To the Graduate Council:

I am submitting herewith a dissertation written by Nancy Rabalais DeTrana entitled "Development of a Real-Time PCR Assay for Detection and Quantification of *Escherichia coli* O157:H7 in Apple Juice." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

David A. Golden, Major Professor

We have read this dissertation
and recommend its acceptance:

P. Michael Davidson

F. Ann Draughon

Gina M. Pighetti

Accepted for the Council:

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

(Original signatures are on file with official student records)

Development of a Real-Time PCR Assay for Detection and Quantification of *Escherichia coli* O157:H7 in Apple Juice

A Dissertation Presented for
the Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Nancy Rabalais DeTrana
May 2007

DEDICATION

This dissertation is dedicated to my husband and daughter

Alexander and Elizabeth DeTrana

whose love and inspiration enabled me to see this to completion.

ACKNOWLEDGMENTS

I wish to express my sincere gratitude and appreciation to my major professor, Dr. David Golden, for giving me the opportunity to further my education at the University of Tennessee. I would like to thank him for his guidance, patience, encouragement, and for his friendship. I also thank my doctoral committee members Dr. F. Ann Draughon, Dr. P. Michael Davidson, and Dr. Gina Pighetti for the assistance they provided and the ear they would often lend when I needed it most.

I would like to acknowledge my colleagues–past and present--Erika Bible, Jeff Boland, Sarah Malone Boland, Kristina Carter, Coesha Fairley, Brandy Knox, Ashley Pedigo, David Rasmussen, Harry Richards, Lilia Santiago, and Vivian Ann Rash Shaver for their wonderful friendships and assistance in the laboratory. I especially thank Kimberly Stanley and Faith Critzer (aka "McGyver") for their never-ending support, guidance, and friendship. What would I have done without the two of you?

I express my sincere thanks and love to my parents, Leon and Cristie Rabalais, who gave everything they could to ensure I received an excellent education. Thank you for always listening and for your constant encouragement. I thank my sister, Jamie Mitchell, for always providing love, support and encouragement throughout my graduate studies. I also express gratitude to the rest of my family for their endless inspiration.

I wish to thank my husband and best friend, Alex, for always believing in me (even when I didn't) and for his unconditional love and support. Thank you for keeping that "light at the end of the tunnel" in my sight! I could not have done this without you. Finally, I thank my daughter, Elizabeth, for her smiles and unconditional love. Love you!

ABSTRACT

Apple cider/juice contaminated with *Escherichia coli* O157:H7 has been implicated in several foodborne illness outbreaks, but due to the presence of reaction inhibitors, detection by polymerase chain reaction (PCR) is often difficult. The studies presented in this dissertation were conducted to evaluate techniques to improve detection of *E. coli* O157:H7 in apple juice using a fluorogenic probe-based real-time PCR assay without prior enrichment. Two commercial DNA extraction and purification procedures, GenElute™ Bacterial Genomic DNA Kit and PrepMan® Ultra Sample Preparation Reagent, were combined with two real-time PCR chemistries, SYBR® Green I dye and TaqMan® probes for potential use in the apple juice assay. After real-time PCR, no significant differences were observed in cycle threshold values (C_t) ($p>0.05$) among the methods. The PrepMan/TaqMan method was subsequently combined with a real-time PCR assay based on detection of the *stx1, stx2,* and *uidA* genes. Apple juice was inoculated individually with nine strains of *E. coli* O157:H7 (1.0 log CFU/ml to 4.0 log CFU/ml) and plated to verify initial inocula. For particulate removal, apple juice was vacuum filtered twice (Whatman No. 4 and Whatman No. 1), followed by a distilled water wash. Samples were plated again to obtain post-filtration inocula. Filtered juice was centrifuged, pellets were resuspended in 1 ml phosphate buffer, and *E. coli* O157:H7 cells were concentrated by immunomagnetic separation. PrepMan Ultra was added to the magnetic bead/*E. coli* O157:H7 complex for DNA extraction. Extracts were combined as appropriate with primers, probe and other reagents, and real-time PCR was performed. Average *E. coli* O157:H7 inoculum levels of 0.3, 2.2, 3.3 and 4.3 log CFU/ml in apple

juice were detected at average C_t values of 41.22, 37.54, 34.69 and 31.81 (*stx1*); 43.13, 38.74, 35.21 and 32.58 (*stx2*)*;* and 44.13, 41.54, 37.81 and 34.06 *(uidA*). Across all *E. coli* O157:H7 strains, populations as low as 1.6 (44 CFU/ml) (*stx1*), 1.6 (43 CFU/ml) (*stx2*), and 1.5 (33 CFU/ml) (*uidA*) log CFU/ml could be quantified using the cell concentration/real-time PCR assay. However, *E. coli* O157:H7 was detected on occasion below the quantifiable level at the lowest inoculum level for all strains. This method can be used for rapid detection and quantification (<5 h) of *E. coli* O157:H7 in apple cider/juice and potentially other foods.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PART I

INTRODUCTION

Over the past few decades, food safety has become an increasingly important public health issue. Each year in the United States, foodborne illness leads to extraordinary medical costs and productivity losses. Foodborne pathogens are estimated to cause 76 million illnesses and 5,000 deaths annually, and in 2000 alone, $6.9 billion were spent on foodborne illnesses caused by seven major foodborne pathogens (CDC, 1999; ERS, 2004). Several significant foodborne illness outbreaks and the need to enhance the safety of the U.S. food supply have challenged scientists to develop new and improved, rapid methods for detection of pathogenic microorganisms. These methods for detection and identification of pathogens in foods are critical for control and, more important, prevention of foodborne-related diseases.

Despite the long-standing use of traditional culture techniques for detection of *E. coli* O157:H7 in these foods, there are limitations associated with these methods. Traditional methods for detection and identification of this organism in foods generally involve growth on culture media, isolation, and biochemical tests for identification (FDA, 1998). These procedures are time-consuming and frequently take days to obtain a positive identification.

In recent years, DNA-based technologies have been developed, overcoming many of the obstacles which have plagued conventional assays. DNA-based technologies provide several benefits over traditional microbiological assays, including increased sensitivity and specificity. These technologies target known nucleotide sequences in a bacterial genome, enabling researchers to accurately confirm the presence of a pathogen on a molecular level rather than on phenotypic properties (Malorny et al., 2003).

Recently, researchers have studied the benefits of new pathogen detection technologies such as optical biosensors and oligonucleotide microarrays. These methods have been shown to have varying levels of success for pathogen detection (Tait et al., 2004; Kakinuma et al., 2003), yet problems such as reduced sensitivity and expense have limited their use. Recent studies have shown that real-time PCR is an effective tool for detection of low numbers of pathogens in foods such as meats, fruits, vegetables, and milk (Fortin et al, 2001; Reischl et al., 2002; Bhagwat, 2004).

JUSTIFICATION

Apple cider and juice have been implicated in several significant foodborne illness-related outbreaks (Besser et al., 1993; Hilborn et al., 2000). Fruit juices contain endogenous polyphenols and acids which often suppress amplification of target DNA sequences altogether (Ogunjimi et al., 1999; Grant, 2003; Park et al., 2004). Research is needed in this area to reduce the presence of inhibitory substances, improve cell extraction from complex food matrices and to reduce or eliminate altogether the need for an enrichment step prior to real-time PCR.

Conventional PCR has commonly been used for detection of pathogens in miscellaneous food products (Gilgen et al., 1998; McKillip et al., 2000; Al-Gallis et al, 2002). During PCR, a DNA molecule is subjected to a series of heating and cooling cycles resulting in strand separation, annealing of oligonucleotide primers, and extension by *Taq* DNA polymerase. In theory, this cycling results in exponential amplification of DNA identical to the initial starting template strand (Walker, 2002). Post amplification,

PCR product is visualized and identified by size through gel electrophoresis.

Although conventional PCR has proved advantageous for detection of foodborne pathogens, end-point results obtained through electrophoresis are quite limiting. During the exponential phase of a conventional PCR, a theoretical doubling of product occurs. In the second phase, the linear phase, reaction components are being exhausted and the reaction is decelerating. Finally, in the plateau phase or end-point, the reaction has stopped, PCR product is no longer produced and the potential for product degradation exists. With this end-point method, replicates which contain the same starting template quantity can reflect different results when visualized on a gel. Therefore, conventional PCR may not be capable of accurately measuring the amount of starting DNA template that may be present in a food sample.

In order to reduce or alleviate many of the problems associated with end-point detection, scientists developed an adaptation to conventional PCR. (Higuchi et al., 1993). The earliest real-time machinery utilized a video camera to monitor multiple PCR reactions simultaneously. The video camera could detect double-stranded DNA (dsDNA) accumulation in each PCR through detection of increased fluorescence of ethidium bromide once bound to dsDNA. Researchers determined that the increase in fluorescence was directly related to the starting number of DNA copies. Consequently, the fewer cycles needed to detect fluorescence, the larger the number of starting template sequences. This technique, now known as "quantitative PCR" or more commonly "real-time PCR", has the capability of monitoring the DNA amplification process, while eliminating end-point analysis (Walker, 2002).

OBJECTIVES

The objectives of this research were:

1. to assess DNA extraction methods and fluorescence chemistries, and determine primer and probe set specificity for *E. coli* O157:H7 in cross-reactivity studies with other enterohemorrhagic *E. coli* and related genera for use in a real-time polymerase chain reaction.

2. to develop and optimize a method to enhance detection of *E. coli* O157:H7 in apple juice through combination of centrifugation, filtration, immunomagnetic separation and real-time PCR.

3. to apply the real-time polymerase chain reaction assay for detection and quantification of low numbers (1.0 log CFU/ml to 4.0 log CFU/ml) of *E. coli* O157:H7 in apple juice without an enrichment step .

REFERENCES

Al-Gallis, N., R. Ben Aissa R., T.A. Annabi, O. Bahri, A. Boudabous. 2002. Isolation and characterization of shiga-toxin producing *Escherichia coli* from meat and dairy products. Food Microbiol. 19:389-398.

Besser, R.E., S.M. Lett, J.T. Weber, M.P. Doyle, T.J. Barrett, J.G. Wells, P.M. Griffin. 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. J. Am. Med. Assoc. 269:2217-2220.

Bhagwat, A.A. 2004. Rapid detection of *Salmonella* from vegetable rinse-water using real-time PCR. Food Microbiol. 21:73-78.

Centers for Disease Control and Prevention. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5:607-625.

ERS. 2004. Foodborne pathogens. http://www.ers.usda.gov/Topics/View.asp?T=102820. Accessed: August 20, 2006.

FDA. 1998. Bacteriological Analytical Manual. 9th Ed. AOAC International, Gaithersburg, MD.

Fortin, N.Y., A. Mulchandani, W. Chen. 2001. Use of real-time polymerase chain reaction and molecular beacons for the detection of *E. coli* O157:H7. Anal. Biochem. 289:281-288.

Gilgen, M., P. Hübner, C. Höfelein, J. Lüthy, U. Candrian. 1998. PCR-based detection of verotoxin-producing *Escherichia coli* (VTEC) in ground beef. Res. Microbiol. 149:145-154.

Grant, M.A. 2003. Evaluation of methods to improve detection of *Escherichia coli* O157:H7 in fresh produce by multiplex polymerase chain reaction. J. Food Prot. 66:18-24.

Higuchi, R., C. Fockler, G. Dollinger, R. Watson. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology. 9:1026-1030.

Hilborn, E.D., P.A. Mshar, T.R. Fiorentino, Z.F. Dembek, T.J. Barrett, R.T. Howard, M.L. Cartter. 2000. An outbreak of *Escherichia coli* O157:H7 infections and haemolytic uraemic syndrome associated with consumption of unpasteurized apple cider. Epidemiol. Infect. 124:31-36.

Kakinuma, K., M. Fukushima, R. Kawaguchi. 2003. Detection and identification of *Escherichia coli*, *Shigella,* and *Salmonella* by microarrays using the *gyrB* gene. Biotechnol. Bioeng. 83:721-728.

Malorny, B., P.T. Tassios, Rådström, N. Cook, M. Wagner, J. Hoorfar. 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. Int. J. Food Microbiol. 83: 39-48.

McKillip, J.L., L.A. Jaykus, M.A. Drake. 2000. A comparison of methods for the detection of *Escherichia coli* O157:H7 from artificially-contaminated dairy products using PCR. J. Appl. Microbiol. 89:49-55.

Ogunjimi, A.A., P.V. Choudary. 1999. Adsorption of endogenous polyphenols relieves the inhibition by fruit juices and fresh produce of immuno-PCR detection of *Escherichia coli* O157:H7. FEMS Imm. Med. Microbiol. 23:213-220.

Park, S., R.A. Durst. 2004. Modified immunoliposome sandwich assay for the detection of *Escherichia coli* O157:H7 in apple cider. J. Food Prot. 67:1568-1573.

Reischl, U., M.T. Youssef, J. Kilwinski, N. Lehn, W.L. Zhang, H. Karch, N.A. Strockbine. 2002.Real-time fluorescence PCR assays for detection and characterization of shiga toxin, intimin, and enterohemolysin genes from shiga toxin-producing *Escherichia coli*. J. Clin. Microbiol. 40:2555-2565.

Taitt, C.R., Y.S. Shubin, R. Angel, F.S. Ligler. 2004. Detection of *Salmonella enteria* serovar Typhimurium by using a rapid, array-based immunosensor. Appl. Env. Microbiol. 70:152-158.

Walker, N.J. 2002. A technique whose time has come. Science. 296:557-558.

PART II

LITERATURE REVIEW

APPLE CIDER/ JUICE

Apple cider is a fermented form of apple juice created by yeasts naturally present in the juice (Jay et al., 2005). Cider differs from apple juice in that cider has not gone through a process to remove suspended solids, is unclarified, and is darker in color (Semanchek and Golden, 1996). Fresh apple cider is often unpasteurized, whereas apple juice is typically pasteurized (Zhao et al., 1993). In the United States, sweet cider refers to a non-fermented product, while hard cider refers to a product which has been fermented (Downing, 1989). Since sweet cider has not gone through a fermentation process, it is more susceptible to microbial contamination and thus has been implicated in several outbreaks of juice-borne *E. coli* O157:H7 infection.

Cidermaking generally begins when apples are ripe, usually in the late summer or fall. Sound fruits are selected, often of different varieties, and stored for a week to ten days to allow softening (Proulx and Nichols, 2005). Prior to grinding, apples are washed with clean water to remove debris, including insects, insecticide residue, twigs and bacteria (Proulx and Nichols, 2005). Moldy or rotten apples are discarded. Once washed, apples, with peel intact, are ground into a fine pulp, either with a hand- or power-driven home mill or at a commercial cider mill (Proulx and Nichols, 2005). Freshly ground pulp, or pomace, is placed in a cider press, exerting pressure on the pomace until the maximum amount of juice is extracted. Types of modern pressing equipment include hydraulic, spiral screw, hand presses, basket and belt, all of which are either continuous or batch presses (Proulx and Nichols, 2005). Once pressed, the liquid is strained and poured into a collecting vessel where sedimentation occurs. At this point, the liquid is

considered (unpasteurized) cider.

According to Besser et al. (1993), a survey on cider manufacturing was conducted with New England cider producers at a trade show and yielded responses from 36 of 45 producers in attendance. These 36 respondents reported producing approximately 9 million gallons of cider in 1991, and all acknowledged use of "drops" (apples collected from the ground) during cider-making season. Only 33% (12 respondents) routinely washed and brushed apples prior to the pressing process. According to this survey, approximately 2.5 million gallons of cider were produced by mills that did not always brush and wash the fruit before processing. Preservatives were never used by most processors, 12 sometimes used one (sodium benzoate or potassium sorbate), and two always used a preservative (Besser et al., 1993). Another survey conducted by the U.S. Apple Association in 1996 revealed that of 450 respondents, 94% produced cider seasonally with less than 5,000 gallons per year (46%); 53% of the cider producers reported using both tree-picked apples and drops for production of cider, and 2% used drops only (Daly, 1997).

Besser et al. (1993) suggested contamination of cider with organisms such as *E. coli* O157:H7 and *Salmonella typhimurium* could result from using drops, using manure to fertilize orchards, and improperly brushing and washing apples prior to processing. Birds, rodents, insects and poor-worker hygiene can also serve as sources for surface contamination of apples. It has been reported that *E. coli* O157:H7 that are introduced onto the surface of an apple can internalize into apple tissue through breaks in the skin and through the flowering end of the apple (Burnett et al., 2000, Janes et al., 2005) .

Janes et al. (2005) reported that *E. coli* O157:H7 can grow inside apples due to degradation of cellular components in apple tissue.

In 1997, the U.S. Food and Drug Administration (FDA) conducted inspections of 237 facilities in 32 states where unpasteurized apple cider was produced. Upon inspection, 84% of processors washed apples prior to pressing, 2% reported brushing alone, and 14% reported no washing procedures at all (FDA, 1999). This report found that contamination of apples is most likely to occur during the growing and harvesting of the fruit, but may occur at any time between the orchard and consumption. This prompted the FDA to propose Hazard Analysis Critical Control Point (HACCP) regulations to address all points during production (FDA, 1998).

Juice HACCP–

After several foodborne illness outbreaks were strongly associated with juices, the FDA proposed and subsequently announced a final rule in January, 2001, designed to improve the safety of fruit and vegetable juices. Juice processors are required to evaluate manufacturing processes to determine possible microbiological, chemical, or physical hazards that could contaminate product. If potential hazards are identified, control measures must be taken to prevent, reduce, or eliminate these hazards. Under the HACCP regulations, processors are required to achieve a 5-log reduction in the numbers of the most resistant pathogen, such as *E. coli* O157:H7 or *Crytosporidium parvum,* in their finished product. Retail producers who process and sell directly to consumers and not to wholesalers or other businesses, are not covered by the HACCP regulation.

However, juices that have not been processed to reduce or eliminate pathogens, including those sold by retail-only producers, must include the following statement: "WARNING: This product has not been pasteurized and, therefore, may contain harmful bacteria which can cause serious illness in children, the elderly, and person with weakened immune systems".

ESCHERICHIA COLI O157:H7

In 1982, two outbreaks of hemorrhagic colitis led to the recognition of *Escherichia coli* O157:H7 as a human pathogen (LeBlanc, 2003; Leyer et al, 1995). Symptoms of illness caused by *E. coli* O157:H7 include severe abdominal cramps and periodical fever and nausea and generally appear within five days after ingestion of a contaminated food. Development of bloody diarrhea and dehydration approximately 48 hours after initial symptoms begin may be indicative of hemorrhagic colitis (Leblanc, 2003). A more life-threatening condition caused by *E. coli* O157:H7, hemolytic uremic syndrome (HUS), is the principal cause of renal failure in children and is characterized by renal insufficiency, thrombocytopenia and hemolytic anemia (Paton and Paton, 1998; Doyle et al, 1997). Death is a possible outcome of HUS, but in North America the death rate is less than 5% (Doyle et al, 1997).

E. coli O157:H7 can be transmitted by food and water and directly from person-to-person. Most outbreaks associated with food have been linked to products which arise from cattle, such as ground beef and raw milk (Doyle et al, 1997; Jay, 2000). However, acidic foods such as unpasteurized apple juice and cider, dry-cured salami, mayonnaise,

and Cheddar cheese have also been implicated in *E. coli* O157:H7-associated outbreaks (Besser et al, 1993; Clavero and Beuchat, 1996; Reitsma and Henning, 1996; Zhao and Doyle, 1994). In late 2006, a 23-state outbreak associated with raw spinach sickened 204 people with 1 confirmed death. Twenty-seven of those affected experienced HUS. To the date of this publication, cases were still being reported to the Centers of Disease Control and the investigation was considered ongoing (FDA, 2006). Finally, in November-December 2006, the CDC reported 62 cases of *E. coli* O157:H7 infection across six states. Among the ill, 49 (78%) were hospitalized and 7 (11%) developed HUS. Although the investigation is ongoing, preliminary reports indicate produce from a fast food restaurant may be the implicated food (CDC, 2006).

Virulence factors–

The ability of *E. coli* O157:H7 serotype to induce illness depends on distinct virulence factors (LeBlanc, 2003). Although unnecessary for replication or commensal relations with other *E. coli*, these virulence factors do provide the organism with an improved ability to colonize host surfaces, circumvent host defense systems, activate a host inflammatory response, and injure host cells and tissue, all of which may contribute to disease (Johnson, 2002). The secretion and regulation of these virulence factors is dependent on genes that have been identified in the organism. These genes are thought to have been acquired from numerous recombination events, such as horizontal transfer, bacteriophagic transfers, or natural transformations with foreign DNA from other species (Buchanan and Doyle, 1997; LeBlanc, 2003). Virulence genes are usually carried on

discrete genomic or plasmid regions known as pathogenicity islands (LeBlanc, 2002; Johnson, 2002).

All EHEC carry virulence genes that encode for production of Shiga toxin 1 (*stx1*) and/or Shiga toxin 2 (*stx2*) (Buchanan and Doyle, 1997; Johnson, 2002). Shiga toxins, hypothesized to have been acquired from *Shigella* via bacteriophage, are cytotoxins important in the mediation of hemorrhagic colitis (HC) and HUS (Doyle et al, 1997; Jay et al, 2005; Thorpe et al, 2002). *Stx1* and *stx2* are approximately 50% homologous at the protein level (Thorpe et al, 2002). Although similar in mode of action, *stx2* is thought to be 1000 times more cytotoxic to endothelial cells (Law, 2000). Both toxins have an AB structure, consisting of a single enzymatically active A subunit and multiple B subunits (Jay et al, 2005; LeBlanc, 2002). For the toxins to be cytopathogenic, the B subunit must bind to a specific glycolipid receptor, globotriaosylceramide (Gb_3), on a sensitive host cell (Jay et al, 2005). Once bound, the A subunit inactivates the 28S ribosome through N-glycosidase activity, blocking synthesis of proteins (Paton and Paton, 1998; LeBlanc, 2002; Jay et al, 2005). This action results in cell death.

***Survival in acidic conditions*–**

E. coli O157:H7 possesses the ability to survive in acidic environments. Researchers have studied its survival in various foods such as apple cider (Besser et al, 1993; Miller and Kaspar, 1994), fruit pulps (Marques et al, 2001), mayonnaise (Raghubeer et al, 1995), sausages (Hinkens et al, 1996), and yogurt (Bachrouri et al, 2002). These foods have emerged as unexpected vehicles for transmission of *E. coli*

O157:H7 due to their acidic properties.

The acidity of apple juice alone, approximately pH 3.6, is an insufficient means to kill *E. coli* O157:H7. Several investigators have reported that *E. coli* O157:H7 can persist in apple juice, especially at refrigeration temperatures (i.e., 4 to 10°C). Zhao et al (1993) reported that the organism is able to survive in apple cider for 20 days at 8°C, while only 2 to 3 days at 25°C. In apple cider, Miller and Kaspar (1994) reported that *E. coli* O157:H7 was able to survive for 21 days at 4°C even in the presence of preservatives and acidity. Other investigators concluded *E.* coli O157:H7 was detectable in apple juice concentrate after 12 weeks at -23°C (Oyarzábal et *al,* 2003). Since very low numbers of *E. coli* O157:H7 (<100) in juice can be sufficient for an infective dose, survival of even a few could cause disease. Therefore, sensitive detection methods for *E. coli* O157:H7 in apple cider or juice could assist in foodborne illness investigation or possibly prevent foodborne illness altogether**.**

JUICE-ASSOCIATED OUTBREAKS

During the past thirty years, foodborne illness outbreaks attributed to unpasteurized apple cider/juice have been caused by several enteric pathogens, including *Salmonella* Typhimurium, *Cryptosporidium,* and *E. coli* O157:H7 (CDC, 1975; CDC, 1996). Two specific outbreaks associated with *E. coli* O157:H7 and unpasteurized apple cider created a national concern about its safety. In the fall of 1991 in southeastern Massachusetts, an outbreak of illnesses was attributed to *E. coli* O157:H7 (Besser et al., 1993). Between October and November, 23 cases of *E. coli* O157:H7 infection were

identified and substantial evidence pointed to consumption of unpasteurized apple cider generated at one, local mill. Patients ranged from 2 to 70 years old and reported a variety of symptoms, including diarrhea (96%), abdominal pain (87%), bloody diarrhea (70%), vomiting (35%) and fever (17%). Although no deaths were associated with this outbreak, four children were diagnosed with hemolytic uremic syndrome (HUS). Environmental samples from the cider mill (collected 2 months after the outbreak), stool specimens from mill workers and cattle raised near the mill, and a sample of fermented cider recovered from a patient's refrigerator did not yield *E. coli* O157:H7 isolates. Nevertheless, inspection of the mill revealed violations of food manufacturing regulations, including failure to wash or sanitize apples and the use of drops for 90% of cider production, which established a strong association with the outbreak (Besser et al, 1993).

The mill associated with the 1991 outbreak was a small operation, providing cider locally. Conversely, in 1996, cases of *E. coli* O157:H7 infection in Washington state were linked to unpasteurized apple juice and apple juice blends from a state-of-the-art producer, Odwalla Inc. (Cody et al., 1999). All in all, 70 cases of infection were identified in California (26 cases), Colorado (5 cases), Washington (29 cases), and British Columbia (10 cases), of which one case led to death. Inspection of the Odwalla processing facility did not reveal an *E. coli* O157:H7 contamination route within the plant. Although Odwalla had a policy in place to accept only hand-picked apples from suppliers, this could not be reliably verified. Contaminated apples (drops) were introduced into the facility for processing despite the plant's efforts (Cody et al., 1999).

Even after media attention highlighted the association between unpasteurized

apple cider/juice and *E. coli* O157:H7, cases have still occurred. In 1998, 14 cases of *E. coli* O157:H7 infection occurred in Ontario and were strongly associated with apple cider made from drops. Seven cases of infection were reported in Oklahoma in 1999, where drop apples were used to made cider (FDA, 2001). Additionally, in 2005, 4 cases of infection were associated with persons who consumed unpasteurized cider purchased from a mill in the Durham region of Ontario, Canada (Anonymous, 2005).

