DETECTION OF VIABLE *ESCHERICHIA COLI* O157:H7 IN FOOD BY PROPIDIUM MONOAZIDE REAL-TIME POLYMERASE CHAIN REACTION

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

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DETECTION OF VIABLE *ESCHERICHIA COLI* O157:H7 IN FOOD BY PROPIDIUM MONOAZIDE REAL-TIME POLYMERASE CHAIN REACTION

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ABSTRACT

Escherichia coli O157:H7 associated with food has caused many serious public health problems in recent years. However, only viable cells of this pathogen can cause infections, and false-positive detection caused by dead cells can lead to unnecessary product recalls. The objective of this study was to develop and optimize a method that combines propidium monoazide (PMA) staining with real-time PCR to detect only viable cells of *E. coli* O157:H7. PMA is a dye that can penetrate dead cells and bind to cellular DNA, preventing its amplification via a subsequent PCR. Compared with ethidium monoazide (EMA), another DNA-binding dye, PMA has been reported to exert less influence on DNA amplification from viable cells. Three strains of *E. coli* O157:H7 (505B, G5310 and C7927) was prepared separately and serially diluted to generate cell suspensions ranging from 10 to 10^8 CFU/mL. Dead cells were obtained by heating the suspensions at 85^{o}C for 15 min. Suspensions were then treated with PMA. The optimized assay was then applied to artificially contaminated apple juice and ground beef. DNA was extracted and amplified by TaqMan® real-time PCR targeting the *uid*A gene to detect only viable *E. coli* O157:H7 cells. Plasmid pUC19 was included in each

reaction as an internal amplification control (IAC) to monitor the efficiency of real-time PCR. Results showed that a treatment of 25 µM PMA with a 10-min light exposure on ice was sufficient to eliminate DNA from 10^8 CFU/mL dead *E. coli* O157:H7 cells. The optimized assay could detect viable *E. coli* O157:H7 at as low as 10^2 CFU/mL in pure culture, 10^4 CFU/mL in apple juice and 10^5 CFU/g in ground beef, in the presence of 10^6 CFU/mL or g dead cells. With 8 h enrichment, viable *E. coli* O157:H7 of 1 CFU/mL or g in apple juice or ground beef was detectable without interference from 10^6 CFU/mL or g dead cells. In conclusion, the PMA real-time PCR assay can effectively prevent amplification of DNA in dead cells of *E. coli* O157:H7 and differentiate viable *E. coli* O157:H7 from dead cells in apple juice and ground beef.

CHAPTER 1

INTRODUCTION

Escherichia coli O157:H7 is one of the most notorious foodborne pathogens, with an infectious dose of as low as a few hundred cells (Karmali 2004). Beef products, dairy products, juices and fresh produce products are common foods associated with *E. coli* O157:H7 outbreaks. *E. coli* O157:H7 infections can lead to nonspecific diarrhea, hemorrhagic colitis, and even hemolytic uremic syndrome (HUS) (Banatvala and others 2001). The Centers for Disease Control and Prevention (CDC) has estimated that *E. coli* O157:H7 is responsible for 73,480 illnesses annually in the United States, resulting in more than 2,000 hospitalizations and 60 deaths each year (Mead and others 1999). It has been estimated that the annual cost of *E. coli* O157:H7 infections was $405 million from 1996 to 2004, including $370 million for premature deaths, $30 million for medical care, and $5 million in lost productivity (Frenzen and others 2005). Thus, accurate and sensitive methods to detect *E. coli* O157:H7 in food products are urgently needed.

Conventional culture-based methods, involving enrichment, isolation and confirmation steps, are widely used due to their sensitivity, low cost, ease of use, and ability to monitor cell viability (Murakami 2012). However, it requires four to five days to obtain results. On the other hand, the polymerase chain reaction (PCR), which is a nucleic acid- based technique, can identify target species within 3 h. Nevertheless, conventional PCR cannot differentiate viable cells from dead cells (Wang and Levin

2006). DNA from dead cells can yield false-positive results in PCR, leading to unnecessary product recalls and economic losses.

Propidium monoazide (PMA), a DNA intercalating dye, can only penetrate compromised membranes of dead cells and covalently bind to cellular DNA through photolysis. Consequently, the covalent link will render the DNA insoluble and inhibit PCR amplification of DNA from dead cells (Nocker and others 2006). PMA has been successfully used in combination with real-time PCR to detect viable lactic acid bacteria (Garcia-Cayuela and others 2009), T4 virus (Fittipaldi and others 2010), *Campylobacter* (Josefsen and others 2010), *Salmonella* (Liang and others 2011) and *E. coli* (Yang and others 2011; Taskin and others 2011) in environmental and food samples. Ethidium monoazide (EMA) is another dye that binds covalently to cellular DNA. Both PMA and EMA have specific advantages and disadvantages. Compared with PMA, EMA penetrates dead cells more effectively and exerts greater inhibitory effect in a PCR (Lee and Levin 2006). However, PMA has not been found to inhibit amplification of DNA from viable cells, while EMA at high concentrations penetrates into viable cells and leads to false-negative results in PCR (Nocker and others 2006).

Real-time PCR is a quantitative PCR method. Fluorescence intensity is accumulated during a real-time PCR process which is proportional to the amount of target DNA in samples. Hence, the measurement of incremental fluorescence intensity will provide an accurate estimate of the amount of target cells in samples. Real-time

PCR is sensitive, rapid, accurate, and allows quantification of microorganisms of interest in samples. However, there is a limitation, which is substances naturally found in environmental and food samples may inhibit the amplification of target DNA in real-time PCR. To solve this problem, internal amplification control (IAC) is intentionally added in a real-time PCR reaction to monitor the PCR efficiency and prevent false-negative results due to inhibitors in food samples (Murphy and others 2007).

The objective of this research was to combine PMA with real-time PCR for detection and estimation of only viable *E. coli* O157:H7 cells in apple juice and ground beef.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Microbial contamination of food

The availability of safe food is a basic human right and guarantees the health of people. Safe food is a prerequisite for health, productivity and development. The importance of safe food has drawn much more attention recently since the growing number of reported foodborne diseases. With rapid development of globalization of food distribution, contaminated food products become potential threats to the entire world. It has been estimated that approximately 30% of infections over the world during the past 60 years were transmitted via food (Jones and others 2008). According to Tauxe and others (2010), "The identification of one single contaminated food ingredient can lead to the recall of literally tons of food products, to considerable economic losses in production and from trade embargoes, as well as damage to the tourist industry." Foodborne diseases spread worldwide significantly affect the health of individuals as well as the development of societies.

2.2 Economic losses caused by food contamination

In the United States, it has been estimated that foodborne diseases lead to 76 million illnesses, 325,000 hospitalizations and 5000 deaths each year (Mead and others 1999), resulting in medical costs and productivity losses between 6.6 billion and 37.1 billion (Buzby and Roberts 1996). The problems caused by foodborne diseases are likely

to be even worse in developing countries (Käferstein and Abdussalam 1999). As reported, food and waterborne diarrheal diseases alone cause approximately 2.1 million deaths each year in less developed countries (WHO 2001).

2.3 Spectrum of foodborne pathogens

A broad spectrum of microbial pathogens can contaminate food and water supplies, and cause illness after they or their toxins are consumed by people. The pathogenic spectrum includes a variety of bacteria, fungi, viruses, parasites, and dinoflagellates. Well-known foodborne pathogenic bacteria include *Salmonella*, *Campylobacter*, *E. coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium perfringens*. Some of these pathogens require humans as hosts while others naturally exist in the environment or in an animal's intestinal tract. Some of them are always transmitted via food, while others can be transmitted by several different routes (Tauxe 2002).

2.4 Symptoms caused by foodborne pathogens

The most common symptoms caused by bacterial foodborne pathogens are gastrointestinal diarrhea and vomiting. The other consequences include arthritis, kidney and liver failure, brain and neural disorders, paralysis and even death (Schlundt 2002).

2.5 *E. coli* O157:H7

2.5.1 Characteristics of *E. coli* O157:H7

E. coli belongs to the Enterobacteriaceae family, whose members are gram-negative, facultatively anaerobic, rod-shaped, and non-spore forming bacteria. Serotypes of *E. coli* are determined based on somatic (O), flagellar (H) and capsule (K) antigens (Feng 2001). While most *E. coli* strains are normal human gut microflora and harmless to human beings, five virulence groups of *E. coli* have been recognized. These are enteroaggregative (EAggEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC), and enterotoxigenic (ETEC) (Levine 1987). Enterohemorrhagic *E. coli* (EHEC) are a group of strains that cause severe human diseases, including bloody diarrhea and hemolytic uremic syndrome (HUS). Among all of the EHEC serogroups, *E. coli* O157:H7 is the one most often involved in EHEC infections in the US (Rasmussen and Casey 2001).

E. coli O157:H7 cannot ferment sorbitol and is glucuronidase-negative, which differentiates it from other *E. coli* strains. Unlike most strains of *E. coli*, the O157:H7 serotype does not grow at 44.5˚C, but can survive for 35 days at 5^{o}C (Weagant and others 1994). D-values of *E. coli* O157:H7 at 55 to 70^{o}C are from 19.05 to 0.038 min, indicating that the organism is not heat resistant (Murphy and others 2004). *E. coli* O157:H7 are acid-resistant strains which can grow under pH 3.6 to 4.0. However, high

concentrations of salt (≥ 8.5%) show an inhibitory effect on the growth of *E. coli* O157:H7 (Jay 2000).

2.5.2 Disease and virulence of *E. coli* O157:H7

E. coli O157:H7 was first recognized as a human pathogen in Michigan and Oregon in 1982, when 47 people developed bloody diarrhea after consuming contaminated hamburgers (Riley and others 1983). The infectious dose of *E. coli* O157:H7 is very low, less than 100 organisms (Karmali 2004). Clinical manifestations after infection with *E. coli* O157:H7 include asymptomatic carriage, diarrhea, hemorrhagic colitis (HC), HUS, and thrombotic thrombocytopenic purpura, leading to substantial morbidity and mortality (Su and Brandt 1995). *E. coli* O157:H7 mainly affects young children, elderly people and immunocompromised individuals. Approximately 10 to 15% of patients infected with *E. coli* O157:H7 develop HUS (Tarr and others 2005; Scheiring and others 2008) while up to 40% of patients with HUS develop long-term renal dysfunction, and about 3 to 5% of patients die during the acute phase of the disease (Fitzpatrick and others 1991; Siegler and others 1991).

Virulence genes of *E. coli* O157:H7 are Shiga toxins (*stx*1, *stx*2), intimin (*eae*), translocated intimin receptor (*tir*), enterohemolysin (*ehly*), long polar fimbriae (*lpf*), and other pathogenic genes (Rasmussen and Casey 2001). Among these, Shiga toxins (*stx*1, *stx*2) are believed to play a critical role for the development of severe clinical symptoms. *E. coli* O157:H7 isolated from patients with HUS usually express both *stx*1 and *stx*2 or

only *stx*2 (Rasmussen and Casey 2001). Both *stx*1 and *stx*2 have the same cell receptor and the same intracellular mechanism of action *in vitro*. Globotriaosylceramide (Gb3), which is highly expressed in the cortex of the human kidney and is found in primary human endothelial cells, is the major receptor cells for both *stx*1 and *stx*2 (Su and Brandt 1995). After binding to the Gb3 receptor on the surface of the endothelial cells, Shiga-like toxins inhibit protein synthesis by N-glycosidase cleavage at a specific site of an adenine residue on the 28s ribosomal subunit, leading to endothelial-cell damage and pathogenesis of HUS (Su and Brandt 1995; Karmali 2004).

