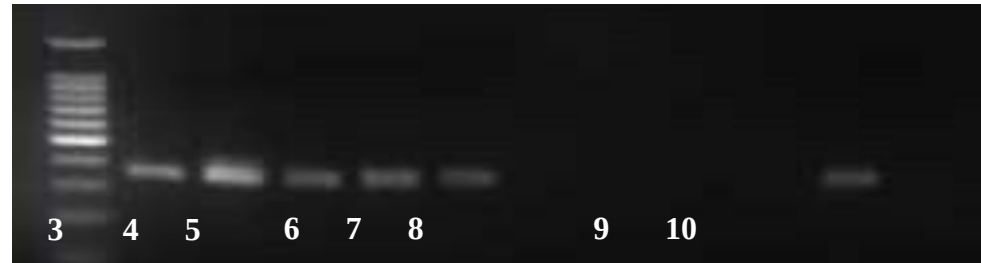


Figure 12: Agarose gel electrophoresis of rt-PCR amplified products (347bp) from Tomatoes spiked with *Salmonella* Typhimurium without enrichment. Lane (M): 100 bp ladder marker. Lane (1-8): products from Tomatoes spiked with 10^8 to 10^1 CFU of *S. Typhimurium*. Lane (9): positive control of RNA extracted from *S. Typhimurium* pure culture. Lane (10): negative control (water)

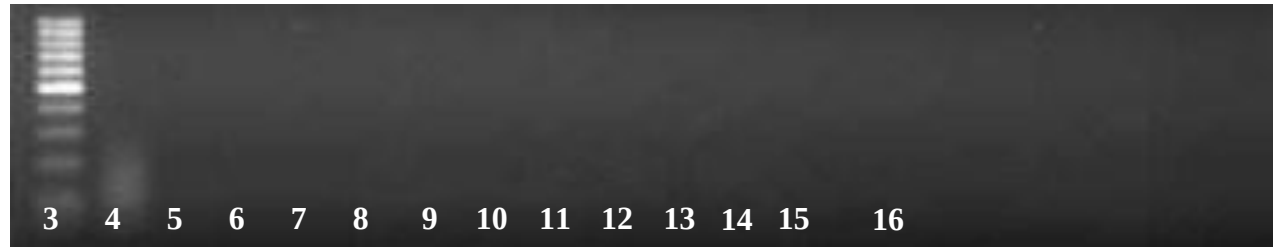


Figure 13: Agarose gel electrophoresis of PCR products using RNA treated with DNase I. Lane (M): 100 bp ladder marker. Lane (1-7): products from *Salmonella Typhimurium*. Lane (8-11): products from Spiked Lettuce (10^7 , 10^6 , 10^5). Lane (12-15): products from Spiked Tomatoes (10^7 , 10^6 , 10^5). Lane (16): negative control (water)



Figure 14: Agarose gel electrophoresis of reverse-transcriptase PCR treated with DNase I. Lane (M): 100 bp ladder marker. Lane (1-7): products from *Salmonella Typhimurium*. Lane (8-11): products from Lettuce. Lane (12-15): products from Tomatoes. Lane (16): positive control of *S. Typhimurium* from RNA extraction. Lane (17): negative control (water)

CHAPTER III
Real-time reverse-transcriptase PCR for *salmonella*
***typhimurium* detection for jalapeño and Serrano peppers**

PCR Detection of *S. Typhimurium* from Peppers

Real-time PCR for *Salmonella* Typhimurium detection from Jalapeño and Serrano
Peppers

Nathan D. Miller and Doris H. D'Souza

University of Tennessee, 2605 River Drive Room 100 FSPB, Knoxville, TN 37996

Key words: *Salmonella* Typhimurium, Real-time PCR, Jalapeño and Serrano Peppers

Doris H. D'Souza. Tel: (865)974-2753; Fax: (865)974-7332
E-mail: ddsouza@utk.edu

Abstract

Outbreaks of *Salmonella* linked to fresh produce emphasize the need for rapid detection methods to curb their spread. Reverse-transcriptase-PCR (RT-PCR) detects the presence of mRNA (shorter half-life than DNA), with greater potential of detecting viable pathogens. Use of real-time PCR eliminates the need for gel electrophoresis and significantly enhances the speed of detection (< 1 day) compared to traditional methods (> 5 days). The objectives of this research were to apply real-time PCR to detect *Salmonella* from Jalapeño and Serrano peppers spiked with low and high inocula. Approximately 25g peppers were inoculated with 10^8 to 10^1 CFU *Salmonella Typhimurium*. Inoculated and un-inoculated peppers were rinsed with water, dried under ultraviolet light for 10 minutes, and hand massaged in 0.05M glycine saline-buffer (0.05% Tween, 3% beef extract) for optimal recovery of bacteria. A short pre-enrichment step of 6 hours in buffered peptone water for detection at low inocula. Portions were plated on XLT4 and portions used for RNA extraction using the Qiagen RNeasy® Mini Kit. RT-PCR was carried out using SYBR Green one step RT-PCR with *invA* gene primers and an internal amplification control. Detection limits were 10^4 CFU/25g from enriched and 10^7 CFU/25g on un-enriched inoculated peppers. Even though this method included a 6 hour incubation period, the results were still obtainable in one day. This method shows promise for applications in routine testing during outbreaks and real-world scenarios.

Introduction

The demand for chili peppers has been on the rise over the past 20 years as trends in consumption of various cuisines increase. Two types of chili peppers, Jalapeño and Serrano originating from a farm in north eastern Mexico, were recently implicated in an outbreak of salmonellosis caused by *Salmonella* Saintpaul (CDC, 2008). The source of the bacteria was determined to be irrigation water that was used on the crops (CDC, 2008). According to the CDC, approximately 1438 people in 43 U.S. states and in Canada became infected with *Salmonella* Saintpaul linked to the peppers (CDC, 2008).

With the demand for fresh chili peppers comes a challenge in maintaining and distributing safe products. In the mid 90's, the US imported about 200 million pounds of fresh chili peppers which increased to just under 675 million pounds in recent years. From 1995 to 2000, these numbers grew about 188 percent and increased by an estimated 72 percent during 2000 to 2007. The per capita consumption of chili peppers are up about 1 pound since 2000 with the total consumption about 6.1 pounds (Lucier and Dettmann, 2008). Since many of our peppers are imported, safety has become more of a concern because of the various contamination sources and the security at the farm level.

Salmonella, a gram negative bacterium causing gastroenteritis, is mainly transmitted through the fecal-oral route. Produce can become contaminated with *Salmonella* through various routes. Some of these ways are the utilization of manure instead of chemical fertilizers and the utilization of untreated sewage or irrigation water

(Beuchat and Ryu, 1997). According to Beuchat et. al. (1997), wild birds have also been known to carry pathogenic bacteria in their feces. Besides direct fecal contamination, some of the pre-harvest points of contamination are soil, irrigation water, inadequately composted manure, air/dust, and human handling. A few of the pre-harvest points of contamination also coincide with the post-harvesting conditions for contamination. Some of the post-harvesting points of contamination are feces, human handling, harvesting equipment, transport containers, air/dust, wash and rinse water, processing equipment, ice, transport vehicles, cross-contamination, improper storage temperatures, and improper handling at the retail level (Beuchat, 1996). Contamination can then occur through various modes from the farm to the table. Rapid sensitive methods of detection may contribute to reduction in contaminated products being sold in the market.