ESCHERICHIA COLI O157:H7 DETECTION METHODS

Traditional detection methods–

Traditionally, detection of *E. coli* O157:H7 in foods requires an enrichment step followed by an isolation procedure (Feng and Weagent, 2002). Enrichment is often conducted in an EHEC enrichment broth (EEB) which is thereafter plated on a selective medium, such as Tellurite-cefixime-sorbitol MacConkey (TCSMAC) agar. This agar allows differentiation of *E. coli* O157:H7 from most strains based on its inability to ferment sorbitol. Presumptive screening can be carried out on TCSMAC isolates, followed by a confirmation screening to positively identify *E. coli* O157:H7.

Selective enrichment steps and/or incubation periods have been employed by researchers to increase *E. coli* numbers in foods to detectable levels. Silk and others (1997) tested Eosin methylene blue agar (EMBA) and Petrifilm *E. coli* Count Plates to screen apple cider samples for *E. coli.* Use of Petrifilm *E. coli* Count Plates yielded presumptive *E. coli* colonies after a 24 to 48 h incubation period. EMBA plates were incubated for 24 h and yielded poor results; the acidity of the apple cider changed the

color of the medium resulting in the inability to distinguish *E. coli* from other species (Silk et al., 1997). Further, Heuvelink and others (1997) studied the use of selective enrichment and plating media for isolation of *E. coli* O157:H7 from pure culture and minced beef. Low numbers of the organism were inoculated into minced beef; however, enrichment steps from 6 to 20 h were needed to increase numbers to detectable levels (Heuvelink et al., 1997). In a study to detect prevalence of *E. coli* O157:H7 in dairy herds, direct plating methods were compared to selective enrichment methods (Zhao et al., 1995). Results from a part of the study indicated that *E. coli* O157:H7 could be detected in all 31 *E. coli* O157:H7- positive calves by enrichment, whereas the organism was only isolated from 16 of the 31(51.6%) of the calves by direct plating without enrichment (Zhao et al., 1995).

There are several problems associated with traditional detection methods. These methods frequently fail to detect the presence of low levels of *E. coli* O57:H7 in foods without an enrichment step. Often, high levels of competing microflora in food samples enhance the difficulty of isolating *E. coli* O157:H7 (Vold et al., 2000). Under high-acid conditions, stressed or sublethally injured cells may not grow on selective media (Silk and Donnelly, 1997; Brashears et al., 2001). Finally, these methods can take more than 48 hours to complete.

In recent years, DNA-based technologies have been developed, overcoming many of the obstacles which have plagued conventional assays. DNA-based technologies such as polymerase chain reaction (PCR), provide several benefits over traditional microbiological assays, including increased sensitivity and specificity. These

technologies target known nucleotide sequences in a bacterial genome, enabling researchers to accurately confirm the presence of a pathogen on a molecular level rather than on phenotypic properties (Malorny et al., 2003). Researchers have also reported results using optical biosensors and oligonucleotide microarrays for pathogen detection in foods (Tait et al., 2004; Kakinuma et al., 2003). These methods have been shown to have varying levels of success for pathogen detection, yet problems such as reduced sensitivity and expense have limited their use.

Polymerase chain reaction–

PCR, first conceived by Kary Mullis in 1983, has been called one of the most important and influential scientific discoveries in the last one hundred years (Walker, 2002). PCR has most commonly been used as a tool for selective amplification of a unique region from a highly complex DNA or RNA template (Mullis et al., 1994). PCR has enhanced the sensitivity of conventional fractionation techniques because it can be initiated from even a single molecule of DNA or RNA (Mullis et al., 1994).

PCR is a three step process which, generally, includes a denaturation step, an annealing step, and an extension step. For initiation of PCR for amplification of DNA, double-stranded DNA (dsDNA) must be denatured to single strands through a heating step to approximately 95˚C in a thermocycler. Once heated, single strands are cooled to approximately 55˚C in the presence of oligonucleotide primers which anneal to single-stranded DNA. Finally, in the extension step, DNA polymerase adds nucleotides

(dNTPs) from the 5' end to the 3', resulting in two, double stranded DNA molecules. This process is repeated until millions of copies of the original DNA template have been produced (Jay et al., 2005). The PCR product can be identified by size using agarose gel electrophoresis where the product is then compared to a DNA ladder of known fragment sizes.

PCR is widely used as a tool for detection and identification of bacteria and viruses in foods (Jay et al., 2005). Assays have been developed to identify bacteria including, but not limited to, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* in a variety of foods, such as meats, dairy products and fruits and vegetables (Gilgen et al., 1997; McKillip et al., 2000; Shearer et al., 2001; Kingsley et al., 2002; Aslam et al., 2003). The sensitivity, specificity and commercial availability of PCR-based kits have added to its use and popularity in food science-based research (Jay et al., 2005).

PCR results can be skewed or impeded in several ways. The complex nature of food components presents challenges in the application of PCR to rapid detection of pathogens in a food. For example, researchers have found that collagen in meats (Kim et al., 2000), bean sprout homogenates (Hill, 1996), components in oysters (Hill, 1996), soft cheeses (Wernars et al., 1991), and fruit juices (Ogunjimi et al., 1999) have been shown to inhibit PCR amplification. Amplification inhibition may also occur if incorrect temperatures and times are chosen for DNA denaturation, primers are incompatible with the gene target, or an insufficient number of cycles is chosen (Meng et al., 1996). Other PCR limitations have been reported. For example, post-PCR analysis of DNA may introduce opportunities for carry-over contamination of PCR products (Fratimaco et al.,

2001a).

Cultural enrichment–

Although nucleic acid amplification methods, such as PCR, are increasingly being used for pathogen detection in foods, limits of detection seldom exceed 10^2 to 10^3 CFU/g or ml in a food product (Swaminathan and Feng, 1994; Wilson, 1997). When researchers have reported detection limits of ≤10 CFU/ml or CFU/g, these have been accomplished almost always after an enrichment period of 8-48 hours, often performed to increase cell numbers to detectable levels or to overcome the problem of PCR inhibitors (Liming and Bhagwat, 2004). In addition, enrichment steps often limit the ability for same-day analysis of *E. coli* in foods. Gilgen and others (1998) developed a PCR for detection of verotoxin-producing *E. coli* in ground beef. Enriched sample results were compared to those of non-enriched samples and it was found that the enrichment step (6 hours) enhanced the detection limit of *E. coli* by a factor of 100. In this study, without an enrichment step, detection was possible only up to a dilution of $1x10^6$ CFU/10g ground beef. In another study comparing traditional culture methods against rapid methods, researchers were able to detect *E. coli* O157:H7 inoculated in a variety of meat products (10^{-1} to 10^{-9}) using PCR (Bohaychuk et al., 2005). Although the researchers reported detection levels of *E. coli* O157:H7 of less than <1 CFU/25 g, meat samples had been enriched through a 35˚C incubation for 24 hours prior to PCR (Bohaychuk et al, 2005). Fratamico and Bagi (2001) applied an 18 hour enrichment step to a *TaqMan* real-time

PCR assay to enhance detection of *E. coli* O157:H7 from spent irrigation water collected during alfalfa sprouting. Recently, a modified immunoliposome sandwich assay was developed for the detection of *E. coli* O157:H7 in apple cider. As few as 1 CFU/ml were detected after an 8 hour enrichment, however, the lower limit of detection without enrichment was $7x10^3$ CFU/ml (Park and Durst, 2004).

Real-time polymerase chain reaction–

In the early 1990s, Holland and others (1991) reported that the 5' nuclease activity of *Taq* DNA polymerase could be exploited as a way to indirectly estimate the level of DNA amplification with the use of specific fluorescent probes, eliminating the need for electrophoresis. Soon after, Higuchi and others (1993) demonstrated that real-time monitoring of DNA amplification within a PCR reaction tube during PCR could be achieved by using fluorescent DNA binding dyes (kinetic PCR). The combination of these two processes laid the foundation for the technology of fluorescence detection real-time PCR

In the last decade, advancements have taken place in PCR technology which include the development of real-time PCR instrumentation and chemistries. In contrast to standard PCR, real-time PCR utilizes optical detection systems which collect fluorescent signals from labeled PCR products during each cycle of the PCR (Hanna et al., 2005; Ginzinger, 2002). A computer records and subsequently displays the fluorescence in relative fluorescence units (RFU). The number of cycles needed to yield enough

fluorescent signal to exceed a defined threshold is known as the threshold cycle, or C_t, and is used as an indicator of successful target amplification (Mackay, 2004).

Real-time PCR can be used for both quantification of a target as well as basic detection since amplification is monitored during the entire reaction. The time at which a sample's fluorescence crosses the threshold depends on the number of copies of target DNA present in the original sample. Higher numbers of DNA will cause an earlier crossing, while fewer will cross later in the real-time PCR run. The C_t from a sample can be compared to a standard curve derived from known amounts of target DNA for determination of initial starting copy number (Ibekwe et al., 2002).

Detection chemistries–

There are multiple fluorescence-based chemistries commonly utilized in real-time PCR assays, which include but are not limited to, SYBR green I dye, molecular beacons, and TaqMan probes (Walker, 2002; Schaad et al., 2002; Ginzinger, 2002). SYBR green I is a dye which only emits a fluorescent signal when bound to double-stranded DNA (Walker, 2002; Hanna et al., 2005). As more double-stranded DNA amplicons increase, SYBR green I dye signal increases (Fig. 1). The accumulation of fluorescence bound to amplified DNA is then measured by real-time PCR machinery. Since probes are not used in this type of chemistry, specificity of the PCR reaction depends upon the melting temperature (T_m) of the obtained amplicon. Products of different lengths and sequences

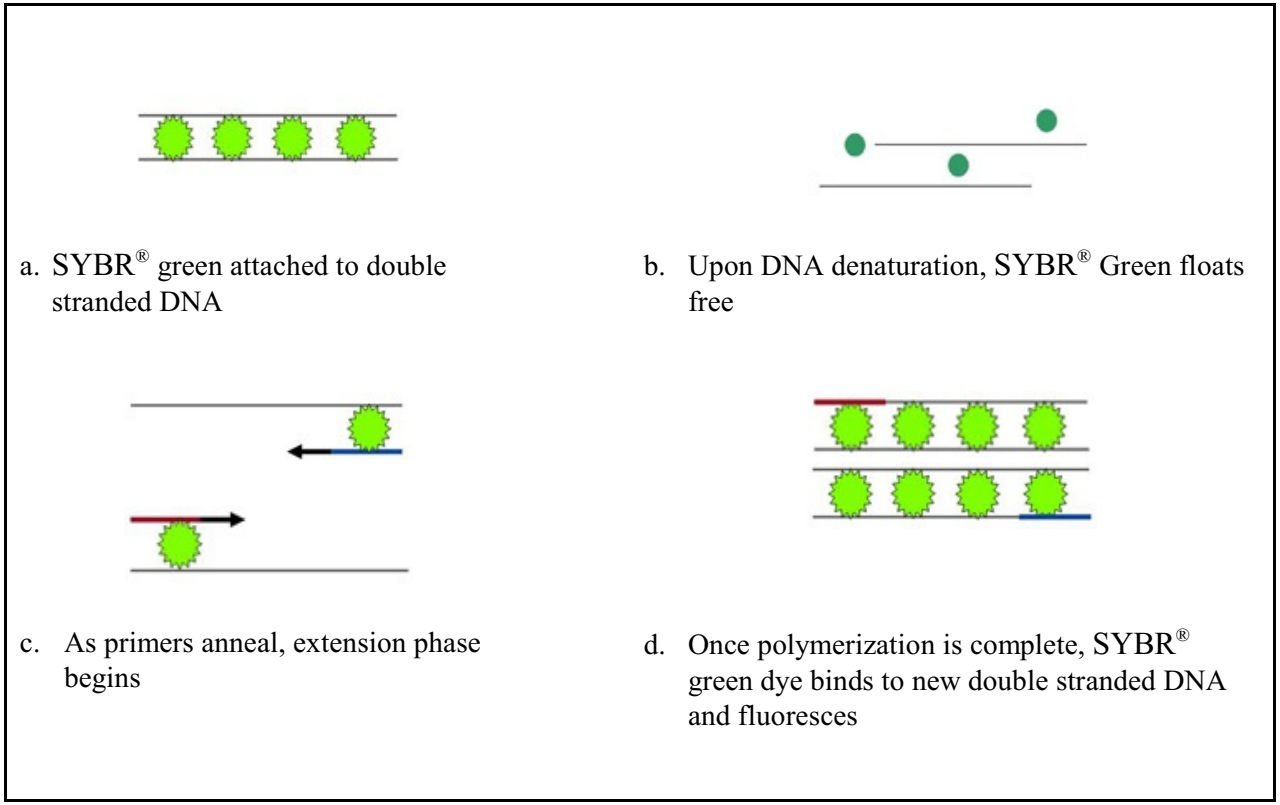


Figure 1. Schematic of SYBR® Green dye I chemistry

will produce distinct peaks via melting curve analysis. Although SYBR green I is relatively inexpensive and easy to use, its disadvantage is that the dye will bind to any double-stranded DNA (dsDNA) in the reaction. This may include primer-dimers and other non-specific products, leading to an inflated estimation of the starting template when used for quantification. However, melting curve analysis can aid in distinguishing desirable from undesirable product. The longer a dsDNA and the higher the G-C content, the higher the melting point. Through analysis of melting temperatures at which DNA strands separate, a distinction can be made between desired and undesired product, or between organisms in a multiplex reaction (deMedici et al, 2003; Wang et al., 2004). *Campylobacter jejuni* has been detected on chicken skin using a SYBR Green I real-time PCR assay at levels of 1 or 10 CFU per 10g after a 24 hour enrichment period. Melting curve analysis was used to distinguish *C. jejuni* from primer-dimers (Oliveria et al., 2005). Researchers have also used SYBR Green I real-time PCR assays for simultaneous detection of organisms in foods. Following an overnight enrichment, Jothikumar and others (2003) were able to detect both *Listeria monocytogenes* and *Salmonella* serovars at levels of 1 cell and 2.5 cells, respectively, using a SYBR Green I real-time PCR assay. The researchers were able to identify *Listeria monocytogenes* and *Salmonella* serovars based upon individual melting temperatures (Jothikumar, 2003).

Molecular beacons are short (between 25 and 35 bases in length), single-stranded oligonucleotide molecules which, in their most common formation, are in a “hairpin” loop (Fortin et al., 2001). While in this formation, the 3' (quencher) and 5' (fluorescent) ends are in close proximity, causing suppression of fluorescence. When the beacon binds

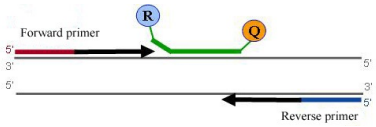
to a complementary target, the hairpin formation is forced to flatten, releasing fluorescence (Hanna et al., 2005). Development of beacons must be extremely accurate for the application, as one mismatched base could cause the beacon to remain in the hairpin formation, suppressing fluorescent and subsequently, detection (Hanna et al., 2005). Molecular beacons were first described in a food microbiology application for the detection of *E. coli* O157:H7 in skim milk (McKillip and Drake, 2000). Fortin et al. (2001) tested the specificity and sensitivity of a molecular beacon real-time PCR assay in milk and apple juice. After a selective enrichment of 6 hours, *E. coli* O157:H7 could be detected in raw milk samples as low as 1 CFU/ml. However, 11 hours of enrichment was needed to detect 1 CFU/ml in apple juice. The researchers hypothesized the reduced sensitivity in the apple juice was possibly due to polyphenolic compounds (Fortin et al., 2001). Other researchers have developed a molecular beacon-real-time PCR assay for detection of *Salmonella* species on artificially contaminated whole and fresh-cut fruits and vegetables (Liming and Bhagwat, 2004). The molecular beacon assay was able to detect *Salmonella* spp. from fruit and vegetable samples at levels of 1 to 3 CFU/g. This experiment proved that a molecular beacon assay could reduce detection time for *Salmonella* spp. to 18 hours from 3 to 4 days using conventional methods (Liming and Bhagwat, 2004).

Recent studies have shown that TaqMan probes in combination with real-time PCR is an effective tool for detection of low numbers of pathogens in foods, such as fruits, vegetables, juices, dairy products, seafood and meats (Fortin et al, 2001; Reischl et al., 2002; Bhagwat, 2004). TaqMan probes are oligonucleotides which attach to a desired

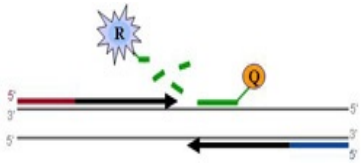
sequence within an amplicon. Designed with a reporter dye on the 5' end and a quencher dye on the 3' end, the reporter remains suppressed by the quencher until it is cleaved by the 5' nuclease activity of the enzyme. Once cleaved, the 5' reporter dye becomes free, preventing the quencher from suppressing the fluorescence. Fluorescence emissions are subsequently recorded by the optical module (Giuletti et al., 2001) (Fig. 2). Ellingson and others (2004) developed a real-time PCR for detection of *Salmonella* in raw and ready-to-eat meat products. After a 6 h enrichment period and a cell concentration step, *Salmonella* could be detected down to 1 CFU/ml in meat samples through the use of fluorescent probes. They concluded *Salmonella* (*Sip B* and *SipC*-encoded proteins) could be detected using this methods within 12 hours of receipt of a meat sample (Ellingson et al., 2005). Hsu et al. (2005) developed a duplex TaqMan real-time PCR for detection of Shiga-like toxin-producing *E. coli* O157:H7 ($rfb_{E.}$ and *stx2* genes) in milk, beef and apple juice using fluorescent probes. The detection sensitivity of the assay ranged from 10^3 to 10^9 CFU/ml in milk, 10^4 to 10^9 CFU/ml for apple juice and 10^5 to 10^9 CFU/g for beef samples without enrichment. However, detection limits were markedly lower in all food products after enrichment with a range of 10^0 to 10^3 CFU/ml in modified tryptic soy broth. These researchers found that their detection success depended on DNA extraction efficiency and removal of PCR inhibitors in the food substances tested (Hsu et al., 2005).

Well-designed primers and probes are vital to the success of a real-time PCR. A compatible primer and probe design reduces the chance of non-specific products during real-time PCR. Primer pairs should produce small amplicons between 50-150 bp, promoting high-efficiency assays. Primer T_m should be in the range of 58-60°C and

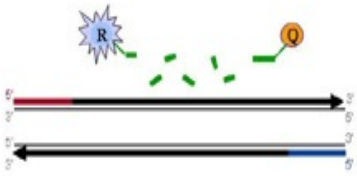
a. Polymerization: Primers and TaqMan® probe anneal to DNA and extension begins



b. Displacement: When the enzyme reaches the probe, the 5' nuclease activity of the enzyme begins to cleave the probe



c. Cleavage: As the probe is cleaved, the reporter fluoresces as it is separated from the quencher



d. Polymerization is complete

Figure 2. Schematic of TaqMan® probe chemistry

probe T_m should be significantly higher (7-11°C) than the primer T_m to ensure full hybridization during primer extension. Ideally, primers should possess a low G-C content on the 3' end, assuring denaturation during thermal cycling. Probes with a G-C content between 30-80% without runs of identical nucleotides are desirable to prevent a less efficient reaction.

Other food applications--

Researchers have described using real-time PCR as a tool for detection of allergens in foods (Stephan and Vieths, 2004). In a recent study, Hird et al. (2003) reported detection of peanut, a possible food allergen, down to 2 mg/kg in spiked biscuits using real-time PCR. Stephan and Vieths (2004) also described a study for detection of trace amount of peanuts in processed food using real-time PCR. These researchers found detection limits to be 10 ppm of peanut in whole milk and chocolate. It is suggested that these methods could be useful for evaluating the effectiveness of cleaning procedures from food processor production lines, which in turn, may help prevent protect consumers from ingestion of hidden allergens (Stephan and Vieths, 2004).

Real-time PCR has been evaluated as a means for detecting genetically modified (GM) DNA content in foods (Garcia-Canas et al., 2005). This is of particular importance in Europe, where the European Union (EU) has set a limit of 0.5% GMO in foods. Kunert et al. (2006) used a TaqMan probe-based assay with real-time PCR for detection and quantification of genetically modified organisms (GMO) in maize flour. Based on a

plasmid standard curve, these researchers were able to detect and quantify (on average) 25 copies of 35S promoter in 200 ng of genomic DNA, resulting in 0.07% GM maize in a cornmeal sample. Real-time PCR has also been utilized for successful detection of genetically modified wheat in foods (Iida et al., 2005).

Research to determine the efficacy of real-time PCR for detection of spoilage organisms has mainly focused on beverages such as fruit juices and wine. Casey and Dobson (2004) reported detection of spoilage yeasts in artificially contaminated apple juice as low as $4x10^4$ CFU ml^{-1} using a SYBR Green I dye assay with real-time PCR. In wine samples, researchers used a real-time PCR to detect and quantify *Saccharomyces cerevisiae*, a common spoilage organism. *S. cerevisiae* could be detected and quantified in artificially contaminated red and sweet wines for concentration values between $3.8x10^5$ to 3.8 CFU/ml and $5x10^6$ to 50 CFU/ml, respectively (Martorell et al., 2005). However, the researchers stated that the number of cells quantified was likely underestimated due to the presence of PCR inhibitors in the wine samples. It is probable that an additional step to remove PCR inhibitors prior to DNA extraction would enhance detection limits.

Cell concentration methods for improved detection--

Traditionally, real-time PCR assays have depended upon enrichment procedures to increase low bacterial numbers to detectable levels. Although real-time PCR allows for quantification of a target, enrichment creates a variation in cell numbers which does not reliably represent the initial sample. Methods such as filtration, immunomagnetic

separation (IMS) and centrifugation have been reported to improve detection and quantification of microorganisms in food samples without an enrichment step (Fratimaco, 2001b). Recent studies omitting enrichment steps have involved high bacterial concentrations or a small sample volume (1 to 2 ml), eliminating the need for an increase in bacterial numbers (Amavisit et al, 2001; Inglis and Kalischuk, 2004; Rudi et al., 2004). Few studies have successfully used larger sample volumes (100 to 250 ml) for detection of very low numbers of pathogenic organisms in a food sample.

Rodrígues-Lázaro et al. (2004) described a method for the quantitative detection of *Listeria monocytogenes* in meat products based on filtration, DNA purification, and real-time PCR. They reported that a filtration step followed by a Chelex-100-based DNA purification prior to real-time PCR considerably increased the sensitivity of the assay. Standard curves were constructed for quantification of *L. monocytogenes,* demonstrating that this method could consistently detect the organism down to 10^3 CFU/g without prior enrichment. Furthermore, in 50% of cooked ham samples, organisms could be detected in replicates containing 10^2 CFU/g. These finding suggest that the combination of filtration and DNA purification allow for rapid quantification of *L. monocytogenes* down to 1,000 CFU/g and detection of down to 100 CFU/g in meat products. Fu et al. (2005) developed a technique combining IMS and real-time PCR for enhanced detection of *E. coli* O157:H7 in buffer and ground beef samples. Magnetic beads were used to capture *E. coli* O157:H7 from bacterial suspensions and ground beef where subsequently, cells were eluted from the beads, lysed by heating, and subjected to real-time PCR. Although cell capture efficiency and real-time PCR efficiency was reduced (likely due to meat-

associated inhibitors), a minimum detection limit of 1.3 x 10^4 cells g^{-1} in ground beef samples resulted without an enrichment step. The researchers emphasized that this assay could likely be improved with further DNA concentration and removal of meat-associated inhibitors through a filtration step.

Johnston et al. (2005) reported a method for direct detection of *Salmonella* and *E. coli* O157:H7 from raw alfalfa sprouts and spent irrigation water using PCR without an enrichment step. Upon seeding alfalfa sprouts and spent irrigation water with 10^{-1} to 10^{-6} CFU/g or CFU/ml of *Salmonella enteritica* serovar Typhimurium and *E. coli* O157:H7, respectively, samples were blended and centrifuged at high speeds to concentrate the bacterial population. This concentrate was used for DNA isolation, amplification of PCR product and amplicon confirmation. In irrigation water, both pathogens were detected as low as 100 CFU/liter of water and in blended sprouts, both pathogens could be detected at concentrations as low as 10 CFU/g. This indicates that pre-concentration could result in detection of low numbers of pathogens, while possibly omitting an enrichment step. Lampel et al. (2000) reported application of concentrated food washes to FTA filters to directly extract DNA for real-time PCR detection of *Shigella flexneri, Salmonella enterica* serotype Typhimurium, and *Listeria monocytogenes.* The sensitivities of detection with FTA filter-based PCR were 30 to 50 CFU for *Shigella* and *Salmonella*, and 200 CFU for *Listeria*. After concentrating bacteria from food washes on FTA filters and subjecting the filters to PCR, researchers determined detection limits were similar to pure culture dilutions. These studies confirm the potential benefits of sample purification and concentration in enhancing detection limits of PCR and real-time PCR.

REFERENCES

Anonymous. 2005. Health department identifies an additional case of *E. coli* O157:H7 associated with unpasteurized apple cider products. The Regional Municipality of Durham Health Department, Ontario, Canada. http://www.region.durham.on.ca/news.asp?nr=dnews/health/2005/oct1305.htm. Accessed February 7, 2006.

Amavisit, P., Browning, G.F., Lightfoot, D., Church, C., Anderson, G.A., Whithear, K.G., and Markham, P.F. 2001. Rapid PCR detection of Salmonella in horse faecal samples. Vet. Microbiol. 79:63-74.

Aslam, M., Hogan, J., and Smith, K.L. 2003. Development of a PCR-based assay to detect Shiga toxin-producing *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* in milk. Food Microbiol. 20:345-350.

Batt, C.A. 1997. Molecular diagnostics for dairy-borne pathogens. J. Dairy Sci. 80:220-229.

Bachrouri, M., Quinto, E.J., and Mora, M.T. 2002. Survival of *Escherichia coli* O157:H7 during storage of yogurt at different temperatures. J. Food Sci. 67:1899-1903.

Benjamin, M.M. and A.R. Datta. 1995. Acid tolerance of enterohemorrhagic *Escherichia coli*. Appl. Environ. Microbiol. 61:1669-1672.

Besser, R.E., S.M. Lett, J.T. Weber, M.P. Doyle, T.J. Barrett, J.G. Wells, P.M. Griffin. 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. J. Am. Med. Assoc. 269:2217-2220.