2.5.3 Transmission and outbreak of *E. coli* O157:H7

Natural reservoirs of *E. coli* O157:H7 are intestinal tracts of animals, especially cattle and other ruminants. Food remains the predominant transmission route for *E. coli* O157:H7, which caused 183 out of 350 (52%) outbreaks from 1982 to 2002 in the U.S. (Rangel and others 2005). Other transmission routes include water, person-to-person contact, and direct contact with animals (Aruscavage and others 2006). The major food vehicle for *E. coli* O157:H7 is meat, especially ground beef contaminated with ruminant feces, and the others include unpasteurized milk, apple cider, yogurt, and vegetables (Beuchat 2002). The *E. coli* O157:H7 infection typically occurs from June through September (Ostroff and others 1991), and it mainly affects young children and the elderly (Griffin and others 1988).

It has been estimated that *E. coli* O157:H7 infections cause 73,480 illnesses annually in the U.S., leading to approximately 2,168 hospitalizations and 61 deaths each year (Mead and others 1999), resulting in an annual cost of $405 million ($370 million for premature deaths, $30 million for medical care, and $5 million in lost productivity) (Frenzen and others 2005).

2.5.4 Prevention of *E. coli* O157:H7 infections

Currently, the United States Department of Agriculture (USDA) has "zero tolerance" towards *E. coli* O157:H7 in ground beef and beef trimmings, which means that the absence of *E. coli* O157:H7 is required in these types of food products (USDA 2012). However, there are no specific measures for preventing or treating *E. coli* O157:H7 infections at present. Hazard analysis and critical control points (HACCP) is an accepted management system for delivering safe food. The implementation of HACCP on food processing can be expected to decrease the risk for cross-contamination (Pennington 2010). The Food and Drug Administration (FDA) has recommended a minimum internal temperature of 155^{O}F for cooked hamburger, which decreases the risk of cross contamination (Line and others 1991). Animals are the primary reservoirs of *E. coli* O157:H7, thus, it might be effective to eliminate or reduce excretion of *E. coli* O157:H7 from animal reservoirs by vaccination or using probiotics for competitive exclusion (Gyles 1998).

2.6 Detection of *E. coli* O157:H7 in food

The detection of pathogens in food is often difficult because of the complexity of food matrices (such as natural microbial background, nutrient components, pH, and water activity) and the low infectious dose of pathogens. Many approaches have been developed to isolate and identify *E. coli* O157:H7 in food, and these can generally be divided into three categories: (i) conventional methods using biochemical characteristics specific to *E. coli* O157:H7, (ii) immunological detection methods, and (iii) nucleic acid based detection methods.

2.6.1 Conventional methods

Culture-based methods have been used for over a century to detect and identify bacterial pathogens. Culture-based methods are specific but labor-intensive and time-costing. The procedure usually involves enrichment, isolation and confirmation, which takes about four to five days to obtain final results. Enrichment is a step to allow the number of pathogenic cells to multiply to detectable levels. Several liquid media for the enrichment of *E. coli* O157:H7 have been reported and some are listed in Table 1. With the help of nutrients and antibiotics in such media, *E. coli* O157:H7 produces more colonies than other bacteria in the samples. After enrichment, selective media are usually used to specifically isolate target *E. coli* O157:H7 in the samples. The most widely used selective medium for the isolation of *E. coli* O157:H7 is sorbitol MacConkey agar with cefixime and tellurite (CT-SMAC). Typical *E. coli* O157:H7 colonies are colorless or neutral/gray with a smoky center and 1-2 mm in diameter on CT-SMAC. Some of the

selective agars used to isolate *E. coli* O157:H7 are listed in Table 2. The FDA recommends two newer chromogenic agars in addition to CT-SMAC agar for the isolation of *E. coli* O157:H7 in their Bacteriological Analytical Manual (BAM), Rainbow® Agar O157 and R&F® *E. coli* O157:H7 Agar (FDA 2011). Due to the fact that *E. coli* O157:H7 cannot ferment sorbitol and is β-glucuronidase negative, *E. coli* O157:H7 colonies should appear as black to blue-black colonies on Rainbow® Agar O157 or R&F® *E. coli* O157:H7 agar. Moreover, several other biochemical tests can be used to differentiate *E. coli* from other members of Enterobacteriaceae. Commercially available kits, such as MICRO-ID® (REMEL, Lenexa, KS), BBL® Enterotube™ II (Becton Dickinson, Sparks, MD), and API systems (bioMerieux, Paris, France) can identify enterobacteria in 4 h based on the results of a series of biochemical tests (Deisingh and Thompson 2004). The presumptive positive isolates which are identified by selective agars or biochemical tests are subsequently confirmed by serotyping, based on the presence of the O157 and H7 antigens. RIM *E. coli* O157:H7 Latex Test (Remel, Lenexa, KS) is a widely used commercial antisera kit for this kind of confirmation.

Table 1 Enrichment liquid media for *E. coli* O157:H7 (adapted from Vernozy-Rozand 1997).

Designation	Composition (L^{-1})
mTSB	Trypticase soy broth, 30 g; Bile salts 3, 1.5 g; K_2HPO_4, 1.5 g; Novobiocin, 20 mg.
dm TSB-CA	Trypticase soy broth, 30 g; Bile salts 3, 1.5 g; K_2HPO_4, 1.5 g; Casamino acids, 10 g; Acriflavine-HCl, 10 mg.
BPW-VCC	Buffered peptone water; Vancomycin, 8 mg; Cefixime, 0.05 mg; Cefsulodin, 10 mg.
mECn	Tryptone, 20 g; Bile salts 3, 1.12 g; Lactose, 5 g; K_2HPO_4, 4 g; KH_2PO_4, 1.5 g; NaCl, 5 g; Novobiocin, 20 mg.

Table 2 Solid selective media for isolation of *E. coli* O157:H7 (adapted from Vernozy-Rozand 1997).

Designation	Composition
SMAC	MacConkey agar; D-Sorbitol, 1%.
CT-SMAC	MacConkey Sorbitol agar; Cefixime, 0.05 mg L^{-1} Potassium tellurite, 1 mg L^{-1}.
MSA-MUG	MacConkey Sorbitol agar; MUG, 0.01%.

2.6.1.1 Immunomagnetic separation

In order to decrease the interference from food debris and background microorganisms, there has been much interest to separate the target microorganisms directly from the enriched samples prior to the isolation step. Immunomagnetic separation (IMS) is one of these approaches. Basically, paramagnetic beads coated with antibodies (anti-O157) are suspended in sample suspension, and the antibodies capture the target cells (*E. coli* O157 cells) by binding with antigens (O157 antigen) on the surface of specific target cells. Then, the beads bound with target cells are separated from the suspension by a magnetic separator, the remaining suspension is removed and the beads are washed several times (Safarik and others 1995). Subsequently, the beads bound with target cells can be plated on selective media. IMS has been successfully used to facilitate the detection of *E. coli* O157:H7. Wang and others (2007) reported that the detection limit of *E. coli* O157:H7 can be improved from 10^5 CFU/g to 10^3 CFU/g in ground beef when combining IMS with real-time PCR.

2.6.2 Immunological detection methods

Numerous immunological detection methods have been developed for detection and enumeration of *E. coli* O157:H7. They are all based on the use of monoclonal or polyclonal anti-O157 antibodies or anti-*Stx* antibodies to capture the targets. Labeled antibodies are more often used while non-labeled antibodies are uncommon due to low sensitivity (Tokarskyy and Marshall 2008). Enzymes, luminescent compounds, fluorophores and conductive polymers have been used as antibody labels for *E. coli*

O157:H7 detection. With the help of these labeled antibodies, *E. coli* O157:H7 can be identified and enumerated by measuring the fluorescence, chemiluminescence, or electrochemical properties of reaction products (Tokarskyy and Marshall 2008).

2.6.2.1 Enzyme-linked-immunosorbent-assay (ELISA)

Enzyme-linked-immunosorbent-assay (ELISA) has been the most common technique in detecting foodborne pathogens among all of the immunological methods, due to its specificity, simplicity and sensitivity. There are four kinds of ELISA, which are direct ELISA, indirect ELISA, direct sandwich ELISA, and indirect sandwich ELISA (Crowther 2008). Among these, the sandwich ELISA is most commonly used to detect pathogens in food. The sandwich ELISA is performed with a solid-phase coated with excess antibodies. Antigens contained in samples will react with antibodies during incubation. After washing, the bound antigens are treated with enzyme-labeled antibodies which cause a chromogenic or fluorogenic substrate to produce detectable and quantitative signals.

Padhye and Doyle published a commercial ELISA kit, EHEC-TEK (Organon Teknika, Durham, NC), for the detection of *E. coli* O157:H7 in food in 1991. In this assay, horseradish peroxidase-labeled monoclonal antibodies (MAb 4E8C12) are used to detect serogroups O157 and O26 in samples (Padhye and Doyle 1991). Later, it was reported that the target antigens of MAb 4E8C12 were also present in *E. coli* serogroups other than O157 and O26, such as O22:H8, O26:H11, O46:H38, O88:H49, O91:H21, O103:H2

and O111:H11 (Johnson and others 1995). Based on this finding, the protocol of EHEC-TEK was subsequently modified by incorporating IMS, followed by culturing on CT-SMC. Currently, more ELISA kits for detection of *E. coli* O157:H7 in food are available in the market, such as 3M™ Tecra™ *E. coli* O157 Visual Immunoassays (3M, St. Paul, MN) and MaxSignal® *E. coli* O157:H7 ELISA Test Kit (Bio Scientific Corporation, Austin, TX). It has been reported that 1 CFU of *E. coli* O157:H7 were detectable in 10 mL of milk by using integrated ELISA (EiaFoss™) (Reinders and others 2002).

2.6.3 Nucleic acid based detection methods

Nucleic acid based detection methods can be made very sensitive, and their targets can be made very specific by using different probes. Despite their relatively recent application in food analysis, nucleic acid based methods have significantly improved the detection efficiency for foodborne pathogens. A large number of nucleic based methods have been developed for detecting pathogens in food products.

2.6.3.1 Hybridization

Hybridization, which was developed in the early 1980s, is the first nucleic acid based method for detecting foodborne pathogens (Hill 1981). It detects DNA or RNA targets using complementary labeled nucleic acid probes. Virulence-associated genomic genes and 16S rRNA are commonly targeted in hybridization assays to detect foodborne pathogens. Hybridization has two formats, solid-phase hybridization (colony hybridization) and liquid-phase hybridization. Solid-phase hybridization involves transfer of colonies on a primary isolation plate to a solid support, such as nitrocellulose or nylon

membranes. In liquid-phase hybridization, hybridization products can be formed in solution and detected directly in the reaction tube. Although the hybridization assay has various advantages, such as ease of use and many options for probe labels, its major disadvantage is that it is time-consuming. Hybridization assays require 10^4 to 10^5 bacterial cells to yield a positive result, so it usually takes 18 to 48 h for enrichment, and several more hours to complete the hybridization itself (Swaminathan and Feng 1994).