In recent years, the use of molecular technologies such as polymerase chain reaction (PCR) and real-time polymerase chain reaction (RT-PCR) have enabled the rapid detection of foodborne pathogens to enhance food safety. PCR uses gel electrophoresis to visualize the presence or absence of the target product, while RT-PCR uses a melt temperature of the target DNA to visualize the presence or absence of the target product. Reverse-transcription PCR (rt-PCR) is a method for potentially detecting viable bacteria by targeting the mRNA of the cell. This is useful because mRNA has a shorter half-life than DNA (Maurer, 2006). This could allow for the detection of live cells or recent contamination rather than cells that have been killed due to implemented

control measures to eliminate bacteria from the food product or environment. Rapid detection technology such as real-time RT-PCR can help the producer recall the fresh produce from the market at a much faster rate than previously done by traditional methods that can take ≥ 5 days. Real-time RT-PCR can also be applied to foods to potentially detect the presence of viable organisms.

Previous studies involving RT-PCR or PCR detection of *Salmonella* from produce were able to detect low limits of 1-10 CFU/ml and 100 CFU / ml from mixed salad (Bhagwat, 2004); 1 CFU/ml, 10 CFU/ml, and 100 CFU/ml from leaf lettuce and apples (Shearer and others 2001); and 1 CFU/ml mixed salad, cilantro, broccoli, cauliflower, and cabbage (Bhagwat, 2003). Much of these lower limits of detection were aided by a pre-enrichment media process of no more than 24 hours. For example, incubating poultry meat for 24 hours allowed a detection limit of 10^3 CFU/ml (Kanki and others 2009) using PCR. A detection limit of 10^3 CFU/g was demonstrated for buffalo meat trimmings using an 18 hour incubation period (Biswas and others 2008). Finally using RT-PCR, a detection limit of less than 5 CFU/25g was shown for alfalfa sprouts, cantaloupe, mixed salad and cilantro when incubated for 24 hours (Liming and Bhagwat, 2004).

The purpose of this study was to determine if a method could be developed for the detection of *Salmonella* from Jalapeño and Serrano peppers within one day using rt-PCR. High (10^8 to 10^6 CFU/ml) and low (10^2 to 10^0 CFU/ml) inocula were used and, for the latter, short enrichment times of 6-hours were applied. Previously described *invA*

primers were used that targeted the chromosomally located *invA* gene specific for *Salmonella* species in a SYBR green I RT-PCR one-step assay.

Materials and Methods:

Growth of *Salmonella enterica* serovar Typhimurium

Salmonella enterica serovar Typhimurium (DT104 2486) was grown in 10 ml of Trypticase Soy Broth (TSB; Difco, Becton Dickinson and Co.; Sparks MD) at 35°C for 24 h. The cultures were transferred two consecutive times at 24 h intervals before use. A ten-fold serial dilution of an overnight culture (1-ml to 9 ml of peptone buffer (Oxoid LTD; Basingstoke Hampshire, England) per tube), was plated on Trypticase Soy Agar (TSA; Difco, Accumedia Lansing, MI) and Xylose Lactose Tergitol 4 agar (XLT4) and incubated to determine counts.

Preparation of Food Source

Chili peppers (Jalapeño and Serrano peppers) were purchased at a local grocery store. Before inoculation, the chili peppers were washed for 1 minute using tap-water and placed in an open sterile Petri plate in a hood to dry under ultra violet light for 10 min at room temperature. The chili peppers (25g) were inoculated with 0.1 ml *Salmonella enterica* serovar Typhimurium high inocula (10^6 to 10^4) and low inocula (10^2 to 10^0) and allowed to dry for 10 min at room temperature under aseptic conditions in a BSL-2 hood. Washed and dried un-inoculated peppers were used as controls. Each experiment was replicated at least twice.

Extraction from Inoculated Food Source

Bacteria were eluted from peppers by washing with 10 ml of 0.05 glycine-0.14N saline buffer (pH 9.0) (glycine, Fisher Scientific, Fair Lawn, New Jersey) and Tween-20 (Promega; Madison, WI) to aid in removal of bacterial cells. To improve bacterial recovery, 0.05% Tween-20 added to ten-ml of glycine-saline buffer as well as glycine-saline with 3% beef extract and 0.05% Tween-20 were individually tested and added to the chili pepper in a sterile stomach bag. The samples were hand rubbed for 1 minute and a 10 ml sample was taken and centrifuged at 8000 x g for 10 min at 4°C (Sorvall Legend 23 R; Thermo Electron Corporation; Asheville, NC). The supernatant was decanted and the cell pellet was resuspended in 1 ml of 0.1% peptone buffer (Oxoid) before proceeding with plate counting and the isolation of RNA.

Enumeration of the Bacteria

For low inocula levels, short-enrichment times were needed. Peppers were placed in 10 ml of sterile peptone water (Oxoid) and incubated for 6 h at 35°C. After the 6 h incubation period, a 10 ml sample was removed and placed in a 15 ml centrifuge tube and spun at 8000 x g for 10 min at 4°C (Sorvall Legend 23 R; Thermo Electron Corporation; Asheville, NC). The supernatant was decanted and the cell pellet was resuspended in 1 ml of peptone buffer (Oxoid). Serial dilutions of enriched and un-enriched samples were plated on XLT4 agar and incubated at 37°C for 24 to 48 h to enumerate typical black colonies for *Salmonella* detection.

Isolation of Total RNA from Bacteria

RNAlater Stabilization Reagent (Qiagen; Valencia, CA) (1.2 ml) was added to 0.6 ml of each sample. The combination of stabilization reagent and bacteria culture/extract was incubated at room temperature for 10 min and vortexed several times. The cells were centrifuged (Eppendorf 5417C; New York, NY) for 10 min at 8000 x g at 4°C and the pellet was stored at -80°C or used directly for extraction. The RNeasy Mini Purification Kit (Qiagen) was used to extract RNA from *Salmonella* Typhimurium, enriched and un-enriched inoculated pepper samples, un-inoculated pepper controls, and peptone water.