Bhagwat, A.A. 2004. Rapid detection of *Salmonella* from vegetable rinse-water using real-time PCR. Food Microbiol. 21:73-78.

Bohaychuk, V.M., Gensler, G.E., King, R.K., Wu, J.T., and McMullen, L.M. 2005. Evaluation of detection methods for screening meat and poultry products for the presence of foodborne pathogens. J. Food Prot. 68:2637-2647.

Brashears, M.M., Amezquita, A., and Stratton, J. 2001. Validation of methods used to

recover *Escherichia coli* O157:H7 and *Salmonella* spp. subjected to stress conditions. J. Food Prot. 64:1466-1471.

Buchanan, R.L. and Klawitter, L.A. 1992. The effect of incubation temperature, initial pH, and sodium chloride on the growth kinetics of *Escherichia coli* O157:H7. Food Microbiol. 9:185-196.

Buchanan, R.L. and Doyle, M.P. 1997. Foodborne disease significance of *Escherichia coli* O157:H7 and other enterohemorrhagic *E. coli.* Food Tech. 51:69-76.

Burnett, S.L., Chen, J., and Beuchat, L.B. 2000. Attachment of *Escherichia coli* O157:H7 to the surfaces of apples as detected by confocal scanning laser spectroscopy. Appl. Env. Microbiol. 66:4679-4687.

Casey, G.D. and Dobson, A.D.W. 2004. Potential of using real-time PCR-based detection of spoilage yeast in fruit juice–a preliminary study. Intl. J. Food Microbiol. 91:327-335.

Centers for Disease Control and Prevention. 1975. *Salmonella* Typhimurium outbreak traced to commerical apple cider–New Jersey. Morbid. Mortal. Weekly Rep. 24:87-88.

Centers for Disease Control and Prevention. 1996. Outbreaks of *Escherichia coli* O157:H7 infection and cryptosporidiosis associated with drinking unpasteurized apple cider–Connecticut and New York, October 1996. Morbid. Mortal. Weekly Rep. 46:4-8.

Centers for Disease Control and Prevention. 2002. Multistate oubreak of *Escherichia coli* O157:H7 infections associated with eating ground beef–United States, June-July 2002. MMWR. 51(29):637-639.

Centers for Disease Control and Prevention. 2006. Multistate oubreak of *Escherichia coli* O157 infections, November-December 2006. http://www.cdc.gov/ecoli/2006/december/120806.htm. Accessed February 8, 2007.

Clavero, M.R.S. and L.R. Beuchat. 1996. Survival of *Escherichia coli* O157:H7 in broth and processed salami as influenced by pH, water activity, and temperature and suitability of media for its recovery. Appl. Environ. Microbiol. 62:2735-2740.

Cody, S.H., Glynn, M.K., Farrar, J.A., Cairns, K.L., Griffin, P.M., Kobayashi, J., Fyfe, M., Hoffman, R., King, A.S., Lewis, J.H., Swaminathan, B., Bryant, R.G., Vugia, D.J. 1999. An outbreak of *Escherichia coli* O157:H7 infection from unpasteurized commercial apple juice. Ann. Intern. Med. 130:202-209.

Daly, J. 1997. Fresh apple cider: past, present and future. J. Assoc. Food Drug Off. 61:60-65.

De Medici, D., Croci, L., Delibato, E., Pasquale, S., Filetici, E., and Toti, L. 2003. Evaluation of a SYBR Green I real-time PCR to detect *Salmonella entericaI* serotype *enteritidis* in poultry. Appl. Env. Microbiol. 69:3456-3461.

Doyle, M.P., Zhao, T., Meng, J., and Zhao, S. 1997. *Escherichia coli* O157:H7, p. 171-191. In M.P. Doyle, L.R. Beuchat and T.J. Montville (ed.), *Food Microbiology: Fundamentals and Frontiers.* ASM Press, Washington D.C.

Downing, D. 1989. Apple Cider. P. 169-188. In D.L. Downing (ed.), *Processed Apple Products,* Van Nostrand Reinhold, New York.

Ellingson, J.L.E., Anderson, J.L., Carlson, S.A., and Sharma, V.J. 2005. Twelve hour real-time PCR technique for the sensitive and specific detection of *Salmonella* in raw and ready-to-eat meat products. Mol. Cell Probes. 18:51-57.

FDA. 1998. Critical controls for juice safety. http://www.cfsan.fda.gov/~dms/fdjuice.html. Accessed February 1, 2006.

FDA. 1999. Report of 1997 Inspections of fresh, unpasteurized apple cider. http://www.cfsan.fda.gov/~dms/ciderrp2.html. Accessed: February 1, 2006.

FDA. 2001. Analysis and evaluation of preventive control measures for the control and reduction/elimination of microbial hazards on fresh and fresh-cut produce. http://www.cfsan.fda.gov/~comm/ift3-4o.html. Accessed February 3, 2006.

FDA. 2006. FDA statement on foodborne *E. coli* O157:H7 outbreak in spinach. http://www.fda.gov/bbs/topics/NEWS/2006/NEW01460.html. Accessed September 22, 2006.

Feng, P. and Weagent, S.D. 2002. Diarrheagenic *Escherichia coli*. http://www.cfsan.fda.gov/~ebam/bam-4a.html. Food and Drug Administration bacteriological analytical manual. Accessed: February 3, 2006.

Fitzmaurice, J., Glennon, M., Duffy, G., Sheridan, J.J., Carroll, C., Maher, M. 2004. Application of real-time PCR and RT-PCR assays for the detection and quantitation of VT1 and VT2 toxin genes in *E. coli* O157:H7. Mol. Cell. Probes. 18:123-132.

Fortin, N.Y., A. Mulchandani, W. Chen. 2001. Use of real-time polymerase chain reaction and molecular beacons for the detection of *E. coli* O157:H7. Anal. Biochem. 289:281-288.

Fratamico, P.M., and Bagi, L.K. 2001a. Comparison of an immunochromatographic method and the Taqman® *E. coli* O157:H7 assay for detection of *Escherichia coli* O157:H7 in alfalfa sprout spent irrigation water and in sprouts after blanching. J. Ind. Microbiol. Biotechnol. 27:129-134.

Fratamico, P.M. 2001b. Applications of the polymerase chain reaction for detection, identification, an typing of foodborne microorganisms, P.95-99. In Wilson, C.L., Droby, S. (ed.), Microbial Food Contamination. New York, CRC Press.

Garcia-Canas, V., Cifuentes, A., and Gonzalez, R. 2004. Detection of genetically modified organisms in foods by DNA amplification techniques. Crit. Rev. Food Sci. Nut. 44:425-436.

Gilgen, M., Hübner, P., Höfelein, C., Lüthy, and Candrian, U. 1998. PCR-based detection of verotoxin-producing *Escherichia coli* (VTEC) in ground beef. Res. Microbiol. 149:145-154.

Ginzinger, D.G. 2002. Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. Exp. Hematol. 30:503-512.

Giuletti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., and Mathieu, C. 2001. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods 25:386-401.

Grant, M.A. 2003. Evaluation of methods to improve detection of *Escherichia coli*

O157:H7 in fresh produce by multiplex polymerase chain reaction. J. Food Prot. 66:18-24.

Hanna, S.E., Connor, C.J., and Wang, H.H. 2005. Real-time polymerase chain reaction for the food microbiologist: Technologies, applications, and limitations. J. Food Sci. 70:R49-R53.

Heuvelink, A.E., Zwartkrukis-Nahuis, J.T.M., and DeBoer, E. 1997. Evaluatio of media and test kits for the detection and isolation of *Escherichia coli* O157 from minced beef. J. Food Prot. 60:817-824.

Higuchi, R., Fockler, C., Dollinger, and Watson, R. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotech. 9:1026-1030.

Hilborn, E.D., P.A. Mshar, T.R. Fiorentino, Z.F. Dembek, T.J. Barrett, R.T. Howard, and M.L. Cartter. 2000. An outbreak of *Escherichia coli* O157:H7 infections and haemolytic uraemic syndrome associated with consumption of unpasteurized apple cider. Epidemiol. Infect. 124:31-36.

Hill, W.E. 1996. The polymerase chain reaction: applications for the detection of foodborne pathogens. Crit. Rev. Food Sci. Nutr. 36:123-173.

Hinkens, J.D., Faith, N.G., Lorang, T.D., Bailey, P., Buege, D., Kaspar, C.W., and Luchansky, J.B. 1996. Validation of pepperoni process for control of *Escherichia coli* O157:H7. J. Food Prot. 59:1260-1266.

Hird, H., Lloyd, J., Goodier, R., Brown, J., Reece, P. 2003. Detection of peanut using real-time polymerase chain reaction. Eur. Food Res. Technol. 217:265-268.

Holland, P.M., Abramson, R.D., Watson, R., and Gelfand, D.H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'–3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. USA. 88:7276-7280.

Hsu, C.F., Tsai, T.Y., and Pan, T.M. 2005. Use of the duplex TaqMan PCR system for detection of shiga-like toxin-producing *Escherichia coli* O157:H7.

Ibekwe, A.M., Watt, P.M., Grieve, C.M., Sharma, V.K. Lyons, S.R. 2002. Multiplex fluorogenic real-time PCR for detection and quantification of *Escherichia coli* O157:H7 in dairy wastewater wetlands. Appl. Environ. Microbiol. 68:4853-4862.

Iida, M., Yamashiro, S., Yamakawa, H., Hayakawa, K., Kuribara, H., Kodama, T., Furui, S., Akiyama, H., Maitani, T., and Hino, A. 2005. Development of taxon-specific sequences of common wheat for the detection of genetically modified wheat. J. Agric. Food Chem. 53:6294-6300.

Inglis, G.D., and Kalischuk, L.D. 2004. Direct quantification of *Campylobacter jejuni* and *Campylobacter lanienae* in feces of cattle by real-time quantitative PCR. Appl. Environ. Microbiol. 70:2296-2306.

Janes, M.E., Kim, K.S., and Johnson, M.G. 2005. Transmission electron microscopy study of enterhemorrhagic *Escherichia coli* O157:H7 in apple tissue. J. Food Prot. 68:216-224.

Jay, J.M. Loessner, M.J., and Golden, D.A. 2005. Foodborne gastroenteritis caused by *Escherichia coli* O157:H7, p. 531-547. In *Modern Food Microbiology,* 7th ed. Springer Science, New York, NY.

Johnson, J.R. 2002. Evolution of Pathogenic *Escherichia coli*, p. 55-77. In M.S. Donnenberg, *Escherichia coli Virulence Mechanisms of a Versatile Pathogen.* Academic Press, San Diego, CA.

Johnston, L.M., Elhanafi, D., Drake, M.A., and Jaykus, L.A. 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. J. Food Prot. 68:2256-2263.

Jothikumar, N., Xiaowen, W., and Griffiths, M.W. 2003. Real-time multiplex SYBR green I-based PCR assay for simultaneous detection of *Salmonella* serovars and *Listeria monocytogenes.* J. Food Prot. 66:2141-2145.

Kakinuma, K., M. Fukushima, R. Kawaguchi. 2003. Detection and identification of *Escherichia coli*, *Shigella,* and *Salmonella* by microarrays using the *gyrB* gene. Biotechnol. Bioeng. 83:721-728.

Kim, S., Labbe, R.G., and Ryu, S. 2000. Inhibitory effects of collagen on the PCR for detection of Clostridium perfringens. Appl. Env. Microbiol. 66:1213-1215.

Kingsley, D.H., Meade, G.K., and Richards, G.P. 2002. Detection of both hepatitis A and Norwalk-like virus in imported clams associated with food-borne illness. Appl. Env. Microbiol. 68:3914-3918.

Kunert, R., Gach, J.S., Vorauer-Uhl, K., Engel, E., and Katinger, H. 2006. Validated method for quantification of genetically modified organisms in samples of maize flour. J. Agric. Food Chem. 54:678-681.

Lampel, K.A., Orlandi, P.A., and Kornegay, L. 2000. Improved template preparation for PCR-based assays for detection of food-borne bacterial pathogens. Appl. Env. Microbiol. 66:4539-4542.

Law, D. 2000. Virulence factors of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*. J. Appl. Microbiol. 88:729-745.

LeBlanc, J.J. 2003. Implication of virulence factors in *Escherichia coli* O157:H7 pathogenesis. Crit. Rev. Microbiol. 29:277-296.

Leyer, G.J., Wang, L., and Johnson, E.A. 1995. Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. Appl. Environ. Microbiol. 61:3752-3755.

Liming, S.H. and Bhagwat, A.A. 2004. Application of a molecular beacon-real-time PCR technology to detect *Salmonella* species contaminating fruits and vegatables. Intl. J. Food Microbiol. 95:177-187.

Mackay, I. 2004. Real-time PCR in the microbiology laboratory. Clin. Microbiol. Infect. 10:190-212.

McKillip, J.L., and Drake, M.A. 2000. Molecular beacon polymerase chain reaction detection of *Escherichia coli* O157:H7 in milk. J. Food Prot. 63:855-859.

McKillip, J.L., Jaykus, L.A., and Drake, M.A. 2000. A comparison of methods for the detection of *Escherichia coli* O157:H7 from artificially-contaminated dairy products

using PCR. J. Appl. Microbiol. 89:49-55.

Malorny, B., P.T. Tassios, Rådström, N. Cook, M. Wagner, J. Hoorfar. 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. Int. J. Food Microbiol. 83: 39-48.

Marques, P.A.H.F., Worcman-Barninka, D., Lannes, S.C.S., and Landgraf, M. 2001. Acid tolerance and survival of *Escherichia coli* O157:H7 inoculated in fruit pulps stored under refrigeration. J. Food Prot. 64:1647-1678.

Martorell, P., Querol, A., and Fernández-Espinar, M.T. 2005. Rapid identification and enumeration of *Saccharomyces cerevisiae* cells in wine by real-time PCR. Appl. Env. Microbiol. 71:6823-6830.

Meng, J., Zhao, S., Doyle, M.P., Mitchell, S.E., Kresovich, S. 1996. Polymerase chain reaction for detecting *Escherichia coli* O157:H7. Intl. J. Food Microbiol. 32:103-113.

Miller, L.G., and Kaspar, C.W. 1993. *Escherichia coli* O157:H7 acid tolerance and survival in apple cider. J. Food Prot. 57:460-464.

Mullis, K.B., Ferre, F., Gibbs, R.A. 1994. The Polymerase Chain Reaction. Birkhäuser, Boston, MA.

Ogunjimi, A.A., P.V. Choudary. 1999. Adsorption of endogenous polyphenols relieves the inhibition by fruit juices and fresh produce of immuno-PCR detection of *Escherichia coli* O157:H7. FEMS Imm. Med. Microbiol. 23:213-220.

Oliveira, C.R.M., Barbut, S., and Griffiths, M.W. 2005. Detection of *Campylobacter jejuni* in naturally contaminated chicken skin by melting peak analysis of amplicons in real-time PCR. Intl. J. Food Microbiol. 104:105-111.

Oyarzábal, O.A., Nogueria, M.C.L., and Gombas, D.E. 2003. Survival of *Escherichia coli* O157:H7, *Listeria monocytogenes,* and *Salmonella* in juice concentrates. J. Food Prot. 66:1595-1598.

Park, S., R.A. Durst. 2004. Modified immunoliposome sandwich assay for the detection

of *Escherichia coli* O157:H7 in apple cider. J. Food Prot. 67:1568-1573.

Paton, J.C., and Paton, A.W. 1998. Pathogenesis and diagnosis of shiga toxin-producing *Escherichia coli* infections. Clin. Microbiol. Rev. 11:450-479.

Pollard, D.R., Johnson, W.M., Lior, H., S.D. Tyler, and Rozee, K.R. 1990. Rapid and specific detection of verotoxin genes in *Escherichia coli* by polymerase chain reaction. J. Clin. Microbiol. 28:540-545.

Proulx, A., and Nichols, L. 2005. Cider: Making, using & enjoying sweet & hard cider. 3rd ed. Maryland: Storey Publishing. 219 p.

Raghubeer, E.V., Ke, J.S., Campbell, M.L., and Meyer, R.S. 1995. Fate of *Escherichia coli* O157:H7 and other coliforms in commercial mayonnaise and refrigerated salad dressing. J Food Prot. 58:13-18.

Reischl, U., M.T. Youssef, J. Kilwinski, N. Lehn, W.L. Zhang, H. Karch, and N.A. Strockbine. 2002. Real-time fluorescence PCR assays for detection and characterization of shiga toxin, intimin, and enterohemolysin genes from shiga toxin-producing *Escherichia coli*. J. Clin. Microbiol. 40:2555-2565.

Reitsma, C.J. and Henning, D.R. 1996. Survival of enterohemorrhagic *Escherichia coli* O157:H7 during the manufacture and curing of Cheddar cheese. J. Food Prot. 59:460-464.

Rodríguez-Lázaro, D., Jofré, A., Aymerich, T, Hugas, M, Pla, M. 2004. Rapid Quantitative detection of *Listeria monocytogenes* in meat products by real-time PCR. Appl. Env. Microbiol. 70:6299-6301.

Rudi, K., Hoidal, H.K., Katla, T., Johansen, B.K., Nordal, J., and Jakobsen, K.S. 2004. Direct real-time PCR quantification of *Campylobacter jejuni* in chicken fecal and cecal samples by integrated cell concentration and DNA purification. Appl. Environ. Microbiol. 70:790-797.

Schaad, N.W., and Frederick, R.D. 2002. Real-time PCR and its applications for rapid plant disease diagnostics. Can. J. Plant Pathol. 24:250-258.

Semanchek, J.J. and Golden, D.A. 1996. Survival of *Escherichia coli* O157:H7 during fermentation of apple cider. J. Food Prot. 59:1256-1259.

Sharma, V.K. Detection and quantitation of enterohemorrhagic *Escherichia coli* O157, O111, and O26 in beef and bovine feces by real-time polymerase chain reaction. J. Food Prot. 65:1371-1380.

Shearer, A.E.H., Strapp, C.M., and Joerger, R.D. 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. J. Food Prot. 64:788-795.

Silk, T.M., Ryser, E.T., and Donnelly, C.W. 1997. Comparison of methods for determining coliform and *Escherichia coli* levels in apple cider. J. Food Prot. 60:1302-1305.

Silk, T.M., and Donnelly, C.W. 1997. Increased detection of acid injured *Escherichia coli* O157:H7 in autoclaved apple cider by using non-selective repair on trypticase soy agar. 60:1483-1486.

Stephan, O., and Vieths, S. 2004. Development of a real-time PCR and a sandwich ELISA for detection of potentially allergenic trace amounts of peanut (*Arachis hypogaea*) in processed foods. J. Agric. Food Chem. 54:3754-3760.

Swaminathan, B., and Feng, P. 1994. Rapid detection of food-borne pathogenic bacteria. Ann. Rev. Microbiol. 48:401-426.

Taitt, C.R., Y.S. Shubin, R. Angel, F.S. Ligler. 2004. Detection of *Salmonella enteria* serovar Typhimurium by using a rapid, array-based immunosensor. Appl. Env. Microbiol. 70:152-158.

Thorpe, C.M., Ritchie, J.M., and Acheson, D.W.K. 2002. Enterohemorrhagic and other Shiga-toxin-producing *Escherichia coli*, p. 119-154. In M.S. Donnenberg, *Escherichia coli Virulence Mechanisms of a Versatile Pathogen.* Academic Press, San Diego, CA.

Vold, L., Holck, A., Wasteson, Y., and Nissen, H. 2000. High levels of background flora inhibits growth of *Escherichia coli* O157:H7 in ground beef. Intl. J. Food Microbiol.

56:219-225.

Walker, N.J. 2002. A techniques whose time has come. Science. 296:557-559.

Wang, X., Jothikumar, N., and Griffiths, M. 2004. Enrichment and DNA extraction protocols for the siumltaneous detection of *Salmonella* and *Listeria monocytogenes* in raw sausage meat with multiplex real-time PCR. J. Food Prot. 67:189-192.

Werners, K., Hevvelman, C.J., Chakraborty, T., and Notermans, S.H.W. 1991. Use of the polymerase chain reaction for direct detection of *Listeria monocytogenes* in soft cheeses. J. Appl. Bacteriol. 70:121-126.

Wilson, I.G. 1997. Minireview: inhibition and facilitation of nucleic acid amplification. Appl. Env. Microbiol. 63:3741-3751.

Wolffs, P., Norling, B, and Rådström. 2004. Rapid uantification of *Yersinia enterocolitica* in pork samples by a novel sample preparation method, flotation, prior to real-time PCR. J. Clin. Microbiol. 42:1042-1047.

Zhao, T., Doyle, M.P., and Besser, R.E. 1993. Fate of Enterohemorrhagic *Escherichia coli* O157:H7 in apple cider with and without preservatives. Appl. Env. Microbiol. 59:2526-2530.

Zhao, T. and Doyle, M.P. 1994. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in commercial mayonnaise. J. Food Prot. 57:780-783.

Zhao, T., Doyle, M.P., Shere, J., and Garber, L. 1995. Prevalence of enterohemorrhagic *Escherichia coli* O157:H7 in a survey of dairy herds. Appl. Env. Microbiol. 61:1290-1293.

PART III

PRELIMINARY STUDIES

GENE TARGET SELECTION AND PRELIMINARY PCR

Prior to initiation of studies described in this dissertation, gene targets, DNA extraction techniques, and PCR conditions for detection of enterohemorrhagic *Escherichia coli* in apple cider were examined for potential use in a real-time PCR. Originally, two strains of *E. coli* O157:H7 (ATCC 43889, ATCC 43894) and a non-pathogenic *E. coli* strain (K-12, ATCC 12435) were used for PCR studies. Four DNA extraction techniques/kits [InstaGene Matrix (Bio-Rad, Hercules, CA), FTA® (Whatman Inc., Clifton, NJ), GenElute™ Bacterial Genomic Kit (Sigma-Aldrich Co., St. Louis, MO), and PrepMan Ultra Sample Preparation Reagent™ (Applied Biosystems, Foster City, CA)] were applied to the pure cultures according to the manufacturers' protocol.

Since the goal of these studies was to produce a rapid method without enrichment for detection of *E. coli* O157:H7 in apple juice, the DNA extraction methods were studied for ease of use and time consumption. DNA from strains ATCC 43889, ATCC 43894, and K-12 was extracted with four methods for use in a preliminary PCR. The InstaGene Matrix kit, a 45 minute DNA extraction procedure, involved centrifugation steps, addition of the Instagene Matrix, an incubation period, and a boil step. Similarly to InstaGene, PrepMan Ultra™ involved centrifugation steps and a boil step. However, unlike InstaGene, it was originally developed for DNA preparation from Gram-negative pathogens present in food and required less time (approximately 15 minutes). FTA cards, fibrous filter-type papers, are infused with chelators which trapped bacterial cells, and chemicals (proprietary) which lysed cells, captured DNA and protected DNA from

nuclease damage. After inoculating the card with 100 µl of culture, a series of washes and a thorough drying step (1 h) were performed according to the manufacturer's instructions. A 2 mm "punch" was taken from the card and was directly added to a PCR reaction tube for amplification. Although the procedure took over an hour, it did lack the additional purification steps of the other methods. Finally, the GenElute™ kit, required a lysis step, followed by a step which bound DNA to a silica-based column. The column was washed to remove impurities, then washed with a buffer solution to elute DNA into a collection tube. The procedure took approximately 2 hours to complete.

Upon extraction of DNA by each method, it was quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA) to measure yield. The manufacturer's protocol was followed and double-stranded DNA (dsDNA) measured on a TBS-80 fluorometer (Turner Biosystems, Sunnyvale, CA) (measured in ng/ml). InstaGene and PrepMan produced similar DNA yield. It was observed that GenElute produced a higher yield of DNA for all three *E. coli* strains, likely due to the additional purification steps in the protocol (Table 1). After consultation with Whatman representatives, a preliminary protocol (unpublished) to elute DNA from FTA cards was carried out to measure yield. However, after several attempts, DNA could not be eluted in measurable amounts.

Once DNA was obtained from *E. coli* samples, it was subjected to PCR using both TaKaRa Taq™ DNA polymerase kit (Takara Bio., Otsu, Shiga, Japan) and SYBR Green (Invitrogen, Carlsbad, CA), in a final volume of 50 µl in a 0.2 ml microfuge tube. Test primers for several genes found in *E. coli*, including *stx1* (virulence gene), *stx2* (virulence gene), *eae* (encodes for intimin), *tir* (encodes for intimin receptor), and *uidA*

Table 1. DNA yield (ng/ml) for *E. coli* strains from four commercial DNA isolation methods.

	DNA yield (ng/ml) for:			
Strain	Instagene	PrepMan	GenElute	FTA
ATCC 43889	8.22	8.21	11.59	ND
ATCC 43894	8.53	7.45	15.94	ND
ATCC 12435 (K-12)	6.14	6.83	12.98	ND

ND: Not detected by PicoGreen dsDNA Assay Kit

Data were derived from three separate extraction procedures

(encodes for β-D-glucoronidase activity), were used in the initial studies (Table 2). The *stx1, stx2,* and *uidA* primers were adapted from Jinneman et al. (2003), and the primers for *eae* and *tir* were obtained from Dr. Harry Richards, University of Tennessee. For TaKaRa reactions, 25.0 µl water, 0.25 µl *Taq*, 5.0 µl 10X buffer, 5.0 µl dNTP, 5.0 µl of 0.5 µM forward primer, 5.0 µl of 0.5 µM reverse primer, and 5.0 µl template DNA were added per reaction tube. For TaKaRa reactions with FTA, the amounts were modified to 29.75 µl water, 0.25 µl *Taq*, 5.0 µl 10X buffer, 5.0 µl dNTP, 5.0 µl of 0.5 µM forward primer, 5.0 µl of 0.5 µM reverse primer, and one 2 mm FTA punch which contained DNA template. SYBR reactions were made up of 25 µl SYBR Green dye, 2.5 µl of 0.5 µM forward primer, 2.5 µl of 0.5 µM reverse primer, 10 µl water, and 10 µl DNA. SYBR reactions with FTA were made up of 25 µl SYBR Green dye, 2.5 µl of 0.5 µM forward primer, 2.5 µl of 0.5 µM reverse primer, 20 µl water, and one 2 mm FTA punch which contained DNA template. DNA was initially denatured at 95°C for 2 minutes followed by amplification through 50 cycles of denaturation, annealing and polymerization. Denaturation was performed at 95° C for 1 minute, primer annealing at 48° C for 1 minute, and DNA extension at 72° C for 1 minute, respectively. The final extension was carried out at 72° C for 3 minutes. For detection of amplified product, a 10 µl aliquot of the PCR product was mixed with 5 µl dye and examined by electrophoresis through a 2% agarose gel in a 1X TBE buffer (90V for 2h). Identification of bands was established by comparison of the band size with two molecular weight markers, ϕX174 DNA-Hae digest (72 - 1,353 bp) and pBR322 DNA-BstNI digest (121 - 1,875 bp) (New Englad Biolabs, Ipswich, MA), after staining with ethidium bromide.