2.6.3.2 Polymerase Chain Reaction

2.6.3.2.1 Conventional polymerase chain reaction

Polymerase Chain Reaction (PCR) remains the most popular method among nucleic acid based detection assays and it has been used extensively to detect pathogens in food, fecal and environmental samples. The PCR technique was invented by Kary Mullis and initially reported in 1985 (Saiki and others 1985; Mullis and others 1986). PCR is an *in vitro* assay that utilizes a DNA polymerase enzyme and designed oligonucleotide primers to amplify specific regions of target DNA during repeated temperature cycles (Levin 2009). Virulence-associated genes and ribosomal RNA are primary targets for detection of foodborne pathogens using PCR (Swaminathan and Feng 1994). The reaction reagents usually include a thermostable polymerase enzyme, excess deoxyribonucleoside triphosphates (dNTPs), a pair of designed primers targeting specific regions of a target DNA, magnesium chloride, and template DNA (Gorski and Csordas 2009). A typical thermal cycle of PCR consists of three steps: denaturation,

annealing and extension. In the denaturation step, double-stranded DNA is separated into single strands at a temperature of 95^{o}C. During the annealing step, the temperature is lowered to allow two specific primers to hybridize to their complementary sequences in the single strands of target DNA. In the extension step, the temperature is increased to 72^{o}C. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs to the 3' ends of two primers. Cycles are repeated 40 times in a typical PCR reaction, taking 60 to 120 min. The number of target copies will increase in an exponential manner in PCR reaction. To check whether PCR generates and amplifies target DNA fragments, agarose gel electrophoresis is usually used to separate PCR products based on their sizes (Swaminathan and Feng 1994). Figure 1 shows the basic principle of conventional PCR and Figure 2 shows a result of conventional PCR with gel.

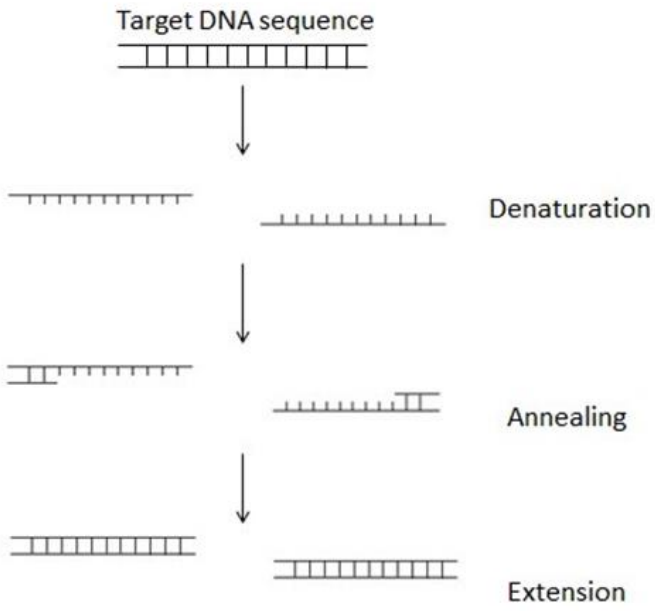

Figure 1 Basic principle of conventional PCR *(adapted from Entis and others 2001).*

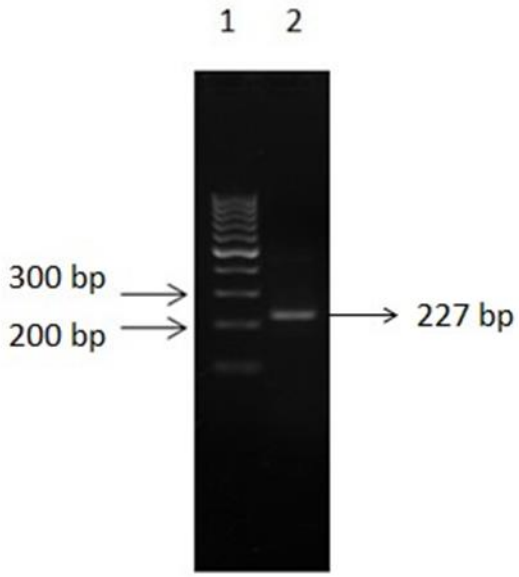

Figure 2 A result of conventional PCR with gel. Lane 1, DNA ladder; lane 2, PCR products with a size of 227 bp.

2.6.3.2.2 Internal amplification control

A negative PCR result can indicate that there is no target sequence present in the sample, but it can also indicate that the PCR reaction itself failed. Inhibitors present in sample matrices (especially when PCR is used to test food samples), malfunction of the thermal cycles, as well as poor polymerase activity can lead to false-negative results of PCR (Hoorfar and others 2003). In order to avoid the uncertainty of such negative results in diagnostic PCR, the addition of an internal amplification control (IAC) in a PCR reaction is required. An IAC is a nontarget DNA sequence present in the same reaction tube, and is amplified regardless of whether or not a target sequence is present in the

sample (Hoorfar and others 2003). In a PCR with an IAC, IAC should always be amplified and yield a positive result. Therefore, negative results of both IAC and target DNA indicate a failed PCR reaction.

2.6.3.2.3 Real-time PCR

Conventional PCR requires separate instruments for amplification and evaluation of DNA products. It is necessary to open the reaction tube and remove the PCR products to verify by agarose gel electrophoresis, which increases the possibility for carryover contamination (Gorski and Csordas 2009). Real-time PCR assay combines amplification and quantitation of PCR products in one step in a "real-time" manner. In a real-time PCR reaction, signals generated from a fluorescent reporter are readily recorded and analyzed by a computer. The fluorescence intensity increases in direct proportion to the amount of PCR products in a real-time PCR reaction. The cycle threshold (*Ct*) is defined as the number of cycles needed for a fluorescent signal to cross through a specified threshold. *Ct* is determined by the concentration of target DNA contained in original samples, the higher the initial concentration, the smaller the *Ct* value and the sooner the signal crosses the threshold. Plotting fluorescence against cycle number generates a curve that represents the accumulation of PCR products during the reaction (Figure 3). A standard curve can be generated by plotting known concentrations of target DNA against corresponding *Ct* values (Figure 4). Thus, the amount of target DNA in unknown samples can be derived by plugging their *Ct* values into the standard curve. In this way, quantitation of PCR products can be realized by real-time PCR.

A number of fluorescent systems have been employed in real-time PCR. Generally, they are in two formats: nonspecific double-stranded DNA binding dyes and specific probes that generate signals only in the presence of the target DNA.

Nonspecific DNA binding dyes are less expensive and easy to use. SYBR Green is a widely used nonspecific DNA binding dye in real-time PCR. It is a thermostable dye that binds to the minor grooves of nonspecific double-stranded DNA and emits fluorescence once it binds (Gorski and Csordas 2009). An additional step is added after PCR to verify the specificity of products by slowly raising the temperature. Fluorescence will decrease as the double-stranded DNA melts and SYBR Green dissociates. Melting temperature (*Tm*) is the temperature at which half of the double-stranded DNA have broken into single-stranded DNA. Because each product has its unique melting temperature (*Tm*) due to their different sequences and lengths, products can be distinguished from each other (Fratamico and Bayles 2005).

Probe-based real-time PCR is more specific since probes are designed to bind to a specific region of target DNA. It is also less time-consuming since it does not require the analysis of melting temperature. TaqMan® probes have been excessively used based on the 5' exonuclease activity of *Taq* polymerase. A TaqMan® probe is a short single-stranded DNA whose sequences are designed to bind to a specific region between two primers on a target DNA. A TaqMan® probe is labeled with a fluorescent reporter dye at one end and with a quencher at the other end. The fluorescence of the reporter dye is

suppressed by the quencher when the TaqMan® probe is intact. During the annealing step, the probe hybridizes to its complementary sites on the target DNA. During extension, the bound probe is disassembled and replaced with dNTPs due to the 5' exonuclease activity of *Taq* polymerase, separating the fluorescent reporter dye from the quencher. During this process, the fluorescence emitted from the reporter dye increases exponentially and is in direct proportion to the amount of PCR products. Figure 5 shows the basic principle of TaqMan® real time PCR.

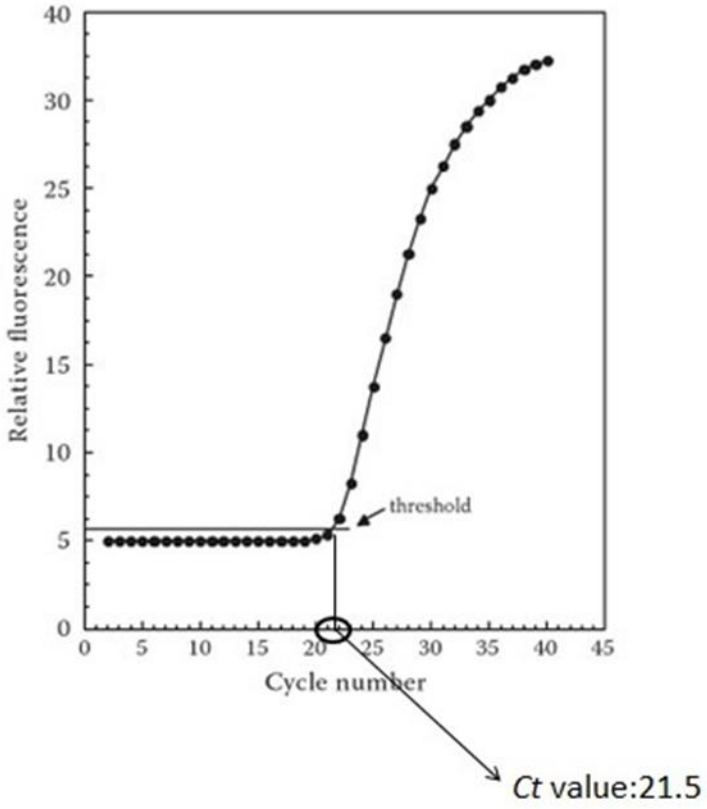

Figure 3 Typical real-time PCR result with a Ct value of 21.5 (adapted from Levin 2009).

