NOVEL PCR-BASED RAPID DETECTION STRATEGIES FOR *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA* IN MEAT PRODUCTS

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

NOVEL PCR-BASED RAPID DETECTION STRATEGIES FOR *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA* IN MEAT PRODUCTS

Presented by Luxin Wang

A candidate for the degree of Doctor of Philosophy.

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

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Sitting in front of my computer, the first day of my elementary school is still so clear in my mind. A 6 year old girl looked at those "big girls" coming out of the classroom; graduating from elementary school was such painful and tough goal for me. I was seated at the left side of the classroom against the window. The first day, classes were so long and I didn't get any word from the teachers. I stared at the front door of the school all day and waited for my parents to take me home. In addition, I was very unhappy about my new name. My parents changed my name from "Xiaopu" to "Luxin", because they thought "Xiaopu" sounded like a tiny girl and they wanted a name that would fit me when I grow up. Thus, September 1st 1989 is not only my first day of school but also an official day for my name "Luxin Wang".

I never thought I would be Dr. Luxin Wang, because that was too far from a child's imagination. The idea of getting a Ph.D. in a foreign country was even more remote. My elementary school and middle school were 0.3 mile away from my home, my high school was 1.0 mile away and my college was again 0.3 mile away. The longest distance I had to go for my study before I was 20 was 1.0 mile. How far is it between Taian City and Columbia? Roughly 7,000 miles.

Until today, I still can not believe that I will graduate in another 5 months. I am excited to move on to new things, but also sad to leave the people and places I've come to know these past five years. I still remember the first day I met Dr. Mustapha. It was August 5, 2004. Dr. Mustapha was in a pink shirt and her eyes were so bright and they

impressed me deeply. Since that day, Dr. Mustapha has been giving me tremendous help, care and support. She gave me the freedom to pursue various projects and provided insightful advice and suggestions whenever I needed her. She is lively, enthusiastic and energetic. These traits encouraged me and the other students in her lab. I have been amazingly fortunate to have an advisor like Dr. Mustapha. Her warm heart and her emotional support brought me through many difficulties I encountered in my study and personal life. Since English is my second language, I easily run out of words to express my gratitude toward her.

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This is the place with tons of laughs and sometimes tears…

This is the place I feel more mature and grew up…

This is the place I will remember and miss all my life…

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NOVEL PCR-BASED RAPID DETECTION STRATEGIES FOR *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA* IN MEAT PRODUCTS

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ABSTRACT

Cultural-based detection method takes at least four days to complete. With the use of TaqMan® probes, the real-time PCR technique is a rapid and sensitive way to detect foodborne pathogens. However, DNA-based PCR techniques cannot differentiate between DNA from live and dead cells, while RNA-based PCR can. Ethidium bromide monoazide (EMA) is a dye that can bind to DNA of dead cells and prevent its amplification by PCR. An EMA staining step prior to real-time PCR allows for the effective inhibition of DNA contamination from dead cells. During the EMA treatment process, samples were stained with EMA for 5 min, iced for 1 min and exposed to bright visible light for 10 min prior to DNA extraction, to allow EMA binding of the DNA from dead cells. DNA was then extracted and amplified by TaqMan® real-time PCR to detect only viable *E. coli* O157:H7 and *Salmonella* cells. An internal amplification control (IAC), consisting of 0.25 pg of plasmid pUC19, was added in each reaction to prevent the occurrence of false negative results. The aim of this study was to use this EMA real-time PCR method to detect only viable *Salmonella* and *Escherichia coli* O157:H7cells

from poultry and beef products. In addition, the sensitivity of this new designed EMA staining coupled real-time PCR was compared to that of an RNA-based reverse transcription (RT)-real-time PCR. With an optimized EMA staining step, the detection range of a subsequent real-time PCR was 10^3 to 10^9 CFU/ml for pure cultures, 10^5 to 10^9 CFU/ml for artificially contaminated poultry samples, and 10^8 to 10^4 CFU/g for ground beef samples. These detection ranges proved that EMA real-time PCR has better detection efficiency than RT-real-time PCR. After a 12-h enrichment step, EMA combined real-time PCR (EMA real-time PCR) could detect as low as 10 CFU/ml *Salmonella* from chicken rinses and egg broths, as well as 10 CFU/g *E. coli* O157:H7 from ground beef. The use of EMA real time PCR can successfully prevent false positive results from dead cells and represent a simple, yet accurate detection tool for enhancing the safety of food.

Accurate and fast detection methods for foodborne pathogens from various food samples have always been important goals for scientists from many research areas. Quantum dots (QDs) are a family of nanosized particles with a 1 to 10 nm in radius. It has long-term stable photostability, high quantum yield, broad absorption spectra, narrow emission spectra and high signal-to-noise ratio. QD has been used in cell detection, imaging and DNA hybridization. In this study, EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide) and protein A were used to build crosslinkers for making QD antibody conjugates. In order to minimize the interference generated from magnetic beads, a FlowComp™ Dynabeads with DSB-X™ biotin protein labeling kit (D-20655) was used to isolate the cells from the food matrix and the beads were removed

after isolation. Detection signals were dramatically increased with the usage of the bead free isolation method. When bead free QD facilitated detection method was used to detect *Salmonella* and *E. coli* O157:H7 cells from pure cultures, it can detect as low as 10 CFU/ml cells. When it was applied to artificially contaminated ground beef, it can detect 10^6 CFU/g cells. After enrichment, it can detect as low as 10 CFU/g *Salmonella* cells from ground beef. The bead free QD facilitated detection method developed in this study is the first research that combines the bead free isolation method and QD labeling technique together to detect *E. coli* O157:H7 and *Salmonella* from ground beef. Further studies which can improve the detection range and specificity will be worth to try.

CHAPTER 1

INTRODUCTION

Although the United States has one of the safest food supplies in the world, foodborne disease outbreaks are still happening. A study done in 2003 found that, in the US, 3.6% of raw meat and poultry samples were contaminated with *Salmonella* (Lawley and others 2008) and according to Mead and others (1999), *Escherichia coli* O157:H7 causes approximately 74,000 illnesses, 2,200 hospitalizations and 61 deaths every year. Good manufacturing practices (GMP), good agricultural practices (GAP) and hazard analysis critical control points (HACCP) start to play more important roles in food safety control.

The effect and efficiency of GAP, GMP and HACCP need proper evaluation, which, in turn, directly decide the food safety results. Conventional microbiological analyses involve enrichment, isolation and confirmation steps, which usually take about 3 to 5 days. Polymerase chain reaction, due to its ability to identify one species from another within 3 hours, had attracted attention from both the food industries and scientists for a long time.

Stressful conditions, such as storage under refrigeration temperature, can induce and enhance the acid tolerance of *E. coli* O157:H7 (Buchanan and Doyle, 1997). The formation of viable but nonculturable (VBNC) cells has been proven in several studies. Species like *Salmonella enterica*, *Campylobacter jejuni*, *Vibrio cincinnatiensis* and *E. coli* O157:H7 can all enter the VBNC status under certain stressful environments.

Detection and enumeration of all viable cells (including VBNC cells) will effectively reflect the efficiency of various intervention methods and GMP, GAP and HACCP plans.

One primary objective of this study was to establish a PCR-based assay to detect only viable cells from artificially contaminated products. Our targets were *E. coli* O157:H7 and *Salmonella* Typhimurium. Initially, reverse transcription PCR was evaluated, because the template in RT-PCR is RNA which is present only in live cells. Then, the efficiency of RT-PCR was compared with a novel PCR method (ethidium monoazide staining [EMA] combined with PCR). EMA is a dye that can bind to the dead cells' DNA and prevent their amplification during PCR processes. Thus, this EMA combined PCR can differentiate the live cells from the dead cells. EMA combined PCR was optimized in this study, for detecting *E. coli* O157:H7 from ground beef and for identifying *Salmonella* from chicken carcasses and raw eggs.

Another objective of this study was to develop a novel quantum dot (QD)-facilitated detection method. QD is a nanoparticle that can be used as a replacer for traditional dyes. It is small (around 20 nm in diameter), but has high photostability and fluorescence. Efforts were invested to optimize the antibody QD conjugation protocol and a bead-free cell isolation method was developed. Finally, a bead-free QD-facilitated novel detection method was developed and successfully applied in *E. coli* O157:H7 detection from ground beef.

REFERENCES

Buchanan RL, Doyle MP. 1997. Foodborne disease significance of *Escherichia coli* O157:H7 and other enterohemorrhagic *E. coli*. Food Technol 51(10):69-76.

Lawley R, Curtis L, Davis J. 2008. The Food Safety Hazard Guidebook. Cambridge, UK: RSC Publishing. 432p.

Mead PS, Slutsker L, Dietz V, MeCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV. 1999. Food-related illness and death in the United States. Emerg Infect Dis 5:607-25.

CHAPTER 2

LITERATURE REVIEW

"Food safety" covers a wide range of topics, from personal hygiene training, contaminant removal, to produce safety analysis. According to The Food Safety Hazard Guidebook (Lawley and others 2008), the practices of food safety can be divided into three parts, "protection of the food supply from harmful contamination", "prevention of the development and spread of harmful contamination" and "effective removal of contamination and contaminants" (Lawley and others 2008). Food safety hazard can be any factor that causes harm to consumers, including biological, chemical and physical objects and the harm includes illness, injury or both.

Biological hazards can be an immediate threat to consumers and have been involved in several foodborne outbreaks. Bacteria, viruses, parasites and prions all belong in this category. Chemical hazards include chemicals like pesticides, veterinary drugs, plant toxins, and environmental contaminants. The injury caused by chemical hazards is usually less immediate than the one caused by bacteria. Physical objects (hazards) that might be involved in food safety problems are objects like metals or stones left in the foods during or after processing. Last but not least, food allergy, another important food safety issue, has been growing in importance for the food industry as the number of people affected by allergy symptoms increased (Lawley and others 2008).