Table 2. Primer sequences used for detection of *E. coli* O157:H7 in preliminary PCR tests.

Primer	Sequence (5' to 3')	Target
Forward	GTGGCATTAATAATACTGAATTGTCATCA	*stx1*[a]
Reverse	GCGTAATCCCACGGACTCTTC	
Forward	GATGTTTATGGCGGTTTTATTTGC	*stx2*[b]
Reverse	TGGAAAACTCAATTTTACCTTTAG	
Forward	GACCCGGCACAAGCATAA	*eae*[c]
Reverse	CCACCTGCAGCAACAAGAGG	
Forward	GTGGTGGATTACGCCATGACATGGGAGGATTAACGG GGGGGAGTAATAGC	*tir*[d]
Reverse	GTAAACTACCTGGAGAATCCACGGGCATAACCGATT CCGCATTAT	
Forward	CAGTCTGGATCGCGAAAACTG	*uidA*[e]
Reverse	ACCAGACGTTGCCCACATAATT	

[a, b, c]Adapted from Jinneman et al., 2003

[d, e]Obtained from Dr. Harry Richards, University of Tennessee

Each experiment was carried out in triplicate.

If the three test strains (ATCC 43889, ATCC 43895, ATCC 12435) were positive for the genes tested by PCR, bands would be present at 109 bp (*stx1*), 83 bp (*stx2*), 106 bp (*eae*), 50 bp (*tir*), and 143 bp (*uidA*). The ATCC 43889 had bands present for *stx2, uidA, tir,* and *eae.* This organism did not have a band present for *stx1,* which is expected since this strain of *E. coli* does not carry the gene. ATCC 43894 had bands present for all genes tested. The non-pathogenic strain, K-12, did not have bands present at *stx1, stx2, tir* and *eae.* However, a band was visible for *uidA*. These results were similar (band visualization) across all DNA extraction methods and chemistry types (data not shown).

Although many genes, such as the ones in the preliminary studies, have been the focus of research in the detection of *E. coli* O157:H7, *stx1, stx2,* and *uidA* genes have been well studied in PCR-based food science applications. Shiga toxins produced by *E. coli* O157:H7 are typically considered the chief virulence factor associated with causation of illness, therefore, these toxin genes were chosen for further studies. The gene *uidA* was chosen because it is present in approximately 94% of *E. coli* strains and could be used as a "control" (Feng et al., 1991). Although *E. coli* O157:H7 does not exhibit β-glucuronidase (GUD) activity which is encoded by the *uidA* gene, they do possess the entire gene (Monday et al., 2001).

REAL-TIME POLYMERASE CHAIN REACTION MACHINERY

The ABI Prism® 7000 Sequence Detection System is a complete real-time PCR system capable of detection and quantification of DNA sequences through fluorescent-based PCR chemistries. This instrument, capable of accommodating 96 samples at once, combines thermal cycling and fluorescence detection in a single instrument. The instrument is attached to a notebook computer in which the sequence detection system (SDS) software is loaded.

The ABI unit is a tungsten-halogen lamp-based system which utilizes a single excitation filter and four individual emission filters. These filters are capable of detecting fluorescent emissions over a range of 500 nm-650 nm. Each filter is specific to the emission maxima of different dyes: filter A for FAM and SYBR®; filter B for VIC and JOE; filter C for NED and TAMRA; and filter D for ROX (Table 3). These dyes can be designed onto a probe as a reporter or quencher for use in fluorescence detection of a desired DNA sequence.

Fluorescence detection on the ABI Prism® 7000 Sequence Detection System is carried out by a Peltier-cooled charged-coupling device (CCD). The data collected during a real-time experiment is analyzed by "multicomponenting," a process through which the contribution of individual dyes to an overall raw signal can be accurately determined. Determination is through a series of equations that compare raw signal in a plate well or tube to pure spectral fingerprints stored on the computer hard drive. This allows accurate measurement of levels of a particular dye by the 7000 instrument.

Table 3. Fluorescent dye labels detected by the ABI Prism 7000 Sequence Detection System

Dyes	Chemical Name	Absorbance[a]	Emission[b]
FAM	Carboxyfluoroscein	500	520
JOE	6-carboxy-4',5'-dichloro-2',7-dimethoxyfluoroscein	529	555
NED	Proprietary Applied Biosystems	553	575
ROX	Carboxy-X-rhodamine	588	608
TAMRA	Carboxytetramethylrhodamine	559	583
VIC	Proprietary Applied Biosystems	538	554

[a,b]Absorbance and emission maxima in nanometers

Through either SYBR® green I dye or TaqMan® probe (FAM, JOE, NED ROX, TAMRA, VIC dyes) chemistries, the ABI Prism® 7000 Sequence Detection System has the capability of not only detection, but quantification of a target nucleic acid sequence using a standard curve method. This allows determination of the quantity of a single nucleic acid target sequence within an unknown sample. For quantification, DNA standards diluted over several orders of magnitude are loaded into a reaction plate along with unknown samples of target. During a real-time PCR run, the instrument records the fluorescence emission either from cleavage of a TaqMan® probe or an increase in fluorescence of SYBR® green dye bound to ds-DNA. Afterward, the software in the ABI Prism 7000 processes the raw fluorescence data to produce threshold cycle (C_t) value for each sample. A standard curve is figured from the C_t values of the diluted standards and software extrapolates quantities for the unknown samples based on their C_t values.

PRIMER AND PROBE SELECTION FOR REAL-TIME PCR

Primers for successful detection of *Escherichia coli* O157:H7 in foods using PCR have been documented **(**Heller et al., 2003; Ibwekwe et al., 2002; Sharma, 2002; Al-Gallas et al., 2002; Radu et al., 2001; Fortin et al., 2001; Miyamoto, 2002; Jinneman et al., 2003). The primers and probes chosen for the studies in this dissertation were adapted from Jinneman et al. (2003) **(**Table 4**).** The original reporter dyes on the *stx1* (ROX)*, stx2* (FAM)*,* and *uidA* (TET) probes were modified to 6FAM, VIC, and NED for *stx1, stx2,* and *uidA*, respectively, for optimal detection in the ABI Prism 7000. The

Table 4. Primer and probe sequences used for detection of *E. coli* O157:H7 in a real-time PCR

Primer or probe	Sequence (5' to 3')	Target
Forward	GTG GCA TTA ATA ATA CTG AAT TGT CAT CA	*stx1*
Reverse	GCG TAA TCC CAC GGA CTC TTC	
Probe	6FAM-TGA TGA GTT TCC TTC TAT GTG TCC GGC GCA GAT-MGBNFQ[a]	
Forward	GAT GTT TAT GGC GGT TTT ATT TGC	*stx2*
Reverse	TGG AAA ACT CAA TTT TAC CTT TAG CA	
Probe	VIC-TCT GTT AAT GCA ATG GCA ATG GCG GCG GGA TT-MGBNFQ	
Forward	CAG TCT GGA TCG CGA AAA CTG	*uidA*
Reverse	ACC AGA CGT TGC CCA CAT AAT T	
Probe	NED-ATT GAG CAG CGT TGG-MGBNFQ	

Primers and probes adapted from Jinneman et al., 2003

quencher dyes, originally Black Hole Quenchers, were replaced with non-fluorescent quenchers (NFQ) (Applied Biosystems, Foster City, CA). Unlike other commonly used quencher dyes, the NFQ enhances the performance of a probe because it is effective over a broad wavelength range and does not emit a detectable fluorescent signal of its own. This improves spectral discrimination and makes interpretation of data easier.

In addition to the NFQ, the probes for preliminary real-time PCR experiments have an integrated minor groove binder (MGB) molecule. When the TaqMan® probe hybridizes, the MGB stablilizes annealing because it folds into the minor groove of the DNA duplex created between the probe and target sequence. The MGB is attached at the 3' end of the probe in conjunction with the NFQ dye.

STANDARD CURVE GENERATION FROM PURE BACTERIAL CULTURE

Initial primer testing was carried out by extracting genomic DNA from multiple microorganisms with the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO) because of its ability to produce a high DNA yield, and PrepMan Ultra (Applied Biosystems, Foster City, CA) because of its cost-effectiveness (~$1.00 per sample) and ability to extract PCR quality DNA from Gram-negative foodborne organisms. *E. coli* O157:H7 ATCC 43889, ATCC 43890, ATCC 43894 and ATCC 43895 (all Shiga toxin-producers), and *E. coli* K-12 ATCC 12435 and ATCC 10798 (non-Shiga toxin producers) were evaluated for presence of *stx1, stx2,* and *uidA* gene sequences.

Shigella flexneri and *Salmonella* Montevideo DNA was also extracted and evaluated to ensure primers did not react with non-*E. coli* species. DNA measurements were read on a TBS-380 mini-fluorometer (Turner Biosystems, Sunnyvale, CA) in ng/µl and 10-fold DNA dilutions were made for standard curve construction (Tables 5 and 6). DNA yield was representative of approximately 7.0 log CFU/ml, serially diluted 10-fold over four orders of magnitude (Figs. 3, 4, 5 and 6). For these initial studies, 50 µl volumes were used for all reactions. For SYBR plates, thermocycler conditions were 95°C for 15 minutes (hotstart), followed by 40 cycles of denaturation, annealing, and polymerization. The hotstart enabled a withholding of vital reagents until after the annealing temperature is reached, which reduces primer-dimers and premature annealing. Denaturation was performed at 95°C for 1 minute, primer annealing at 48°C for 1 minute, and DNA extension at 72°C for 1 minute, respectively. All conditions were similar for TaqMan probes except for the initial denaturation step, which was lowered to 95°C for 2 min. Real-time PCR product was removed from the TaqMan plate and visualized on an agarose gel for comparison purposes (Fig. 5).

All *E. coli* O157:H7 tested positive for the presence of either *stx1* or *stx2* and *uidA*. *E. coli* K-12 strains, *S. flexneri*, and *S.* Montevideo tested negative for *stx1* and *stx2* gene sequences. Further cross-reactivity studies indicated cross reactivity with enterohemorrhagic *E. coli* O26 and O111. This was true for experiments utilizing either SYBR green or TaqMan probes, with either GenElute or PrepMan. The standard curves generated in these experiments indicated that the selected primers could be successful for detection and possible quantification of enterohemorrhagic *E. coli* in apple cider.

Table 5. DNA yield (ng/ml) for *E. coli* strains and non-*E. coli* strains using GenElute Bacterial Genomic DNA kit.

E. coli O157:H7		*E. coli* K-12		Non-*E. coli*	
Strains	DNA Yield[a]	Strains	DNA Yield	Strains	DNA Yield
ATCC 43889	13.87	ATCC 12435	13.07	*S. flexneri*	12.36
ATCC 43890	10.54	ATCC 10798	11.34	*S.* Montevideo	15.6
ATCC 43894	10.15				
ATCC 43895	12.97				

[a]DNA measurements are averages of three separate extraction procedures.

Table 6. DNA yield (ng/ml) for *E. coli* strains and non-*E. coli* strains using PrepMan Ultra Sample Preparation Reagent.

E. coli O157:H7		*E. coli* K-12		Non-*E. coli*	
Strain	DNA Yield[a]	Strains	DNA Yield	Strains	DNA Yield
ATCC 43889	9.45	ATCC 12435	9.31	*S. flexneri*	10.23
ATCC 43890	8.33	ATCC 10798	7.55	*S.* Montevideo	11.49
ATCC 43894	7.87				
ATCC 43895	8.83				

[a]DNA measurements are averages of three separate extraction procedures.

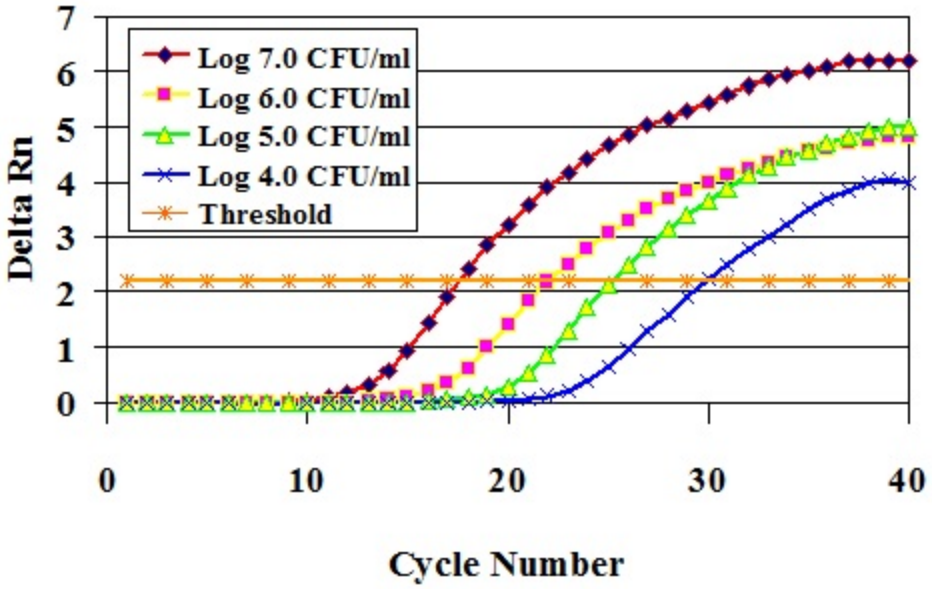


Figure 3. Typical amplification plot generated by real-time PCR using GenElute Bacterial DNA Extraction Kit and TaqMan Universal PCR Master Mix (ATCC strain 43895, *stx2* gene, 12.97 ng/ml diluted 10-fold over four orders of magnitude).

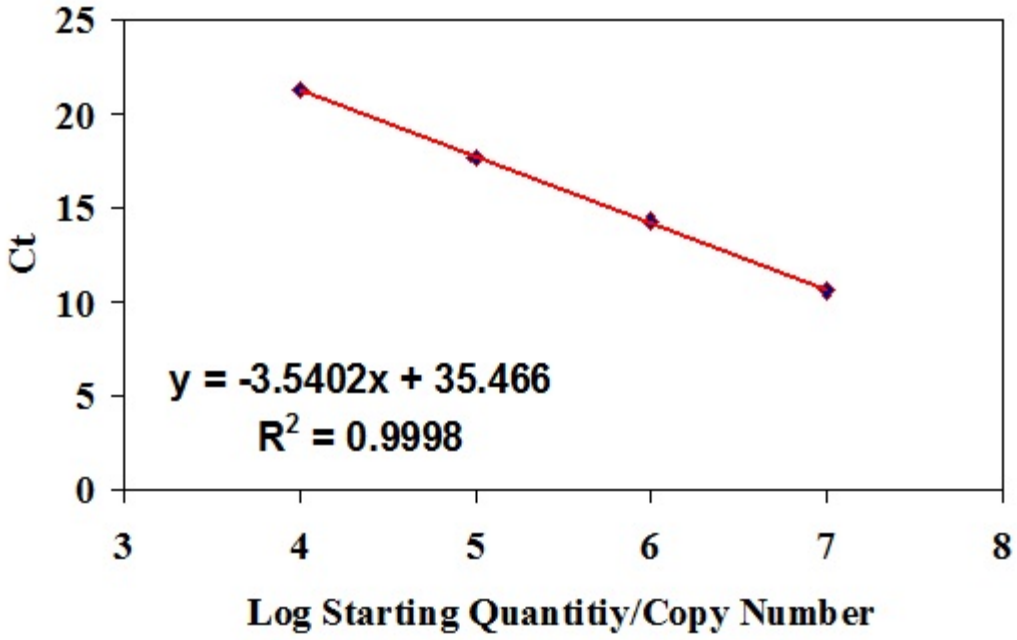


Figure 4. Typical standard curve generated by real-time PCR using GenElute Bacterial Genomic DNA kit and PrepMan Universal PCR Master Mix (ATCC strain 43895, *stx2* gene, 12.97 ng/ml diluted 10-fold over four orders of magnitude).

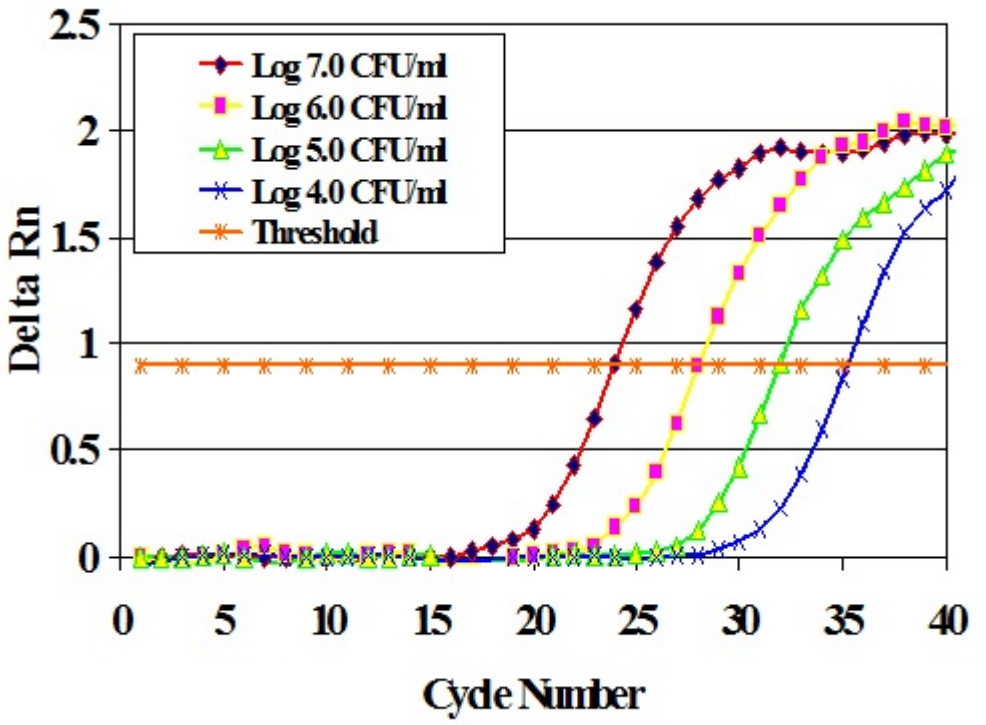


Figure 5. Typical amplification plot generated by real-time PCR using PrepMan Ultra Sample Preparation Reagent and TaqMan Universal PCR Master Mix (ATCC strain 43895, *stx2* gene, 12.97 ng/ml diluted 10-fold over four orders of magnitude).

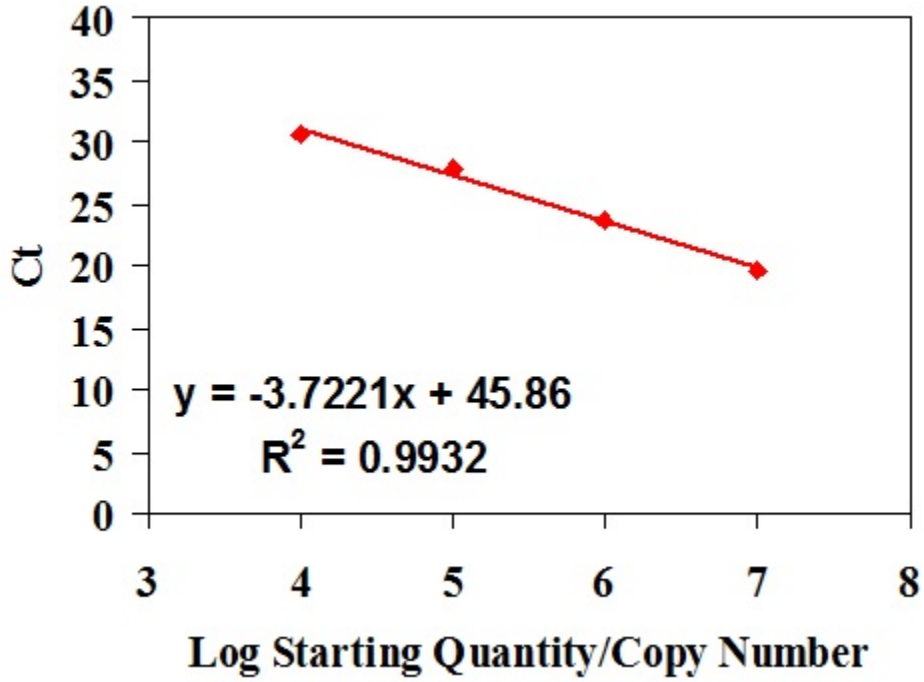


Figure 6. Typical standard curve generated by real-time PCR using GenElute Bacterial Genomic DNA kit and PrepMan Universal PCR Master Mix (ATCC strain 43895, *stx2* gene, 12.97 ng/ml diluted 10-fold over four orders of magnitude).

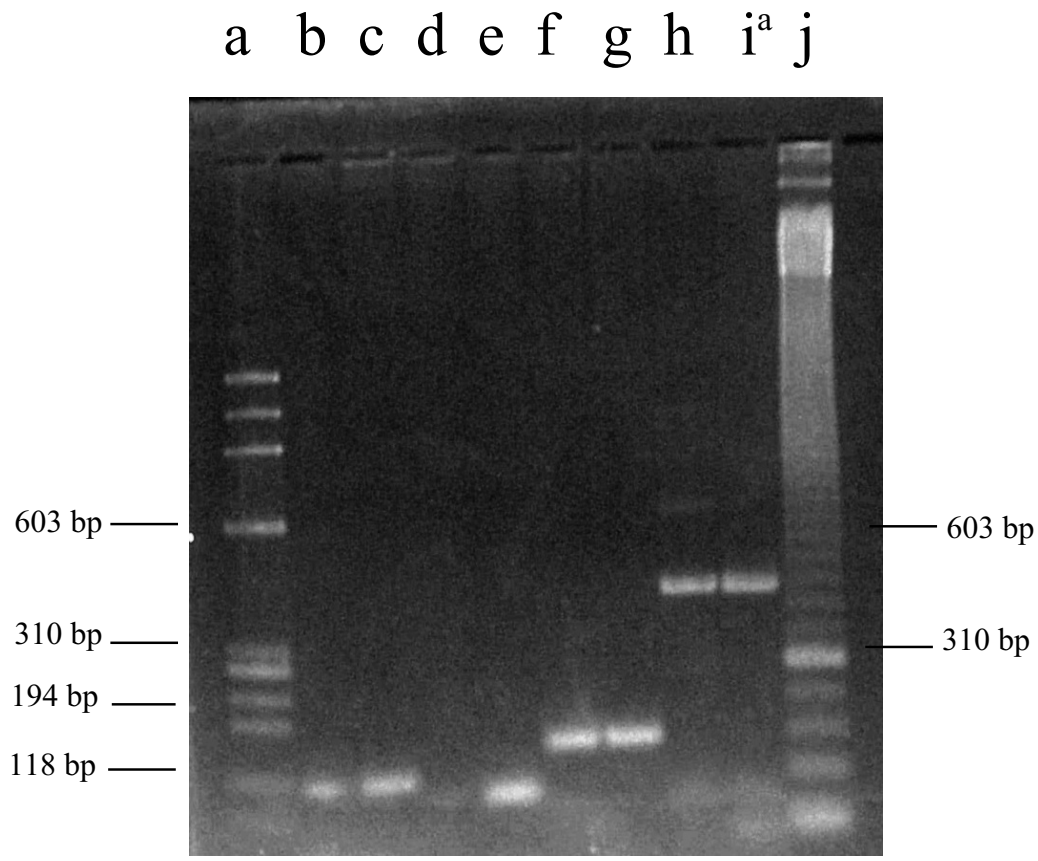


Figure 7. PCR amplification of DNA from *E. coli* O157:H7 ATCC Strain 43895. Lanes: (a) ϕX174 DNA-Hae III digest ladder (New England Biolabs, Ipswich, MA); (b) and (c) *stx1*, 109 bp; (d) and (e) *stx2*, 83 bp; (f) and (g) *uidA*, 143 bp; (h) and (i) *eaeA*, 450 bp; (j) pBR322 DNA-BstNI digest ladder (New England Biolabs, Ipswich, MA).

[a]*eaeA* was omitted from further study.

REFERENCES

Al-Gallas, N., Ben-Aissa, R.R., Attia-Annabi, T., Bahri, O., and Boudabous, A. 2002. Isolation and characterization of Shiga toxin-producing *Escherichia coli* from meat and dairy produts. Food Microbiol. 19:389-398.

Feng, P., Lum, R., and Chang, G.W. 1991. Identification of *uidA* gene sequences in β-D-Glucoronidase-Negative *Escherichia coli*. Appl. Environ. Microbiol. 57:320-323.

Fortin, N.Y., A. Mulchandani, W. Chen. 2001. Use of real-time polymerase chain reaction and molecular beacons for the detection of *E. coli* O157:H7. Anal. Biochem. 289:281-288.

Heller, L.C., David, C.R., Peak, K.K., Wingfield, D., Cannons, A.C., Amuso, P.T., and Cattani, J. 2003. Comparison of methods for DNA isolation from food samples for detection of Shiga toxin-producing *Escherichia coli* by real-time PCR. Appl. Environ. Microbiol. 69:1944:1846.

Ibwekwe, A.M., Watt, P.M., Grieve, C.M., Sharma, V.K. Lyons, S.R. 2002. Multiplex fluorogenic real-time PCR for detection and quantification of *Escherichia coli* O157:H7 in dairy wastewater wetlands. Appl. Environ. Microbiol. 68:4853-4862.