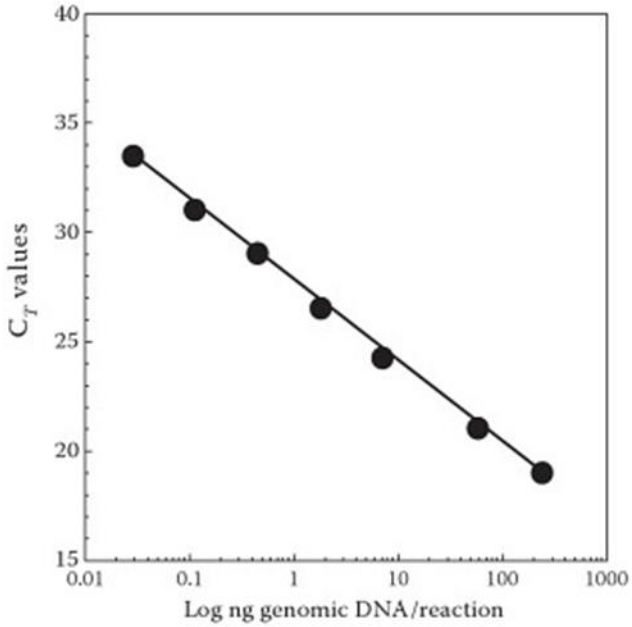

Figure 4 A typical standard curve yielded by real-time PCR (adapted from Levin 2009).

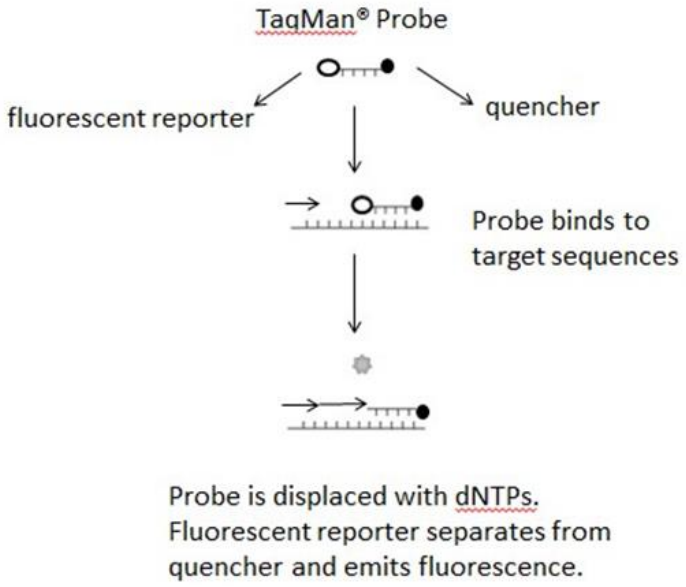

Figure 5 Basic principle of TaqMan® probe (adapted from Gorski and Csordas 2009).

2.6.3.2.4 Multiplex PCR

In reality, one food product could be contaminated by various pathogenic bacteria at the same time. It is more convenient if multiple suspect pathogens can be analyzed at the same time. In multiplex PCR assays, simultaneous amplification of multiple targets can be achieved by using more than one pair of primers in a single reaction. In this way, multiplex PCR assays can help to save much time, labor and cost. However, optimization of reaction parameters, such as annealing temperature and reagent concentrations are crucial for multiplex PCR. All of the satisfactory conditions must be met to realize simultaneous amplification of multiple targets. Therefore, multiplex PCR assays might require a long time to be established and optimized. To distinguish between different PCR products, gel electrophoresis is used to differentiate between targets with different sizes in multiplex conventional PCR. In a multiplex real-time PCR using nonspecific double-stranded DNA binding dyes, target sequences are designed to have different melting temperatures. In a probe-based multiplex real-time PCR, fluorescent reporter dyes with different excitation and emission wavelengths can be used to differentiate PCR products. A multiplex TaqMan® real-time PCR assay was developed for the simultaneous quantitation of *E. coli* O157:H7, *Salmonella*, and *Shigella* in ground beef. With an immunomagnetic separation (IMS) step prior to PCR, the detection limit was 10^3 CFU/g for both pathogens (Wang and others 2007).

2.6.3.2.5 Reverse transcription PCR

In reverse transcription PCR (RT-PCR), RNA is reverse transcribed into complementary DNA by the enzyme reverse transcriptase, and the complementary DNA is subsequently amplified. Food processing, such as heating, UV light and high pressure, may destroy bacteria. However, DNA persists even after cell death, leading to a false-positive result in DNA-based PCR. On the other hand, RNA is only present in live cells, providing a promising way to differentiate live cells from dead cells by using RT-PCR. Unfortunately, mRNA is not stable and is difficult to extract from pathogens in complex food matrices, making RT-PCR less practical for detection of viable pathogens in food. McIngvale and others (2002) developed a RT-PCR assay to detect viable Shiga toxin-producing *E. coli* in cooked ground beef. The results showed that 1 CFU of viable Shiga toxin-producing *E. coli* in 25 g of beef was detectable after 12 h enrichment.

2.6.3.3 Isothermal amplification assays

Isothermal amplification assays do not require complicated equipment for thermal cycling, and they may work with a simple water bath. There are several enzymes and more than one set of primers involved in an isothermal amplification assay to accomplish amplification of the target sequences. Many isothermal amplification assays have been developed, such as loop mediated amplification (LAMP) and nucleic acid sequence based amplification (NASBA).

LAMP was developed in 2000 and it utilized a DNA polymerase isothermally at 60 to 65^{o}C and four primers that recognize six distinct sequences on the target DNA, allowing for the generation of 10^{9} target sequences in less than 1 h (Notomi and others 2000). Hara-Kudo and others (2007) developed a LAMP assay for the detection of *E. coli* O157:H7 in ground beef. Nine CFU of *E. coli* O157:H7 in 25 g of ground beef was detectable after an 18-h enrichment by this LAMP assay, whose sensitivity was 100-fold higher than that of a conventional PCR.

A NASBA assay can amplify an RNA target isothermally at approximately 40^{o}C with the help of two specific primers and three enzymes, including T7 RNA polymerase, RNaseH and a reverse transcriptase (Compton 1991). There is no interference from DNA in a NASBA assay since RNA is amplified below the melting temperature of DNA during the process. Recently, Won and others (2010) published a NASBA assay using a hair-loop type reporting probe with FAM and DABCYL for detection of *E. coli* O157:H7 in ground beef. As a result, this assay could detect as low as 100 CFU of *E. coli* O157:H7 in 1 g of beef.

2.7 Detection of viable foodborne bacterial pathogens

Culture-based methods have been mostly used to monitor cell viability because of their ease of use and low cost. However, many types of bacteria can enter a viable-but-nonculturable (VBNC) state and are unable to form colonies on culture media (Roszak and Colwell 1987). But VBNC cells can maintain their metabolic activity and

resuscitate to a virulent state under appropriate conditions, posing risks to consumer health. *E. coli* O157:H7 was found to be able to enter the VBNC state (Asakura and others 2008) and return to the infectious state after passing in an animal host (Rivers and Steck 2001). Moreover, culture-based methods can be hard to operate due to the complexity of food matrices. Therefore, there is an increasing interest in the development of rapid methods for the detection of only viable cells.

2.7.1 Dye-based methods

Several dyes which have been used to differentiate live from dead cells and their principles are shown in Table 3.

Direct microscopic examination techniques can be used to observe the results. However, counting and examining cells under microscopes are labor-intensive and time-consuming. Flow cytometry is an alternative for rapid result reading. In flow cytometry, cells pass through a light beam in a directed fluid stream. And the interaction of each cell with the beam can be recorded automatically. Depending on the dyes used, multiple cellular parameters of each single cell, such as structures and functions, can be measured simultaneously by flow cytometry (Comas-Riu and Rius 2009).

Table 3 Criteria and principles of selected dyes for the assessment of viability of microorganisms (adapted from Breeuwer and Abee 2000; Comas-Riu and Rius 2009).

Criterion	Examples of dyes	Principle
Membrane integrity	Ethidium bromide (EB) Propidium iodide (PI) SYTO 9	Viable cells have intact membrane. Membrane-permeant SYTO 9 labels live bacteria with green fluorescence; membrane-impermeant EB and PI label dead bacteria with red fluorescence.
Respiration	5-cyano-2,3-ditolyl tetrazolium chloride (CTC)	Respiring cells can reduce CTC to its formazan products CTF which is fluorescent.
Enzyme activity	Fluorescein diacetate (FDA)	Cells with esterase activity can accumulate the fluorescein and form fluorescent products.
Membrane potential	Rhodamine 123, carboxycyanine dyes, oxonals	Cells which have membrane potential (negative inside) are viable. They accumulate the rhodamine 123 and carboxycyanines, whereas oxonals are excluded. Rhodamine 123 and carboxycyanines can emit green fluorescence.
pH gradient	2',7'-Biscarbpoxyehtyl-5,6-carboxyfluorescein (BCECF)	Viable cells maintain pH gradient. The amount of dye retained by cells depends on their intracellular pH.

2.7.1.1 Propidium monoazide

Propidium monoazide (PMA) and ethidium monoazide (EMA) are high affinity photoreactive DNA binding dyes with molecular weights of 511 and 420, respectively. The dyes are weakly fluorescent by themselves but become more fluorescent after binding to nucleic acids. They both preferentially bind to double-stranded DNA with high affinity. PMA was used in this study. The photoreactive azido group on the dye is converted to a highly reactive nitrene radical upon photolysis. The nitrene radical reacts with any hydrocarbon moiety at the binding site to form a stable covalent nitrogen-carbon bond, thus resulting in permanent DNA modification. The dye is nearly completely cell membrane-impermeable, and thus can be selectively used to modify only exposed DNA from dead cells while leaving DNA from viable cells intact. This feature makes the dye highly useful in the selective detection of viable pathogenic cells by quantitative real-time PCR in the presence dead cells whose DNA has been PMA-modified and thus cannot be amplified.

2.7.2 PCR-based methods

2.7.2.1 RT-PCR

As mentioned above, RT-PCR can be used to distinguish between viable and dead cells because DNA persists after cell death whereas RNA does not. Therefore, cell viability can be assessed directly by the detection of specific mRNA using RT-PCR since it can only be found in live cells. However, mRNA is easy to be denatured during

processing and is difficult to extract, making accurate measurements of bacterial numbers difficult by using RT-PCR.

2.7.2.2 Ethidium monoazide and propidium monoazide DNA-based PCR

Ethidium monoazide (EMA) and propidium monoazide (PMA) have been used as dyes for microscopic differentiating between live and dead cells for many years. Recently, EMA or PMA combined with real-time PCR was introduced as diagnostic methods to differentiate between live and dead cells (Nogva and others 2003; Nocker and Camper 2006; Nocker and others 2006). EMA and PMA are DNA-intercalating dyes, which can only penetrate dead cells with compromised cell membranes but not live cells with intact membranes. Following photolysis by bright visible light, EMA or PMA can produce a nitrene that forms a covalent link with DNA. This covalent link will render the bound DNA insoluble and subsequently inhibit the amplification of DNA from dead cells during PCR. At the same time, the unbound excess EMA or PMA will react with water and is no longer able to bind with DNA extracted from intact cells. EMA-PCR and PMA-PCR have been used for detection of viable foodborne bacterial pathogens, such as *E. coli* O157:H7, *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter*, and *Cronobacter sakazakii* (Pan and Breidt 2007, Wang and others 2009, Liang and others 2011, Banihashemi and others 2012, Minami and others 2012).