2.1 Development of biological hazard risk assessment

Although the scientifically-based definition and development of hazard risk assessment started in the 1990s, risk assessment had already been used in food processing back in the late 1800s. The work done by van Ermengen in 1896 which clarified the etiology of botulism in humans can be recognized as risk assessment work. In the 1990s, both the government and industry committed to developing an internationally accepted methodology for biological, especially microbiological, risk assessment. One reason for this commitment was due to the well-publicized outbreaks of foodborne diseases in the United States and Europe (Brown and Stringer 2002). Those outbreaks highlighted the need for new hazard identification, assessment and management of existing microbiological food safety risks and necessary dialogues with consumers about microbiological safety (Pennington 1997; Tuttle and others 1999; Brown and Stringer 2002). In addition, globalization of the food supply underlined the importance to harmonize food safety principles around the world. In 1995, the sanitary and phytosanitary (SPS) agreement was published by member countries of the World Trade Organization (WTO) (Brown and Stringer 2002). This agreement proposed the requirements to ensure equivalent food safety levels from different nations.

The Codex Alimentarius Commission (CAC) defines microbiological risk assessment as a scientifically-based process involving four key steps. They are hazard identification, hazard characterization, exposure assessment and risk characterization. The risk assessment procedure starts with identifying relationships between illnesses and microbes (hazard identification), followed by dose response analysis (hazard characterization) and exposure probability evaluation (exposure assessment). After these

three stages, the evaluation process is summarized by estimating the overall risk level for one particular food product and specific groups of people (Brown and Stringer 2002).

2.1.1 Introduction of GMP and HACCP

Good Manufacturing Practice (GMP) is one of the first quality assurance systems developed by food industries. GMP is a summary of long practical experience. It is a quality approach to minimize or eliminate instances of contamination, mix-ups, and errors that could happen during food processing. "GMP regulations address issues including recordkeeping, personnel qualifications, sanitation, cleanliness, equipment verification, process validation, and complaint handling (GMP Institute 2009)". Most GMP requirements are open-ended with flexible rules, allowing manufacturers to decide the best control method for their plants and interpret the requirements that make more sense for their own businesses.

The Hazard Analysis Critical Control Points (HACCP) is a systematic approach to identify, assess and control hazards in one particular food operation. The purpose of HACCP is to identify and prevent the risk before it happens. The concept arose from a collaboration between the Pillsbury Company, the US Army Natick Research and Development Laboratories, and the US National Aeronautics and Space Administration. In 1972, HACCP was first introduced in a food protection meeting. The concept included three principles at the beginning: "hazard identification and characterization", "identification of critical control points" and "monitoring of the CCPs". After that, modifications and additional requirements were added to complete the establishment of the current HACCP plan. By 1973, the FDA made the use of HACCP principles

mandatory for low-acid canned food production (FDA 1973) and it became a legal requirement for all food products in the European Union after that.

2.1.2 Trends in food safety control

To check food quality and safety, samples are usually randomly selected and checked by both food producers and regulatory agencies. Although this approach can confirm whether the food meets the criteria at the point of sampling, it does not guarantee the absence of potential changes in the following handling and storage steps. Food safety control is increasingly dependent on a more prospective approach, involving the application of GMP and HACCP principles (Notermans and Barendsz 2002). The use of predictive microbiology is being proved as valuable as it showed previously in microbial heat inactivation model establishment. Thus, one trend in food safety control is that predictive model receives more attention.

In 2001, participants at the International Association of Food Protection (IAFP) Congress in Minneapolis proposed that an appropriate level of protection (ALOP) should be added to microbial risk assessment plans. ALOP is the level of protection that a country decides is necessary to protect health and life against the harmful effects of hazards. If ALOP cannot be met, the production of the food must cease. The addition of ALOP in a risk assessment and management plan is another trend in food safety field.

2.2 Sample microbiological risk assessment

2.2.1 *Salmonella*

2.2.1.1 Hazard identification

Salmonella is one of the causes of foodborne gastroenteritis worldwide. The salmonellosis it causes is a zoonotic infection which can be transferred from animals to humans. There are two main species of *Salmonella*, *S. enterica* and *S. bongori*. The subspecies most important in foodborne disease is *S. enterica* subspecies *enterica*.

Food animals can be infected with *Salmonella* from both the feed and the environment. In 2005, a Europe-wide study found that about one in five large-scale commercial egg-producing facilities had hens infected with *Salmonella*. In the United States, a study done in 2003 found that 3.6% of raw meat and poultry samples were contaminated with *Salmonella* (Lawley and others 2008). Fresh produce, like tomatoes, lettuce, sprouting seeds, melons, and products like fruit juice have already been involved in *Salmonella* outbreaks (Lawley and others 2008).

2.2.1.2 Hazard characterization

Some *Salmonella* serotypes have limited host spectra, such as *S.* Typhimurium and *S.* Paratyphi which can cause typhoid fever only in humans. Symptoms usually include diarrhea, abdominal pains, nausea, vomiting and chill. The death rate of non-typhoid salmonellosis is <1% (the number might higher in certain groups, like the elderly and immunocompromised patients). The incubation time is usually 12-36 h. Small numbers (10-100 cells) can cause illness in young or the elderly, while it is thought that

high numbers (10^5 – 10^6 cells) need to be taken in order to make healthy people sick (Lawley and others 2008).

2.2.1.3 Exposure assessment

Because *Salmonella* can be shed in feces, soil and surface waters which may become contaminated with *Salmonella* can induce contamination in produce, seeds, nuts, and other commodities. Poultry and pigs are considered to be significant reservoirs of *Salmonella*. In addition, *Salmonella*'s ability to survive in some food processing and storage environments lead to more exposure chances of humans to *Salmonella*-contaminated foods.

Salmonella serotypes can grow between 7-48^{o}C. Some reports found that they can grow at temperatures as low as 4^{o}C, although most *Salmonella* serotypes cannot grow in refrigeration temperatures. *Salmonella* can survive for quite a long time in the refrigerator, can grow over a wide range of pH values (from 3.7 to 9.5), and can survive A_w down to 0.94 (or 0.93). *Salmonellae* are facultative anaerobes, being able to grow with or without oxygen. They can even grow in atmospheres containing high levels of carbon dioxide. Their D values are 1-10 min at 60^{o}C and <1 min at 70^{o}C, while their z-value is 4-5^{o}C.

2.2.1.4 Control points

Fresh produce and animal-derived raw materials, like eggs, poultry and pork, need to be examined for potential *Salmonella* contamination. *Salmonella* can be effectively controlled by relatively mild heat processing, and the key point during food processing is to prevent cross contamination. Various detection methods for *Salmonella* are listed in Table 2.3.

2.2.1.5 Epidemiological and genetic information of *Salmonella*

In the United States, 40,000 cases of *Salmonellosis* are reported annually (Wray and Davies 2003) and the estimated annual cost has ranged from billions of dollars in the US (Todd 1989). Approximately 2,500 different *Salmonella* serovars have been described and the number is increasing (Wray and Davies 2003). *S*. Dublin and *S*. Typhimurium are usually found from cattle, while *S.* Derby, *S.* Typhimurium, *S.* Agona are usually found in swine; *S.* Enteritidis is commonly detected in poultry products (Ferris and Miller 1996; MAFF 2000). *Salmonella* contamination can be cased by sources like animal feed, farm building, manure, water, as well as other animals. *Salmonella* can be transmitted through the fecal-oral route, aerosol, dust-borne and ovarian transmission (Wray and Davies 2003).

Several genes are involved in the survival and invasion of *Salmonella*. For example, its acid tolerance response is regulated by the alternative sigma factor σ which is encoded by *rpo*S, two-component sensor regulator PhoP/Q and the ferric uptake regulator *Fur* (Gahan and Hill 1999). Its adhesion is based on different kinds of fimbriae and is also controlled by bovine colonization factor (*Bcf*) (Patterson and Isaacson 2003). The infection caused by *Salmonella* is encoded by *spv*B and *spv*C genes (Matsui and others 2001)

2.2.2 Shiga toxin-producing *Escherichia coli*

2.2.2.1 Hazard identification

Escherichia coli are normal human gut microflora and most of them are not pathogenic. The ones that are commonly involved in foodborne diseases are Shiga toxin-

producing *E. coli* (STEC) and Enterotoxigenic *E. coli* (ETEC). ETEC are the causes of traveler's diarrhea. STEC are usually associated with foods derived from cattle, like ground beef. They have also been identified from some fresh produce and products like apple cider (Lawley and others 2008). Comparisons between *Salmonella* and STEC are listed in Table 2.1.

2.2.2.2 Hazard characterization

The incubation time for STEC is between 1 to 14 days. The infective dose is as low as 10 cells. The common symptoms are hemolytic uremic syndrome (HUS). The mortality rate of HUS in the UK and in North America is 3-5% (Lawley and others 2008).

2.2.2.3 Exposure assessment

Cattle, as well as sheep and camels, are main carriers of STEC. Irrigation water and fertilizers contaminated with STEC are the main sources causing secondary contamination in fresh produce. The optimal growth temperature of STEC is 37^{o}C, but it can survive and grow over the temperature range of 7-46^{o}C. *E. coli* O157, the most notorious STEC, can grow under low pH (4.0 to 4.4) and can stay alive for up to 2 months at 4^{o}C with a pH of 4.5. Salt solution (8.5%) and 100% CO_2 have shown an inhibition effect on *E. coli* O157:H7 growth. D_{57oC} value is 5 min and D_{63oC} is 0.5 min.

2.2.2.4 Control points

Internal cooking temperature has been set up for meat products. The purpose of checking internal cooking temperature is to set a standard to make sure no live STEC is in foods. Usually, the internal temperature of medium rare is approximately 145^{o}F, 160^{o}F for medium and 170^{o}F for well done. Strict recall policies are available for

potentially contaminated beef products. Table 2.2 shows standard internal temperatures for some meat products. Various detection methods for *E. coli* O157:H7 are listed in Table 2.3.

2.2.2.5 Epidemiological and genetic information of *E. coli* O157:H7

In the United States, *E. coli* O157:H7 infection is a leading cause of HUS and the most common cause of acute kidney failure in children (Altekruse and others 1997). Surveillance of laboratory-confirmed *E. coli* O157-related illnesses in the selected regions of the US from 1996-2000 showed 2.1 to 2.9 diagnosed infections per 100,000 populations (CDC 2001). A research of 139 cases (1982-1996) showed that 28 out of 139 outbreaks were associated with person-to-person contact, 53 out of 139 were attributed to consumption of beef or unpasteurized milk, 19 out of 139 were associated with consumption of produce, 10 out of 139 were related with swimming in pools or ponds and 3 out of 139 were associated with contaminated water (Sparling 1998). *E. coli* O157:H7 was first recognized as a foodborne pathogen in 1982, due to fast-food hamburgers (Riley and others 1983). It is quite acid tolerant and can survive for weeks in pH 4.32 (Glass and others 1992; Attenborugh and matthews 2000). Potential contamination sources can be cattle, bovine, swine, poultry, sheep, environmental sources (feces, water, feed, insects, wildlife) (Sargeant and Smith 2003).