Jinneman, K.C., Yoshitomi, K.J., and Weagant, S.D. 2003. Multiplex real-time PCR method to identify Shiga toxin genes *stx1* and *stx2* and *Escherichia coli* O157:H7/H$^-$ serotype. Appl. Environ. Microbiol. 69:6327-6333.

Monday, S.R., Whittam, T.S., and Feng, P.C.H. 2001. Genetic and evolutionary analysis of mutations in the *gusA* gene that cause the absence of β-glucoronidase activity in *Escherichia coli* O157:H7. J. Infect. Dis. 184:918-921.

Radu, S., Ling, O.W., Rusul, G., Abdul-Karim, M.I., and Nishibuchi, M. 2001. Detection of *Escherichia coli* O157:H7 by multiplex PCR and their characterization by plasmid profiling, antimicrobial resistance, RAPD and PFGE analysis. J. Microbiol. Meth. 46:131-139.

Sharma, V.K. 2002. Detection and quantitation of enterohemorrhagic *Escherichia coli* O157, O111, and O26 in beef and bovine feces by real-time polymerase chain reaction. J. Food Prot. 65:1371-1380.

PART IV

A COMPARISON OF REAL-TIME POLYMERASE CHAIN REACTION DETECTION CHEMISTRIES IN COMBINATION WITH DIFFERENT DNA EXTRACTION METHODS FOR DETECTION OF *ESCHERICHIA COLI* O157:H7 AND CROSS-REACTIVITY EXAMINATION

ABSTRACT

DNA extraction methods (GenElute and PrepMan) in combination with real-time PCR detection chemistries (SYBR and TaqMan) were evaluated for detection of *E. coli* O157:H7 in a real-time PCR assay. Twelve, pure *E. coli* O157:H7 cultures (1 ml), along with twelve, non-*E. coli* O157:H7 cultures (1 ml), were individually diluted in 0.1% peptone water to approximately 4.0 log CFU/ml and subjected to two commercial DNA extraction kits, GenElute bacterial genomic kit and PrepMan Ultra. Template (5 µl) from each extraction method was used in a real-time PCR using both SYBR Green I dye and TaqMan probe chemistries for detection of *E. coli* O157:H7. The DNA extraction methods and real-time PCR chemistries were evaluated on the whole for simplicity, speed of result production and reproducibility. After 40 cycles in the ABI Prism 7000 Sequence Detection System, C_t values were evaluated for the four combinations (SYBR/PrepMan, SYBR/GenElute, TaqMan/PrepMan, TaqMan/GenElute). Across all methods, no significant differences ($p>0.05$) were found among the C_t values. Cross-reactivity with *stx1* and *stx2* primers and probes was observed with non-O157 strains, including *E. coli* O26 and O111, and *S. sonnei* G129. Non-O157 strains and *Shigella* strains exhibited amplification with *uidA*, but this results was not unexpected since these organisms also possess the gene. This study indicates that DNA extracted from pure culture with PrepMan Ultra in combination with TaqMan chemistry in a real-time PCR may potentially provide a successful method for detection of *E. coli* O157:H7 in apple juice.

INTRODUCTION

In the United States, foodborne illness is estimated to cost $10 to $83 billion each year (FDA, 2001). Historically, detection and identification of pathogenic microorganisms in foods has presented challenges. Initial contamination levels in foods are generally low, often creating sampling difficulty (Jaykus, 2003). Nutrients and specific ingredients present in foods can create a favorable environment for growth of microorganisms, yet at the same time, these ingredients can inhibit the enzymes involved in PCR detection (Rossen et al., 1992). High numbers of background microoganisms in foods can make detection more difficult. Consequently, development of specific and sensitive methods for detection of foodborne microorganisms in foods is crucial for a safe food supply.

Traditional culture and biochemical methods for detection and identification of pathogens, despite long-standing use, have limitations. For example, these cultures methods generally involve growth and isolation of the organism, plus biochemical tests for positive identification (FDA, 1998). These techniques are often time-consuming and can take days to obtain a positively identified sample. In order to address this issue, scientific researchers have developed molecular methods for detection and identification of pathogenic microorganisms in foods which are more rapid and specific than conventional culture methods (Feng, 2001; Rijpens and Herman, 2002; Smith et al., 2000).

Real-time polymerase chain reaction utilizes fluorescence-based chemistries

which measure the progress of amplification of labeled PCR products in "real-time". SYBR Green I dye and TaqMan probes are two available chemistries for use in a real-time PCR assay. SYBR green I dye, a non-specific dye suitable for real-time PCR, fluoresces after interfacing with double-stranded DNA. The specificity of the reaction is determined by the melting temperature (T_m) of the amplicon obtained. SYBR green is relatively easy to use without the requirement to design a probe; it can be used with any primer set; and is more cost effective than probes, such as those used in TaqMan assays. However, the limitations are its inability to discriminate between different dsDNA segments because it binds to all dsDNA, including primer-dimers and amplification of nontarget DNA. Hein et al. (2001) reported that SYBR green I could be used in real-time PCR for detection and quantification of *Staphylococcus aureus* in cheese. Liu et al. (2006) developed a SYBR Green real-time PCR for detection of *Enterobacter sakazakii* in infant formula. Yoshitomi et al. (2006) investigated a SYBR Green I-based real-time multiplex PCR method to detect *E. coli* grown in brain heart infusion broth (BHI).

TaqMan probes are a specific real-time chemistry based on a dual-labeled probe, bound to a specific region on a template strand of DNA. *Taq* polymerase cleaves the probe, releasing a fluorescent signal. TaqMan probe-based real-time PCR has been used in a variety of food applications. Ward and Bej (2006) developed a TaqMan probed-based real-time PCR for detection of *Vibrio parahaemolyticus* in shellfish. These researchers successfully detected an initial inoculum of 1 CFU/ml of *V. parahaemolyticus* after an overnight enrichment. Fu and others (2005) could detect at least $<5x10^2$ CFU/ml

E. coli O157:H7 in buffer and $1.3x10^4$ CFU/ml in ground beef samples with a fluorogenic probe-based real-time PCR assay. Researchers have also utilized TaqMan chemistry for detection of *E. coli* O157:H7 in raw milk (Buerk et al., 2002).

The successful detection of a target pathogen relies on application of template preparation and DNA extraction techniques. DeMedici et al. (2003) reported that two DNA extraction methods (boiling and Nucleospin) were successful for detection of *Salmonella enterica* in poultry samples when applied to a SYBR Green I real-time PCR. Lampel et al. (2000) investigated use of FTA filters for extraction of DNA from *S. flexneri, S.* Typhimurium, and *Listeria monocytogenes* in pure culture, and further testing was performed for detection of *Shigella* in food products by PCR. The procedure was successful for detection of *Shigella* at levels as low as 50 CFU/ml in a variety of food products. Higgins et al. (2001) evaluated four methods/kits (Prepman, Instagene, Xtra Amp, Isocode paper) for extraction of PCR-ready DNA from *E. coli* O157:H7 and *Cryptosporidium parvum* oocysts, in which all methods produced PCR-quality DNA.

Real-time PCR has been evaluated as a tool for detection of microorganisms from a variety of food products, including meat products, juices, dairy products and produce (Fortin et al., 2001; Buerk et al., 2002; Bhagwat, 2004; Fu et al., 2005). The objective of this study was to compare two DNA extraction methods (GenElute and PrepMan Ultra) for effectiveness and then combining this method with a real-time PCR based on either SYBR Green I dye or TaqMan probes for detection of *E. coli* O157:H7. Cross-reactivity with other shiga toxin-producing *E. coli* and non-*E. coli* strains were also examined.

MATERIALS AND METHODS

Strains and preparation of inoculum–

Twelve strains of *E. coli* O157:H7 (ATCC strains 43888, 43889, 43890, 43894, 43895; cider, apple cider; 994, salami; JITB, ground beef-associated outbreak; environmental samples A4, C9, F2, B5), two strains of *E. coli* O26 (#1, bovine isolate; 9B, human isolate), two strains of *E. coli* O111 (2145, human isolate; 2146 human isolate), two strains of *E. coli* K-12 (ATCC strains 10798 and 12435), three strains of *S. sonnei* (G129, 10304, 10305), as well as, *S.* Typhimurium, *S.* Enteritidis, and a non-pathogenic *E. coli* (NPEC) strain were used in this study. All *Shigella* cultures were obtained from Dr. Larry Beuchat, University of Georgia, and *E. coli* O111 and O26 strains were obtained from Dr. Stephen P. Oliver, University of Tennessee.

All cultures of each strain were grown separately in Luria-Bertani broth (LB) (Difco; Becton-Dickinson; Sparks, MD) at 37°C and transferred at 24-h intervals, with the exception of the *Salmonella* strains, which were grown at 35°C for 24-h. Twenty four-hour cultures were diluted 10-fold in 0.1% peptone water (PW) (Bacto Peptone; Difco; Becton-Dickinson; Sparks, MD) to approximately 4.0 log CFU/ml prior to DNA extraction. Cell counts were verified by surface plating (0.1 ml) in duplicate onto TSA and incubing at either 35°C or 37°C for 24 h. The count of 4.0 log CFU/ml was chosen since it would be the highest concentration inoculated into apple cider in further studies.

DNA isolation–

DNA extraction was carried out for each organism using both GenElute Bacterial Genomic Kit (Sigma-Aldrich Co., St. Louis, MO) and PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA). For GenElute extraction,1.5 ml of each bacterial broth was centrifuged (13,000 x *g,* 2 minutes–Eppendorf Microcentrifuge 5417C; Eppendorf North America; Westbury, NY) in a 2 ml microfuge tube, the spent PW was decanted, and the cell pellet resuspended in 180 µl lysis solution T (proprietary). RNase A (20 µl) was added to the solution which was then incubated at room temperature (2 min.). Proteinase K (20 µl) was added to the cell suspension, briefly vortexed to mix, and incubated at 55°C (30 min.). Lysis solution C (proprietary) (200 µl) was added to the cell suspension, briefly vortexed once again to mix, and incubated at 55°C for an additional 10 min. Ethanol (200 µl) was added to the lysed cells and the entire mixture transferred to a binding column, which was centrifuged (8,000 x *g*, 1 min.). The removable column was transferred to a new collection tube, wash solution (500 µl) was added, followed by centrifugation. This step was carried out twice (centrifugation was 8000 x *g*, 1 min and 13,000 x *g*, 3 min., respectively) to dry the column. Finally, the dry column was added to a new collection tube, where an elution solution (200 µl) was added, followed by centrifugation (8,000 x *g,* 1 min.). The eluate which contained DNA was stored at 4°C until further use. The GenElute extraction procedure took approximately two hours.

For DNA extraction with PrepMan Ultra, 1.5 ml PW containing bacterial culture was added to a 2 ml microfuge tube. Samples were centrifuged (16,000 x *g*, 3 min.), the

spent PW was decanted, and the cell pellet was resuspended in 200 µl PrepMan Ultra Sample Preparation Reagent. The suspension was briefly vortexed then heated in a boiling water bath for 10 min. Samples were once again centrifuged (16,000 x *g*, 3 min.), and the supernatant transferred to a new microfuge tube. Tubes which contained DNA were stored at 4°C until further use. The PrepMan Ultra procedure took approximately fifteen minutes.

Primers and probes–

The primers used for detection of *stx1*, *stx2,* and *uidA* have previously been described by Jinneman et al. (2003), and were designed to amplify sequences of 109 bp, 83 bp, and 143 bp, respectively. The probes chosen for the studies in this dissertation were adapted from Jinneman et al. (2003) **(**Table 2**).** The original reporter dyes on the *stx1* (ROX)*, stx2* (FAM)*,* and *uidA* (TET) probes were modified to 6FAM, VIC, and NED for *stx1, stx2,* and *uidA*, respectively, for optimal detection in the ABI Prism 7000. The quencher dyes, originally Black Hole Quenchers, were replaced with non-fluorescent quenchers (NFQ) (Applied Biosystems, Foster City, CA)

Real-time PCR conditions–

The DNA samples were subjected to real-time PCR using both SYBR Green I dye and TaqMan probe detection chemistries. The assays were performed using either GenElute or PrepMan in combination with SYBR Green PCR Master Mix or TaqMan

Universal PCR Master Mix (Applied Biosystems, Foster City, CA) (four combinations). For SYBR Green dye assays, each reaction mixture consisted of 12.5 µl SYBR Green PCR Master Mix (contains SYBR Green I dye, AmpliTaq Gold DNA polymerase, dNTPs, passive reference dye, and optimized buffer components), 5.0 µl RNase-free water, 5.0 µl DNA, and 1.25 µl (0.5 µM) each forward and reverse primer, for a 25 µl total reaction volume. For TaqMan assays, each reaction mixture consisted of 12.5 µl TaqMan Universal PCR Master Mix (contains AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs, passive reference dye, and optimized buffer components), 0.5 µl probe, 4.5 µl RNase-free water, 5.0 µl DNA, and 1.25 µl (0.5 µM) each forward and reverse primer, for a 25 µl total reaction volume. Amplification and detection were carried out in optical grade 96-well plates in an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA), with an initial denaturation at 95°C for 10 min followed by amplification through 40 cycles of denaturation, annealing and polymerization. Denaturation was performed at 95°C for 1 min, primer annealing at 48°C for 1 min, and DNA extension at 72°C for 1 min, respectively. All plates contained a negative control for each gene target without target DNA. All extractions were performed in triplicate. The reaction conditions for amplifications and the parameters for fluorescence data collection were programmed into a Dell Latitude laptop computer linked directly to the ABI Prism 7000 sequence detection system with SDS 1.1 application software (Applied Biosystems; Foster City, CA). After real-time data acquisition, the threshold was determined. The C_t value was manually set so that it intersected the amplification curves in the linear region of the semilog plot. Each

experiment was repeated in triplicate.

Statistical analysis

All experiments were performed in triplicate. Comparison of C_t values from DNA extraction/real-time PCR methods among *stx1, stx2,* and *uidA,* and when analyzing the genes individually, were statistically analyzed using the mixed procedure (PROC MIXED) of SAS version 9.1 (SAS Institute, Cary, NC). The statistical models were randomized block designs with sampling, blocked on method and strain. This same procedure was utilized for the examination of C_t values between *E. coli* O157:H7 and cross-reactive strains. The model for these studies was a randomized block design with sampling, blocked on method and strain. All numbers were converted to log units for statistical analysis.

RESULTS

In this study, two DNA extraction methods in combination with two real-time PCR chemistries were tested for use in a real-time PCR. A 109 bp fragment of the *stx1* gene, an 83 bp fragment of the *stx2* gene, and a 143 bp fragment of the *uidA* gene were chosen as targets for detection in *E. coli* cells, as well as non-*E. coli* cells for cross-reactivity examination. The primers and probes for this study have previously been described by Jinneman and others (2003) for amplification and identification of *stx1, stx2,* and *uidA*. The probes were modified slightly to complement the filters in the ABI Prism

7000 SDS. Our preliminary studies proved these oligonucleotides to be suited for real-time PCR.

DNA was extracted and purified using two commercially available kits, GenElute Bacterial Genomic Kit and PrepMan Ultra Sample Preparation Reagent and subsequently combined with a SYBR or TaqMan assay. For the SYBR samples, detection of the amplicon was verified by examination of the melting temperatures (T_m) (dissociation curve) of the amplicon after real-time PCR. The T_ms were 78.2°C, 74.4°C, and 80.6°C for *stx1, stx2,* and *uidA*, respectively. Non-specific products and primer dimers exhibited peaks lower than the T_ms of the genes of interest (Fig. 8). Unlike SYBR, the TaqMan assay did not detect any unspecific product (Fig. 9).

Amplification of *stx1, stx2,* and *uidA* gene targets occurred using all four combinations. No amplification was observed in the negative control samples. Average C_t values for *E. coli* O157:H7 strains which included the genes of interest were 18.05, 17.62, 19.49, and 16.87 for *stx1*; 17.25, 15.69, 18.12, and 17.72 for *stx2*; and 16.74, 16.02, 18.31, and 18.79 for *uidA* for SYBR/PrepMan, SYBR/GenElute, TaqMan/PrepMan, and TaqMan/GenElute combinations, respectively. The combination of SYBR Green and GenElute had the highest efficiency (lowest C_t) across all methods. Although there were slight variations in the C_t values across O157:H7 strains, no statistical differences ($p>0.05$) were found among the chemistry/DNA extraction method combinations for *stx1* or *stx2.* For *uidA*, statistical differences ($p<0.05$) were found

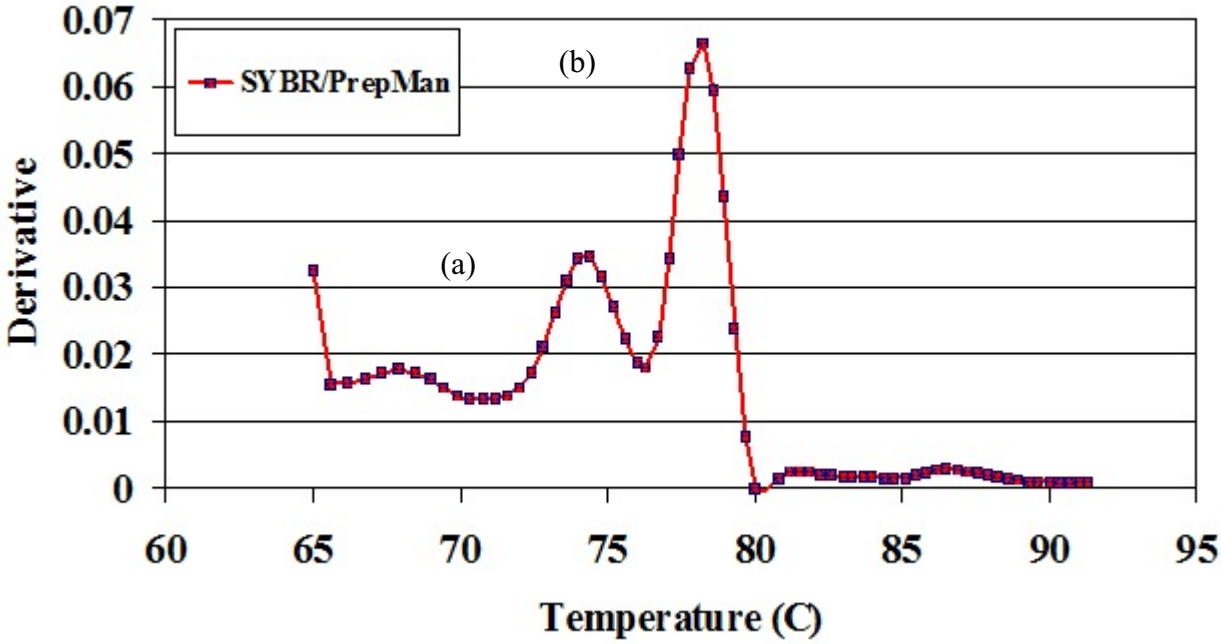


Figure 8. Example dissociation curve for SYBR/PrepMan method exhibiting a peak for (a) non-specific product with T_m of 74.3˚C, and (b) *stx1* target with T_m of 78.4˚C.

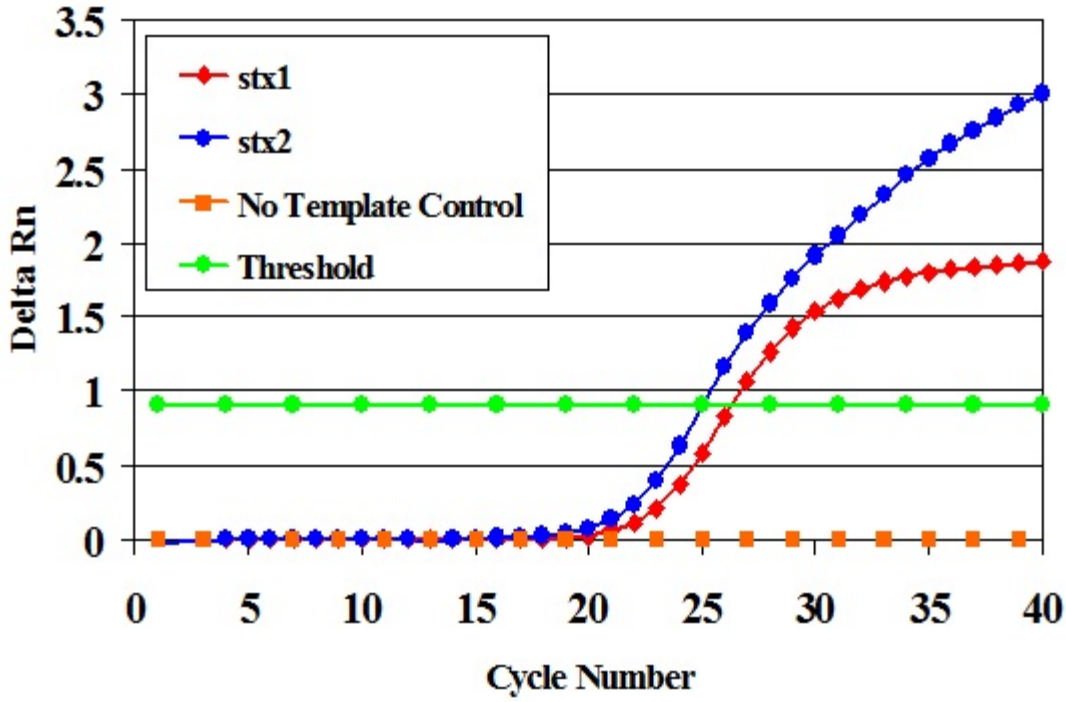


Figure 9. Typical amplification plot obtained with a TaqMan probe-based real-time PCR

between the C_t values for SYBR methods and TaqMan methods; however, no statistical differences ($p>0.05$) were found within SYBR (PrepMan or GenElute) or within TaqMan (PrepMan or GenElute) (Tables 7-9).

Upon examination of results, cross-reactivity was noted with several non-O157 strains. For *stx1,* amplification occurred with *E. coli* 0111 (2146), *E. coli* O26 (#1), *E. coli* O26 (9B), and *S. sonnei* (G129). *Stx2* cross-reactivity occurred with *E. coli* O111 (2146) and *S. sonnei* (G129). Finally, *uidA* was detected in the non-pathogenic *E. coli* strains, *E. coli* O26 (#1) and O26 (9B), *E. coli* O111 (2145) and O111 (2146), as well as *S. flexneri* and *S. sonnei* 10305, 10304 and G129. The C_t values for the cross-reactive strains did not statistically differ ($p>0.05$) from those of the target *E. coli* O157:H7 strains. No false-positives were noted for *stx1* or *stx2* for the NPEC strain, the two K-12 strains, or the two *Salmonella* test strains.

DISCUSSION

PCR methodologies have been proven successful for food analysis, mostly because of their high specificity, sensitivity and rapidity. However, the presence of inhibitory substances in food matrices has been a limiting factor in the detection of microorganisms (Wilson, 1997). Therefore, DNA extraction and isolation techniques for use in a PCR must restrict inhibitory compounds that may suppress amplification of the desired target (Jinneman et al., 1995; DiPinto et al., 2007). In this study, both the GenElute Bacterial DNA Extraction Kit and PrepMan Ultra Sample Preparation reagent

Table 7. Mean C_t values for *stx1* at 4.0 log CFU/ml in *E. coli* O157:H7 strains.

Strain	Real-time PCR chemistry/Extraction method (Mean C_t ±SD)			
	SYBR/PrepMan	SYBR/GenElute	TaqMan/PrepMan	TaqMan/GenElute
ATCC 43888	NA	NA	NA	NA
ATCC 43889	NA	NA	NA	NA
ATCC 43890	17.27±0.08	16.32±0.35	18.55±0.24	15.65±0.03
ATCC 43894	17.35±0.10	13.32±0.27	18.66±0.07	15.46±0.01
ATCC 43895	16.45±0.37	14.15±0.52	19.12±0.06	15.43±0.03
Cider isolate	16.99±0.22	14.79±0.59	19.81±0.08	16.03±0.08
JITB isolate	17.14±0.07	15.98±0.14	19.95±0.01	16.06±0.03
Salami isolate	17.29±0.06	27.96±0.68	20.24±0.08	20.18±0.87
A4	18.59±0.21	17.22±0.67	19.74±0.09	18.47±0.02
C9	25.44±0.07	24.11±0.28	20.69±0.32	19.96±0.07
F2	16.85±0.16	14.54±0.23	19.87±0.10	15.80±0.06
B5	17.08±0.11	17.79±1.72	18.28±0.09	15.61±0.04

Table 8. Mean C_t values for *stx2* at 4.0 log CFU/ml in *E. coli* O157:H7 strains.

	Real-time PCR chemistry/Extraction method (Mean C_t ±SD)			
Strain	SYBR/PrepMan	SYBR/GenElute	TaqMan/PrepMan	TaqMan/GenElute
ATCC 43888	NA	NA	NA	NA
ATCC 43889	13.31±0.47	11.27±0.26	16.24±0.12	15.45±0.03
ATCC 43890	NA	NA	NA	NA
ATCC 43894	18.01±0.04	13.72±0.36	17.99±0.19	16.19±0.35
ATCC 43895	14.39±0.42	13.82±0.99	17.34±0.33	16.15±0.25
Cider isolate	18.32±0.13	14.95±0.58	18.36±0.04	17.09±0.03
JITB isolate	18.73±0.19	16.52±0.71	18.84±0.25	16.87±0.36
Salami isolate	18.06±0.06	24.54±0.08	19.36±0.30	24.80±1.98
A4	19.63±0.06	19.23±0.98	19.92±0.27	19.00±0.26
C9	17.18±0.04	12.37±0.32	17.83±0.18	19.65±0.28
F2	17.55±0.05	13.19±0.28	18.22±0.07	16.29±0.10
B5	17.31±0.06	17.33±1.13	17.07±0.09	15.70±0.34

Table 9. Mean C_t values for *uidA* at 4.0 log CFU/ml in *E. coli* O157:H7 strains.