However, both EMA and PMA seem to have specific advantages and disadvantages. The greatest concern with EMA has been its toxicity to live cells. It has

been reported that EMA could also penetrate live cells of some bacterial species and cause partial DNA loss for subsequent PCR (Nocker and others 2006). Moreover, some studies showed incomplete suppression of the dead cell signal using EMA-PCR (Flekna and others 2007; Wagner and others 2008; Kobayashi and others 2009). Compared to EMA, PMA was shown to be less toxic to live cells, which is thought to be mainly due to the higher positive charge of PMA than EMA (Nocker and others 2006). However, the greatest concern with PMA has been the generation of false-positive results because PMA was not effective enough to remove all the signals from dead cells (Pan and Breidt 2007; Kralik and others 2010; Løvdal and others 2011).

Long amplicon sizes (>1kb) were reported to improve the efficacy of EMA-PCR and PMA-PCR to distinguish between live and dead cells. Recent studies using EMA-PCR or PMA-PCR have shown that the size of the amplification product was important when applied to heat-killed bacteria (Luo and others 2010; Contreras and others 2011; Soejima and others 2011; Banihashemi and others 2012).

CHAPTER 3

MATERIALS AND MATHODS

3.1 Preparation of viable and dead *E. coli* O157:H7 cells

E. coli O157:H7 strains G5310, C7927, and 505B were provided by the Food Microbiology Laboratory at University of Missouri, Columbia, MO. Each of the three strains was grown in tryptic soy broth supplemented with 0.5% yeast extract (TSBY; Difco Labs., BD Dignostic Systems, Sparks, MD, USA) at 37^{o}C overnight (~10^{9} CFU/mL). One milliliter of each strain was then mixed together, harvested by centrifugation at 13,400 × *g* for 5 min, washed and serially diluted in 0.1% peptone water to yield cell suspensions ranging from 10^{0} to 10^{8} CFU/mL. To obtain dead cells, the cell suspensions were heated at 85^{o}C for 35 min. The viability of cells was checked by plating in plate count agar (PCA; Difco Labs., BD Diagnostic Systems, Sparks, MD, USA).

3.2 Minimum PMA amount needed to bind dead cell DNA

Propidium monoazide (PMA) powder was purchased from Biotium Inc. (Hayward, CA, USA), and a 20 mM stock solution was prepared in sterile distilled water and stored in the dark at -20^{o}C. Six dead *E. coli* O157:H7 samples (10^{8} CFU/mL each) were prepared as described previously. Different amounts of PMA (0, 0.5, 1.25, 2.5, 5, 10 µL) were added to 1 mL of each sample to reach PMA concentrations of 0, 10, 25, 50, 100, and 200 µM, respectively. Following a 5-min incubation period in the dark at room temperature on a Labquake Rotisserie (Barnstead International, Dubuque, Iowa, USA),

the samples were exposed to a 650-W halogen light for 10 min. The samples were placed on ice to avoid excessive heating and placed about 20 cm from the light source. After photo-induced cross-linking, cells were collected by centrifugation at 13,400 × *g* for 5 min, and washed in sterile distilled water under the same centrifugation conditions prior to DNA extraction.

3.3 Influence of PMA concentration on amplification of DNA from viable cells

Fresh viable *E. coli* O157: H7 cells (10^8 CFU/mL) were prepared as described previously. Varying amounts of PMA (0, 10, 25, 50, 100, and 200 µM) were added to 1 mL of each cell suspension. PMA staining was conducted as described earlier (5 min in the dark followed by a 10-min light exposure). Cell pellets were collected by centrifugation at 13,400 × *g* for 5 min and washed with sterile distilled water under the same centrifugation conditions. After DNA extraction, the influence of PMA concentration on DNA amplification from viable cells was evaluated by real-time PCR.

3.4 Influence of light exposure time on DNA amplification from viable cells

Six viable *E. coli* O157:H7 samples (10^8 CFU/mL each) were prepared as described previously. PMA stock solution in the amount of 1.25 µL was added to each 1 mL of cell suspension to achieve a final concentration of 25 µM. Samples were incubated in the dark at room temperature for 5 min and exposed to a 650-halogen light

for varying lengths of time (0, 2, 5, 10, 15, 20 min). After PMA staining, cell pellets were collected by centrifugation at 13,400 × *g* for 5 min and washed with sterile distilled water. The influence of light exposure time on DNA amplification from viable cells was evaluated by real-time PCR after DNA extraction.

3.5 DNA extraction

Cell pellets were resuspended in 100 µL of PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, Calif., USA). Cell suspensions were vortexed for 10 s and boiled for 20 min. Upon centrifugation at 13,400 × *g* for 3 min, DNA extracts were recovered in the supernatants.

3.6 Real-time PCR

Primers and probe targeting *E. coli* O157:H7 were designed and successfully used by Wang and others (2007; 2009). The sequence of *E. coli* O157:H7 primer 1 is 5'-TTGACCCACACTTTGCCGTAA-3', and that of *E. coli* O157:H7 primer 2 is 5'-GCGAAAACTGTGGAATTGGG-3'. The sequence of *E. coli* O157:H7 probe is 5'-HEX-TGACCGCATCGAAACGCAGCT-BHQ1-3'. pUC 19 was used as an internal amplification control. Primers and probe targeting an internal amplification control (IAC) were designed by Fricker and others (2007). The sequence of the IAC primer 1 is 5'-GCAGCCACTGGTAACAGGAT-3', and that of the IAC primer 2 is 5'-GCAGAGCGCAGATACCAAAT-3'. The sequence of the IAC probe is 5'-6FAM-AGAGCGAGGTATGTATGTAGGCGG-BHQ1-3'. A 7500 real-time PCR system (Applied

Biosystem) was used. A PCR reaction of 50 µL contained 12.5 µL of 2× TaqMan™ Universal PCR Master Mix (Applied Biosystem), 0.5 µM of each *E. coli* O157:H7 primer, 0.4 µM of each IAC primer, 0.2 µM of *E. coli* and IAC probe, 0.25 pg of pUC19 (8.62×10^4 copies; Promega, Madison, WI, USA), and 3 µL DNA extract. For apple juice samples, 10 µL DNA extract was used for each reaction. Nuclease-free water (Promega) was used to adjust the reaction volume to 50 µL. The real-time PCR program consisted of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

3.7 Application of PMA real-time PCR to viable *E. coli* O157:H7 cells

One milliliter of viable *E. coli* O157:H7 cells (10^1 to 10^8 CFU/mL) was treated or untreated with PMA. For PMA staining, 1.25 µL of PMA stock solution (25 µM) was added to each sample, and samples were incubated in the dark for 5 min and exposed to a 650-W halogen light for 10 min. DNA extraction and real-time PCR with IAC were conducted as described earlier. Standard curves were constructed by plotting *Ct* values generated from real-time PCR against *E. coli* O157:H7 cell concentrations (log CFU/mL).

3.8 Application of PMA real-time PCR to mixed viable and dead *E. coli* O157:H7 cells

One milliliter of dead *E. coli* O157:H7 cells (10^6 CFU/mL) was mixed with 1 mL of fresh viable cells with different concentrations (10^1 to 10^8 CFU/mL) and centrifuged at 13,400 × *g* for 5 min. The mixed cell pellets were resuspended in 1 mL of 0.1% peptone water and untreated or treated with PMA, as was optimized previously. DNA extraction

and real-time PCR with IAC were conducted as described earlier. Real-time PCR with IAC was conducted to check the results.

3.9 Application of PMA real-time PCR to artificially contaminated food

Apple juice and ground beef with two different fat contents (10% and 27%) were purchased from a local food store. They were determined to be free of *E. coli* O157:H7 using standard cultural methods (FDA 1995) and real-time PCR. Twenty-five grams or milliliters of each food sample was placed in sterile stomacher bags containing filter membranes (Filtra-Bag®,VWR International, Edmonton, AB, Canada). Two sets of food samples were prepared. The first set of nine samples was prepared by adding only viable *E. coli* O157:H7 cells at different concentrations (10^0 to 10^8 CFU/g or mL) into each food sample. The second set of nine samples was prepared by adding dead *E. coli* O157: H7 cells (10^6 CFU/g or mL) and various concentrations of viable *E. coli* O157:H7 cells (10^0 to 10^8 CFU/g or mL) into each 25 g of ground beef or 25 mL of apple juice sample. Samples were individually mixed with 225 mL TSBY and homogenized for 2 min by stomaching. One milliliter of homogenized suspension from each sample was taken for DNA extraction with prior PMA staining, as was optimized earlier. Real-time PCR was conducted as described previously.

3.10 Detection of low concentrations of viable *E. coli* O157:H7 in food samples with enrichment by PMA real-time PCR

Apple juice and ground beef with two different fat contents (10% and 27%) were purchased from a local food store and determined to be free of *E. coli* O157:H7. Twenty-five grams or milliliters of each sample was inoculated with 10^6 CFU/g or mL of dead E. *coli* O157: H7 cells, and viable *E. coli* O157:H7 cells at levels of 10^0, 10^1, 10^2, 10^3 and 10^4 CFU/g for ground beef and 10^0, 10^1, 10^2, and 10^3CFU/mL for apple juice. Each sample was added to 225 mL TSBY and homogenized for 2 min by stomaching. Samples were incubated in a shaking incubator at 200 rpm at a temperature of 37^{o}C. Ten milliliters of each sample were collected at different enrichment times (0, 2, 4, 6, and 8 h). Ground beef suspensions were centrifuged at 2,000 × *g* for 2 min to precipitate the meat tissues and fat. Cells were collected by centrifuging at 13,400 × *g* for 5 min. Cell pellets were washed and resuspended in 1 mL of 0.1% peptone water. PMA staining, DNA extraction and real-time PCR were conducted as described previously.

3.11 Statistical data analysis

The SAS GLM procedure (SAS 9.2, Copyright 2002-2007; SAS Institute Inc., Cary, NC, USA) was used to evaluate the effect of PMA staining on dead and viable cells. Tukey's test was applied to determine differences between various PMA staining and light exposure treatments. Differences were compared at a significance level of 0.05.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Minimum PMA amount needed to bind dead cell DNA

Various amounts of PMA were applied to 10^8 CFU/mL dead *E. coli* O157:H7 cell suspensions. DNA was extracted and subjected to real-time PCR with an IAC. DNA from 10^8 CFU/mL of dead cells without PMA treatment was also included in real-time PCR as a positive control. As shown in Figure 6, DNA from dead cells that were not stained with PMA generated false-positive results in real-time PCR. The *Ct* value of 18.82, generated from 10^8 CFU/mL of dead cells without PMA treatment, confirmed the facts that DNA persists after cell death and PCR cannot differentiate live from dead cells. By staining dead cells with various concentrations of PMA, it was shown that the concentration of PMA higher than 10 µM was necessary to stain as much as 10^8 CFU/mL dead cells. Starting from a PMA concentration of 25 µM, no *Ct* value was yielded from 10^8 CFU/mL of dead cells, indicating a concentration of 25 µM was sufficient to completely prevent the DNA amplification from 10^8 CFU/mL dead cells by real-time PCR (Figure 6). The amplification of the IAC (pUC19) in each reaction successfully monitored the efficiency of the PCR reaction.