Genes that are involved in *E. coli* O157:H7 attachment are *tox*B locus of pO157 (Tatsuno and others 2001), *iha* gene (Tarr and others 2000) and *bfp* gene (Benson 2003). The main toxin genes in it are *stx*1 and *stx*2, similar to *Shigella* (Paton and Paton 1998). It has been found that *stx*2 is more toxic than *stx*1 (Boerlin and others 1999).

Table 2.1 Characteristics of *Salmonella* and Shiga toxin-producing *Escherichia coli* (STEC).

Bacteria	Gram Stain	Sporeforming	Shape	Family	Diseases	Incubation time
Salmonella	G-	None	rod	*Enterobacteriaceae*	Salmonellosis	6-48 h
STEC	G-	None	rod	*Enterobacteriaceae*	Hemorrhagic colitis	1-14 days

Table 2.2 Internal cooking temperature for common meat products (Adapted from National Restaurant Association 2009).

	Beef streak	Duck	Hamburger patties	Fish	Eggs	Lamb
Internal temperature	145°F	165°F	155°F	145°F	145°F	145°F
Duration	15 s	15 s	15 s	15 s	15 s	4 min

Table 2.3 Detection methods for *Salmonella* and *E. coli* O157:H7.

	Detection methods
Salmonella	1. Non-selective enrichment: Trypticase soy broth; buffered peptone water. 2. Selective enrichment: Rappaport-vassiladis and Tetrathionate broth. 3. Selective agar: Xylose Lysine Dexoycholate agar (XLD); Xylose Lactose Tergitol™ 4 (XLT4); Hektoen Enteric Agar (HE). 4. Immunocapture method: Vitek Immunodiagnostic Assay System (VIDAS); immunomagnetic bead separation method (IMS); Enzyme-linked immunosorbent assay (ELISA). 5. Polymerase Chain Reaction (PCR) based detection method. Target genes: *oriC, ompC,* 16S *rRNA, rfbS, sefA*, *orf6e, fliC, mdh, sdiA, Stn, ttrBCA, Prot6e*
***E. coli* O157:H7**	1. Sorbitol-MacConkey agar 2. ß-glucuronidase tests 3. sorbitol-fermenting tests 4. immunomagnetic separation (IMS) 5. buffered peptone water with vancomycin, cefixime and cefsulodin for selective enrichment 6. immofluorescence and enzyme-linked immunosorbent assay (ELISA) 7. Polymerase Chain Reaction (PCR) based detection method. Target genes: *eae*A, *stx*, *ehx*, *uid*A, *fim*A, *fli*C, *rfb*E

2.3 Microbiological hazard identification guide

The meat and poultry subcommittee of the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) developed the microbiological hazard identification guide for small plants. This is very important for development of effective HACCP plans for small companies. This guide consists of tables that identify typical microbiological hazards in various products. Those tables were adapted and summarized in Table 2.4.

2.3.1 Microbiological hazards in meat and poultry

2.3.1.1 Bacteria

Bacteria cause a large proportion (approximately 90%) of all foodborne illnesses. Pathogens most likely to be found in slaughtered livestock (cattle, sheep, and swine) and poultry (chicken and turkey) are *Salmonella*, *Campylobacter, E. coli* O157:H7 and *Listeria monocytogenes*.

2.3.1.2 Toxin

Toxins produced by *Clostridium botulinum*, *Clostridium perfringens*, *Bacillus cereus* and *Staphylococcus aureus* can also be found in meat and poultry.

Table 2.4 Microbiological hazard guide in meat and poultry (Adapted from FSIS 1999).

Process category	General product samples	Species	*Salmonella*	*E. coli* O157:H7	*Campylobacter*	*Listeria monocytogenes*	*Staphylococcus aureus*	*Clostridium perfringens*	*Clostridium botulinum*
Slaughter-all species	Carcasses, carcass parts, and variety meats	Beef	+	+					
		Lamb	+						
		Pork	+						
		Poultry	+		+				
Raw product-Ground	Ground product	Beef	+	+					
		Lamb	+						
		Pork	+						
		Poultry	+		+				
Raw product-not ground	Steaks, roasts, chops	Beef	+	+					
		Lamb	+						
		Pork	+						
		Poultry	+		+				

Table 2.4 Microbiological hazard guide in meat and poultry (Adapted from FSIS 1999) (Cont.).

Process category	General product samples	Species	*Salmonella*	*E. coli* O157:H7	*Campylobacter*	*Listeria monocytogenes*	*Staphylococcus aureus*	*Clostridium perfringens*	*Clostridium botulinum*
Fully cooked-not shelf stable	Bologna, Deli meats, patties, hot dogs	Beef	+	+		+		+	+
		Lamb	+			+		+	+
		Pork	+			+		+	+
		Poultry	+		+	+		+	+
Heat treated but not fully cooked	Partially cooked patties	Beef	+	+					
		Lamb	+						
		Pork	+						
		Poultry	+		+				
Product with secondary inhibitors-not shelf stable	Fermented sausage	Beef	+	+			+		
		Lamb	+				+		
		Pork	+				+		
		Poultry	+		+		+		

Table 2.4 Microbiological hazard guide in meat and poultry (Adapted from FSIS 1999) (Cont.).

Process category	General product samples	Species	*Salmonella*	*E. coli* O157:H7	*Campylobacter*	*Listeria monocytogenes*	*Staphylococcus aureus*	*Clostridium perfringens*	*Clostridium botulinum*
Thermally-processed-commercially sterile	Canned beef	Beef							+
		Lamb							+
		Pork							+
		Poultry							+
Not heat treated-shelf stable	Country cured products, unrefrigerated	Beef				+	+		
		Lamb				+	+		
		Pork				+	+		
		Poultry				+	+		
Heat treated-shelf stable	Lard	Beef							
		Lamb							
		Pork							
		Poultry							

2.3.2 Survival of foodborne pathogens

Water activity, pH, temperature, oxygen and nutrition are all important growth factors that influence and control the survival of foodborne pathogens in foods. In order to control their growth, various intervention methods have been developed and used in the food industry. The purpose of these intervention methods is to control or kill pathogens via provide unfavorable environment.

"Foodborne pathogenic bacteria sicken more than 76 million Americans and many of these cases are caused by the consumption of foodstuffs produced from animals. Although postharvest intervention strategies are targeted at reducing pathogens from the abattoir to the table, the continuous outbreaks of foodborne illnesses suggested that preharvest intervention strategies are needed to effectively reduce human foodborne illness" (Callaway and others 2002). Several preharvest intervention strategies have been invented and are currently under investigation or usage. Vaccination, competitive exclusion, substrate-adapted competitive exclusion, usage of probiotics and prebiotics, application of bacteriophage, exploitation of the physiology of specific pathogens and usage of antibiotics have shown promising results.

In addition to preharvest food safety intervention methods, postharvest intervention methods, like pasteurization, ultra high temperature, high hydrostatic pressure, irradiation, fermentation, play important roles in food safety control as well.

2.3.2.1 Viable but non-culturable

The viability of bacterial cells is traditionally determined by their ability to grow and produce colonies. However, the sprouting of one concept, "viable but non-culturable" (VBNC) provided the traditional plating method with a challenge. VBNC

represents a status of cells that are alive, but can't form colonies on nutrient agar. By activating survival mechanisms, bacteria can enter a VBNC status when they are under environmental stress (Roszak and Colwell 1987). Although the physiological significance of this phenotype is unclear, VBNC cells have become an important safety concern in public health risk assessments. Pathogenic bacteria like *E. coli* have been found to enter the VBNC state and return to an infectious state after passing in an animal host (Rivers and Steck 2001).

There are two groups of ideas that tried to explain the VBNC phenomenon. One group proposed that VBNC is part of an adaptive response aimed at long term survival under adverse conditions (Mukamolova and others 2003), while another group argued that it is a consequence of stochastic cellular deterioration and VBNC cells are on their way to death (Bogosian and Bourneuf 2001).

The presence of VBNC cells has been proven in many studies. One of them was presented by Roszak and Colwell (1987). By using direct viable count, Roszak and Cowell (1987) constructed direct count and culturable count curves, from which the presence of VBNC cells was proven (Roszak and Cowell 1987) (Figure 2.1). *S. enterica* has been shown to enter the VBNC state in response to environmental stresses (Reissbrodt and others 2000; Oliver 2005), as well as *Campylobacter jejuni* (Klančnik and others 2009), *Vibrio cincinnatiensis* (Zhong and others 2009), and *E. coli* O157:H7 (Asakura and others 2008).

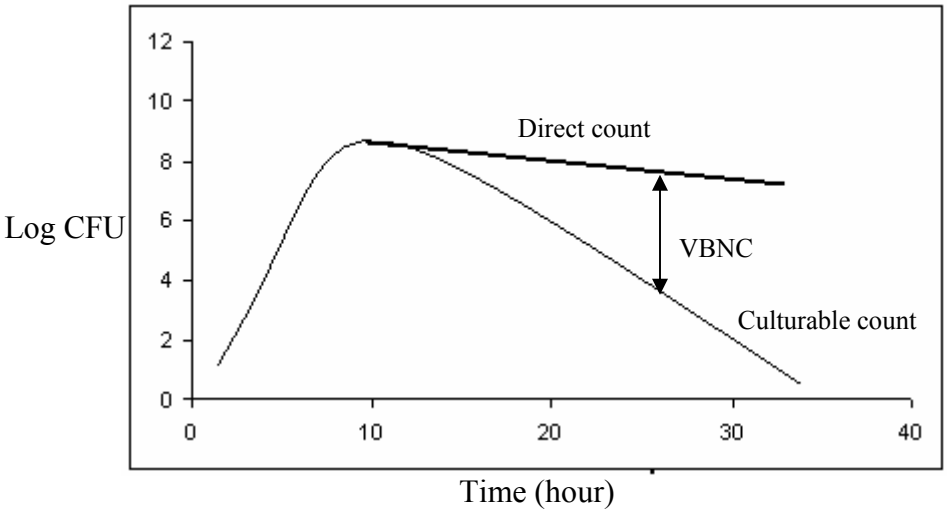

Figure 2.1 Comparison between culturable count and direct count. The difference between direct and culturable counts represented the population of cells that were viable but not culturable (adapted from Roszak and Cowell 1987).