	Real-time PCR chemistry/Extraction method (Mean C_t ±SD)			
Strain	SYBR/PrepMan	SYBR/GenElute	TaqMan/PrepMan	TaqMan/GenElute
ATCC 43888	16.39±0.22	15.34±0.07	18.14±0.15	18.16±0.13
ATCC 43889	15.52±0.29	14.21±0.11	17.61±0.07	18.30±0.05
ATCC 43890	16.72±0.09	15.55±0.47	18.80±0.23	18.41±0.14
ATCC 43894	17.04±0.03	16.85±0.17	17.29±0.03	18.30±0.18
ATCC 43895	17.97±0.06	15.98±0.01	18.82±0.14	18.22±0.12
Cider isolate	16.71±0.06	15.35±0.26	18.17±0.14	19.26±0.16
JITB isolate	15.63±0.73	15.07±0.19	17.19±0.06	18.88±0.08
Salami isolate	17.25±0.35	16.86±0.67	19.29±0.19	19.75±0.58
A4	18.18±0.05	19.42±0.08	18.14±0.22	18.39±0.12
C9	16.23±0.04	15.10±0.04	18.24±0.12	19.96±0.07
F2	16.55±0.08	16.48±0.09	19.49±0.14	19.10±0.19
B5	16.73±0.06	15.20±0.13	18.56±0.26	18.81±0.28

Table 10. Mean C_t values for *stx1* and *stx2* at 4.0 log CFU/ml in cross-reactive strains.

Gene	Strain	Real-time PCR chemistry/Extraction method (Mean C_t ±SD)			
		SYBR/PrepMan	SYBR/GenElute	TaqMan/PrepMan	TaqMan/GenElute
stx1	O26 #1	17.20±0.03	11.41±0.34	20.23±0.30	15.56±0.02
	O26 9B	17.22±0.07	10.53±0.08	18.10±0.62	15.47±0.04
	O111 2146	16.48±0.05	12.74±1.09	19.46±0.12	16.83±0.04
	S. Sonnei (G129)	15.22±0.14	12.14±0.06	20.45±0.08	18.78±0.11
stx2	O111 2146	17.51±0.30	16.82±0.21	20.25±0.12	16.03±0.34
	S. Sonnei (G129)	17.21±0.12	14.84±0.71	20.54±0.08	19.34±0.24

were evaluated for amplification capacity and ease of use, in combination with SYBR green dye and TaqMan probes.

For the first approach in this study, SYBR green was used in combination with DNA extracted via PrepMan or GenElute for detection of approximately 4.0 log CFU/ml of each test strain. In this study, successful qualitative amplification occurred for organisms containing the genes of interest when both GenElute and PrepMan were combined with the SYBR green assay. Unlike TaqMan, SYBR green allows detection of fluorescence from only double-stranded DNA, including fluorescence from unspecific products and primer-dimers. A dissociation curve was produced after each real-time PCR run which allowed specific amplicons to be identified based on their specific melting temperatures. Unspecific amplicons, resulting in more than one dissociation curve, were generated in several of the real-time PCR runs in this study, including those using DNA from both GenElute and PrepMan, for all three gene targets. The dissociation curves showed that the unspecific amplicon melting temperature (73˚C) was lower than those characteristic of the specific amplicons. Although SYBR Green can produce successful results, for a quantitative assay, these non-specific products could hinder accurate quantification of *E. coli* O157:H7 when the assay is applied to food samples since any double-stranded DNA will be quantified (Hein et al., 2001; Arlorio et al., 2007).

Unlike SYBR, TaqMan probes are designed with a reporter dye and a quencher dye attached to the 5' and 3' ends, respectively. The reporter dye is quenched until the extension phase of PCR where the polymerase enzyme cleaves the probe, allowing the reporter dye to fluoresce. Since the cleavage occurs only when the probe is hybridized to

the target sequence, the detected fluorescence originates from a specific amplification. In this study, successful qualitative amplification occurred for organisms containing the genes of interest when both GenElute and PrepMan were combined with the TaqMan probe assay.

These results indicate that either SYBR green or TaqMan probes, in combination with GenElute or PrepMan, could potentially be used for detection of *E. coli* O157:H7 in apple juice. Some researchers have found that the sensitivity of both SYBR Green and TaqMan assays are comparable (Malinen et al., 2003; Salm and Geider, 2004; Ravva and Stanker, 2005). However, Hein and others (2001) reported that accurate quantification of low numbers of the *nuc* gene of *S. aureus* in cheese was inhibited due to accumulation of primer dimers and unspecific side products which were bound to SYBR green. Since no significant differences ($p>0.05$) were noted among the C_t values in the current study, and because TaqMan produced more specific results than SYBR Green, a TaqMan probe-based real-time PCR was chosen to be applied to further study in apple juice.

In the current study, two methods were used for DNA extraction. Although GenElute, combined with either SYBR Green or TaqMan probes, showed slightly lower C_t values (generally, 1 to 3 cycles), no statistical differences ($p>0.05$) were noted among the methods. Differences in GenElute and PrepMan extraction efficiencies are likely due to GenElute's column-based system and additional purification steps. However, PrepMan is less time-consuming and less technically demanding than GenElute. In the current study, PrepMan proved to be a simple and reliable extraction method for detection of *E. coli*. Bürk and others (2002) reported that the use of DNA extraction methods other

than PrepMan, resulted in false positives in a TaqMan assay developed for detection of verotoxin genes in raw milk. PrepMan has been evaluated against other DNA extraction kits in food applications and has been reported to be an easy method which produces results similar to other commercial kits such as Instagene Matrix, Xtra Amp Tube and Isocode paper (Higgins et al., 2001; Heller et al., 2003). In the current study, GenElute took approximately 1 ½ to 2 h for DNA extraction, versus approximately 15 minutes for PrepMan. PrepMan was chosen for DNA extraction in combination with TaqMan probe chemistry for further study in apple juice.

Cross-reactivity was noted with several non-O157 *E. coli* and *Shigella* strains with all combinations. Both *stx1* and *stx2* exhibit very similar characteristics as Shiga toxin found in *Shigella* spp., including biological activities and biochemical modes of action (Strockbine et al., 1996). Furthermore, Shiga toxin and *stx1* are virtually identical, differing by only one amino acid substitution in the A subunit (Strockbine et al., 1988). These similar characteristics are the possible explanations for the cross-reactivity with *Shigella* spp. and non-O157 strains. Cross-reactivity was observed with the non-O157 strains, *E. coli* O26 and O111, also Shiga-like toxin producers. *S. sonnei* (G129) was found to cross-react with the *stx1* and *stx2* primers. Cleuziat and Robert-Baudouy (1990) confirmed the presence of the *uidA* gene in both *E. coli* and *Shigella* spp. (*S. boydii, S. dysentariae, S. flexneri, S. sonnei*). Since the *uidA* gene was included in the study as a "control" gene, cross-reactivity was not unexpected. Repeating the assays did not change the results.

This study demonstrates that combining PrepMan Ultra for DNA extraction and

TaqMan probe chemistry in a real-time PCR assay is useful for detection of *E. coli* O157:H7 in pure culture. This combination proved to be a quick, relatively simple method for detection of *E. coli* O157:H7 while, at the same time, not differing significantly from other methods tested in this study.

REFERENCES

Arlorio, M., Cereti, E., Coïsson, J.D., Travaglia, F., and Martelli, A. 2007. Detection of hazelnut (*Corylus* spp.) in processed foods using real-time PCR. Food Control. 18:140-148.

Bhagwat, A.A. 2004. Rapid detection of *Salmonella* from vegetable rinse-water using real-time PCR. Food Microbiol. 21:73-78.

Buerck, C., Braumiller, I.G.B., Becker, H., and Maertlbauer, E. 2002. Nuclease fluorescence assay for the detection of verotoxin genes in raw milk. Lett. Appl. Microbiol. 35:153-156.

Bürk, C., Braumiller, I.G.B., Becker, H., and Märtlbauer, E. 2002. Nuclease fluorescence assay for the detection of verotoxin genes in raw milk. Lett. Appl. Microbiol. 35:153-156.

Cleuziat, P. and Robert-Baudouy, J. 1990. Specific detection of *Escherichia coli* and *Shigella* species using fragments of genes coding for beta-glucuronidase. FEMS Microbiol. Lett. 60:315-322.

DiPinto, A.D., Forte, V.T., Guastadisegni, M.C., Martino, C., Schena, F.P., Tantillo, G. 2007. A comparison of DNA extraction methods for food analysis. Food Control. 18:76-80

FDA. 1998. Bacteriological Analytical Manual. 9th Ed. AOAC International, Gaithersburg, MD.

FDA. 2001. 2001 Food Code. Washington, D.D.:Food and Drug Administration. Available from: http://www.cfsan.fda.gov/~dms/fc01-toc.html. Accessed June 10, 2006.

Feng, P. 2001. Development and impact of rapid methods for detection of foodborne pathogens, p. 775-796. *In* M.P. Doyle, L.R. Beuchat, and T.J. Montville (ed.), Food microbiology. Fundamentals and frontiers. ASM Press, Washington D.C.

Fortin, N.Y., Mulchandani, A., and Chen, W. 2001. Use of a real-time polymerase chain

reaction and molecular beacons for detection of *Escherichia coli* O157:H7. Anal. Biochem. 289:281-288.

Fu, Z., Rogelj, S., and Kieft, T.L. 2005. Rapid detection of *Escherichia coli* O157:H7 by immunomagnetic separation and real-time PCR. Intl. J. Food Microbiol. 99:47-57.

Hein, I., Lehner, A., Rieck, P., Klein, K., Brandl, E., and Wagner, M. 2001. Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this techniques for examination of cheese. Appl. Environ. Microbiol. 67:3122-3126.

Heller, L.C., Davis, C.R., Peak, K.K., Wingfield, D., Cannons, A.C., Amuso, P.T., and Cattani, J. 2003. Comparison of methods for DNA isolation from food samples for detection of Shiga toxin-producing *Escherichia coli* by real-time PCR. Appl. Environ. Microbiol. 69:1844-186.

Higgins, J.A., Jenkins, M.C., Shelton, D.R., Fayer, R., and Karns, J.S. 2001. Rapid extraction of DNA from *Escherichia coli* and *Cryptosporidium parvum* for use in PCR. Appl. Environ. Microbiol. 67:5321-5324.

Jaykus, L. 2003. Challenges to developing real-time methods to detect pathogens in foods. ASM News. 69:341-347.

Jinneman, K.C., Trost, P.A., Hill, W.E., Weagent, S.D., Bryant, J.L., Kaysner, C.A., and Wekell, M.M. 1995. Comparison of template preparation methods from foods for amplification of *Esherichia coli* O157 Shiga-like toxins type I and II DNA by multiplex polymerase chain reaction. J. Food Protect. 58:722-726.

Liu, Y., Cai, X., Zhang, X., Gao, Q., Yang, X., Zheng, Z., Luo, M., Huang, X. 2006. Real time PCR using TaqMan and SYBR Green for detection of *Enterobacter sakazakii* in infant formula. J. Microbiol. Meth. 65:21-31.

Lyon, W.J. 2001. TaqMan PCR for detection of *Vibrio cholerae* O1, O139, non-O1, and non-O139 in pure cultures, raw oysters, and synthetic seawater. Appl. Environ. Microbiol. 67:4658-4693.

Malinen, E., Kassinen, A., Rinttilä, T., and Palva, A. 2003. Comparison of real-time

PCR with SYBR Green I of 5'-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. Microbiol. 149:269-277.

Ravva, S.V. and Stanker, L.H. 2005. Real-time quantitative PCR detection of *Mycobacterium avium* subsp. paratuberculosis and differentiation from other *Mycobacteria* using SYBR green and TaqMan assays. J. Microbiol. Meth. 63:305-317.

Rijpens, N.P. and Herman, L.M.F. 2002. Molecular methods for identification and detection of bacterial food pathogens. J. AOAC Int. 85:984-995.

Rossen, L., Nørskov, Holmstrøm, K., and Rasmussen, O.F. 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Intl. J. Food Microbiol. 17:37-45.

Salm, H. and Geider, K. 2004. Real-time PCR for detection and quantification of *Erwinia amylovora*, the casual agent of fireblight. Plant pathol. 53:602-610.

Smith, T.J., O'Connor, L., Glennon, M., and Maher, M. 2000. Molecular diagnostics in food safety: rapid detection of food-borne pathogens. Irish J. Agric. Food Res. 39:309-319.

Strockbine, N.A., Marques, L.R., Newland, J.W., Williams-Smith, H., Holmes, R.K., and O'brien, A.D. 1986. Two toxin-converting phages from *E. coli* O157:H7 strain 933 encode antigentically distinct toxins with similar biological activities. Infect. Immun. 53:135-140.

Strockbine, N.A., Jackson, M.P., Sung, L.M., Holmes, R.K., and O'brien, A.D. 1988. Cloning and sequencing of genes for Shiga toxin from *Shigella dysentariae* type 1. J. Bacteriol. 170:1116-1122.

Ward, L.N. and Bej, A.K. 2006. Detection of *Vibrio parahaemolyticus* in shellfish by use of multiplexed real-time PCR with TaqMan fluorescent probes. Appl Environ. Microbiol. 72:2031-2042.

Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Env. Microbiol. 63:3741-3751.

Yoshitomi, K.J., Jinneman, K.C., and Weagant, S.D. 2006. Detection of Shiga toxin genes *stx1*, *stx2*, and the +93 *uidA* mutations of *E. coli* O157:H7/H-using SYBR Green I in a real-time multiplex PCR. Mol. Cell Probes. 20:31-41.

PART V

APPLICATION OF CELL CONCENTRATION METHODS AND A REAL-TIME POLYMERASE CHAIN REACTION ASSAY FOR DETECTION AND QUANTIFICATION OF *ESCHERICHIA COLI* O157:H7 IN APPLE CIDER.

ABSTRACT

Bacterial cell concentration methods in combination with a TaqMan-based real-time PCR were evaluated for detection and quantification of low numbers of *E. coli* O157:H7 in apple juice. Nine strains of *E. coli* O157:H7 were individually inoculated (1 ml) into 99 ml apple juice at levels of 1 to 4 log CFU/ml. Samples were withdrawn, diluted in 0.1% peptone water and surface plated on TSA for determination of initial inoculum levels. All juice samples were independently vacuum filtered and washed with 100 ml sterile water. The remaining sample was centrifuged, and the pellet resuspended in sterile water. The samples were vacuum filtered a second time and washed with 30 ml water. Samples were withdrawn, diluted in 0.1% peptone water, and surface plated onto TSA for determination of *E. coli* O157:H7 post-filtration. The remaining sample was centrifuged and the pellet resuspended in 1 ml phosphate buffer. The 1 ml samples were subjected to an immunomagnetic separation (IMS) procedure and DNA extracted from *E. coli* O157:H7. The remaining cell fragments were magnetically separated from the DNA. Cell standard curves were generated for *stx1, stx2*, and *uidA* with average efficiencies of 97.8% (R^2=0.99), 104.6% (R^2=0.99), and 99.2% (R^2=0.99), respectively. Using primers and probes specific for *stx1, stx2,* and uidA, it was possible to detect and quantify *E. coli* O157:H7 in apple juice at concentrations as low as 1.6, 1.6, and 1.5 log CFU/ml cider, respectively. Detection was possible below these numbers, but values that fell outside the range of the standard curves could not be quantified. This study successfully utilized a combination of cell concentration methods, including a two-step filtration, centrifugation and IMS, in association with a real-time PCR for detection and quantification of low

numbers of *E. coli* O157:H7 without an enrichment step. The assay not only produced results that were similar or more sensitive to those of conventional culture assays, but drastically reduced assay time to less than 5 h.

INTRODUCTION

Juice-associated foodborne illness outbreaks have been reported since the 1970s (Anderson, 2001). Moreover, during the 1990s, several foodborne illness outbreaks caused by *Escherichia coli* O157:H7 were traced to unpasteurized apple cider consumption (Besser et al., 1998; Cody et al., 1999; Millard et al., 1994; Mshar et al., 1997). These outbreaks heightened interest in the development of rapid, specific, and sensitive methods for detection of *E. coli* O157:H7 in apple cider and juice. In response, PCR- and real-time PCR-based detection methods have become an important tool for identification of foodborne pathogens and for investigative purposes during a foodborne illness outbreak (Hill, 1996; Hines, 2000).

Real-time PCR fluorogenic probes can be used to not only detect PCR product, but calculate the quantity of product as it accumulates during each PCR cycle. Through the use of suitable standards, real-time PCR can be used to quantify organisms based on a linear relationship between the real-time PCR cycle threshold (C_t) and the log of the initial target copy number. Cycle threshold values for unknown samples are compared to the standard curve to determine the initial target number in a reaction. End-point analysis such as gel electrophoresis, can be eliminated with real-time PCR.

Molecular-based detection methods such as real-time PCR, may be inhibited by complex substances in food matrices, reducing the sensitivity and specificity of the assay (Lantz, 1994; Jinneman et al., 1995). Fruit juices are known to contain PCR inhibitory substances, such as polyphenols, polysaccharides, peptides and free amino acids (Zoecklein et al., 1995; Siebert et al., 1996). Researchers have developed molecular-based methods for detection of *E. coli* O157:H7 in foods such as ground beef, but the application of these methods to apple juice have been less successful due to juice-related inhibitors (Ogunjimi and Choudary, 1999). Fortin et al. (2001) developed a real-time PCR assay for detection of *E. coli* O157:H7 in apple juice, but found an 11 hour enrichment step was needed to increase numbers to detectable levels, as opposed to a 6 h enrichment for milk. The researchers reported that polyphenolic inhibitors were likely responsible for the reduction in sensitivity. Lampel et al. (2000) reported lowered sensitivity in an FTA filter-based PCR when apple cider was the experimental food matrix, and that additional preparation of the samples prior to PCR may reduce inhibition.

Cell separation and/or concentration methods, such as immunomagnetic separation (IMS, centrifugation and filtration), have been used successfully to rapidly separate or concentrate microorganisms from food samples and reduce their inhibitory effects in less time than required for cultural enrichment (Benoit and Donahue, 2003; Stevens and Jaykus, 2004). Immunomagnetic separation has been shown to increase sensitivity and specificity of real-time PCR assays for detection of pathogenic microorganisms in foods (Fu et al., 2005; Mercanolğlu and Griffiths, 2005). Rodrígues-Lázaro and others (2004) reported increased sensitivity for detection of *Listeria*

monocytogenes when filtration and DNA purification were carried out prior to real-time PCR. Johnston et al. (2005) concluded that centrifugation steps for cell concentration prior to real-time PCR enhanced detection of *Salmonella* and *E. coli* O157:H7.

Real-time PCR has been evaluated as a tool for detection and quantification of microorganisms from a variety of food products, including meat products, juices, dairy products and produce (Fortin et al., 2001; Buerk et al., 2002; Bhagwat, 2004; Fu et al., 2005). However, studies which employ separation and concentration of *E. coli* O157:H7 from apple cider or juice prior to a quantitative real-time PCR assay are few. The objective of this study was to apply filtration, centrifugation and an IMS step for *E. coli* O157:H7 separation and concentration from apple juice samples for application to a quantitative TaqMan real-time PCR assay.

MATERIALS AND METHODS

Strains and preparation of inoculum–

Nine strains of *E. coli* O157:H7 (ATCC strains 43888, 43889, 43890, 43894, 43895; environmental strain A4; 994; salami; cider, apple cider; JITB, hamburger-associated outbreak) were used in this study. Cultures were obtained from the Food Science and Technology Department, University of Tennessee.

Cultures of each strain were grown separately in Luria-Bertani (LB) broth (Oxoid; Basingstoke, UK) at 37°C and transferred at 24-h intervals. Ten-fold serial dilutions

were made from each culture ranging from approximately 1.0 to 4.0 log CFU/ml in sterile 0.1% peptone water (PW; Difco; Sparks, MD). Each dilution was spread plated (0.1 ml) in duplicate onto Tryptic Soy Agar (TSA; Difco Becton Dickinson Microbiology Systems; Sparks, MD) and incubated overnight at 37°C. Colonies were counted and duplicates averaged to obtain initial organism levels prior to inoculation of apple juice.

Inoculation of apple juice–

Unfiltered, pasteurized apple juice (pH ~3.6) with no preservatives, sweeteners or artificial color was purchased from a local supermarket. Juice was held at room temperature if unopened, or brought to ambient temperature (~25°C ± 2°C) if held under refrigeration (4°C). Four, 99 ml aliquots of apple juice were transferred to sterile 250 ml screw-cap bottles. Bottles were labeled as number 1, 2, 3 and 4 were inoculated with 1 ml of approximately 1.0 log CFU/ml, 2.0 log CFU/ml, 3.0 log CFU/ml and 4.0 log CFU/ml, respectively. The inoculated juice samples were mixed by slowly inverting the bottle by hand for approximately 30 seconds before sampling.

Prior to cell concentration, initial inoculum levels in the apple juice were verified using conventional culture methods. Each inoculum level was surface plated (0.1 ml) onto TSA and incubated for 24 h at 37°C. *E. coli* O157:H7 colonies were enumerated (CFU/ml) and recorded.

Cell concentration–

Juice samples (100 ml) were individually vacuum filtered through a No. 4 Whatman filter paper (Whatman Inc.; Florham Park, NJ) placed in a sterile Buchner funnel to remove particulate matter in the juice. The filter was washed with 100 ml sterile deionized water and the entire 200 ml was collected in an Erlenmeyer flask. The 200 ml samples were broken into four, 50 ml samples which were subsequently placed in 50 ml centrifuge tubes. Samples were centrifuged (8,000 x g, 11 min) (Biofuge 17-R; Heraeus Sepatech; Germany), the spent liquid decanted, and the cell pellet resuspended in 5 ml phosphate buffer (0.1 M, pH 7.2). The samples were combined for 20 ml total volume and vacuum filtered through a No. 1 Whatman filter paper (Whatman Inc.; Florham Park, NJ) placed in a sterile Buchner funnel. The filter was washed with 30 ml sterile deionized water. Post-filtration, samples were serially diluted in 0.1% peptone water, surface plated (0.1 ml) onto TSA, and incubated at 37°C for 24 h. Colonies were enumerated and compared to initial inoculum numbers to determine organism loss during filtration steps. The sample was subsequently centrifuged (8,000 x g, 11 minutes), the spent liquid decanted, and the cell pellet resuspended in 1 ml 0.1 M, pH 7.2 phosphate buffer (PB; Difco) for neutralization of any remaining juice.

Immunomagnetic separation application–

Dynabeads anti-*E. coli* O157:H7 (Dynal, Lake Success, NY) were used for isolation of *E. coli* O157:H7 by IMS. Twenty µl aliquots of anti-*E. coli* O157:H7 beads

were added to 2 ml microfuge tubes and were subsequently inoculated with the 1 ml samples which were previously concentrated. Each mixture was mixed for 10 min with gentle continuous rotation (30 rpm) on a Dynal Biotech Sample Mixer. The bead/bacteria complexes were placed in a magnetic particle concentrator (Dynal MPC-S) for 3 min to separate the magnetic beads from the liquid. One ml of wash buffer (Phosphate Buffered Saline, Tween 20, pH 7.4) was added to the beads and the tube inverted several times to resuspend the beads. The wash procedure was repeated twice. After the final wash step, the wash buffer was removed leaving only the bead/bacteria complexes in the tubes.

Template preparation–

Two hundred µl PrepMan Ultra Sample Preparation Reagent were added to the bead/bacteria complexes. Samples were briefly vortexed then heated in a 100°C water bath for 10 min. Tubes were returned to the magnetic particle concentrator for 3 min. to draw away the beads, then supernatant containing the DNA was transferred to a sterile 0.5 ml tube under a sanitized class II biological safety cabinet (Fisher Scientific; Pittsburgh, PA). The template DNA obtained was subjected to the real-time PCR assay as described in Figure 10.

Primers and probes--

The primers used for detection of *stx1*, *stx2,* and *uidA* have previously been described by Jinneman et al. (2003), and were designed to amplify sequences of 109 bp,

Figure 10. Summary of *E. coli* O157:H7 template preparation for use in a real-time PCR.

83bp, and 143 bp, respectively. The fluorogenic probes for Taqman assays were adopted from Jinneman et al. (2003); however, reporter dyes were modified to fit those calibrated on the ABI Prism 7000 Sequence Detection System. The fluorogenic probe for *stx1* had the reporter dye, 6FAM coupled at the 5' end, *stx2* had VIC, while *uidA* had NED. All probes had a non-fluorescent quencher (NFQ) combined with a minor groove binder (MGB) at the 3' end (MWG Biotech; High Point, NC).

Preparation of standard curves–

Standard curves were generated for individual *E. coli* O157:H7 strains (*stx1*, *stx2* and *uidA* genes) during each real-time PCR performed. *E. coli* O157:H7 cultures were grown for 24 h at 37°C in LB broth, then subsequently serially diluted in 0.1% peptone water. 0.1 ml of selected dilutions (approximately 1 log CFU/ml to 6 log CFU/ml) were spread plated in duplicate on TSA, incubated for 24 h at 37°C, and the number of CFU/ml determined. From each serial dilution of cells prepared above, 1 ml was removed and added to 2 ml microcentrifuge tubes. Tubes were centrifuged (13,000 rpm, 3 min–Eppendorf Microcentrifuge 5417C; Eppendorf North America; Westbury, NY), the supernatant discarded, and 200 µl PrepMan Ultra Sample Preparation Reagent added to each cell pellet. After the samples were briefly vortexed (20 seconds), the samples were heated for 10 min in a 100°C water bath. The samples were again centrifuged (13,000 rpm, 3 min–Eppendorf Microcentrifuge), and the supernatant containing template DNA was transferred to a new microcentrifuge tube. Real-time PCR was performed

using 5 µl template DNA, at each dilution for each gene target, to generate standard curves by plotting the log CFU/ml (based on the plate counts and dilutions) versus C_t. Each reaction mixture consisted of 12.5 µl TaqMan Universal PCR Master Mix, 0.5 µl probe, 4.5 µl RNase-free water, 5.0 µl DNA, and 1.25 µl of 0.5 µM each forward and reverse primer. Standards were amplified in the same plate as the unknown samples. Standard curve amplification efficiencies were determined according to the following equation (Meuer et al., 2001): PCR Efficiency = $[10^{(-1/\text{slope})} - 1]$.