According to Wang and others (2009), a treatment of 12 µM EMA with a 10-min light exposure on ice was sufficient to eliminate DNA from 10^8 CFU/mL dead *E. coli* O157:H7 cells. In this study, a treatment of 25 µM PMA with the same light exposure

conditions was necessary to bind with DNA from 10^8 CFU/mL dead *E. coli* O157:H7 cells. Considering the price of PMA was five times higher than that of EMA, EMA might be a more economical choice.

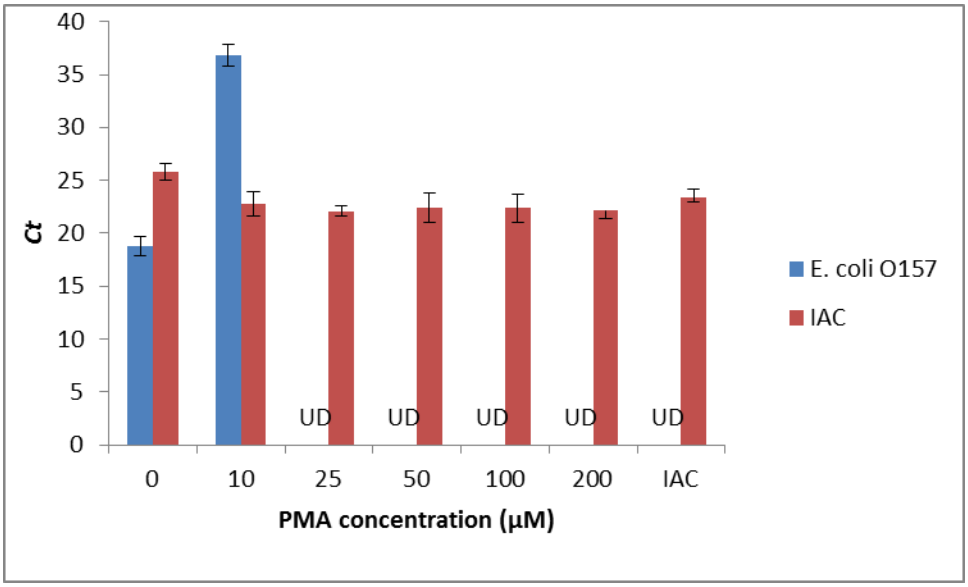

Figure 6 Minimum PMA concentration necessary for binding DNA from 10^8 CFU/mL of dead *E. coli* O157:H7 cells. Results were from two repeated experiments. UD, undetected after 40 cycles in real-time PCR.

4.2 Influence of PMA concentration on amplification of DNA from viable cells

Viable *E. coli* O157:H7 cells, at a concentration of 10^8 CFU/mL, were stained with different amounts of PMA. Figure 7 shows that when the PMA concentration was ≤ 50 µM, no significant differences were found between *Ct* values generated from PMA treated and those from PMA untreated viable cells (P>0.05). However, when the PMA concentration was ≥ 100 µM, the *Ct* values generated from 10^8 CFU/mL of PMA treated viable cells were significantly higher than that generated from PMA untreated cells

($P \leq 0.05$). The results indicated that high concentrations of PMA (≥ 100 μM) could also inhibit the amplification of DNA from viable cells, causing an underestimation of viable cells in real-time PCR. This shows that PMA at high concentrations (≥ 100 μM) is toxic to viable *E. coli* O157:H7 cells. Data from Figure 6 and 7 shows that a PMA concentration of 25 μM could completely exclude the DNA from dead cells, and was found not to inhibit the DNA amplification from viable cells. Thus, 25 μM of PMA was decided to be used for further studies.

Ethidium monoazide (EMA) is another DNA binding dye that has been evaluated. Wang and others (2009) reported that the higher the EMA concentration, the greater its inhibition of DNA amplification from viable *E. coli* O157:H7. EMA at a concentration higher than 120 μM combining with a 10-min light exposure could completely prevent DNA amplification from 10^8 CFU/mL viable *E. coli* O157:H7 cells, generating false-negative results. In this study, although PMA at high concentrations (≥ 100 μM) lead to an underestimation of viable *E. coli* O157:H7 cells, positive results can still be obtained in real-time PCR. By comparing these results, PMA was found to be less toxic to viable *E. coli* O157:H7 cells than the same concentration of EMA.

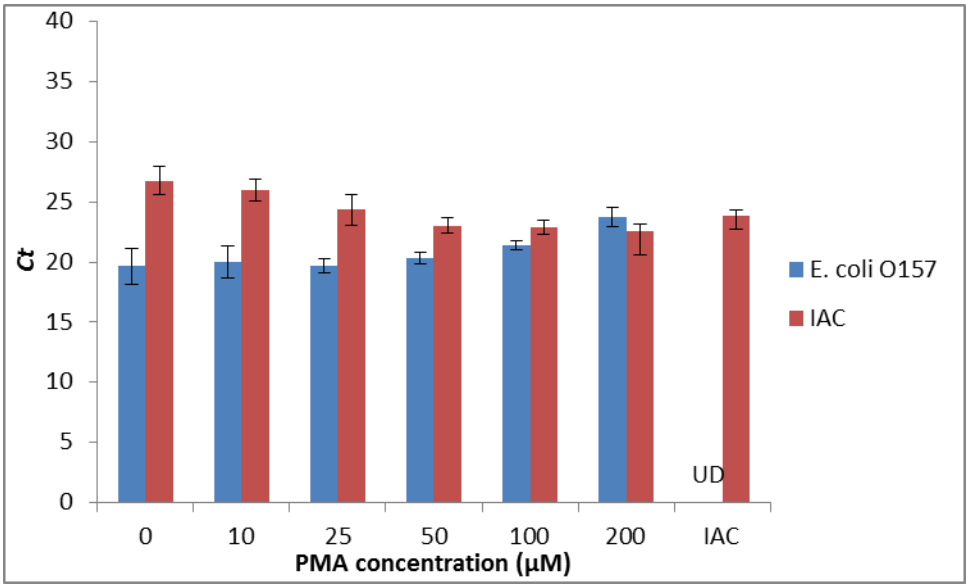

Figure 7 Influence of PMA concentration on amplification of DNA from 10^8 CFU/mL of viable *E. coli* O157:H7 cells. Results were from two repeated experiments. UD, undetected after 40 cycles in real-time PCR.

4.3 Influence of light exposure time on DNA amplification from viable cells

PMA at a concentration of 25 µM was added to each viable *E. coli* O157:H7 suspension. The samples were exposed to light for different lengths of time. Figure 8 shows that the length of light exposure time did not significantly influence the *Ct* values generated from 10^8 CFU/mL viable *E. coli* O157:H7 cells (P>0.05).

Taking into account the different light sources and bacterial species used in different studies, it is not surprising that the literature did not report identical optimal light exposure time. Fittipaldi and others (2012) stated that the light exposure step was import to activate the dye bound with DNA from dead cells and to inactivate the excess dye that had not entered cells. In this study, a light exposure of 10 min was found to be

effective for 25 µM of PMA to completely prevent the DNA amplification from 10^8 CFU/mL *E. coli* O157:H7 dead cells (Figure 6). Figure 8 demonstrates that viable cells treated with a 10-min light exposure showed a *Ct* value close to that of PMA untreated control (P>0.05). Therefore, a 10-min light exposure was decided to be used for future studies.

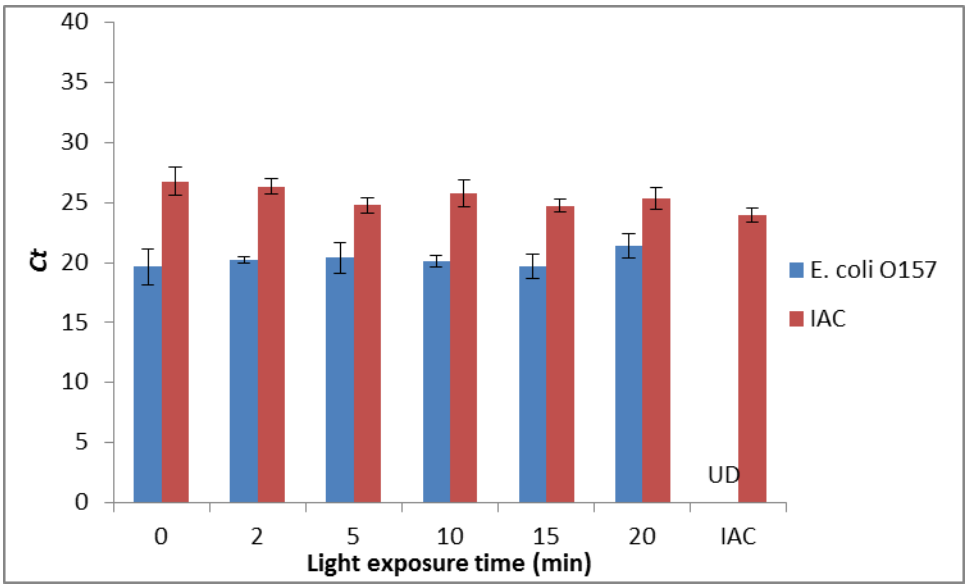

Figure 8 Influence of light exposure time on amplification of DNA from 10^8 CFU/mL of viable *E. coli* O157:H7 cells. Results were from two repeated experiments. UD, undetected.

4.4 Application of PMA real-time PCR to viable *E. coli* O157:H7 cells

Viable *E. coli* O157:H7 cell suspensions of different concentrations were treated or untreated with PMA prior to DNA extraction. Without PMA treatment, the detection range of the real-time PCR assay was from 10^2 to 10^8 CFU/mL (Figure 9b). PMA staining did not influence the detection limit of the real-time PCR assay (Figure 9a). However, a slight increase (approximate 1.5 cycles) in the *Ct* values from PMA-treated samples was

observed. The increase in *Ct* values might be because that a small portion of *E. coli* O157:H7 cells was lost during the washing step after the PMA treatment. According to the results, the PMA treatment, as optimized previously, had no adverse effect on detection of viable *E. coli* O157:H7 by real-time PCR.

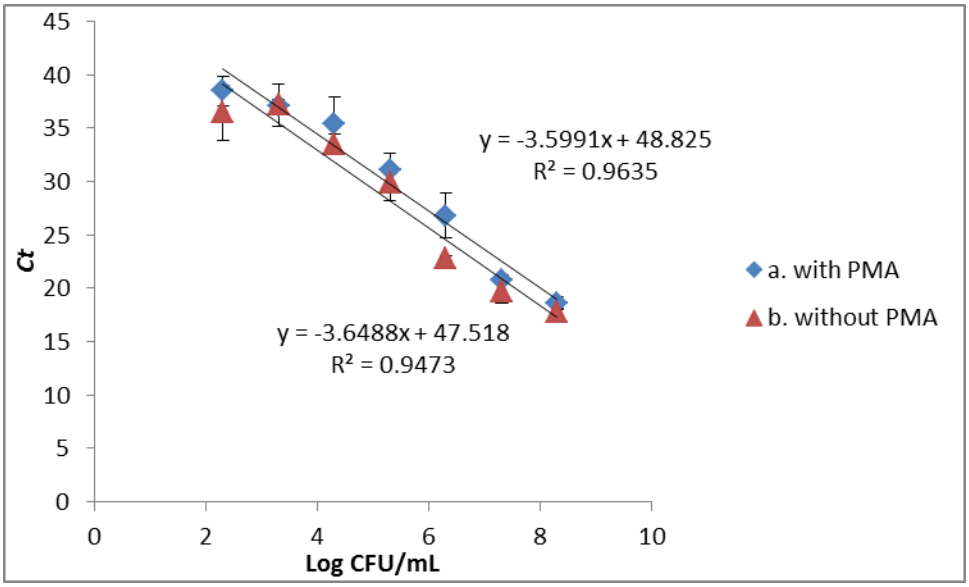

Figure 9 Standard curves for detection of viable *E. coli* O157:H7 cells when only viable cells are present by real-time PCR and PMA real-time PCR. Results were from two repeated experiments.