2.3.2.1.1 Detection of viable but nonculturable cells

Due to the nonculturable property of VBNC cells, an agar-based detection method cannot be applied for their detection. Methods that do not require culturable colonies and resuscitation are needed. These methods are like cell-based staining/imaging, flow cytometry as well as certain molecular DNA/RNA-based detection assays. Detection methods that have been used in viable cell detection (including VBNC cells) will be discussed more in the following "Microbial hazard identification tools" section.

2.4 Microbial hazard assessment tools

Routine tests used in hazard identification include growth characteristic tests of a pathogen on selective medium (colony color, smell, biochemical properties as well as antibiotic resistant properties). Genotype identification and phenotype analysis can be realized by techniques like polymerase chain reaction (PCR) and methods like cellular enzyme electrophoresis, respectively (Brown 2002).

To do dose-response assessment (hazard characterization), mathematical models combined with observational data (like human trails, animal studies) are needed. The development of dose-response models depends on the availability of data that can quantitatively describe the relationship between the levels of the microbe ingested and the frequency and severity of illness. Unfortunately, there are several problems associated with methods used in hazard characterization. Sometimes, problems like "lack of human quantitative data", "lack of methods to extrapolate high doses to low doses", "extrapolation from animal to human" and "conducting severity assessments" are hard to overcome.

Microbiological exposure assessments are models of pathogen or toxin levels in foods moving through the supply chain. The purpose of exposure assessment is to provide an estimate of hazard levels in a product at the point of consumption. There are nine stages in an exposure assessment: "formulation of the food safety problem", "hazard identification", "statement of purpose", "scope", "data collection", "production of a supply chain model", "identification of major risk-determining steps", "description of variability, uncertainty and assumptions" and "presentation of information" (Brown 2002).

Risk characterization uses mathematical terminology often. While exposure assessment estimates the exposure probability of the population to hazards and hazard characterization estimates the harm done to populations by hazards, risk characterization combines these estimates to produce estimates of the harm caused by pathogens under certain exposure probability (Voysey and others 2002).

2.5 Microbial hazard identification tools

2.5.1 Cultural methods

Cultural methods are traditional microbial identification methods. The purpose of cultural methods is to let stressed and injured microbes resuscitate. Pre-enrichment and selective enrichment steps have been developed for most foodborne pathogens. The formulation of pre-enrichment media depends on the level of competing background microbes and the ability of foods to inhibit microbial growth. For example, the FDA bacteriological analytical manual describes 10 different pre-enrichment media just for the enrichment of *Salmonella* from various food samples (Downes and Ito 2001).

In Figure 2.2, there are growth curves of *Salmonella* and competitive microflora in pre-enrichment and selective enrichment broths. A pre-enrichment step is not mandatory when the foods are nutritious and the resuscitation of microflora in foods is not required. It can be seen in Figure 2.2 that although the growth of *Salmonella* was slower than the background microflora during the pre-enrichment process, the number of *Salmonella* increased dramatically during the selective enrichment step.

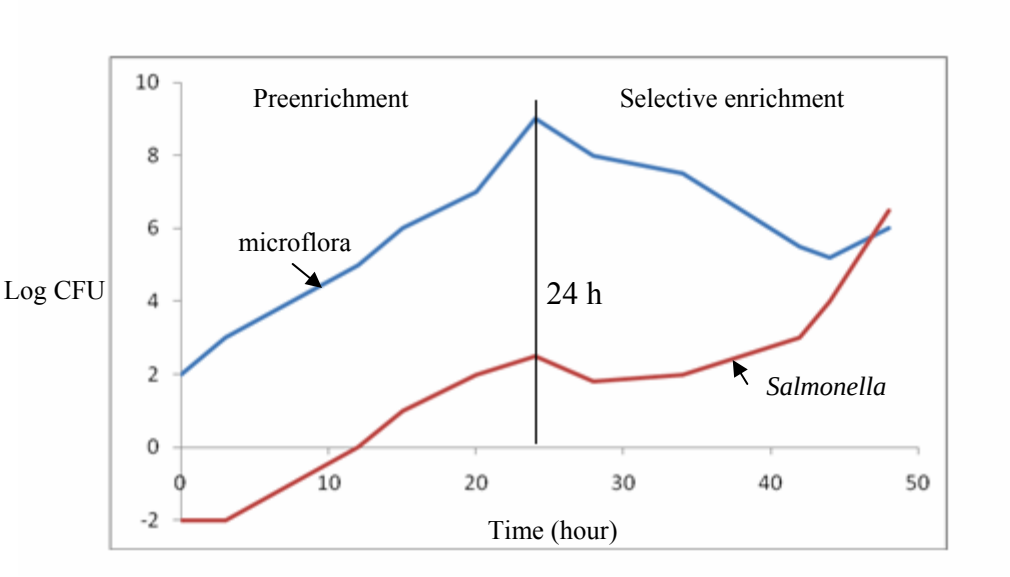

Figure 2.2 Growth of *Salmonella* and competitive microflora during preenrichment and selective enrichment (Adapted from Downes and Ito 2001).

In selective enrichment media, selective factors can be antibiotics, chemicals, anaerobiosis, pH, water activity and temperature. Due to the different biochemical or physical properties of microorganisms, target cells will be selectively enriched under certain circumstances. Besides selective agents, there are chemicals called differential agents. Those agents are added to selective media together with selective agents to make the identification of target microbes easier, usually associated with color changes. Examples of differential agents are pH indicators, H_2S indicators, and egg yolk reaction or blood hemolysis reactions (Downes and Ito 2001).

For a quality assurance plan or HACCP, enumeration of target cells is as important as identification. Plate count method, most probable number (MPN) and membrane filtration are methods used for quantitative examination of food samples. Changes in cell numbers make the standardization of quality assurance plan reliable and easy to check and follow.

2.5.2 Nucleic acid based methods

The sequences of nucleotide bases in DNA are different from species to species. The application of nucleic acid based detection method in food science has significantly improved the identification and detection efficiency for foodborne microbes. From DNA hybridization to gel electrophoresis, nucleic acid based methods can be mainly divided into two groups, qualitative and quantitative. Examples will be given in the following discussion regarding their application in food microbe detection. In Table 2.5 to 2.9, real-time PCR and PCR-based fingerprinting methods are listed and compared.

Colony-based hybridization can be used as a quantitative detection method. By transferring colonies from an agar plate onto a membrane, hybridization results can be recorded as "number of fluorescence colonies" per plate/membrane, which is equal to CFU/ml or CFU/g. Although traditional PCR was initially only used as a qualitative method, with the application of a fluorescence measuring program, it can now be used as a quantitative method as well. Amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), short tandem repeat analysis, denaturing gradient gel electrophoresis (DGGE) are all molecular techniques that can be used to identify and detect target pathogens in foods. Detailed information can be found in Table 2.5 to 2.9. These methods can effectively individualize different species and strains based on their genomic differences. Knowledge of the genome is not required sometimes but, pure colonies are key requirements for successful application of some techniques.

Table 2.5 Hybridization methods.

Method	Primer Probe	Variation	Quantitative detection	Advantage or disadvantage	Application in microbe detection	Reference
Hybridization	Probes	Solid-phase hybridization (colony blotting)	Y	Advantages: 1. Easy to use 2. various labeling methods 3. If single strand probes are used, no additional denaturation step is needed Disadvantages: 1. Reanealing during hybridization may decrease probe availability 2. Pure colony is needed 3. Enrichment is needed	*E. coli*, *Shigella*, *Listeria*, *Staphylococcus*, *Vibrio*, *Yersinia*	Downes and Ito 2001; Montgomery 2002
		Liquid-phase hybridization	N		*Salmonella*, *Listeria*, *E. coli*, *Staphylococcus*, *Campylobacter*, *Yersinia*	

Table 2.6 PCR methods.

Method	Primer Probe	Variation	Quantitative detection	Advantage or Disadvantage	Application in microbe detection	Reference
Polymerase Chain Reaction	Primers	DNA-based	Y	Advantages: 1 Easy to use 2 High specific 3 Low detection limit 4 Enrichment may or may not be needed 5 Results within 4h Disadvantages: 1 DNA from background microflora inhibits amplification of target cells 2 RNA is not stable and hard to extract 3 DNA-based PCR cannot separate dead cells from live cells	*E. coli*, *Shigella*, *Salmonella*, *Listeria*. *Stapylococcus*, *Campylobacter*, *Yersinia*, *Vibrio*, *Bacillus*,	Mustapha and Li 2006
		RNA-based	Y			

Table 2.7 Real-time PCR methods.

Method	Primers Probes	Variations	Quantitative detection	Advantages Or Disadvantages	Application in microbe detection	Reference
Real-time Polymerase Chain Reaction	Primers Probes	DNA based	Y	Advantages: 1 Easy to use 2 High specific 3 Low detection limit 4 Enrichment may or may not needed 5 Results within 3h 6 Various labeling dye choices Disadvantages: 1 DNA from background microflora may inhibit the amplification of target cells. 2 RNA is not stable and hard to extract. 3 DNA based PCR cannot separate dead cells from live cells.	*E. coli, Shigella, Salmonella, Listeria. Stapylococcus, Campylobacter Yersinia Vibrio, Bacillus*	Mustapha and Li 2006
		RNA based	Y			

Table 2.8 Amplified fragment length polymophisms and randomly amplified polymorphic DNA.

Method	Primers Probes	Variations	Qualitative detection	Advantages Or Disadvantages	Application in microbe detection	Reference
PCR-based fingerprinting	Primers	amplified fragment length polymophisms (AFLP)	Y	Advantages: 1. Analyze and individualize multiple species 2. No previous knowledge of the genome is necessary. Disadvantages: 3 Inability to differentiate cells from mixed samples. Pure colony is needed.	*E. coli, Shigella, Salmonella, Listeria. Stapylococcus, Campylobacter Yersinia Vibrio, Bacillus*	Monna 1994
		randomly amplified polymorphic DNA (RAPD)	Y	Advantages: 1. Low amount of pure DNA is needed. 2. Generate genomic markers for future PCR usage		

Table 2.9 Short tandem repeat analysis and denaturing gradient gel electrophoresis.