Real-time PCR conditions--

From the 25 bacterial strains tested in the preceding section, 9 *E. coli* O157:H7 were chosen for further evaluation to determine the sensitivity of the real-time PCR assay. Real-time PCR was carried out on juice samples after each cell concentration step, including IMS, to verify the need for further cell concentration or removal of PCR inhibitors. Each reaction mixture consisted of 12.5 µl TaqMan Universal PCR Master Mix, 0.5 µl probe, 4.5 µl RNase-free water, 5.0 µl DNA, and 1.25 µl of 0.5 µM each forward and reverse primer. Amplification and detection were carried out in optical grade 96-well plates in an ABI Prism 7000 sequence detection system (Applied Biosystems; Foster City, CA), with an initial denaturation at 95°C for 10 min followed by amplification through 50 cycles of denaturation, annealing and polymerization to increase the likelihood the lowest inoculated cell levels would be detected . Denaturation was performed at 95°C for 1 min, primer annealing at 48°C for 1 min, and DNA extension at

72°C for 1 min, respectively. All plates contained a negative control without target DNA. The reaction conditions for amplifications and the parameters for fluorescence data collection were programmed into a Dell Latitude laptop computer (Dell linked directly to the ABI Prism 7000 sequence detection system with SDS 1.1 application software (Applied Biosystems, Foster City, CA)). After real-time data acquisition, the threshold was determined. The C_t value was manually set so that it intersected the amplification curves in the linear region of the semilog plot. Real-time PCR assays for each organism were carried out in triplicate.

Statistical analysis–

All experiments were performed in triplicate. Numbers of *E. coli* O157:H7 post-filtration obtained by direct plating and those predicted by real-time PCR were statistically analyzed using the mixed procedure (PROC MIXED) of SAS version 9.1 (SAS Institute, Cary, NC). For analysis of pre-filtration versus post-filtration plate counts for *E. coli* O157:H7, the experimental design was a randomized block design with sampling, blocked on dilution. For analysis of plate count results versus real-time PCR computer predicted values, the experimental design was a randomized block design with sampling, blocked on strain. All numbers were converted to log units for statistical analysis.

RESULTS

In this study, three primer sets and three TaqMan probes were used, including primers for *stx1*, *stx2* and *uidA*, to identify DNA sequences of 109 bp, 83 bp, and 143 bp, respectively, in *E. coli* O157:H7 strains. Standard curves for quantification of DNA in artificially contaminated juice samples were constructed for each strain, with a range of concentrations from approximately 1.0 to 6.0 log CFU/ml *E. coli* O157:H7 (Fig. 11 to 19). Analysis of the standard curves across nine *E. coli* O157:H7 strains had amplification efficiency (AE) ranges of 93.1%-100%, with an average AE of 97.8% (R^2=0.99) for *stx1*; 77.8%-168.0%, with an average AE of 104.6% (R^2=0.99) for *stx2*; and 93.4%-104.8%, with an average AE of 99.2% (R^2=0.99) for *uidA*.

Initially, preliminary studies for real-time PCR optimization were conducted on pure cultures of *E. coli* O157:H7. However, when these methods were applied to DNA extracted from *E. coli* O157:H7 in apple juice, the assay was inhibited entirely (data not shown). Therefore, a filtration, centrifugation and immunomagnetic separation technique was evaluated to overcome the juice-associated inhibitors.

The four initial inoculum levels (approximately 1.0, 2.0, 3.0 and 4.0 log CFU/ml) in apple juice prior to filtration were determined by plate count and averaged 0.6, 2.5, 3.4, and 4.4 log CFU/ml across the nine experimental strains in a 100 ml sample volume. After a two-step filtration procedure to remove larger, then smaller particulate matter in the juice, plate count averages were 0.3, 2.2, 3.3, and 4.3 log CFU/ml, with an average loss of 0.3 (50%), 0.3 (12.0%), 0.1 (2.9%), and 0.1 (2.3%) log CFU/ml, respectively.

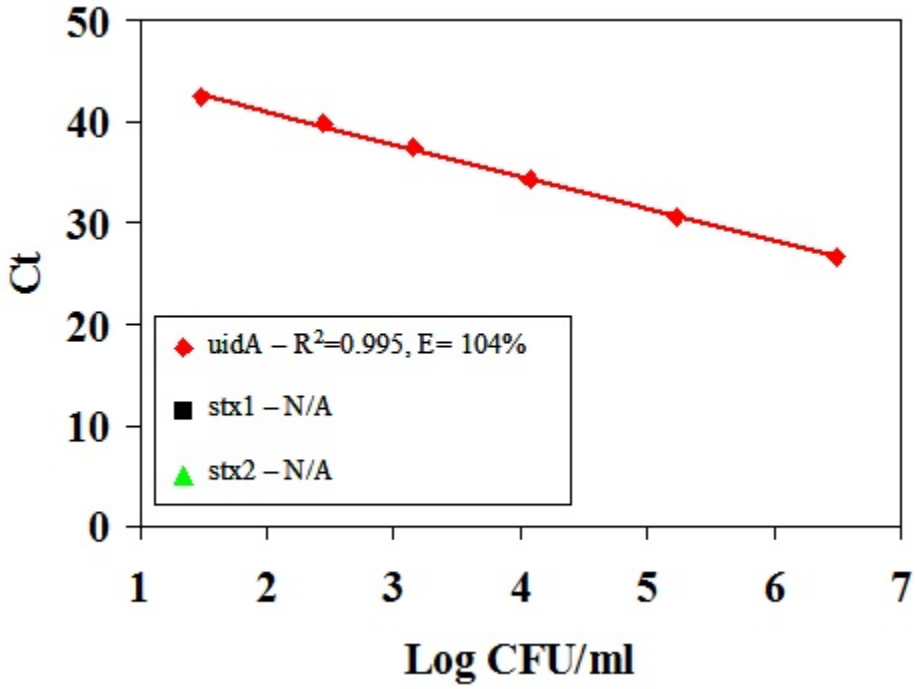


Figure 11. Standard curve for a ten-fold dilution series of *E. coli* O157:H7 ATCC 43888 cells (1.0 to 6.0 log CFU/ml per assay, in triplicate) plotted as threshold cycle on the *Y*-axis, against the target number (Log CFU/ml) per assay on the *X*-axis.

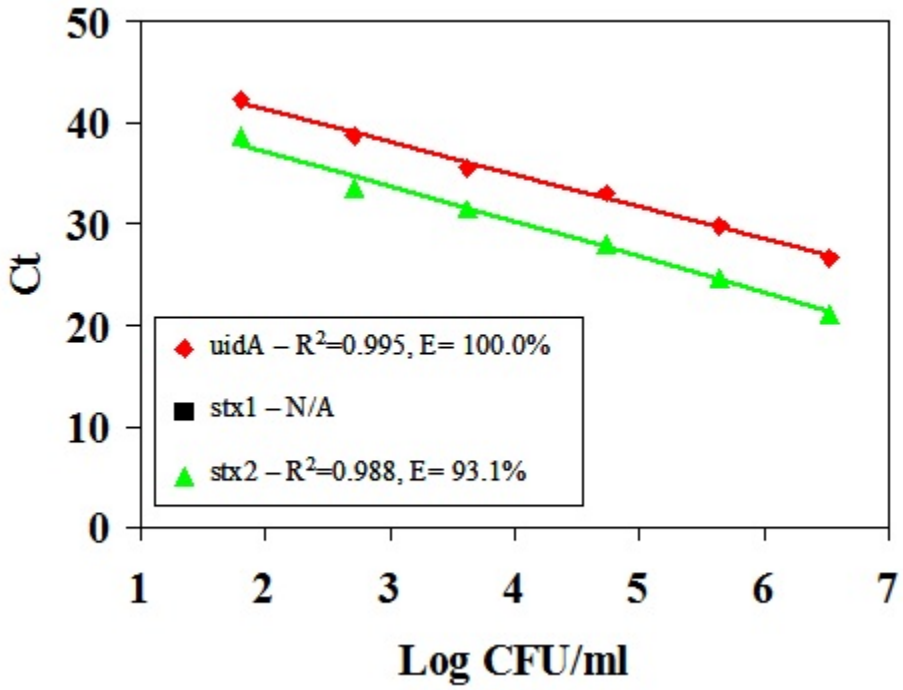


Figure 12. Standard curve for a ten-fold dilution series of *E. coli* O157:H7 ATCC 43889 cells (6 to 1 Log CFU/ml per assay, in triplicate) plotted as threshold cycle on the *Y*-axis, against the target number (Log CFU/ml) per assay on the *X*-axis.

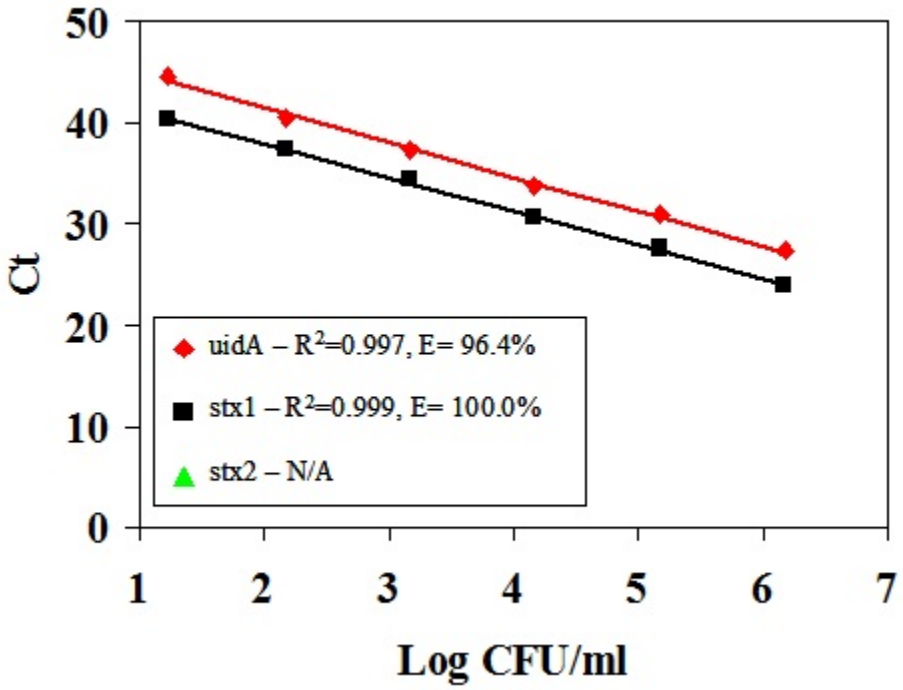


Figure 13. Standard curve for a ten-fold dilution series of *E. coli* O157:H7 ATCC 43890 cells (1.0 to 6.0 log CFU/ml per assay, in triplicate) plotted as threshold cycle on the *Y*-axis, against the target number (Log CFU/ml) per assay on the *X*-axis.

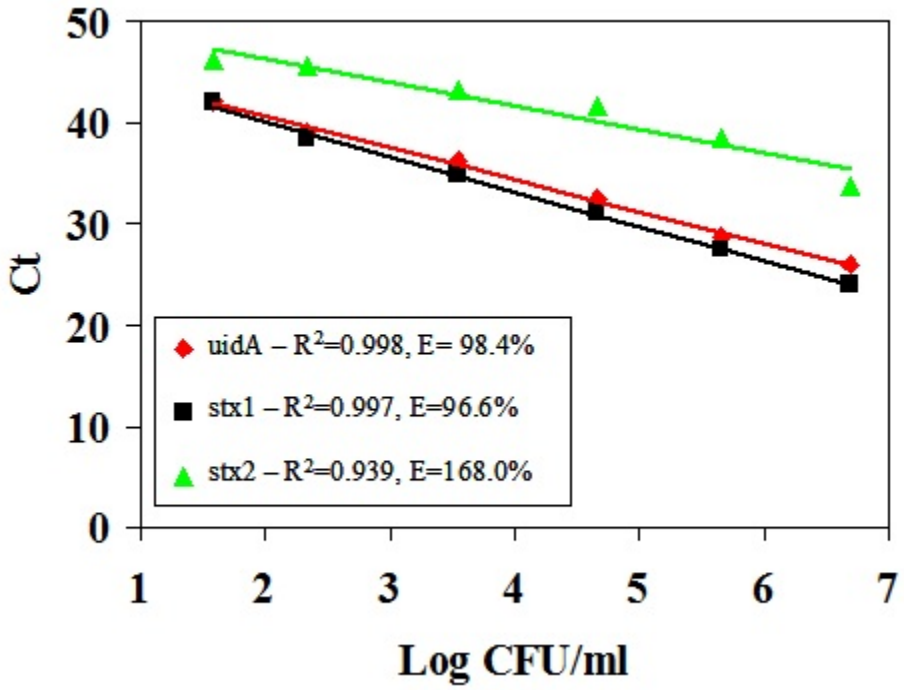


Figure 14. Standard curve for a ten-fold dilution series of *E. coli* O157:H7 ATCC 43894 cells (1.0 to 6.0 log CFU/ml per assay, in triplicate) plotted as threshold cycle on the *Y*-axis, against the target number (Log CFU/ml) per assay on the *X*-axis.

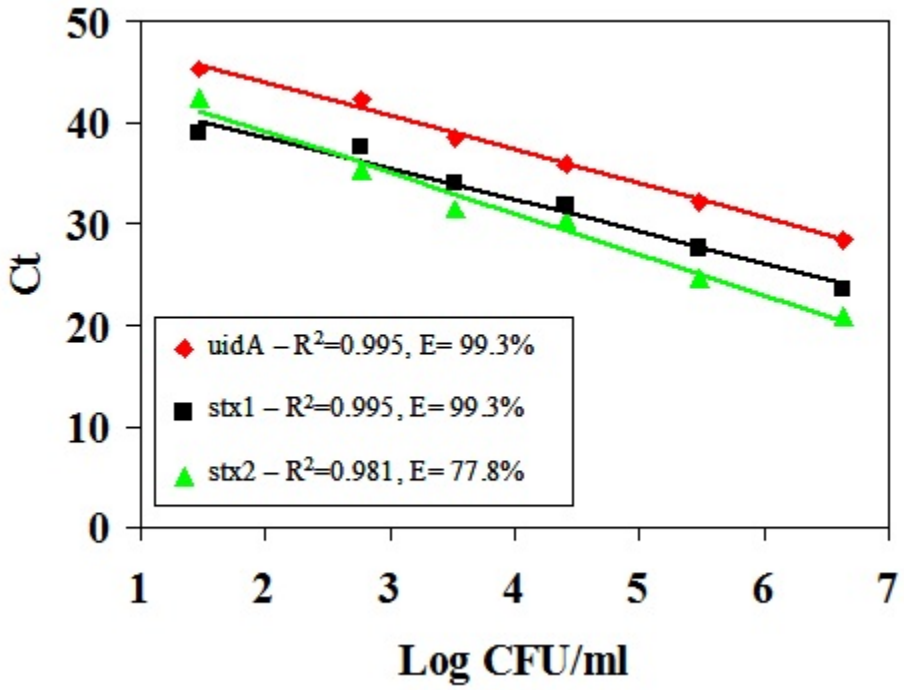


Figure 15. Standard curve for a ten-fold dilution series of *E. coli* O157:H7 ATCC 43895 cells (6 to 1 log CFU/ml per assay, in triplicate) plotted as threshold cycle on the *Y*-axis, against the target number (Log CFU/ml) per assay on the *X*-axis.

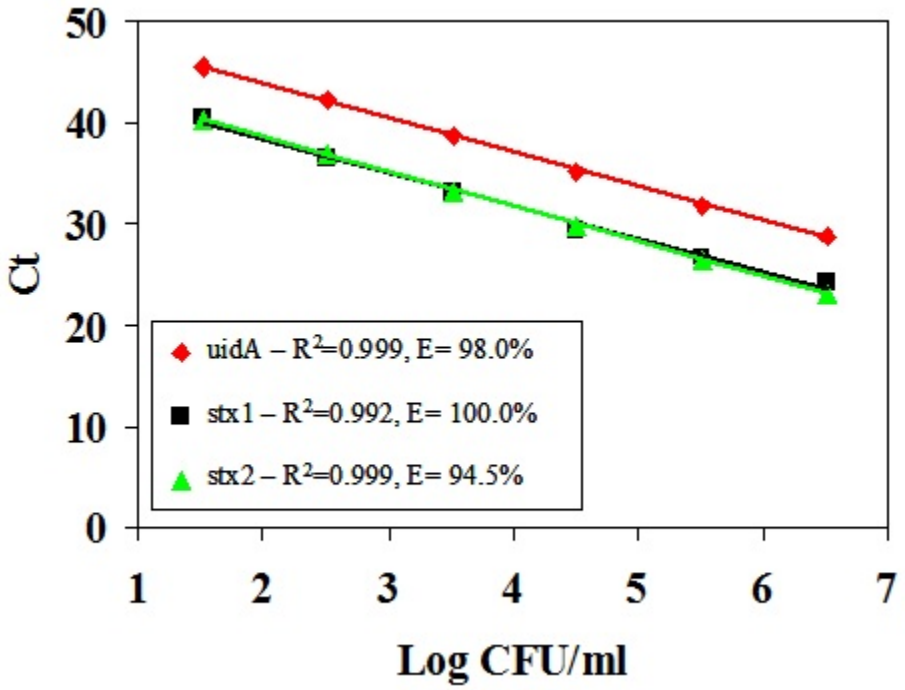


Figure 16. Standard curve for a ten-fold dilution series of *E. coli* O157:H7 A4 cells (1.0 to 6.0 log CFU/ml per assay, in triplicate) plotted as threshold cycle on the *Y*-axis, against the target number (Log CFU/ml) per assay on the *X*-axis.

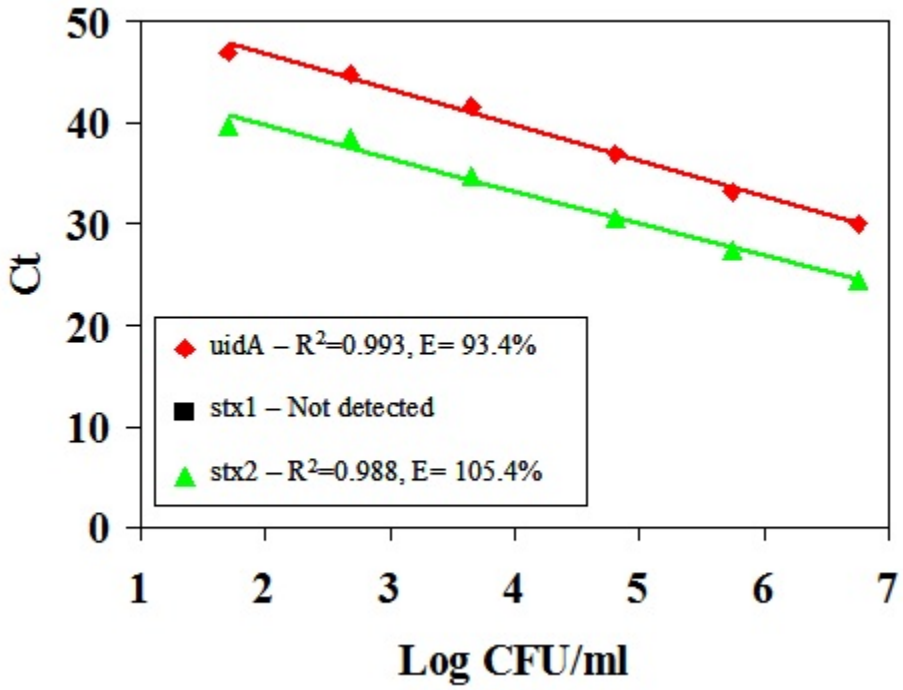


Figure 17. Standard curve for a ten-fold dilution series of *E. coli* O157:H7 Cider cells (1.0 to 6.0 log CFU/ml per assay, in triplicate) plotted as threshold cycle on the *Y*-axis, against the target number (Log CFU/ml) per assay on the *X*-axis.

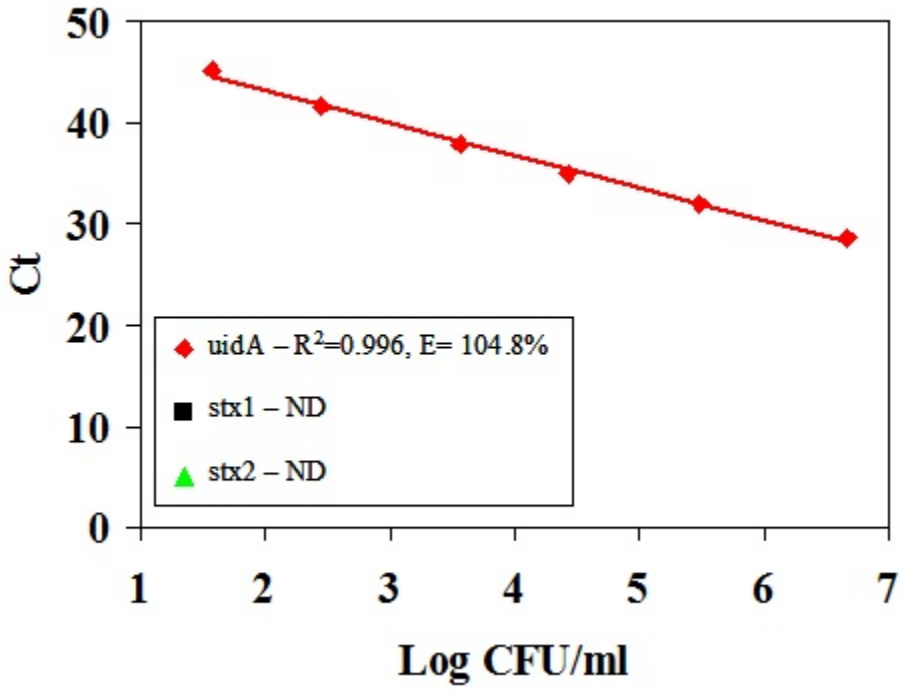


Figure 18. Standard curve for a ten-fold dilution series of *E. coli* O157:H7 JITB strain cells (1.0 to 6.0 log CFU/ml per assay, in triplicate) plotted as threshold cycle on the *Y*-axis, against the target number (Log CFU/ml) per assay on the *X*-axis.

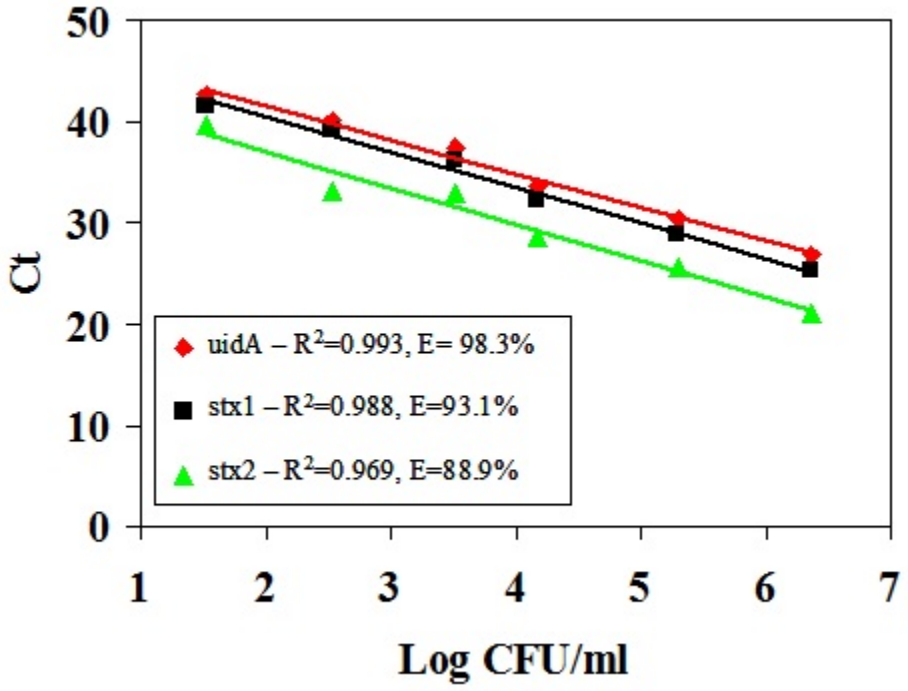


Figure 19. Standard curve for a ten-fold dilution series of *E. coli* O157:H7 Salami cells (1.0 to 6.0 Log CFU/ml per assay, in triplicate) plotted as threshold cycle on the *Y*-axis, against the target number (Log CFU/ml) per assay on the *X*-axis.

Pre- and post-filtration counts did not statistically differ ($p>0.05$) among the approximate 2.0, 3.0 and 4.0 log CFU/ml counts, however, the values did differ ($p<0.05$) for the 1.0 log CFU/ml values. The filtration steps resulted in a two-fold sample volume concentration (100 ml to 50 ml), while the centrifugation step resulted in an additional fifty-fold sample volume concentration, for a final volume of 1.0 ml.

Regardless of the *E. coli* O157:H7 strain, amplification of the target (approximately 1.0, 2.0, 3.0 and 4.0 log CFU/ml) in apple juice by real-time PCR was suppressed when both filtration steps were omitted. If the first filtration or second filtration step alone was omitted, the assay was also inhibited. Similarly, when IMS step was omitted, fluorescence from samples did not exceed the threshold. Non-specific PCR products were not detected in any of the real-time experiments in this study. The broad range of specificities of the *stx1*, *stx2*, and *uidA* primer and probes have previously been determined by Jinneman and others (2003).

The ABI Prism 7000 SDS was used for detection and quantification of *E. coli* O157:H7 in artificially contaminated apple juice. Generally, with the exception of the Cider and JITB strains, gene targets for *stx1*, *stx2* and *uidA* (where applicable) were detected and quantified by real-time PCR at each inoculum level for each *E. coli* O157:H7 strain. Across all strains, cell numbers as few as 1.6 (44.0 CFU/ml) (*stx1*), 1.6 (43.0 CFU/ml) (*stx2*), and 1.5 (33 CFU/ml) (*uidA*) log CFU/ml sample could be quantified by the ABI Prism 7000 SDS using the cell concentration assay in this study. However, several *E. coli* O157:H7 strains were detected on occasion below the quantifiable level at the lowest inoculum level (Table 11).

Table 11. Detection of *E. coli* O157:H7 below quantifiable levels in apple juice using three gene targets.