4.5 Application of PMA real-time PCR to mixed viable and dead *E. coli* O157:H7 cells

Dead cells at a concentration of 10^6 CFU/mL were mixed with different concentrations of viable cells. The mixed cell suspensions were treated or untreated with PMA, as optimized above. Without PMA treatment, the real-time PCR was unable to differentiate between DNA from viable *E. coli* O157:H7 cells and that from dead cells (Figure 10a). Because DNA from both viable and dead cells served as templates for real-

time PCR, the number of viable cells was significantly overestimated. For example, when 10^1 CFU/mL of viable cells were present together with 10^6 CFU/mL of dead cells, PMA real-time PCR assay could not detect *E. coli* O157:H7 in the mixture (Figure 10b), whereas the real-time PCR without PMA treatment generated a *Ct* value of 25.8 (Figure 10a). This *Ct* value corresponded to the *Ct* value yielded from 10^6 CFU/mL of viable cells by using only real-time PCR (Figure 9b). These results prove that PMA staining prior to DNA extraction can effectively prevent false-positive results in real-time PCR that was caused by the presence of DNA from dead cells.

With the PMA treatment, the PMA real-time PCR assay could detect a range of 10^2 to 10^8 CFU/mL viable *E. coli* O157:H7 cells when 10^6 CFU/mL dead cells were also present (Figure 10b). Comparing Figure 10b with Figure 9a, the detection range of PMA real-time PCR for viable cells when both viable and dead cells were present was the same as when only viable cells were present. Although similar slopes of the two standard curves were observed, the *Ct* values from viable and dead cell mixtures ($y = -3.2767 x + 46.42$; $R^2 = 0.9368$) were slightly smaller than that from only viable cells ($y= -3.5991 x + 48.825$; $R^2 = 0.9635$), especially when the concentrations of viable cells were low ($\leq 10^3$ CFU/mL), indicating a slight overestimation of viable cells in the mixture. For example, the average *Ct* value yielded from 10^2 CFU/mL viable and 10^6 CFU/mL dead cells was 36.4, whereas that generated from 10^2 CFU/mL viable alone was 38.5. However, no *Ct* value was yielded from the negative control when only 10^6 CFU/mL dead cells were present (data not shown), which again proved that the PMA treatment

was sufficient to eliminate DNA from dead cells when only dead cells were present. These indicate that the PMA treatment was not able to completely remove signals from a high number of dead *E. coli* O157:H7 cells when both viable and dead cells were present in the sample. Incomplete suppression of the dead cell signal using PMA real-time PCR has also been reported before (Pan and Breidt 2007; Kralik and others 2010; Løvdal and others 2011). Pan and Breidt (2007) stated the number of viable *Listeria monocytogenes* was significantly overestimated when the ratio of dead cells to live cells exceeded 10^4 and the concentration of live cells was less than 10^3 CFU/mL. Løvdal and others (2011) also reported the same critical ratio between dead cells and live cells of 10^4 when PMA real-time PCR was applied to *Listeria innocua*. The presence of a high number of dead cells might be overcome by increasing the dye concentration (Chang and others 2010).

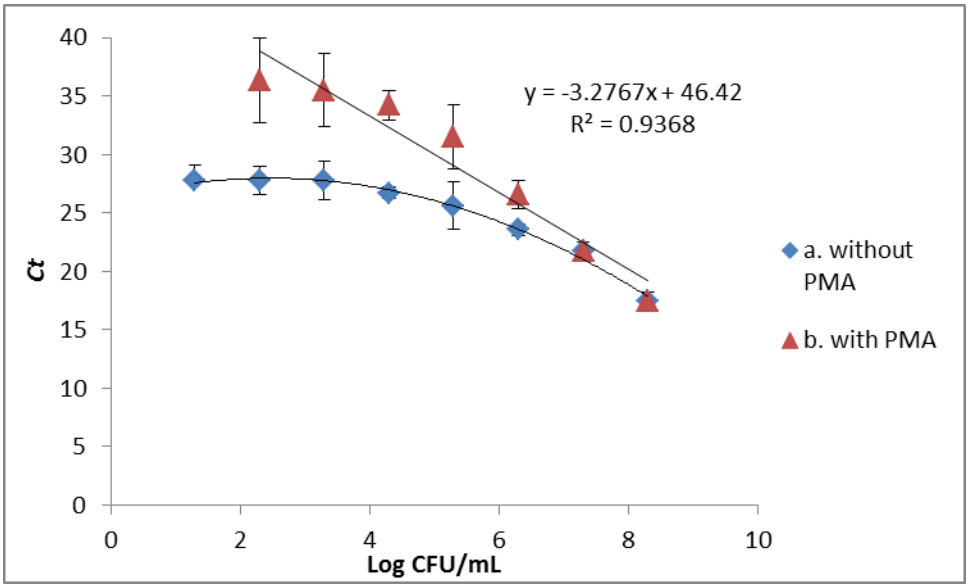

Figure 10 Detection range of *E. coli* O157:H7 cells when viable and 10^6 CFU/mL dead cells are present by real-time PCR and PMA real-time PCR. Results were from two repeated experiments.

4.6 Application of PMA real-time PCR to artificially contaminated food

Apple juice and ground beef (fat content: 10% and 27%, respectively) purchased from the local store was tested by FDA standard cultural-based methods and real-time PCR. Both tests showed that all the food samples were *E. coli* O157:H7-free. The aerobic plate count of apple juice was 0, that of 10% fat content ground beef was 1.7×10^6 CFU/g , and that of 27% fat content ground beef was 1.5×10^5 CFU/g. The PMA real-time PCR assay was applied to food samples inoculated with only viable *E. coli* O157:H7 from 10^1 to 10^8 CFU/mL or g. It was also applied to food samples which were simultaneously inoculated with 10^6 CFU/mL or g of dead *E. coli* O157:H7 cells and different concentrations of viable *E. coli* O157:H7 cells (10^1 to 10^8 CFU/mL or g).

4.6.1 Application of PMA real-time PCR to artificially contaminated apple juice

Figure 11a shows that the PMA real-time PCR with an IAC could detect as low as 10^4 CFU/mL of viable *E. coli* O157:H7 cells in apple juice samples. With the addition of 10^6 CFU/mL dead cells in each apple juice sample, the detection range of viable *E. coli* O157:H7 (Figure 11b) was found to be the same as that for apple juice contaminated with only viable cells (Figure 11a), with the slopes of these two standard curves being similar. These demonstrate that PMA could effectively penetrate dead *E. coli* O157:H7 in apple juice and subsequently prevent the DNA amplification from dead cells in real-time PCR. However, the detection limit of 10^4 CFU/mL in apple juice was not satisfactory considering the low infectious dose of *E. coli* O157:H7. The high detection limit of viable *E. coli* O157:H7 in apple juice was probably caused by the presence of polyphenolic compounds in apple juice that are inhibitory to PCR (Siebert and others 1996).

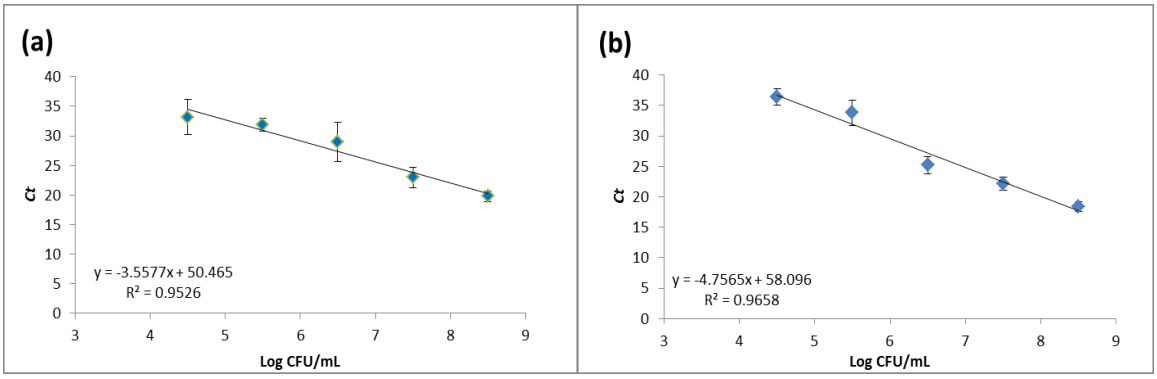

Figure 11 Application of PMA real-time PCR to artificially contaminated apple juice and its detection range. (a) Detection range of *E. coli* O157:H7 cells in apple juice contaminated with only viable cells. (b) Detection range of *E. coli* O157:H7 cells in apple juice contaminated with 10^6 CFU/mL dead cells and different concentrations of viable cells. Results were from two repeated experiments.

4.6.2 Application of PMA real-time PCR to artificially contaminated ground beef

As shown in Figure 12, the PMA real-time PCR could detect a range from 10^5 to 10^8 CFU/g of viable *E. coli* O157:H7 in ground beef samples regardless whether dead cells were present or not. The PMA treatment was proven to be effective to penetrate the dead *E. coli* O157:H7 cells without being negatively influenced by meat tissues and background microflora. Standard curves of 10% and 27% fat content of ground beef exhibited very similar linear relationships between *Ct* values and the concentrations of viable *E. coli* O157:H7 in ground beef. Nonetheless, the detection limit of 10^5 CFU/g in ground beef was still not satisfactory, since the infectious dose of *E. coli* O157:H7 is as low as a few hundred cells (Karmali 2004) and there are millions of cells in one colony forming unit. Therefore, a pre-enrichment step is needed to increase the number of viable cells to a detectable level when the initial concentration is low.