Method	Primers Probes	Variations	Qualitative detection	Advantages Or Disadvantages	Application in microbe detection	Reference
PCR-based fingerprinting	Primers	Short tandem repeat (STR) analysis	Y	Advantages: 1. Identify target cells out of the mixed samples 2. individualize multiple species Disadvantages: 1. DNA from background microflora may inhibit the amplification of target cells. 2. Enrichment is needed for low concentration	*E. coli*, *Shigella*, *Salmonella*, *Listeria*, *Stapylococcus*, *Campylobacter* *Yersinia* *Vibrio*, *Bacillus*	He and others 2009
		Denaturing gradient gel electrophoresis (DGGE)	Y			

2.5.3 Other cell-based methods

Enzyme linked immunosorbent assays (ELISAs) provide an ideal system for a wide range of biological studies. The main reason for its success is its flexibility. The exploitation of ELISA has increased with the development of techniques used for making monoclonal and polyclonal antibodies. There are four kinds of ELISA methods. They are direct ELISA, indirect ELISA, direct sandwich ELISA and indirect sandwich ELISA (Figure 2.3 and Table 2.10)

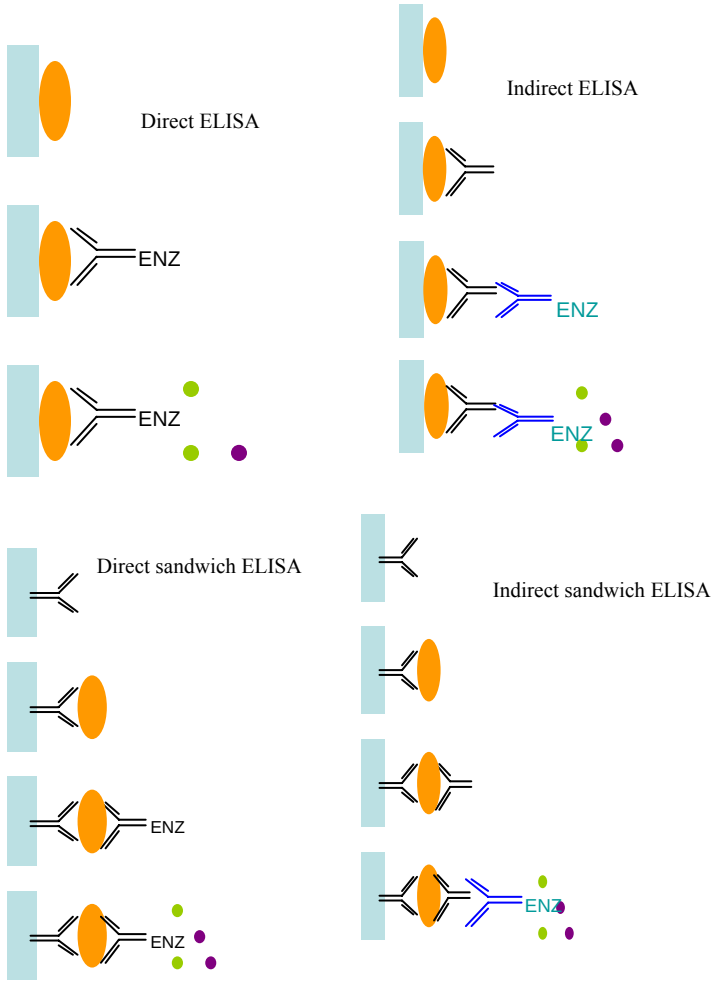

Figure 2.3 ELISA methods (Adapted from Crowther 2008).

2.5.3.1 Immunomagnetic separation (IMS)

Immunomagnetic separation (IMS) is a method used for cell isolation from environmental or food samples. The basic principle of IMS is to use magnetic beads coated with antibodies to catch target cells and collect them by the absorption power between magnet and ion beads. It is a cell based isolation and detection method. IMS has been applied to detect foodborne pathogens from various products. It was usually used by combining with other detection methods like ELISA, real-time PCR, PCR or plating.

In 1996, Chapman and Siddons found that by using IMS, the detection sensitivity of *E. coli* O157:H7 can be improved up to 100%. They first used IMS to catch the cells and then plated the cells on cefixime tellurite sorbitol MacConkey agar (CT-SMAC). At the same time, a control group, in which samples containing *E. coli* O157:H7 were plated directly on to CT-SMAC agar without IMS step, was also prepared. The results showed that while the IMS combined plate count method could detect 25 positive samples, the direct plate count method detected only 12 to 15 positive samples (Chapman and Siddons 1996). The use of IMS can improve the detection sensitivity dramatically.

In our previous researches, improvements done by IMS to real-time PCR had also been found. When the IMS combined real-time PCR was applied to detect *E. coli* O157:H7 from artificially contaminated ground beef, the lowest detection limit of real-time PCR can be improved from 10^5 CFU/g to 10^3 CFU/g (Wang and others 2007).

Table 2.10 Brief descriptions of elements common to ELISAs (Adapted from Crowther 2008).

Elements	Definitions
Solid phase	A plastic microtiter plate well.
Adsorption	A process of adding an antigen (direct ELISA) or antibody (indirect ELISA). Antigen or antibody will attaches passively to the solid phase after certain incubation time.
Washing	Simply flooding and emptying wells with a buffered solution, to separate bound and free reagents.
Antigen	Targets the whole ELISA is designed to detect.
Antibody	Antibodies are produced in response to antigenic stimuli, they are antigenic.
Antispecies antibody	Antibodies obtained when antibodies from one animal are injected into another species. Guinea pig serum injected into a rabbit would elicit a rabbit anti-guinea pig serum.
Enzyme	A substance that acts at low concentration as a catalyst to promote a specific reaction.
Enzyme conjugate	An enzyme attached irreversibly to a protein (like an antibody).
Substrate	A chemical compound on which the enzyme reacts specifically. This is the chemical used to product signals for reading.
Chromophore	A chemical that alters color as a result of enzyme interacting with substrate, allowing the ELISA to be quantified.
Reading	Measurement of the color produced in ELISA.

2.5.4 Novel detection methods - usage of quantum dots

Quantum dots (QDs) appeared in biological studies a few years ago, and many new biological methods using them have since been published. QDs were first known and used as reporter dyes to label probes or proteins. QDs have a crystalline structure, of which the dimensions are very small. This small crystal size can directly manipulate its electronic characteristics and optical properties (Woll and others 2002). QDs are semiconductors and their electronic properties are strongly related to the transitions between the edges of valence bands and conduction bands. As the band gap depends on the size of QDs, different sized QDs have different spectra.

In addition to its size-dependent fluorescence property, another advantage of QDs is that they can provide a high quantum yield and enhanced photostability. One reason that QDs can remain stable at room temperature is because of their coating layers. Coating nanoparticles by another material generates the "core-shell" nanoparticle structure. This outer layer can protect QDs from the external environment. The shell layers can also enhance the fluorescence of QDs by up to 20% (Banin and Millo 2004) and make the modification of QD surfaces easier. Based on these properties, QDs have the potential to be used in the detection of pathogenic cells from food with low concentrations. Colloidal method synthesized CdSe/ZnS nanocrystal is a core/shell structure nanoparticle. CdSe/ZnS core/shell QDs have been the most commonly used and the best understood QDs.

Biological studies using QDs can be divided into two main groups, imaging and detection. In the imaging group, QDs have been used in cell, tissue and *in vivo* live animal imaging. In the detection group, QDs have been used in immunoassays, such as

ELISA, Western blotting, flow cytometry, fluorescence resonance energy transfer (FRET) and array detections (Hotz 2008).

To apply QDs in biological studies, crosslinkers are built to conjugate them with different biological groups. Interactions between biotin and avidin are among the strongest non-covalent affinities. This interaction has a dissociation constant of about 1.3×10^{-15}M and is one of the most popular crosslinkers used for QD biological molecule conjugation. Other crosslinkers, such as zero length crosslinkers, homobifunctional crosslinkers, heterobiofunctional crosslinkers, and trifunctional crosslinkers, are also available for various conjugation purposes (Hermanson 1996).

2.5.5 Detection of VBNC cells

The *Bac*Light™ Live/Dead cell viability kit (Invitrogen, Carlsbad, CA) is one method that can differentiate live cells from dead cells. Through the staining of dead cells red by propidium iodide and live cells green by SYTO 9, VBNC cells can be detected due to their ability to be stained green. Unfortunately, both cell imaging and flow cytometry techniques can only be used for pure cultures and cannot separate target pathogenic cells from the background microflora. Thus, a detection method that can simultaneously separate target cells out of the background microflora and detect all viable cells (including VBNC cells) is needed.

Although debates still exist on the definition and formation of VBNC cells, one thing for sure is that by the time there are VBNC cells, there are also dead cells. Thus, how to prevent the amplification or fall positive results generated from dead cells remains a challenge. The PCR is a rapid and sensitive technique for pathogen detection and identification in food. However, conventional PCR cannot differentiate viable cells from

dead microbial cells (Wang and Levin 2006). DNA from dead cells can serve as a template during PCR amplification (Chaiyanan and others 2001; Nogva and others 2003; Wang and Levin 2006). The amplification of dead cells will result in false-positive data and cause unnecessary product recalls.

2.5.5.1 Reverse transcription PCR

Reverse transcription PCR (RT-PCR) is a PCR process targeting Ribonucleic acid (RNA). The RNA is only present in live cells and it won't persist after the cells die. Using RT-PCR seems a good way to differentiate live cells from dead cells, as dead cells' RNA won't exist and serve as PCR templates.

Unfortunately, the use of RT-PCR in foods is not as common as DNA based PCR, due to the difficulty of extracting undegraded mRNA from pathogens in complex food matrices. Successful applications of RT-PCR in foodborne pathogen detection are limited. McIngvale (2002) established an RT-PCR protocol for Shiga-toxin-producing *E. coli,* the assay was validated in artificially contaminated ground beef and it can detect viable *E. coli* O157:H7 at an initial inoculum of 1 cfu/g in the meat after 12-h enrichment.