One hundred µl of Tris-EDTA (TE; 10 mM Tris-Cl, 1 mM EDTA, pH 8.0; Promega; Madison, WI) containing lysozyme (3 mg/ml; Sigma-Aldrich; St. Louis, MO) was added to each pellet, vortexed, and incubated at room temperature for 10 min with mixing. RLT buffer (350 µl) was added, followed by 250 µl of Absolute-200 proof ethanol (Aaper Alcohol; Shelbyville, KY) and transferred to the RNeasy mini column. The column was centrifuged at 8000 x g for 15 seconds and the collection tube was discarded. RW1 buffer (700 µl) was added to the column and centrifuged for 15 seconds at 8000 x g to wash the column. RPE buffer (500 µl) was added and centrifuged at 8000 x g for 15 seconds and repeated once. The RNA was then eluted off the column with 40 µl of RNase-free water and centrifuging at 8000 x g for 1 minute. The collection tubes containing RNA were capped and stored at -80°C until further analyses.

DNase I Treatment

DNase I treatment was carried out using the TURBO DNA-free™ Kit (Ambion®; Applied Biosystems; Foster City, CA). The procedure consisted of 5 µl of 10x TURBO DNase Buffer, 1 µl of rDNase I DNA-free™, and 34 µl of Nuclease-free Water. The reagents were then mixed with 10 µl of RNA and incubated at 37°C for 30 min. 5 µl of DNase Inactivation Reagent were added after incubation for 30 min and then incubated for 2 min. The sample was centrifuged at 10,000xg for 2 min. The supernatant was removed and placed in a new tube.

Nanodrop Quantification of RNA

After extraction of the RNA, a quick method to quantify the RNA was carried out. Quantification was done using a NanoDrop® Spectrophotometer (NanoDrop® ND-1000 Spectrophotometer; Thermo Scientific; Wilmington, DE). The concentration of the nucleic acid was confirmed using UV-spectrophotometry at 260 nm while, the purity was determined using the ratio 260/280 for determining protein and 260/230 for organic carry-over respectively. The two ratios needed to be above 1.85 to provide a good sample of RNA with little to no contamination.

Real-Time Reverse-Transcriptase Polymerase Chain Reaction (rt-RT-PCR) Procedure

The SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen; Carlsbad, California) was used in the amplification of mRNA to cDNA followed by DNA amplification. Twenty-five µl reactions contained 5 µl RNA extracts in RNase-DNase free

water, Superscript III (SSIII) one-step RT-PCR kit reagents (Invitrogen) with SYBR Green I, 0.02 μ M of each *invA* primer (**F: 5'CACGCTCTTTCGTCTGGCA3'**; **R2: 5'TACGGTTCCTTTGACGGTGCGA3'**) and bovine serum albumin (BSA) with a final concentration of 1.6 μ l and an internal amplification control (IAC) (see below) obtained from Sigma-GenoSys (St. Louis, MO, USA). The thermocycler conditions were carried out as follows, RT at 50°C/30min, denaturation at 95°C/5min, followed by 45 cycles at 95°C/45s, 58°C/45s, 72°C/45s, and final extension at 72°C/7min in a Bio-Rad iCycler (Hercules, CA, USA). After amplification, melt temperature analysis from 50°C to 95°C with 0.5°C increments was carried out. The software provided by iCycler was used to obtain the threshold cycle (C_T) values, melt temperatures (T_M), and the standard curve. The expected melt temperature was ~85°C for the *Salmonella* specific *invA* product and 82°C for the IAC product. Products were separated by agarose gel electrophoresis (2% agarose gels) (in 1X Tris-acid-EDTA (TAE) buffer; Fisher Scientific, Pittsburgh, PA, USA), stained with ethidium bromide, visualized under ultraviolet light, and photographed using the Gel Doc System/Station (Bio-Rad). A DNA, 100 bp (Promega) marker, was also run to determine the presence of the 347 bp target amplified product.

Preparation of the Internal Amplification Control (IAC)

The Internal Amplification Control was added to the One-Step qRT-PCR mix in order to eliminate the concern of false negatives due to PCR inhibitors, enzyme inactivation or instrument malfunction. The Beacon Designer Software (BioRad) was used to help design the *stx 1* primer set from the shiga toxin region of *E. coli* O157:H7

DNA to yield a product of 109 bps. The forward and reverse *invA* primer was coupled to the *stx1* forward and reverse primers respectively to amplify a 182 bp product. A T₇ promoter added to the forward primer and amplified using the MEGAscript T7 Transcription Kit (Ambion). The concentration of the amplified RNA product was diluted in order to reach an optimal concentration of 1.9 fg/μl for the rt-PCR procedure.

Results

Bacterial Cell Recovery from Peppers

Extracting the target *Salmonella* from the pepper skin proved to be much easier than removal of the target from the stem of the pepper. The hard smooth surface of the pepper without any crevices left no room for trapping target bacteria within (Rusin and others 2002). Our recovery of *Salmonella* Typhimurium cells, as determined by plating from the surface of the Jalapeño and Serrano peppers, was about 10³ CFU from the initial inocula of 10⁷ CFU/ml using the optimal buffer containing Tween-20 and beef extract. With 0.1% glycine-saline, the addition of the 0.05% Tween-20 and 3% Beef Extract to the extraction solution, this allowed for an increase in the recovery of the target *Salmonella* cells. The addition of Tween-20 acted as a surfactant to help lift the cells off the pepper's outer surface and into solution in order to be detected. Table 4 shows the CFU counts that were recovered from each of the extraction buffers used in optimizing the extraction buffers from previous research. For inocula levels without enrichment and with initial 10⁷ CFU/ml inoculation, recovery was low ~1.0x10³ CFU

hence enrichment of 6 h was needed. After enrichment with an initial 10^4 CFU/ml inoculation, recovery remained $\sim 1.08 \times 10^3$ CFU.

RNA Extraction Yields from Peppers

RNA extracted using the RNeasy Mini Purification Kit were quantified using a Nanodrop spectrophotometer. For detection of *Salmonella* without enrichment, the 10^7 detection limit contained 6.97 ± 2.03 ng/ μ l. After an enrichment process, with an improved 10^4 detection limit, 263.34 ± 18.39 ng/ μ l of RNA was extracted.

rt-PCR Detection and Specificity

Detection of 10^7 CFU/25g from both inoculated Jalapeño and Serrano peppers without enrichment was achieved using RT-PCR (Figure 15). For low inocula levels, a 6 h enrichment was necessary, which increased the detection limit by three log to 10^4 CFU/25g (Figure 18) for both Jalapeño and Serrano peppers. This showed that the 6 h enrichment did aid in better detection of the target bacteria at lower levels and could still obtain results in 1 day. To determine specificity of the amplified products melt temperature analysis was carried out. T_m curves showed peaks at $\sim 85^\circ\text{C}$ and IAC showed peaks at 82°C . This showed that false negatives were not obtained and that indeed the detection limit was 10^4 CFU/25g (Figure 16 & 19). This is true because of the IAC peak produced at 82°C showed that no inhibitors were present and that there was no equipment malfunction or enzyme failure. Gel electrophoresis revealed the 347 bp target product as expected, with similar detection sensitivity to RT-PCR Ct values of 10^4

CFU/25g. Negative controls did not show any Tm's at 85°C or any product on the gel. Negative controls consisted of water, TSB, peptone, or uninoculated peppers that did show the Tm at 82°C of the IAC (Figure 17 & 20). This showed that false negatives were not obtained.