	Number of positives/replication[a]		
	stx1	*stx2*	*uidA*
Strain	~1.0 log CFU/ml	~1.0 log CFU/ml	~1.0 log CFU/ml
43889	NA	39241	39121
43890	39241	NA	39241
43894	Q	39121	39302
43895	Q	Q	39302
A4	39241	39210	39180
Cider	39090	39149	39180
Salami	39149	39302	Q

[a]Data are from three experiments (triplicate analyses within each experiment)

NA: Not applicable (gene not present)

Q: Quantifiable

The C_t values generated during detection and quantification by real-time PCR varied slightly among *E. coli* O157 strains. C_t values were statistically different ($P<0.05$) across 4.0, 3.0, 2.0 log CFU/ml inoculum levels, however, 2.0 and 1.0 showed similarity ($P>0.05$) for *stx1*. C_t values for *stx2* and *uidA* were statistically different across 1.0, 2.0, 3.0 and 4.0 log CFU/ml inoculum levels ($P<0.05$). Within each inoculum level, C_t values did not differ ($P>0.05$) for *stx1*, *stx2* or *uidA* gene targets.

The accuracy of the real-time PCR method was determined through comparisons of initial inoculum counts (post-filtration) in apple juice determined by cultural plating methods and the real-time PCR predicted values (Table 12). Generally, the numbers of *E. coli* O157:H7 quantified by real-time PCR were of the same order of magnitude as the original bacterial counts in apple juice post-filtration for all gene targets. For the 2.0, 3.0, and 4.0 log CFU/ml inoculum levels, there were no statistical differences ($P>0.05$) between the predicted numbers and initial bacterial numbers determined by plate count. For the lowest inoculum level, 1.0 log CFU/ml, differences were found ($p<0.05$) between the predicted values and plate counts. There were no computer predicted values obtained for *stx1* in the cider and JITB strains at any inoculum level. Predicted values for *stx2* were not generated for the JITB strain at any inoculum level.

DISCUSSION

Over the last decade, studies have shown real-time PCR to be useful for detection and quantification of pathogenic bacteria in food products. In this study, cell

Table 12. Quantification of *E. coli* O157:H7 in inoculated apple juice samples by plate count and real-time PCR

			C_t values and predicted counts (Log CFU/ml) for:					
	E. coli O157:H7 population (Log CFU/ml)±SD[a]		*stx1*		*stx2*		*uidA*	
Strain	Initial inoculum	After filtration	C_t value ±SD	Predicted count ±SD	C_t value ±SD	Predicted count ±SD	C_t value ±SD	Predicted count ±SD
43888	4.1±0.14	3.9 ±0.18	NA[b]	NA	NA	NA	32.68 ± 0.43	4.5±0.13
	3.1±0.19	3.0±0.12					36.99 ± 0.25	3.3±0.18
	1.5±0.24	1.1±0.17					39.81 ± 0.59	2.4±0.18
	0.2±0.53	ND					41.79 ± 0.97	1.7±0.30
43889	4.4±0.10	4.3±0.08	NA	NA	30.21 ± 0.88	4.1±0.25	33.15 ± 0.24	4.3±0.19
	3.4±0.05	3.4±0.04			31.96 ± 0.12	3.5±0.03	35.59 ± 0.18	3.5±0.21
	2.6±0.16	2.3±0.26			35.99 ± 0.26	2.4±0.02	38.57 ± 1.10	2.5±0.26
	0.6±0.52	0.2±0.41			40.52 ± 1.25	NQ[d]	42.89 ± 2.85	NQ
43890	4.4±0.22	4.3±0.16	30.17 ± 0.21	3.9±0.07	NA	NA	32.10 ± 0.17	4.3±0.02
	3.4±0.10	3.2±0.09	32.05 ± 0.37	3.3±0.12			36.17 ± 0.19	3.5±0.06
	2.6±0.11	2.4±0.21	35.01 ± 0.41	2.4±0.13			40.31 ± 0.69	2.3±0.20
	0.5±0.55	0.3±0.52	41.73±1.05	NQ			43.29 ± 0.13	NQ

Table 12. Continued

	E. coli O157:H7 population (Log CFU/ml)±SD[a]		C_t values and predicted counts (Log CFU/ml) for:					
			stx1		*stx2*		*uidA*	
Strain	Initial inoculum	After filtration	C_t value ±SD	Predicted count	C_t value±SD	Predicted count	C_t value±SD	Predicted count
43894	4.5±0.06	4.4±0.10	32.01 ± 0.13	4.3±0.04	42.5 ± 0.95	3.4±0.03	32.39 ± 0.07	4.7±0.023
	3.6±0.06	3.4±0.09	35.49 ± 0.38	3.4±0.11	43.01± 0.23	2.1±0.27	36.38 ± 0.29	3.4±0.09
	2.7±0.12	2.4±0.13	37.15 ± 0.86	2.9±0.25	46.0 ± 0.87	1.4±0.34	38.07 ± 0.37	2.7±0.13
	0.9±0.48	0.5±0.55	40.68 ± 0.52	1.8±0.11	48.67±0.80	NQ	42.12 ± 2.97	NQ
43895	4.4±0.11	4.4±0.17	30.05±0.125	4.4±0.04	29.15±0.02	4.5±0.01	34.01±0.72	4.7±0.22
	3.5±0.12	3.3±0.14	34.16±1.08	3.4±0.23	33.77±0.09	3.3±0.03	38.72±0.61	3.4±0.18
	2.7±0.16	2.5±0.16	36.54±0.75	2.8±0.23	38.07±0.23	2.3±0.02	43.65±0.57	2.1±0.17
	0.7±0.52	0.2±0.41	38.0±0.37	1.8±0.11	42.25±0.34	1.6±0.06	47.50±2.58	NQ
A4	4.4±0.08	4.2±0.04	33.03±0.56	4.6±0.10	32.06±0.35	4.1±0.02	35.61±0.01	4.5±0.01
	3.7±0.19	3.5±0.20	35.05±0.37	3.1±0.12	35.80±0.18	3.0±0.15	39.75±0.2	3.2±0.09
	2.7±0.16	2.5±0.31	37.0±0.76	2.4±0.24	40.15±0.82	2.2±0.11	47.0±0.99	1.4±0.32
	1.1±0.59	0.4±0.60	41.35±1.05	NQ	43.06±0.75	NQ	47.37±0.38	NQ

Table 12. Continued

	E. coli O157:H7, population (Log CFU/ml)±SD[a]		C_t values and predicted counts (Log CFU/ml) for:					
			stx1		*stx2*		*uidA*	
Strain	Initial inoculum	After filtration	C_t value ±SD	Predicted count	C_t value±SD	Predicted count	C_t value±SD	Predicted count
Cider	4.5±0.06	4.4±0.03	ND[c]		31.34±0.07	4.3±0.02	33.66±0.07	4.3±0.03
	3.6±0.04	3.4±0.07			32.44±0.12	3.2±0.08	37.21±0.14	3.4±0.7
	2.9±0.07	2.5±0.18			36.72±0.21	2.6±0.14	40.59±0.35	2.2±0.12
	0.4±0.17	0.2±0.41			40.17±0.38	NQ	44.01±0.24	NQ
Salami	4.3±0.12	4.1±0.12	33.80±0.40	4.0±0.11	30.22±0.05	3.8±0.01	34.58±0.18	4.1±0.06
	3.3±0.10	3.0±0.12	36.71±0.37	3.1±0.11	34.29±0.08	2.8±0.03	38.49±0.50	3.0±0.15
	2.5±0.28	2.2±0.41	42.02±0.32	1.7±0.32	35.52±0.43	2.3±0.13	41.45±0.61	2.1±0.18
	0.4±0.69	0.2±0.41	44.84±1.61	0.9±0.46	40.13±0.31	0.9±0.09	44.04±2.28	1.6±0.68
JITB	4.3±0.12	4.3±0.15	ND	ND	32.61±0.16	NQ	38.4±0.41	4.8±0.12
	3.4±0.09	3.3±0.19			35.06±0.07	NQ	41.0±0.48	4.0±0.14
	2.5±0.34	2.3±0.19			39.52±0.28	NQ	44.4±0.39	3.0±0.11
	0.5±0.55	0.2±0.41			ND	ND	ND	ND

[a]Data are avg. means ± standard deviations from triplicate analysis; [b]NA:Not applicable; [c]ND:Not detected by real-time PCR at this inocula; [d]NQ:Not quantifiable

concentration methods including filtration, centrifugation, and IMS, were combined for use in a TaqMan probe-based real-time PCR assay for detection and quantification of *E. coli* O157:H7 in apple juice. With this methodology, *E. coli* O157:H7 could be detected and quantified in apple juice in less than 5 h without an enrichment step. Of the 5 h assay time, sample preparation took nearly 2 h, while the real-time PCR cycling was approximately 2.5 h. The rapidity of the assay could allow for early recognition of a causative agent during a juice- or cider-associated outbreak.

Other investigators have used real-time PCR or other molecular-based rapid methods in combination with cell concentration techniques for detection of *E. coli* O157:H7 in apple juice or cider. Hsu et al. (2005) developed a TaqMan-based PCR system for quantification of *E. coli* O157:H7 in food samples, including apple juice. The detection sensitivity in apple juice was $1x10^5$ CFU/ml without an enrichment step, while the current study had the capability of detection of <2.0 log CFU/ml (<1 x 10^2 CFU/ml) of the organism in apple juice. Fortin and others (2001) developed a molecular beacon-based real-time PCR which could detect *E. coli* O157:H7 in apple juice at levels as low as 1 CFU/ml, but only after an 11 hour enrichment. DeCory et al. (2005) found that an IMS assay combined with sulforhodamine B-containing immunoliposomes specific for *E. coli* O157:H7 could detect less than 1 CFU/ml. Although these investigators reported detection of low numbers of *E. coli* O157:H7, either enrichment steps were used in the studies, or if no enrichment period was employed, very small sample volumes were used (e.g. 1 ml apple juice). The current study utilized large sample volumes for detection and quantification of low numbers of *E. coli* O157:H7 without the addition of a timely enrichment step.

Apple cider and juice are aqueous matrices known to contain PCR inhibitory substances, particularly, polyphenolic compounds (Siebert et al., 1996). Fortin and others (2002) hypothesized that polyphenolic compound in apple juice reduced the sensitivity of the molecular beacon-based real-time PCR in their studies. This is likely due to the ability of polyphenolic compounds to irreversibly bind nucleic acids, which makes DNA unavailable for PCR reaction (Ogunjimi and Choudary, 1999; Li et al., 2002). When the filtration steps were omitted in this study, amplification of real-time PCR target product was inhibited altogether. The filtration step conceivably reduced the amount of polyphenolic substances prior to DNA extraction, thereby lowering the polyphenolic compound inhibitory capabilities. The IMS step was included in the assay to capture *E. coli* O157:H7 from the concentrated sample (post-filtration and centrifugation) and separate the cells from any remaining inhibitory substances. When the IMS step was omitted, no results were obtained, even after two filtration steps and centrifugation for cell concentration. The *E. coli* O157:H7 DNA was extracted using PrepMan Ultra while the bacteria were attached to the magnetic particles. Therefore, the bacterial components bound to the beads were magnetically separated from the DNA and discarded prior to real-time PCR. Based on data collected from control samples (samples inoculated with beads only), there was no increase in fluorescence after the beads were removed which indicates that the beads did not inflate fluorescence readings in this experiment.

Standard curve efficiencies were generally near 100.0% ± 5.0% for all gene targets for every *E. coli* O157:H7 strain. An efficiency reading of 168.0% was recorded for the *stx2* gene in ATCC strain 43894. This result suggests that at the later cycles

(>40), real-time PCR reagents were already consumed, causing amplification to plateau early and final fluorescence intensity to diminish. A one or two cycle difference in the samples of higher dilutions can often create a skewed slope, artificially inflating the efficiency. The factor most likely to affect real-time PCR efficiency is primer and probe concentration (Ginzinger, 2002).

The TaqMan probe-based real-time PCR assay developed in this study provides the possibility of quantitative detection of *E. coli* O157:H7. The results demonstrated that the assay, when combined with concentration steps and PrepMan Ultra reagent, is sensitive for the detection of *E. coli* O157:H7 to 1.5 log CFU/ml by the ABI Prism 7000 SDS. The detection range was between 1.0 log CFU/ml and 4.0 log CFU/ml. Detection below this range was feasible, but values that lie outside the linear range of the standard curve for quantification can only be expressed as semi-quantitative. Nevertheless, this information can be valuable, as it confirms the presence of toxin-producing genes (*stx1* and/or *stx2*) in the juice sample. The assay detected all three gene targets with success at similar levels in the experimental strains with the exception of the *stx1* gene in the Cider strain, and *stx1* and *stx2* in the JITB strain, none of which were detected in all three experimental replications. This could be due to insufficient starting template, poor-quality reagents, presence of inhibitory substances or pipetting errors. During standard curve generation, *stx1* and *stx2* were detected in the JITB strain, and *stx2* was detected in the Cider strain. A standard curve for *stx1* in the Cider strain could not be generated. Poor quality reagents, pipetting errors, or imprecise cell dilutions could explain this result.

The specificity of this assay relies on both the IMS steps and the real-time PCR. The anti-*E. coli* O157 magnetic beads are coated with a polyclonal antibody against *E. coli* O157. Additionally, the primers and probes in the study target the *stx1* and *stx2* genes of enterohemorrhagic *E. coli* O157:H7. The *stx1* and *stx2* genes also occur in other Shiga-toxin producing *E. coli* such as *E. coli* O26, O111 and O113, and in *Shigella.* Although this could generate false positive results, the O157 specific IMS step should not capture non-O157 serotypes. Future studies which analyze a mixed enterohemorrhagic *E. coli,* such as O26, O111, and O157 culture in apple juice are needed to determine if IMS could eliminate false positives. Futhermore, false positive detection of similar serotypes, such as O26, O111 and O113, may be admissible since these are able to survive in apple juice and are considered a potential health hazard (DebRoy et al., 2004; Drysdale et al., 2004).

The current study demonstrates the importance of cell concentration methods for a successful TaqMan-based real-time PCR for detection of low numbers of *E. coli* O157:H7 in apple juice while eliminating enrichment steps. Even though the current detection levels may not be as low as the detection levels of 1 CFU/ml or < 1 CFU/ml in apple juice or cider samples which have been documented when using real-time PCR combined with enrichment, this proves that detection without cultural enrichment is reaching the same degree of sensitivity and reproducibility as traditional detection methods (Fortin et al., 2001; DeCory et al., 2005).

REFERENCES

Anderson, S. 2001. Recent FDA juice HACCP regulations. Food Saf. Mag. April/May:18-25.

Benoit, P.W. and Donahue, D.W. 2003. Methods for rapid separation and concentration of bacteria in food that bypass time-consuming cultural enrichment. J. Food Prot. 66:1935-1948.

Besser, R., Letts, S., Webber, J., Doyle, M., Barrett, T., Wells, J. And Griffin, P. 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. JAMA 269:2217-2220.

Cody, S.H., Glynn, M.K., Farrar, J.A., Cairns, K.L., Griffin, P.M., Kobayashi, J., Fyfe, M., Hoffman, R., King, A.S., Lewis, J.H., Swaminathan, F., Bryant, R.G., and Vugia, D.J. 1999. An outbreak of *Escherichia coli* O157:H7 infection from unpasteurized commercial apple juice. Ann. Intern. Med. 130:202-209.

DebRoy, C., Roberts, E., Kundrat, J., David, M.A., Briggs, C.E., Fratamico, P.E. 2004. Detection of *Escherichia coli* O26 and O113 by PCR amplification of the *wzx* and *wzy* genes. Appl. Environ. Microbiol. 70:1830-1832.

DeCory, T.R., Durst, R.A., Zimmerman, S.J., Garringer, L.A., Paluca, G., DeCory, H.H., and Montagna, R.A. 2005. Development of an immunomagnetic bead-immunoliposome fluorescence assay for rapid detection of *Escherichia coli* O157:H7 in aqueous samples and comparison of the assay with a standard microbiological method. Appl. Environ. Microbiol. 71:1856-1864.

Drysdale, M., MacRae, M., Strachan, N.J.C., Reid, T.M.S., and Ogden, I.D. 2004. The detection of non-O157 *E. coli* in food by immunomagnetic separation. J. Appl. Microbiol. 97:220-224.

Fortin, N.Y., Mulchandani, A., and Chen, W. 2001. Use of a real-time polymerase chain reaction and molecular beacons for the detection of *Escherichia coli* O157:H7. Anal. Biochem. 289:281-288.

Fu, Z., Rogelj, S., and Kieft, T.L. 2005. Rapid detection of *Escherichia coli* O157:H7 by immunomagnetic separation and real-time PCR. Intl. J. Food Microbiol. 99:47-57.

Ginzinger, D.G. 2002. Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. Exper. Hemat. 30:503-512.

Hill, W.E. 1996. The polymerase chain reaction: applications for the detection of foodborne pathogens. Crit. Rev. Food Sci. Nutr. 36:123-173.

Hines, E. 2000. PCR-based testing: unraveling the mystery. Food Quality. 7:22-28.

Hsu C.F., Tsai, T.Y., and Pan T.M. 2005. Use of the duplex TaqMan PCR system for detection of Shiga-like toxin-producing *Escherichia coli* O157. J. Clin. Microbiol. 43:2668-2673.

Jinneman, K.C., Trost, P.A., Hill, W.E., Weagant, S.D., Bryant, J.L., Kaysner, C.A., and Wekell, M.M. 1995. Comparison of template preparation methods from foods for amplification of *Escherichia coli* O157:H7 Shiga-like toxins type I and II DNA by multiplex polymerase chain reaction. J. Food Prot. 58:722-726.

Johnston, L.M., Elhanafi, D., Drake, M.A., and Jaykus, L.A. 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. J. Food Prot. 68:2256-2263.

Lampel, K.A., Orlandi, P.A., and Kornegay, L. 2000. Improved template preparation for PCR-based assays for detection of food-borne bacterial pathogens. Appl. Env. Microbiol. 66:4539-4542.

Lantz, P.G., Hahn-Hägerdal, B., Rådström, P. 1994. Sample preparation methods in PCR-based detection of food pathogens. Trends Food Sci. Technol. 5:384-389.

Li, Y.X., Su, Z.X., and Chen, F. 2002. Rapid extraction of genomic DNA from leaves and bracts of dove tree (*Davidia involucrata).* Plant Mol. Bio. Report. 20:185a-185e.

Millard, P., Gensheimer, K., Addiss, D., Sosin, D., Beckett, G., Houck-Jankoski, A. And Hudson, A. 1994. An outbreak of cryptosporidiosis from fresh-pressed apple cider. JAMA 272:1592-1596.

Mercanoğlu, B. and Griffiths, M.W. 2005. Combination of immunomagnetic separation with real-time PCR for rapid detection of *Salmonella* in milk, ground beef, and alfalfa sprouts. J. Food Prot. 68:557-561.

Meuer, S., Wittwer, C., Nakagawara, K. (Eds). 2001. Rapid cycle real-time PCR: methods and applications. Heidelberg:Springer Press. 408 p.

Mshar, P.A., Dembek, Z.F., Cartter, M.L., Hadler, J.L., Fiorentino, T.R., Marcus, R.A., McGuire, J., Shiffrin, M.A., Lewis, A., Feuss, J., Dyke, J. van, Toly, M., Cambridge, M., Guzewich, J., Keithly, J., Dziewulski, D., Braun-Howland, E., Ackman, D., Smith, P., Coates, J., Ferrara, J. 1997. Outbreaks of *Escherichia coli* O157:H7 and Crypotsporidiosis associated with drinking unpasteurized apple cider–Connecticut and New York, October 1996. MMWR 46:4-8.

Ogunjimi, A.A. and Choudary, P.V. Adsorption of endogenous polyphenols relieves the inhibition by fruit juices and fresh produce of immuno-PCR detection of *Escherichia coli* O157:H7. FEMS Imm. Med. Microbiol. 23:231-220.

Rodríguez-Lázaro, D., Jofré, A., Aymerich, T, Hugas, M, Pla, M. 2004. Rapid quantitative detection of *Listeria monocytogenes* in meat products by real-time PCR. Appl. Env. Microbiol. 70:6299-6301.

Siebert, K.J., Troukhanova, N.V., and Lynn, P.Y. 1996. Nature of polyphenol-protein interactions. J. Agric. Food Chem. 44:80-85.

Stevens, K.A. and Jaykus, L.A. 2004. Bacterial separation and concentration from complex matrices: a review. Crit. Rev. Microbiol. 30:7-24.

Zoecklein, B.W., Fugelsang, K.C., Gump, B.H., and Nury, F.S. 1995. Fining and fining agents. In: Wine Analysis and Productions, pp. 243-271. Chapman and Hall, New York, NY.

APPENDIX

SAS Programming: Comparison of C_t values from DNA extraction/real-time PCR methods among *stx1, stx2,* and *uidA.*

```
data one;
input strain$ method$ ct;
datalines;

%include 'C:\Documents and Settings\Owner\My Documents\SAS\PDMIX800.sas';
%include 'C:\Documents and Settings\Owner\My Documents\SAS\mmaov.sas';
%mmaov (one, ct, class=method strain, fixed=method);
```

SAS Programming: Comparison of C_t values from DNA extraction/real-time PCR methods for the *stx1* gene.

```
data one;
input stx1 $ dilution ct ;
datalines;

%include 'C:\Documents and Settings\Owner\My Documents\SAS\PDMIX800.sas';
%include 'C:\Documents and Settings\Owner\My Documents\SAS\mmaov.sas';
%mmaov (one, ct, class=method stx1, fixed=method);
```

SAS Programming: Comparison of C_t values from DNA extraction/real-time PCR methods for the *stx2* gene.

```
data one;
input stx2 $ dilution ct ;
datalines;

%include 'C:\Documents and Settings\Owner\My Documents\SAS\PDMIX800.sas';
%include 'C:\Documents and Settings\Owner\My Documents\SAS\mmaov.sas';
%mmaov (one, ct, class=method stx2, fixed=method);
```

SAS Programming: Comparison of C_t values from DNA extraction/real-time PCR methods for the *uidA* gene.

```
data one;
input uidA $ dilution ct ;
datalines;

%include 'C:\Documents and Settings\Owner\My Documents\SAS\PDMIX800.sas';
%include 'C:\Documents and Settings\Owner\My Documents\SAS\mmaov.sas';
%mmaov (one, ct, class=method uidA, fixed=method);
```

SAS Programming: Comparison of C_t values from DNA extraction/real-time PCR methods among *E. coli* O157:H7strains and cross-reactive strains.

```
data one;
input strain$ method ct ;
datalines;

%include 'C:\Documents and Settings\Owner\My Documents\SAS\PDMIX800.sas';
%include 'C:\Documents and Settings\Owner\My Documents\SAS\mmaov.sas';
%mmaov (one, ct, class=method strain, fixed=method);
```

SAS Programming: Comparison of *E. coli* O157:H7 pre- and post-filtration cell counts at 4.0 log CFU/ml, 3.0 log CFU/ml, 2.0 log CFU/ml and 1.0 log CFU/ml.

```
data one;
input strain $ dilution count;
datalines;

%include 'C:\Documents and Settings\Owner\My Documents\SAS\PDMIX800.sas';
%include 'C:\Documents and Settings\Owner\My Documents\SAS\mmaov.sas';
%mmaov (one, strain, class=dilution count, fixed=dilution);
```

SAS Programming: Comparison of *E. coli* O157:H7 C_t values among 4.0 log CFU/ml, 3.0 log CFU/ml, 2.0 log CFU/ml and 1.0 log CFU/ml for *stx1*.

```
data one;
input strain $ dilution ct;
datalines;

%include 'C:\Documents and Settings\Owner\My Documents\SAS\PDMIX800.sas';
%include 'C:\Documents and Settings\Owner\My Documents\SAS\mmaov.sas';
%mmaov (one, strain, class=dilution ct, fixed=dilution);
```

SAS Programming: Comparison of *E. coli* O157:H7 C_t values among 4.0 log CFU/ml, 3.0 log CFU/ml, 2.0 log CFU/ml and 1.0 log CFU/ml for *stx2*.

```
data one;
input strain $ dilution ct;
datalines;

%include 'C:\Documents and Settings\Owner\My Documents\SAS\PDMIX800.sas';
%include 'C:\Documents and Settings\Owner\My Documents\SAS\mmaov.sas';
%mmaov (one, strain, class=dilution ct, fixed=dilution);
```

SAS Programming: Comparison of *E. coli* O157:H7 C_t values among 4.0 log CFU/ml, 3.0 log CFU/ml, 2.0 log CFU/ml, and 1.0 log CFU/ml for *uidA*.

```
data one;
input strain $ dilution ct;
datalines;

%include 'C:\Documents and Settings\Owner\My Documents\SAS\PDMIX800.sas';
%include 'C:\Documents and Settings\Owner\My Documents\SAS\mmaov.sas';
%mmaov (one, strain, class=dilution ct, fixed=dilution);
```

SAS Programming: Comparison of *E. coli* O157:H7 cell counts by plate count and real-time PCR at 4.0 log CFU/ml, 3.0 log CFU/ml, 2.0 log CFU/ml, and 1.0 log CFU/ml.

```
data one;
input strain $ method ct;
datalines;

%include 'C:\Documents and Settings\Owner\My Documents\SAS\PDMIX800.sas';
%include 'C:\Documents and Settings\Owner\My Documents\SAS\mmaov.sas';
%mmaov (one, strain, class=dilution method, fixed=dilution);
```

VITA

Nancy Rabalais DeTrana was born on August 18, 1974, in Metairie, Louisiana. She graduated from Hillcrest Christian School in Jackson, Mississippi, in May 1992. She received a Bachelor of Science degree in Biological Sciences, with a minor in Chemistry, from the University of Southern Mississippi (Hattiesburg, MS) in December 1995. In December 1997, she received a Master of Public Health degree, with an emphasis in environmental health and safety. Nancy accepted a position as a Public Health Environmentalist with the Mississippi State Department of Health in Hattiesburg, Mississippi, in September 1998. In August 2001, she left the MSDH to pursue a Ph.D. in Food Microbiology at the University of Tennessee. The Ph.D. degree was awarded in May 2007.