2.5.5.2 Ethidium bromide monoazide PCR

Ethidium bromide monoazide (EMA) is a dye that has been used for microscopic differentiation of viable and dead cells. It has been suggested as a means to reduce PCR signals from DNA originating from dead cells (Nogva and others 2003; Flekna and others 2007). It can penetrate dead cells via their damaged cell walls and bind to cellular DNA. Following photolysis by bright visible light, EMA can produce a nitrene that forms a covalent link with DNA (Hixon and others 1975; DeTraglia and others 1978; Coffman

and others 1982), rendering the DNA insoluble and inhibit PCR amplification of DNA from dead cells (Nocker and Camper 2006; Nocker and others 2006). On the other hand, DNA in viable cells won't be stained by EMA and can, thus, be used as a template for a subsequent PCR step.

This technique has been used to detect foodborne pathogens including *E. coli* O157:H7, *S. enterica* (Guy and others 2006; Nocker and Camper 2006), *L. monocytogenes* in gouda-like cheeses (Rudi and others 2005b), *C. jejuni* in complex samples (Rudi and others 2005a), and *Vibrio vulnificus* in seafood (Wang and Levin 2006). Results from these studies showed that the addition of an EMA staining step had effectively prevented the amplification from dead cells. An EMA staining step, which takes about 30 min, together with DNA based PCR can be as powerful as reverse transcription PCR in the detection of viable cells.

2.5.5.3 Internal amplification control

An internal amplification control (IAC) is a sequence that is included in the same reaction tube as target DNA templates during PCR. It can prevent the occurrence of false negative results from the PCR reaction and monitor the efficiency of each reaction, without inhibiting the amplification of target sequences (Hoorfar and others 2004, Lambertz and others 1998). Addition of an IAC in each PCR reaction has been proposed to be a requirement for all diagnosed PCR since 2004 (Hoorfar and others 2004). Adding an IAC in the PCR system is as important as adding a negative control.

The IAC sequences can be conservative sequences present in target cells or pure plasmid DNA that is different with target sequences and commercially available. Plasmid pUC19 has been successfully employed as an IAC in a PCR method for

detecting *Bacillus cereus* in foods (Fricker and others 2007). It is a small, high copy number *E. coli* plasmid with a molecular weight of 2686 bp. Its availability on the market makes it a potential universal internal amplification control for foodborne pathogen detections.

REFERENCES

Altekruse SF, Cohen ML, Swerdlow DL. 1997. Emerging foodborne diseases. Emerg Infect Dis 3(3):285-93.

Asakura H, Kawamoto K, Haishima Y, Igimi S, Yanamoto S, Makino S. 2008. Differential expression of the outer membrane protein W (ompW) stress response in enterohemorrhagic *Escherichia coli* O157:H7 corresponds to the viable but non-culturable state. Res Microbiol 159(9-10):708-17.

Attenborough M, Matthews KR. 2000. Food safety through the meat supply chain. J Appl Microbiol Symposium supplement. 88:144s-48s.

Banin U, Millo O. 2004. Properties. In: Schmid G, editor. Nanoparticles. New Jersey: Wiley InterScience press. p 305-08.

Benson A. 2003. Molecular and population genetcis of virulence traits in *Escherichia coli* O157:H7. In: Torrence ME, Isaacson RE, editors. Microbial food safety in animal agriculture-current topics. Ames, IA: Iowa State Press. p 131-36.

Boerlin P, McEwen SA, Boerlin-Petzold F, Wilson JB, Johnson RP, Gyles CL. 1999. Associations between virulence factors of Shiga toxin–producing *Escherichia coli* and disease in humans. J Clin Microbiol 37:497-503.

Bogosian G, Bourneuf EV. 2001. A matter of bacterial life and death. EMBO Rep. 2:770-4.

Brown M. 2002. Hazard identification. In Brown M, Stringer M, editors. Microbiological risk assessment in food processing. Cambridge, UK: CRC press. p 64-70.

Brown M, Stringer M. 2002. Microbiological risk assessment in food processing. Cambridge, UK: CRC press. 280 p.

Callaway TR, Anderson RC, Edrington TS, Elder RO, Genovese KJ, Bischoff KM, Poole TL, Nisbet DJ. 2002. Preharvest intervention strategies to reduce foodborne pathogens in food animals. Accessed on July 8, 2009. Available from: http://www.asas.org/JAS/2002abs/jnabs76.pdf

CDC. 2001. Outbreaks of *Escherichia coli* O157:H7 and Campylobacter among attendees of the Washington county fair-New York, 1999. MMWR 50(15):293-7.

Chaiyanan S, Chaiyanan S, Huq A, Mangel T, Colwell RR. 2001 Viability of the nonculturable *Vibrio cholerae* O1 and O139. Syst Appl Microbiol 24(3): 331-41.

Chapman PA, Siddons CA. 1996. A comparison of immunomagnetic separation and direct culture for the isolation of verocytotoxin-producing *Escherichia coli* O157 from cases of bloody diarrhea, non-bloody diarrhea and asymptomatic contacts. J Med Microbiol 44: 267-71.

Coffman GL, Gaubatz JW, Yielding KL, Yielding LW. 1982. Demonstration of specific high affinity binding sites in plasmid DNA by photoaffinity labeling with ethidium analog. J Biol Chem 257(22): 13205-97.

Crowther J. 2008. Enzyme linked immunosorbent Assay (ELISA). In: Walker JM, Rapley R, editors. Molecular biomethods handbook, 2nd ed. Totowa, NJ: Humana Press. p 657-60.

DeTraglia MC, Brand JS, Tometsko AM. 1978. Characterization of azidobenzamidines as photoaffinity labeling for trypsin. J Biol Chem 253(6): 1846-52.

Downes FP, Ito K. 2001. Microbiological examination of foods. 4th ed. Washington, DC: American Public Health Association. 676 p.

FDA. 1973. Acidified foods and low acid foods in hermetically sealed containers in code of US federal regulations. In: Downes FP, Ito K, editors. Microbiological examination of foods. 4th ed. Washington, DC: American Public Health Association. 600 p.

Ferris KE, Miller DA. 1996. *Salmonella* serovars from animals and related sources reported during July 1995-June 1996. In proceedings of the 104 annual meeting of the United State animal health association. 100:505-26.

Flekna G, Stefanic P, Wagner M, Smulders FJ, Mozina SS, Hein I. 2007. Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. *Res Microbiol* 158(5): 405-12.

Fricker M, Messelhäußer U, Busch U, Scherer S, Ehling-Schulz M. 2007. Diagnostic real-time PCR assays for the detection of emetic *Bacillus cereus* strains in foods and recent food-borne outbreaks. Appl Environ Microbiol 73(6):1892–8.

FSIS. 1999. Microbiological hazards identification guide for meat and poultry components of products produced by very small plants. Accessed online on July 6, 2009. Available from: http://www.fsis.usda.gov/oa/haccp/hidguide.htm.

Gahan CG, Hill C. 1999. The relationship between acid stress response and virulence in *Salmonella typhimurium* and *Listeria monocytogenes*. Int J Food Microbiol 50:93-100.

Glass KA, Loeffelholz JM, Ford JP, Doyle MP. 1992. Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride and in fermented, dry sausage. Appl Environ Microbiol 58(8):2513-6.

GMP institute. 2009. Accessed on June 29 2009. Available from http://www.gmp1st.com/gmp.htm.

Guy, R.A., Kappor, A., Holicka, J., Shepherd, D. and Horgen, P.A. (2006) A rapid molecular-based assay for direct quantification of viable bacteria in slaughterhouses. *J. Food Prot.* 69(6), 1265-72.

He H, Dong J, Lee CN, Li Y. 2009. Molecular analysis of spoilage-related bacteria in pasteurized milk during refrigeration by PCR and denaturing gradient gel electrophoresis. J Food Prot 72 (3):572-7.

Hermanson GT. 1996. Bioconjugate techniques. San Diego, CA: Academic Press. 1202 p.

Hixon SC, White WEJ, Yielding KL. 1975. Selective covalent binding of an ethidium analog to mitochondrial DNA with production of petite mutants in yeast by photoaffinity labeling. J Mol Biol 92(2): 319-29.

Hoorfar J, Cook N, Malorny B, Wagner M, De Medici D, Abdulmawjood A, Fach P. 2004. Diagnostic PCR: making internal amplification control mandatory. J Clin Microbiol 96:221-2.

Hotz CZ. 2008. Quantum Dots. In: Walker JM, Rapley R, editors. Molecular biomethods handbook, 2nd ed. Totowa, NJ: Humana Press. 697 p.

Klančnik A, Guzej B, Jamnik P, Vučković D, Abram M, Možina SS. 2009. Stress response and pathogenic potential of *Campylobacter jejuni* cells exposed to starvation. Res Microbiol [Epub ahead of print].

Lambertz ST, Ballagi-Pordany A, Lindqvist R. 1998. A mimic as internal standard to monitor PCR analysis of food-borne pathogens. Lett Appl Microbiol 26:9-11.
Lawley R, Curtis L, Davis J. 2008. The Food safety hazard guidebook. Cambridge, UK: RSC publishing. 427 p.

MAFF. 2000. *Salmonella* in livestock production. Addlestone, UK: Veterinary Laboratories Agency. 381 p.

Matsui H, Bacot CM, Garlington WA, Doyle TJ, Reberts S, Gulig PA. 2001. Virulence plasmid-borne *spv*B and *spv*C genes can replace the 90-kilobase plasmid in conferring virulence to *Salmonella enterica* serovar Typhimurium in subcutaneously inoculated mice. J Bacteriol 183: 4652-8.

McIngvale SC, Elhanafi D, Drake MA. 2002. Optimization of reverse transcriptase PCR to detect viable Shiga-toxin-producing *Escherichia coli*. Appl Environ Microbiol 68:799-806.

Monna L, Miyao A, Inoue T, Fukuoka S, Yamazaki M, Zhong HS, Sasaki T, Minobe Y. 1994. Determination of RAPD markers in rice and their conversion into sequence tagged sites (STSs) and STS-specific primers. DNA Res 1:139-48.

Montgomery SM. 2002. In site Hybridization. Accessed online on July 7, 2009. Available from: http://osiris.rutgers.edu/~smm/in_situ_hybridization.htm

Mukamolova GV, Kaprelyants AS, Kell DB, Young M. 2003. Adoption of the transiently non-culturable state - a bacterial survival strategy. In: Rose AH, Wilkinson JF, editors. Advances in Microbial Physiology. UK: Elsevier Science Ltd. 450 p.