DNase I Treatment

DNase I treatment was performed in order to determine if amplification is from RNA or carryover DNA. When the treatment was applied to our study, the ability to detect the target RNA from Jalapeño and Serrano peppers was reduced. A one log reduction in the detection limit for Jalapeño and Serrano peppers when applied to rt-PCR was obtained to give 10^8 CFU/25g in un-enriched peppers. Also, PCR on the DNase I treated extracts did not yield any positive PCR products as expected, showing the absence of any DNA carryover (Figure 21 & 22) due to the fact that a one-step qRT-PCR kit was used.

Discussion

The recovery of *Salmonella* from Jalapeño and Serrano peppers were obtained through gently messaging the outer surface of the pepper in an optimal glycine-saline buffer solution containing 3% beef extract and 0.05% Tween-20. Previous research has suggested that the attachment of bacterial cells to the surface of produce could be due to the roughness or hydrophobic properties of the produce surface (Iturriaga and others 2003). This study used a buffer solution that contained a surfactant to aid in removal of

the *Salmonella* cells from the surface of peppers. Our recovery of *Salmonella* Typhimurium cells, as determined by plating from the surface of the Jalapeño and Serrano peppers, was about 10^3 CFU/ml from the initial inocula of 10^7 CFU/ml using the optimal buffer containing Tween-20 and beef extract. Although this percentage is low, other factors may have played a role in the low recovery. According to a study done by Shi and others (2007), the different serovars of *Salmonella* may play a role in how the bacteria may attach to the surface (Shi and others 2007). Another factor that can affect recovery is cell viability. The death or injury of the cell can be attributed to the cells drying time on the produce's surface (Lang and others 2004). Traditional methods use long enrichment steps to increase cell numbers.

The major advantage to real-time-PCR is the speed of detection with results within a 10 hr period. Results can be obtained within one day as compared to traditional culturing methods which can take upwards of about 5-7 days (Feng, 2007). An advantage of using reverse-transcription-PCR is it allows for the detection of viable cells within the food product. This is because one is targeting the mRNA of a certain bacterium. The advantage of using mRNA from the target bacteria is that mRNA has a shorter half life than DNA and therefore has greater potential of detecting primarily viable cells that could cause infection (Maurer, 2006). Since most of the quality control analyses are done on the finished product, this is an excellent way to determine if the critical control points (CCP) are working. Several other studies have shown an 18-24 h enrichment procedure to determine if low levels of bacteria are present (Cheng and

others 2008; Perelle and others 2004). According to a study done by Perelle (2004), an 18 h enrichment process was done prior to DNA extraction with a detection limit of 10^3 CFU/ml (Perelle and others 2004) for *Salmonella* in raw milk. Another study by Cheng (2008), showed a 24 h enrichment process prior to running RT-PCR with a detection limit of 10^3 CFU/ml (Cheng and others 2008) for *Salmonella* in chili powder. Previous studies have shown that detection of *Salmonella* from produce using RT-PCR or PCR are able to detect lower limits. A study done by Bhagwat (2004) showed a lower limit detection of 100 CFU / ml from mixed salad rinse water (Bhagwat, 2004) for *Salmonella*; 1 CFU/ml from leaf lettuce and apples (Shearer and others 2001) for *Salmonella*; and 1 CFU/ml mixed salad, cilantro, broccoli, cauliflower, and cabbage (Bhagwat, 2003) for *Salmonella* Typhimurium. Much of these lower limits of detection were aided by an enrichment process. Bhagwat (2004), used a 5 h enrichment (Bhagwat, 2004) while the study by Shearer and others (2001) had an overnight enrichment process (Shearer and others 2001). Another study using RT-PCR by Bhagwat (2003), used a 20 h enrichment process to further increase the detection sensitivity of the target (Bhagwat, 2003). With our study we have been using a 6 h enrichment process to detect the lower inoculum levels and have been able to detect 10^4 CFU/25g or 400 CFU/g. This has proved to still be a much quicker method than traditional detection procedures.

The simultaneous amplification and detection using fluorescence in rt-PCR, allows for the results to be confirmed by melt temperature (T_m) rather than using gel electrophoresis. A melt temperature is when 50% of the amplified DNA is double

stranded and 50% is single stranded (De Medici and others 2003). The T_m determines if the target amplicon is present or not. Since the T_m is part of the rt-PCR process, it allows for much faster results, is less labor intensive, and is less likely for cross-contamination due to a one-tube single step format. It is also cheaper than using fluorescent probes.

A validation step was established in order to confirm the results of the experiment. The validation step that was applied was an internal amplification control (IAC). The IAC was added to determine if there was any false-negatives that may have been caused by PCR inhibitors (Moreira and others 2009). PCR inhibitors could be lipids, proteins, enzymes, chemical additives, fiber, other bacteria, and pH (Lee and Fairchild, 2006). In our study the size of the IAC was smaller than the target, even though it has been recommended that the IAC be larger (Hoorfar and others 2004). This was done to compensate for the high T_m of the target product to be able to differentiate between the two different product T_m 's. The target and IAC were both amplified using the same primer sets. This was done in order to have only one set of PCR reaction conditions (Hoorfar and others 2004). While we cannot conclusively detect only viable cells using this assay, we can detect *Salmonella* from produce at 10^4 CFU/25g from peppers within 10 h.

A DNase I treatment was performed in order to eliminate the genomic DNA that may have been present (Werbrouck and others 2007). When this treatment was applied to our study, a 1 log loss of detection was observed. This one log reduction went from

10^7 CFU to 10^8 CFU/25g. Even though this procedure was done, it has been expressed by Harper et. al. (2003) that all the genomic DNA may have not been completely removed (Harper and others 2003). It could also be possible that with the loss of 1 log that the treatment could not be 100% free of contamination of RNAses. The addition of DNase I treatment, could degrade some of the RNA present (Werbrouck and others 2007), therefore leading to lower yields. Although this treatment only showed a loss of 1 log, this could still detect 10^8 CFU/25g or ml within one day. Also PCR run on DNase I treated samples did not show any product on the gel showing that DNA was indeed eliminated (Figure 21). DNase I treatment was carried out to determine if the RNA sample contained carryover DNA. The lower yield of RT-PCR after DNase I treatment showed that caution must be used while extracting RNA to prevent carry-over DNA that could lead to erroneous results. However it is also crucial to ensure that DNase I used does not have and RNAses present that could destroy the total RNA as well to decrease yield while keeping RNA pure.