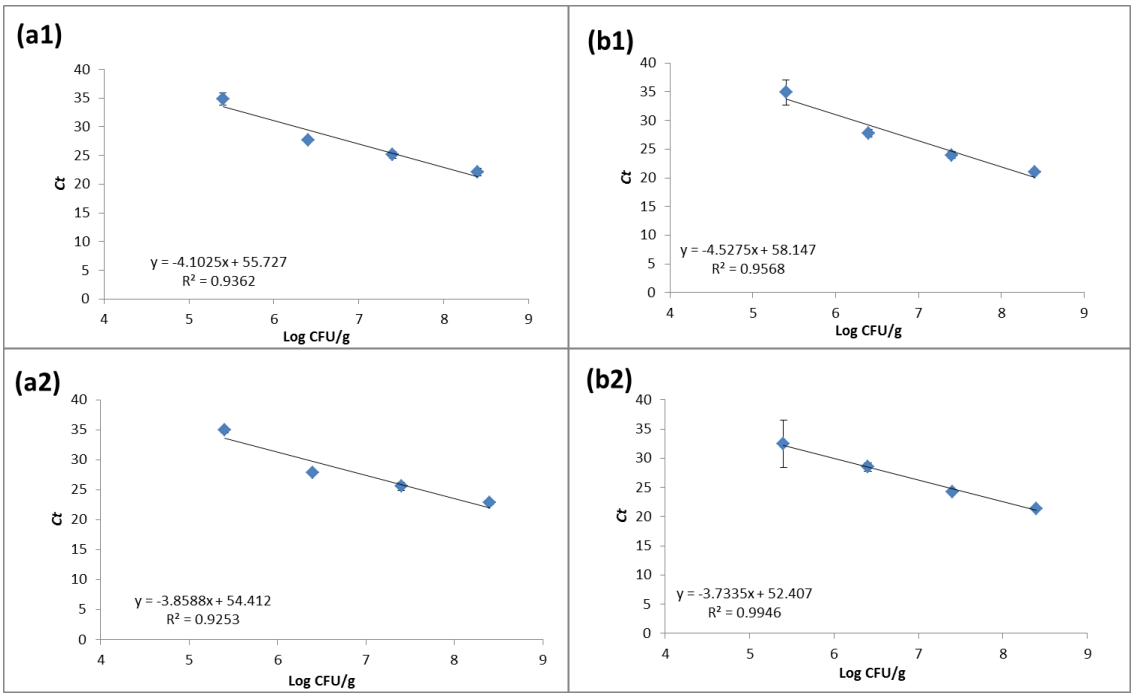

Figure 12 Application of PMA real-time PCR to artificially contaminated ground beef and its detection range. (a1) Detection range of *E. coli* O157:H7 cells in 10% fat content ground beef contaminated with only viable cells. (b1) Detection range of *E. coli* O157:H7 cells in 10% fat content ground beef contaminated with 10^6 CFU/g dead cells and different concentrations of viable cells. (a2) Detection range of *E. coli* O157:H7 cells in 27% fat content ground beef contaminated with only viable cells. (b2) Detection range of *E. coli* O157:H7 cells in 27% fat content ground beef contaminated with 10^6 CFU/g dead cells and different concentrations of viable cells. Results were from two repeated experiments.

4.7 Detection of low concentrations of viable *E. coli* O157:H7 in food samples with enrichment by PMA real-time PCR

To detect low concentrations of viable *E. coli* O157:H7 cells in apple juice and ground beef, enrichment in TSBY was conducted prior to the PMA real-time PCR.

In apple juice, viable *E. coli* O157:H7 at initial concentrations of 10^2 and 10^3 CFU/mL could be detected after a 4-h enrichment. An initial concentration of 10^1

CFU/mL viable *E. coli* O157:H7 in apple juice could be detected by PMA real-time PCR after a 6-h enrichment. With an 8-h enrichment, the concentrations of viable *E. coli* O157:H7 ranging from 10^0 to 10^3 could all be detected in apple juice (Table 4). For ground beef samples, a 6-h enrichment was required to detect initial concentrations of 10^2, 10^3 and 10^4 CFU/g viable *E. coli* O157:H7. After an 8-h enrichment, PMA real-time PCR could detect as low as 1 CFU/g viable *E. coli* O157:H7 in ground beef (Table 5). The results from 10% fat content and those from 27% fat content ground beef were the same. In addition, it was also found that the sample inoculated with only 10^6 CFU/mL or g dead cells generated negative results during the whole enrichment process using PMA real-time PCR, while the same dead cell sample generated false-positive results using real-time PCR without PMA treatment (Tables 4, 5). These results again proved that the addition of PMA prior to DNA extraction effectively eliminated the DNA from *E. coli* O157:H7 dead cells in apple juice and ground beef. Blank food samples without any artificial inoculation of *E. coli* O157:H7 were also enriched, showing that there was no *E. coli* O157:H7 contamination in the original samples. The addition of an IAC in this study successfully monitored the efficiency of real-time PCR, preventing the occurrence of false-negative results. Both viable and dead cells can be present in food products; however, only viable pathogens pose a risk to consumers' health. Therefore, effective detection of only viable bacterial pathogens is urgently needed. A pre-enrichment step is commonly included in pathogen detection methods, allowing the ratio of live cells to dead cells to increase. Thus, the DNA influence from dead cells decreases in subsequent

PCR. However, as shown in this study, enrichment steps alone cannot prevent the false-positive generated from 10^6 CFU/mL or g dead *E. coli* O157:H7 cells during the 8-h enrichment. As shown in this study, after the addition of the PMA staining step prior to DNA extraction, DNA amplification from dead cells in PCR and the false-positive results yielded from dead cells could be successfully prevented (Tables 4, 5).

Hsu and others (2005) reported 1 CFU/mL of *E. coli* O157:H7 in apple juice was detectable after a 10-h enrichment in TSB by a duplex TaqMan® PCR assay. Wang and others (2009) developed an EMA real-time PCR assay to detect viable *E. coli* O157:H7 cells in ground beef, finding that a 12-h enrichment step was required to detect 10^1 CFU/g viable *E. coli* O157:H7 in ground beef. Compared with their results, a shorter enrichment time (8 h) and a better detection limit (1 CFU/mL or g) was achieved in this study. That might be mainly because a shaker was used for enrichment in this study, achieving a faster growth of *E. coli* O157:H7. Additionally, 10 mL of enriched suspensions, instead of 1 mL, were taken to conduct DNA extraction, obtaining more DNA template for the subsequent real-time PCR.

Table 4 Application of PMA real-time PCR to apple juice contaminated with low concentrations of viable *E. coli* O157:H7 and 10^6 CFU/mL dead cells.

Enrichment time (h)	Concentration of viable cells (CFU/mL)	Concentration of dead cells (CFU/mL)	PMA treatment		No PMA treatment	
			E. coli O157:H7	IAC	*E. coli* O157:H7	IAC
2 h	10^3	10^6	-	+	+	+
	10^2	10^6	-	+	+	+
	10^1	10^6	-	+	+	+
	10^0	10^6	-	+	+	+
	0	10^6	-	+	+	+
4 h	10^3	10^6	+	+	+	+
	10^2	10^6	+	+	+	+
	10^1	10^6	-	+	+	+
	10^0	10^6	-	+	+	+
	0	10^6	-	+	+	+
6 h	10^3	10^6	+	+	+	+
	10^2	10^6	+	+	+	+
	10^1	10^6	+	+	+	+
	10^0	10^6	-	+	+	+
	0	10^6	-	+	+	+
8 h	10^3	10^6	+	+	+	+
	10^2	10^6	+	+	+	+
	10^1	10^6	+	+	+	+
	10^0	10^6	+	+	+	+
	0	10^6	-	+	+	+

Table 5 Application of PMA real-time PCR to ground beef (10% and 27% fat content) contaminated with low concentrations of viable *E. coli* O157:H7 and 10^6 CFU/g dead cells.

Enrichment time (h)	Concentration of viable cells (CFU/g)	Concentration of dead cells (CFU/g)	PMA treatment		No PMA treatment	
			E. coli O157:H7	IAC	*E. coli* O157:H7	IAC
2 h	10^4	10^6	-	+	+	+
	10^3	10^6	-	+	+	+
	10^2	10^6	-	+	+	+
	10^1	10^6	-	+	+	+
	10^0	10^6	-	+	+	+
	0	10^6	-	+	+	+
4 h	10^4	10^6	-	+	+	+
	10^3	10^6	-	+	+	+
	10^2	10^6	-	+	+	+
	10^1	10^6	-	+	+	+
	10^0	10^6	-	+	+	+
	0	10^6	-	+	+	+
6 h	10^4	10^6	+	+	+	+
	10^3	10^6	+	+	+	+
	10^2	10^6	+	+	+	+
	10^1	10^6	-	+	+	+
	10^0	10^6	-	+	+	+
	0	10^6	-	+	+	+
8 h	10^4	10^6	+	+	+	+
	10^3	10^6	+	+	+	+
	10^2	10^6	+	+	+	+
	10^1	10^6	+	+	+	+
	10^0	10^6	+	+	+	+
	0	10^6	-	+	+	+

CHAPTER 5

CONCLUSIONS

In this study, PMA was applied to dead, viable and a mixture of dead and live *E. coli* O157:H7 cells, respectively in order to prevent DNA from dead cells being detected by a subsequent PCR. As shown in the results, 25 µM of PMA was enough to bind all the DNA from 10^8 CFU/mL dead *E. coli* O157:H7, preventing generation of false-positive results in real-time PCR. The optimized PMA staining procedure showed no significant inhibitory effects on DNA amplification from 10^8 CFU/mL viable *E. coli* O157:H7. The length of light exposure time was not found to be influential on DNA amplification from viable cells. The influence of high concentrations of PMA on the DNA amplification from viable cells was studied. When the concentration of PMA was higher than 100 µM, amplification of DNA from viable cells was significantly inhibited. Although other studies have claimed that PMA was less toxic than EMA on viable cells, this study proved that PMA at high concentrations also exerts inhibitory effects on amplification of DNA from viable *E. coli* O157:H7 cells. The presence of live cells was found to prevent PMA from completely eliminating signals from dead cells, resulting in an overestimation of viable cells, especially when the concentrations of viable cells were low ($\leq 10^3$ CFU/mL) and the ratio of dead cells to live cells was high. Therefore, quantification of viable *E. coli* O157:H7 cells in dead and viable cell mixtures requires deliberation.

To our knowledge, this study is the first to successfully combine PMA staining with real-time PCR with the addition of an IAC to detect viable *E. coli* O157:H7 in apple juice and ground beef. Overall, a PMA treatment can effectively prevent the amplification of DNA from dead *E. coli* O157:H7 cells in apple juice and ground beef. The PMA real-time PCR assay can selectively detect only viable *E. coli* O157:H7 at as low as 10^2 CFU/mL in pure culture, 10^4 CFU/mL in apple juice and 10^5 CFU/g in ground beef, in the presence of 10^6 CFU/mL or g dead cells. With an 8-h enrichment, the assay could detect as low as 1 CFU/mL or g viable *E. coli* O157:H7 in apple juice and ground beef. The whole process can easily be completed in about 5 h after an 8-h enrichment step. Hence, PMA real-time PCR assay has the potential to be employed as a rapid detection method for the food industry to detect only viable *E. coli* O157:H7 in food products.

Further studies should include the influences of ratio between live and dead cells on detection of viable *E. coli* O157:H7 in various kinds of food. Since effects of PMA were reported to be different among various microbial species, PMA real-time PCR assay developed in this study should be applied to detect other foodborne pathogens in different kinds of food in future studies. Long amplicon sizes have been reported to improve the efficiency of PMA to differentiate viable cells from dead cells (Contreras and others 2011; Banihashemi and others 2012). In this study, the size of the PCR amplicons was 227 bp. Therefore, longer amplicon sizes could be promising to improve the accuracy of the PMA real-time PCR assay.

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VITA

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