Mustapha A, Li Y. 2006. Molecular Detection of Foodborne Bacterial Pathogens. In Maurer J, editor. PCR methods in foods. New York, NY: Springer. 69 p.

National restaurant association educational foundation. 2009. Accessed on July 1 2009. Available from: http://www.nraef.org/foodsafetycenter/downloads/factsheets_activities/minimum_internal.pdf

Nocker A, Camper AK. 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. Appl Environ Microbiol 72(3): 1997-2004.

Nocker A, Cheung CY, Camper AK. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods 67(2), 310-20.

Nogva HK, Dromtorp SM, Nissen H, Rudi K. 2003. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR. Biotech 34(4): 804-13.

Notermans S, Barendsz AW. 2002. The evolution of microbiological risk assessment. In: Brown M, Stringer M, editors. Microbiological risk assessment in food processing. Cambridge, UK: CRC press. 5 p.

Oliver JD. 2005. The viable but nonculturable state in bacteria. J Microbiol 43:93-100.

Paton JC, Paton AW. 1998. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin Microbiol Rev 11: 450-79.

Patterson S, Isaacson R. 2003. Genetics and pathogenesis of *Salmonella*. In Torrence ME, Isaacson RE, editors. Microbial food safety in animal agriculture-current topics. Ames, IA: Iowa State Press. 89 p.

Pennington TH. 1997. The Pennington Group Report on the circumstances leading to the 1996 outbreak of infection with *E. coli* O157 in Central Scotland, the implications for food safety and the lessons to be learned. Edinburgh: The Stationary Office. 64 p.

Reissbrodt R, Heier H, Tschape H, Kingsley RA, Williams PH. 2000. Resuscitation by ferrioxamine E of stressed *Salmonella enterica* serovar Typhimurium from soil and water microcosms. Appl Environ Microbiol 66:4128-30.

Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, Hebert RJ, Olcott ES, Johnson LM, Hargrett NT, Blake PA, Cohen ML. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med 308(12):681-5.

Rivers B, Steck TR. 2001. Viable but nonculturable uropathogenic bacteria are represent in the mouse urinary tract following urinary tract infection and antibiotic therapy. Urol Res 29:60-6.

Roszak DB, Colwell RR. 1987. Survival strategies of bacteria in the natural environment. Microbiol Rev 51:365-79.

Rudi K, Moen B, Dromtorp SM, Holck AL. 2005a. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl Environ Microbiol 71(2): 1018-24.

Rudi K, Naterstad K, Dromtorp SM, Holo, H. 2005b. Detection of viable and dead Listeria monocytogenes on gouda-like cheeses by real-time PCR. Lett Appl Microbial 40(4): 301-6.

Sargeant JM, Smith DR. 2003. The epidemiology of *Escherichia coli* O157:H7. In Torrence ME, Isaacson RE, editors. Microbial food safety in animal agriculture-current topics. Ames, IA: Iowa State Press. 131 p.

Sparling PH. 1998. *Escherichia coli* O157:H7 outbreaks in the United States, 1982-1996. JAVMA. 213(12):1733.

Tarr PI, Bilge SS, Vary Jr. JC, Jelacic S, Habeeb RC, Ward TR, Baylor MR, Besser TE. 2000. *Iha*: a novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. Infect Immun 68:1400-7.

Tatsuno I, Horie M, Abe H, Miki T, Makino K, Shinigawa H, Taguchi H, Kamiya S, Hayashi T, Sasakawa C. 2001. *tox*B gene on pO157 of enterohenorrhagic *Escherichia*

coli O157:H7 is required for full spithielal cell adherence phenotype. Infect Immun 69:6660-9.

Todd ECD. 1989. Preliminary estimates of costs of foodborne disease in the United States. J Food Prot 595-601.

Tuttle J, Gomez T, Doyle M, Wells J, Zhao T, Tauxe R, Griffin P. 1999. Lessons from a large outbreak of *E. coli* O157 infections. Epidemiol Infect 122: 192-5.

Van Ermengem E. 1896. Ueber einem neuen anaeroben *Bacillus* und seine Beziehungen zum Botulismus. English translation (1979). Rev Infect Dis 1: 701-19.

Voysey P, Jewell K, Stringer M. 2002. Risk characterization. In: Brown M, Stringer M, editors. Microbiological risk assessment in food processing. Cambridge, UK: CRC press. 77 p.

Wang S, Levin RE. 2006 Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. J Microbial Methods 64(1): 1-8.

Wang L, Li Y, Mustapha A. 2007. Rapid and simultaneous quantitation of *Escherichia coli* O157:H7, *Salmonella* and *Shigella* in ground beef by multiplex real-time PCR and immunomagnetic separation. J Food Prot 70(6):1366-72.

Woll AR, Rugheimer P, Lagally MG. 2002. Self-organized quantum dots. Int J High Speed El Sys 12(1):45-78.

Wray C, Davies RH. 2003. The epidemiology an ecology of *Salmonella* in meat-producing animals. In: Torrence ME, Isaacson RE, editors. Microbial food safety in animal agriculture-current topics. Ames, Iowa: Iowa State Press. 73 p.

Yang L, Li Y. 2005. Quantum dots as fluorescent labels for quantitative detection of *Salmonella* Typhimurium in chicken carcass wash water. J Food Prot 68(6): 1241-5.

Zhao Y, Ye M, Chao Q, Jia N, Ge Y, Shen H. 2009. Simultaneous detection of multifoodborne pathogenic bacteria based on functionalized quantum dots coupled with immunomagnetic separation in food samples. J Agric Food. Chem 57:517-24.

Zhong L, Chen J, Zhang XH, Jiang YA. 2009. Entry of *Vibrio cincinnatiensis* into viable but nonculturable state and its resuscitation. Lett Appl Microbiol 48(2):247-52.

CHAPTER 3

COMPLETED STUDIES

Three studies were completed for this dissertation. Two projects focused on the detection of only viable cells from artificially contaminated products, while the other one applied quantum dots (QDs) as novel protein labeling components to detect *Escherichia coli* O157:H7 and *Salmonella* from ground beef by a QD-facilitated bead-free detection method.

3.1 Detection of only viable cells by DNA based real-time PCR

Polymerase Chain Reaction (PCR) has successfully been proven to be a powerful tool in food pathogen detection, due to its ability to separate one species from another. Due to this property, PCR was chosen to be the foundation detection method in this study. Reverse transcription PCR (RT-PCR), which can detect only RNA (from live cells), attracted our attention. Testing the efficiency of RT-PCR in food pathogen detection and comparing it to DNA based PCR is one task of this study.

Ethidium monoazide (EMA) is a dye used for cell staining. Recent research discovered its ability to inhibit DNA amplification from dead cells. A DNA based PCR with an additional EMA staining process to separate live cells from dead cells in the amplification process seemed a good replacement for RT-PCR. Thus, applying EMA combined with DNA-based PCR to detect only viable cells from artificially contaminated products was another task of this study.

3.2 Bead-free QD-facilitated detection method

The proposed QD study was a project that combines QDs, ELISA principle, immunomagnetic separation mechanisms, and bioconjugation techniques together. This bead-free QD-facilitated detection method was designed to detect foodborne pathogens from artificially contaminated ground beef products. The immunomagnetic separation (IMS) method is an isolation and purification method that is commercially available. Based on the principle of antibodies vs. antigens, cells can be separated from the background matrix with the help of magnetic beads and a magnet. Conjugation processes and conjugation crosslinkers were optimized, in order to ensure that the QD antibody conjugates were in uniform structures and had stable labeling efficiency.

CHAPTER 4

Running Heads: *E. coli* O157:H7 detection in beef by EMA-real-time PCR

DETECTION OF VIABLE *ESCHERICHIA COLI* O157:H7 By ETHIDIUM MONOAZIDE REAL-TIME PCR

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ABSTRACT

Aims: The aim of this study was to develop and optimize a novel method that combines ethidium bromide monoazide (EMA) staining with real-time PCR for the detection of viable *E. coli* O157:H7 in ground beef. EMA can penetrate dead cells and bind to intracellular DNA, preventing its amplification via PCR.

Methods and Results: Samples were stained with EMA for 5 min, iced for 1 min and exposed to bright visible light for 10 min prior to DNA extraction, to allow EMA binding of the DNA from dead cells. DNA was then extracted and amplified by TaqMan® real-time PCR to detect only viable *E. coli* O157:H7 cells. The primers and TaqMan® probe used in this study targeted the *uid*A gene in *E. coli* O157:H7. An internal amplification control (IAC), consisting of 0.25 pg of plasmid pUC19, was added in each reaction to prevent the occurrence of false negative results. Results showed a reproducible application of this technique to detect viable cells in both broth culture and ground beef. EMA, at a final concentration of 10 μg/ml, was demonstrated to effectively bind DNA from 10^8 CFU/ml dead cells and the optimized method could detect as low as 10^4 CFU/g viable *E. coli* O157:H7 cells from ground beef without interference from 10^8 CFU/g dead cells.

Conclusions: EMA real-time PCR with IAC can effectively separate dead from viable *E. coli* O157:H7 cells and prevent amplification of DNA in the dead cells.

Significance and Impact of the Study: The EMA real-time PCR in this work has the potential to be a highly sensitive quantitative detection technique to assess contamination of viable *E. coli* O157:H7 in ground beef and other meat or food products.

Key words: ethidium monoazide, real-time PCR, *Escherichia coli* O157:H7, ground beef

INTRODUCTION

Escherichia coli O157:H7, which is responsible for approximately 73,500 cases of infections in the United States each year (Mead and others 1999), can colonize the intestinal tract of cattle and be introduced into beef products during slaughtering and subsequent processing. A recent study by Frenzen and others (2005) estimated that "the annual cost of *E. coli* O157:H7 illnesses is $405 million, which includes $370 million for premature deaths, $30 million for medical care and $5 million for lost productivity" (Frenzen and others 2005). Different intervention methods have been used in the meat industry to prevent or decrease *E. coli* O157:H7 contamination from various potential sources. Thermal processing is one of the most common methods applied to foods to inactivate pathogens like *E. coli* O157:H7 (Erickson and Doyle 2007) because this organism is not heat resistant (Kaur and others 1998). However, Smith and others (2001) found that the resistance of *E. coli* O157:H7 to heat varies significantly depending on the beef fat content. These authors found that the *E. coli* O157:H7 cells are more resistant to heat in beef containing 19% fat than that containing 4.8% fat.