Detection limits obtained in this study was similar to results obtained by other researchers using molecular methods for produce. Bhagwat (2003) produced a study that was able to detect 1 CFU/ml using real-time PCR in mixed salad, cilantro, broccoli, cauliflower, and cabbage (Bhagwat, 2003) for *Salmonella* Typhimurium detection. Also a study by Cheng and others (2008) showed a 24 h enrichment process prior to running RT-PCR with a detection limit of 10^4 CFU/ml (Cheng and others 2008) for *Salmonella* in chili powder. Finally Yuk and others (2006) evaluated *Salmonella* on the surface of

tomatoes with a detection limit of 10^2 CFU/tomato with an incubation period of 4 h (Yuk and others 2006). When compared to our study, the results had similar detection limits around 400 CFU/g with a 6 h enrichment step using reverse-transcriptase PCR. The study by Bhagwat (2003) using real-time PCR had a 16-20 h enrichment process (Bhagwat, 2003) and Cheng (2008) using real-time PCR had a 24 h (Cheng and others 2008) enrichment process. Even though Yuk (2006) only used a 4 h enrichment process (Yuk and others 2006), the additional cost of using the flow-through immunocapture system may deter companies from using this technique. Our total detection time with the enrichment process is 10-12 hours whereas the above studies have used longer enrichment times therefore delaying final results. The advantage of using reverse-transcriptase is it targets the mRNA which has a shorter half life than DNA therefore detecting viable cells is much greater. As where PCR and real-time PCR targets DNA and does not distinguish between dead and viable cells. This method can be used to target other *Salmonella* serovars as well. The *invA* gene is presently located on the chromosome and is specific to *Salmonella* (Cheng and others 2008). Invasive A gene is needed for *Salmonella* to target the epithelial cells (Galan and Curtiss, 1989). When our method of reverse-transcriptase PCR is compared to NASBA or LAMP it still requires similar time to get results. This is because one still has to visualize results using gel electrophoresis or electrochemiluminescence. Reverse-transcriptase PCR does amplify and detect the product within one process, therefore reducing the chance of cross-contamination and producing results needed for confirmation.

This method rapidly detected *Salmonella* faster than traditional plating. With continued research on improving detection sensitivity, bacterial recovery, and RNA yields, this method has the potential for routine diagnostic use to ultimately save the food industry millions of dollars as well as costly recalls, and protect public health. This method has allowed for us to obtain results within a 10-12 hrs period rather than the 5-7 day traditional plating and confirmation procedure when compared to the USDA and FDA BAM procedures. Future work should focus on improving bacterial recovery, RNA yields and purity to increase detections sensitivity to $< 1 \log \text{CFU/g}$ peppers within one work day or at the most two work shifts to allow for timely product recall or prevention of release of contaminated product for sale.

List of References

- Beuchat LR. 1996. Pathogenic microorganisms associated with fresh produce. *Journal of Food Protection* 59(2):204-216.
- Beuchat LR & Ryu JH. 1997. Produce handling and processing practices. *Emerging Infectious Diseases* 3(4):459-465.
- Bhagwat AA. 2003. Simultaneous detection of *Escherichia coli* O157 : H7, *Listeria monocytogenes* and *Salmonella* strains by real-time PCR. *International Journal of Food Microbiology* 84(2):217-224.
- Bhagwat AA. 2004. Rapid detection of *Salmonella* from vegetable rinse-water using real-time PCR. *Food Microbiology* 21(1):73-78.
- Biswas AK, Kondaiah N, Bheilegaonkar KN, Anjaneyulu ASR, Mendiratta SK, Jana C, Singh H & Kumar RR. 2008. Microbial profiles of frozen trimmings and silver sides prepared at Indian buffalo meat packing plants. *Meat Science* 80(2):418-422.
- CDC. 2008. Investigation of Outbreak of Infections Caused by *Salmonella* Saintpaul. Centers for Disease Control and Prevention. Centers for Disease Control and Prevention.
- Cheng CM, Lin W, Van KT, Phan L, Tran NN & Farmer D. 2008. Rapid Detection of *Salmonella* in Foods Using Real-Time PCR. *Journal of Food Protection* 71(12):2436-2441.
- De Medici D, Croci L, Delibato E, Di Pasquale S, Filetici E & Toti L. 2003. Evaluation of DNA extraction methods for use in combination with SYBR Green I real-time PCR to detect *Salmonella enterica* serotype enteritidis in poultry. *Applied and Environmental Microbiology* 69(6):3456-3461.
- Feng P. 2007. Rapid Methods for the Detection of Foodborne Pathogens: Current and Next-Generation Technologies. In: Doyle, M. P. & Beuchat, L. R., editors. *Food Microbiology: Fundamentals and Frontiers*, 3rd Ed. Washington, D.C.: ASM Press. p. 911 - 934.
- Galan JE & Curtiss R. 1989. CLONING AND MOLECULAR CHARACTERIZATION OF GENES WHOSE PRODUCTS ALLOW *SALMONELLA-TYPHIMURIUM* TO PENETRATE TISSUE-CULTURE CELLS. *Proceedings of the National Academy of Sciences of the United States of America* 86(16):6383-6387.
- Harper LV, Hilton AC & Jones AF. 2003. RT-PCR for the pseudogene-free amplification of the glyceraldehyde-3-phosphate dehydrogenase gene (*gapd*). *Molecular and Cellular Probes* 17(5):261-265.
- Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M & Fach P. 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. *Journal of Clinical Microbiology* 42(5):1863-1868.
- Iturriaga MH, Escartin EF, Beuchat LR & Martinez-Peniche R. 2003. Effect of inoculum size, relative humidity, storage temperature, and ripening stage on the attachment of *Salmonella* Montevideo to tomatoes and tomatillos. *Journal of Food Protection* 66(10):1756-1761.
- Kanki M, Sakata J, Taguchi M, Kumeda Y, Ishibashi M, Kawai T, Kawatsu K, Yamasaki W, Inoue K & Miyahara M. 2009. Effect of sample preparation and bacterial

- concentration on *Salmonella enterica* detection in poultry meat using culture methods and PCR assaying of preenrichment broths. *Food Microbiology* 26(1):1-3.
- Lang MM, Harris LJ & Beuchat LR. 2004. Evaluation of inoculation method and inoculum drying time for their effects on survival and efficiency of recovery of *Escherichia coli* O157 : H7, *Salmonella*., and *Listeria monocytogenes* inoculated on the surface of tomatoes. *Journal of Food Protection* 67(4):732-741.
- Lee MD & Fairchild A. 2006. Sample Preparation for PCR. In: Maurer, J., editor). *PCR Methods in Foods*. New York, NY: Springer Science+ Business Media, Inc. p. 41 - 50.
- Liming SH & Bhagwat AA. 2004. Application of a molecular beacon - real-time PCR technology to detect *Salmonella* species contaminating fruits and vegetables. *International Journal of Food Microbiology* 95(2):177-187.
- Lucier G & Dettmann RL. 2008. Potato Acreage Down, Prices Up. Economic Research Service/USDA. Vegetables and Melons Outlook.
- Maurer JJ. 2006. The Mythology of PCR: A Warning to the Wise. In: Maurer, J., editor). *PCR Methods in Foods*. New York, NY: Springer Science+ Business Media, Inc. p. 27 - 40.
- Moreira AN, Conceicao FR, Conceicao RDS, Dias CN, Carvalhal JB, Dellagostin OA & Aleixo JAG. 2009. IMS USING IN-HOUSE MONOCLONAL ANTIBODY-COATED MAGNETIC BEADS ASSOCIATED TO PCR ASSAY FOR DETECTION OF *SALMONELLA* TYPHIMURIUM IN RAW MEATS. *Journal of Food Safety* 29(1):59-72.
- Perelle S, Dilasser F, Malorny B, Grout J, Hoorfar J & Fach P. 2004. Comparison of PCR-ELISA and LightCycler real-time PCR assays for detecting *Salmonella* spp. in milk and meat samples. *Molecular and Cellular Probes* 18(6):409-420.
- Rusin P, Maxwell S & Gerba C. 2002. Comparative surface-to-hand and fingertip-to-mouth transfer efficiency of gram-positive bacteria, gram-negative bacteria, and phage. *Journal of Applied Microbiology* 93(4):585-592.
- Shearer AEH, Strapp CM & Joerger RD. 2001. Evaluation of a polymerase chain reaction-based system for detection of *Salmonella enteritidis*, *Escherichia coli* O157 : H7, *Listeria* spp., and *Listeria monocytogenes* on fresh fruits and vegetables. *Journal of Food Protection* 64(6):788-795.
- Shi X, Namvar A, Kostrzynska M, Hora R & Warriner K. 2007. Persistence and growth of different *Salmonella* serovars on pre- and postharvest tomatoes. *Journal of Food Protection* 70(12):2725-2731.
- Werbrouck H, Botteldoorn N, Uyttendaele M, Herman L & Van Coillie E. 2007. Quantification of gene expression of *Listeria monocytogenes* by real-time reverse transcription PCR: Optimization, evaluation and pitfalls. *Journal of Microbiological Methods* 69(2):306-314.
- Yuk HG, Warren BR & Schneider KR. 2006. Preliminary evaluation of flow-through immunocapture followed by real-time PCR for the detection of *Salmonella*

serovars on tomato surfaces within 8 hours. *Journal of Food Protection* 69(9):2253-2257.

Appendix

Table 4: Recovery of *S. Typhimurium* without enrichment from peppers by plating on XLT4 agar and nanodrop readings of RNA extracts

Buffer	Level of Inocula Log Count	Cells Recovered by Plating on XLT4	RNA Extracted ng/ μ l
Glycine-Saline	10^7	$5.5 \times 10^1 \pm 0.70$	7.8 ± 0.7
Glycine-Saline w/ .05% Tween-20	10^7	$8.20 \times 10^2 \pm 111$	7.0 ± 3.18
Glycine-Saline w/ .1% Tween-20	10^7	$7.5 \times 10^2 \pm 93$	4.15 ± 0.07
Glycine-Saline w/ .05% Tween-20 & 3% Beef Extract	10^7	$6.45 \times 10^3 \pm 36$	5.85 ± 0.77

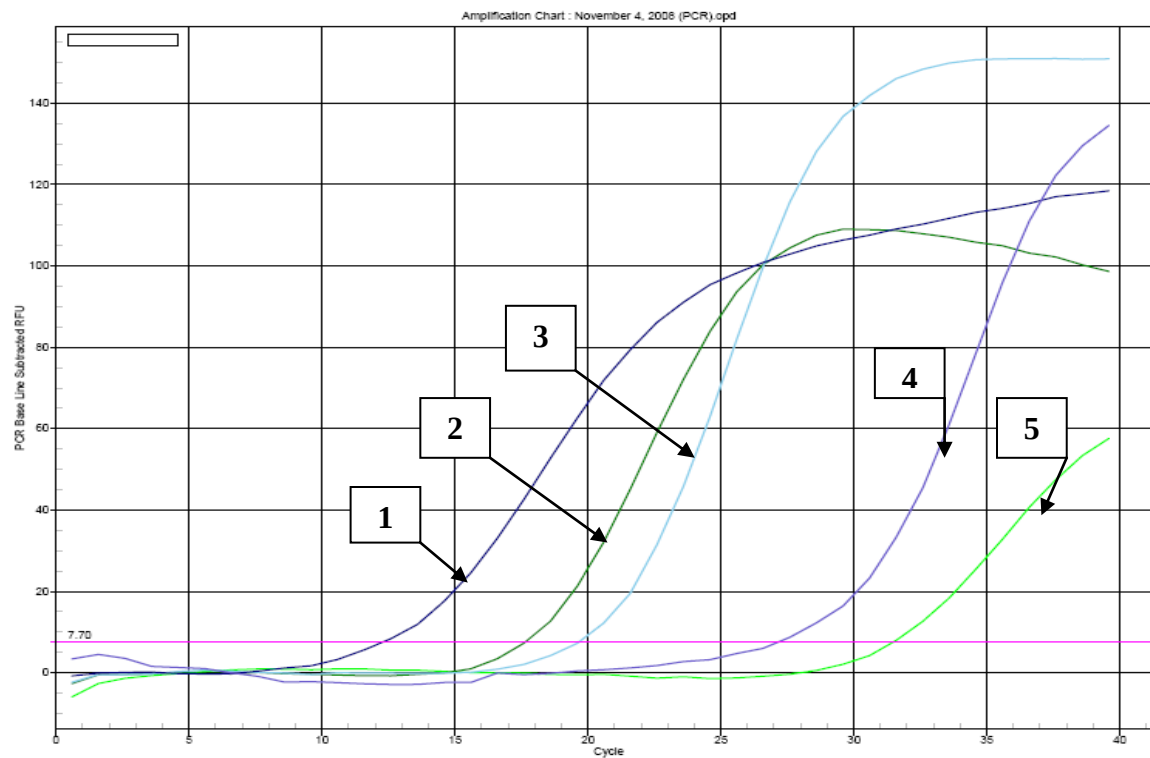


Figure 15: Detection limits of *Salmonella* showing cycle threshold values (Ct) from spiked Jalapeno peppers by rt-PCR without enrichment. Positive Control (1), Log10⁸ (2), Log10⁷ (3), Pepper control no inoculation (4), and Negative Control water (5)

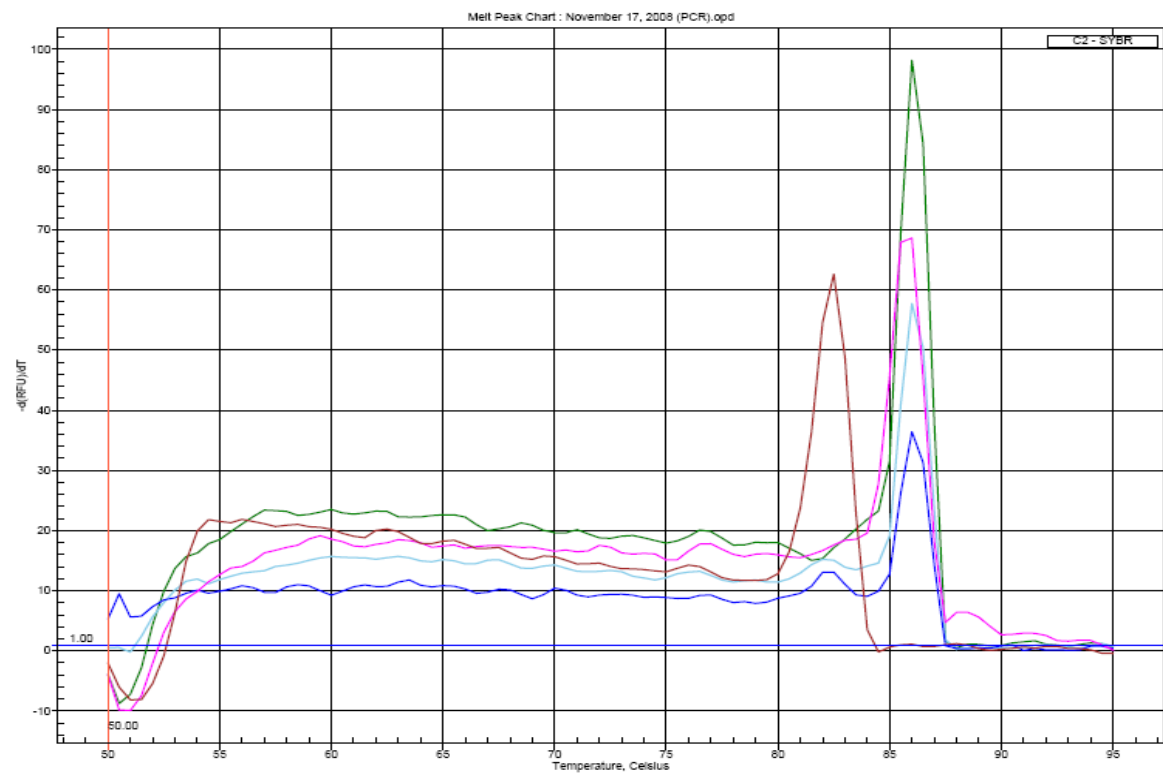


Figure 16: Melt temperature curve depicting specific *Salmonella* amplification products at ~85°C with detection limits of 10^7 without enrichment from Jalapeno peppers.

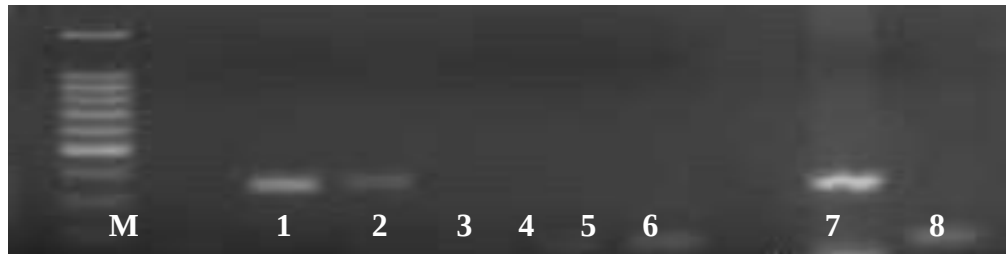


Figure 17: Agarose gel electrophoresis of rt-PCR amplified products (347bp) from Jalapeno peppers spiked with *Salmonella* Typhimurium without enrichment. Lane (M): 100 bp ladder marker. Lane (1-6): products from Jalapeno peppers spiked with 10^8 to 10^3 CFU/ml of *S. Typhimurium*. Lane (7): positive control of *S. Typhimurium* from RNA extraction. Lane (8): negative control (water)

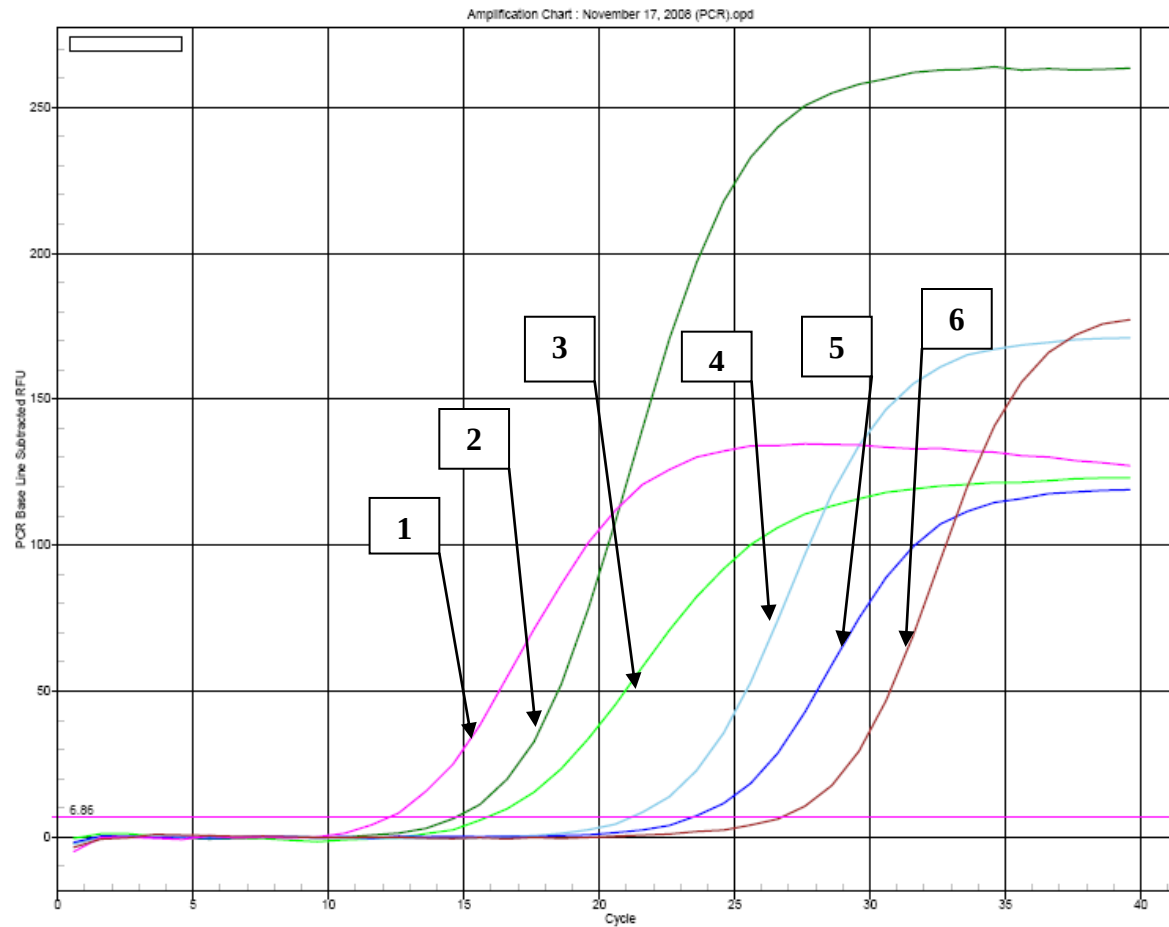


Figure 18: Detection limits of *Salmonella* showing cycle threshold values (Ct) from spiked Jalapeño peppers by rt-PCR with enrichment. Positive Control (1), 10^6 - 10^4 CFU (2-4), Negative Control water (5), and 10^3 CFU (6)

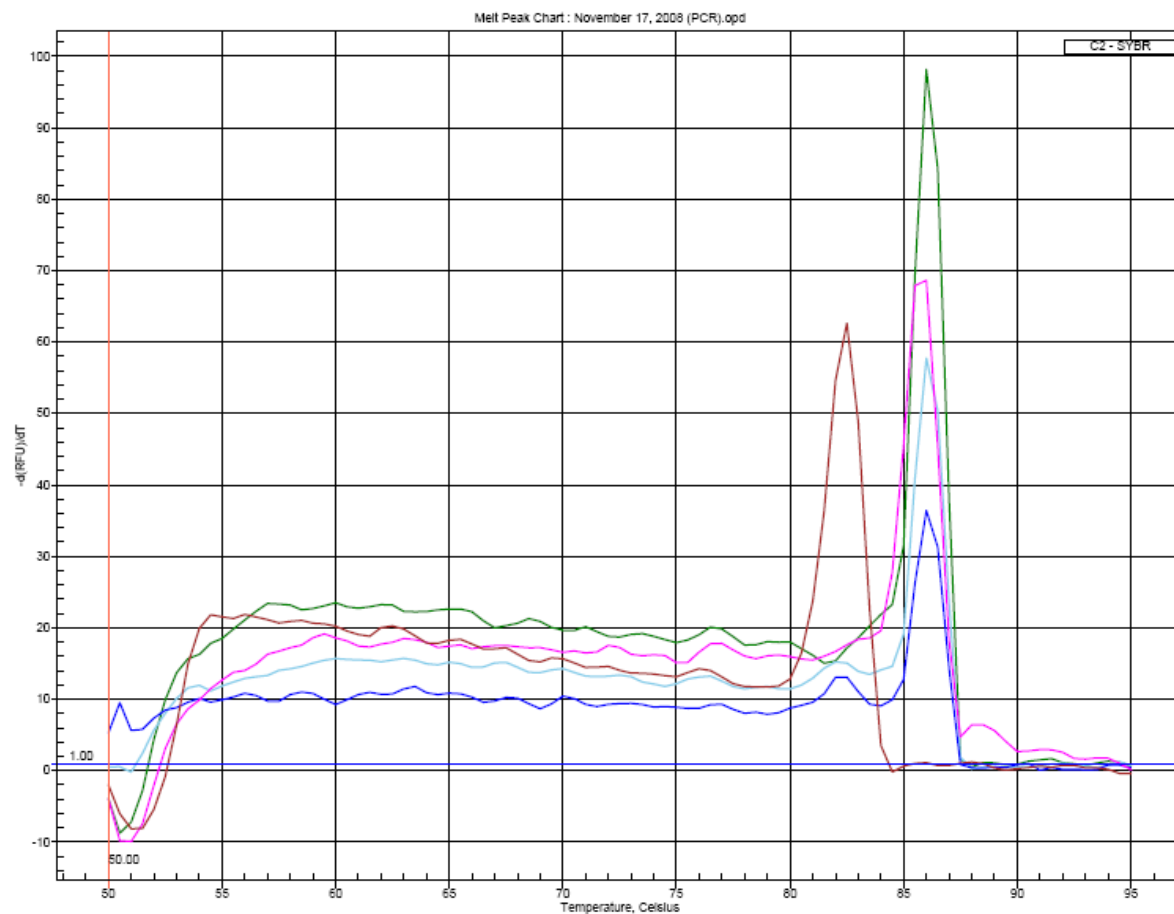


Figure 19: Melt temperature curves depicting specific *Salmonella* amplification products at ~85°C with detection limits of 10^4 with enrichment from Jalapeno peppers



Figure 20: Agarose gel electrophoresis of rt-RT-PCR amplified products (347 bp) from Jalapeno peppers spiked with *Salmonella* Typhimurium after enrichment. Lane (M): 100 bp ladder marker. Lane (1-9): products from Jalapeno peppers spiked with 10^8 to 10^0 CFU/ml of *S. Typhimurium*. Lane (12): positive control of RNA extracted from *S. Typhimurium*. Lane (13): negative control (water)

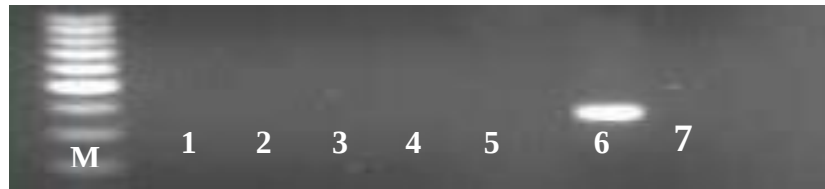


Figure 21: Agarose gel electrophoresis of PCR products (347 bp) using RNA treated with DNase I from Jalapeno peppers spiked with *Salmonella Typhimurium*. Lane (M): 100 bp ladder marker. Lane (1-5): products from Jalapeno peppers spiked with 10^7 to 10^3 CFU/ml of *S. Typhimurium*. Lane (6): positive control of RNA extracted from *S. Typhimurium*. Lane (7): negative control (water)

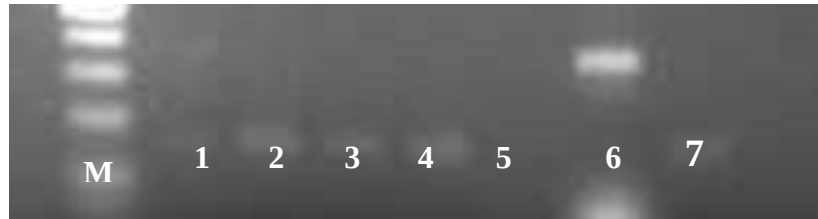


Figure 22: Agarose gel electrophoresis of rt-PCR products (347 bp) using RNA treated with DNase I from Jalapeno peppers spiked with *Salmonella* Typhimurium. Lane (M): 100 bp ladder marker. Lane (1-5): products from Jalapeno peppers spiked with 10^7 to 10^3 CFU/ml of *S.typhimurium*. Lane (6): positive control of RNA extracted from *S.typhimurium*. Lane (7): negative control (water)

Vita

Nathan Duray Miller was born in Nashville, Tennessee, on May 11, 1984. I grew up in Williamson County and graduated from Page High School in 2002. He continued his education at the University of Tennessee at Martin where he earned a B.S. degree majoring in Biology and a M.S degree in Food Science and Technology with a concentration in Food Microbiology from the University of Tennessee. Nathan will pursue a career in the food industry concentrating in Quality Control.