Even though common heating and processing methods will kill many bacteria that may spoil food or cause foodborne diseases, some can survive by activating certain survival mechanisms under environmental stresses (Roszak and Colwell 1987). Surviving pathogens are the ones that can potentially cause foodborne diseases. Thus, a sensitive and accurate method to detect these pathogens in foods, without a false positive result from dead cells, is much needed.

The polymerase chain reaction (PCR) is a rapid and sensitive technique for pathogen detection and identification in food. However, conventional PCR cannot differentiate viable from dead microbial cells (Wang and Levin 2006). DNA from dead cells, killed by processing procedures or other factors, can serve as a template during PCR amplification (Chaiyanan and others 2001; Nogva and others 2003; Wang and Levin 2006). The presence of dead pathogenic cells and the limitation of conventional PCR may result in false-positive results which, in turn, could cause unnecessary product recalls or foodborne illnesses.

Ethidium bromide monoazide (EMA) is a dye that has been used for microscopic differentiation of viable and dead cells. It has been suggested as a means to reduce PCR signals from DNA originating from dead cells (Nogva and others 2003; Flekna and others 2007). The minimum EMA amount needed to prevent DNA from dead cells from being amplified by PCR varies from 1 μg/ml (Lee and Levin 2006) to 100 μg/ml (Rudi and others 2005a) depending on different target species. EMA can penetrate dead cells via their damaged cell wall and bind to cellular DNA. Following photolysis by bright visible light, EMA can produce a nitrene that forms a covalent link with DNA (Hixon and others 1975; DeTraglia and others 1978; Coffman and others 1982). This crosslink will render the DNA insoluble and inhibit PCR amplification of DNA from dead cells (Nocker and Camper 2006; Nocker and others 2006). On the other hand, DNA in viable cells is not usually affected by EMA and can, thus, be used as a template for a subsequent PCR step. This technique has been used to detect foodborne pathogens including *E. coli* O157:H7, *Salmonella enterica* (Guy and others 2006; Nocker and Camper 2006), *Listeria monocytogenes* in gouda-like cheeses (Rudi and others 2005b), *Campylobacter jejuni* in

complex samples (Rudi and others 2005a), and *Vibrio vulnificus* in seafood (Wang and Levin 2006). However, it has been reported that DNA amplification from viable cells of certain bacteria, such as *Enterobacter sakazakii* (Cawthorn and Witthuhn, 2008), *L. monocytogenes* (Flekna and others 2007; Nocker and others 2006; Pan and Breidt 2007), and *C. jejuni* (Flekna and others 2007) can also be negatively influenced by EMA staining.

Compared with conventional PCR, real-time PCR is a more sensitive and rapid DNA detection method. Changes in fluorescence intensity in this technique result in an accurate estimation of bacterial targets present in food products (Sharma 2002). In this study, real-time PCR was combined with EMA staining for selectively detecting viable *E. coli* O157:H7 cells from beef samples.

The addition of an internal amplification control (IAC) in a real-time PCR reaction system allows one to monitor the efficiency of each reaction and prevent false negative results (Lambertz and others 1998; Hoorfar and others 2004; Murphy and others 2007). pUC19 is a small, high copy number *E. coli* plasmid with a molecular weight of 2686 bp. It has been previously used with success as an IAC in real-time PCR (Fricker and others 2007). Further, a negative control (water) was added to ensure that no DNA cross-contamination occurred in the PCR reaction.

The aim of this study was to design a novel real-time PCR system that is coupled to an EMA staining step for detection and estimation of only *viable E. coli* O157:H7 cells in ground beef. To prevent false negative results, a pUC19 IAC was added in each PCR reaction.

MATERIALS AND METHODS

Preparation of viable and dead *E. coli* O157:H7 cells. Fresh *E. coli* O157:H7 was prepared by growing it overnight in tryptic soy broth supplemented with 0.5% yeast extract (TSBY) (Difco Labs., BD Diagnostic Systems, Sparks, MD, USA), and serially diluting to generate cell suspensions representing 10 to 10^8 CFU/ml. To obtain dead *E. coli* O157:H7 cells, freshly grown cultures were washed in distilled water and the cell pellets were resuspended in an equal volume of distilled water. The suspension was heat-treated at 95^{o}C for 30 min. Cell viability was confirmed by plating in Plate Count Agar (Difco Labs.) and by the use of the Live/Dead® *Bac*Light™ bacterial viability kit (Invitrogen™, Eugene, OR, USA).

Minimum EMA amount needed to bind dead cell DNA. Eight dead *E. coli* O157:H7 samples (10^8 CFU/ml each) were prepared as described above. Different amounts of EMA (0, 1, 10, 20, 30, 40, 50, 100 μg) were added to 1 ml of each sample. Samples were stained with EMA in the dark at room temperature for 5 min and exposed to a 650-W halogen light for 10 min. Real-time PCR was conducted with DNA from the EMA-treated samples.

Influence of EMA on DNA amplification from viable cells. Fresh viable 10^8 CFU/ml *E. coli* O157:H7 cells were washed and the cell pellets were resuspended in distilled water. Varying amounts (0, 1, 10, 20, 30, 40, 50, 100 μg) of EMA were respectively added to 1 ml of the cell suspension. Samples were covered with foil, incubated in the dark at room temperature for 5 min and exposed to a 650-W halogen light for varying lengths of time. The influence of EMA on DNA amplification from

viable cells was evaluated by real-time PCR after extracting the DNA by the method of Rudi and others (2005a). DNA from 10^8 CFU viable cells, which was not treated with EMA staining or light exposure, was used as a control to evaluate the EMA effect.

DNA sample preparation after EMA staining. After a 10-min light exposure, EMA-stained samples were centrifuged at 12,000 × *g* for 5 min. Cell pellets were washed once in distilled water and resuspended in 100 µl of PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems-ABI, Foster City, CA, USA). Cell suspensions were incubated at 56°C for 30 min, vortexed for 10 s, and boiled for 8 min (PrepMan® Ultra protocol 1998, ABI, USA; Rudi and others 2005a). Upon centrifugation at 12,000 × *g* for 2 min, DNA extracts in the supernatants were recovered. Ten microliters of each DNA extract were used to perform real-time PCR.

Optimization of EMA staining by ice treatment. To avoid or minimize the influence of heat generated by the 650-W halogen lamp during EMA photolysis, varying ice treatments were applied to viable *E. coli* O157:H7 cell samples during or after EMA staining. The treatment variations were specifically: (1) incubation with EMA on ice for 5 min in the dark, followed by a 10-min light exposure, (2) dark incubation with EMA for 5 min at room temperature followed by a 1-min ice incubation step and a 10-min light exposure, and (3) incubation with EMA in the dark for 5 min at room temperature, followed by a 10-min light exposure on ice. Aluminum foil was used to cover the tubes during the 5-min dark incubation periods. Two controls, one with a 5-min dark incubation and a 10-min light exposure without any ice treatment, and the other without EMA treatment, light exposure, or ice treatment, were also processed.

Primers and probes. Primers and probe targeting *E. coli* O157:H7 were designed and their specificities tested by the same authors (Wang and others 2007). The sequence of *E. coli* O157:H7 primer 1 is 5'-TTGACCCACACTTTGCCGTAA-3', that of *E. coli* O157:H7 primer 2 is 5'-GCGAAAACTGTGGAATTGGG-3', and that of *E. coli* O157:H7 probe is 5'-VIC-TGACCGCATCGAAACGCAGCT-TAMRA-3'. Sequences of the IAC primers and probe were designed by Fricker and others (2007). They are IAC-for 5'-GCAGCCACTGGTAA CAGGAT-3', IAC-rev 5'-GCAGAGCGCAGATACCAAAT-3', and 5'-FAM-AGAGCGAG GTATGTATGTAGGCGG-TAMRA-3', respectively, for forward and reverse primers, and probe sequences.

Real-Time PCR with IAC. Real-Time PCR was carried out in a 96-well ABI Prism® 7700 Sequence Detection System (ABI, Foster City, CA, USA). Each 50 µl reaction mixture contained 25 µl of TaqMan™ Universal PCR Master Mix (2×, ABI, Branchburg, NJ, USA), 0.5 µM *E. coli*-1 primer, 0.5 µM *E.coli-2* primer, 0.2 µM *E. coli* probe and plasmid pUC19 IAC (Fermentas, St. Leon-Rot, Germany), which was diluted to 0.05 pg/µl in nuclease-free water (Promega, USA). In each reaction, 1 µl IAC forward primer (0.4 µM), 1 µl IAC reverse primer (0.4 µM), 1 µl IAC probe (0.2 µM) and 5 µl of diluted pUC19 (8.62×10^4 copies) were added to the real-time PCR system, together with *E. coli* primers, *E. coli* probe and PCR Master Mix. Nuclease-free water (Promega, Madison, WI, USA) was used to adjust the reaction volume to 50 µl. DNA samples (10 µl) were then added to each reaction. The negative control, water, was separately added to one reaction tube containing the Master Mix and all primers and probes, except

pUC19. Real-time PCR was performed through 40 cycles of 95°C for 15 s and 60°C for 1 min after preheat steps of 50°C for 2 min and 95°C for 10 min.

Application of EMA real-time PCR to mixed viable and dead cells. One milliliter of dead *E. coli* O157:H7 cells (10^8 CFU/ml) was mixed with 1 ml of fresh viable cells (10^8 CFU/ml) and centrifuged at 12,000 × *g* for 5 min. The mixed cell pellets were resuspended in 1 ml distilled water. Ten micrograms of EMA were added to reach a final concentration of 10 μg/ml. EMA staining and DNA extraction were conducted as described above. Real-Time PCR with IAC was conducted to check the results.

Application of EMA-real-time PCR to artificially contaminated beef. Ground beef with a fat content of 10% was purchased from a local supermarket. It was determined to be free of *E. coli* O157:H7 by standard cultural methods (FDA 1995) and real-time PCR. Two series of *E. coli* O157:H7-inoculated ground beef samples were prepared. The first series of eight samples was prepared by adding only viable *E. coli* O157:H7 cells at different concentrations (10^1 CFU/g to 10^9 CFU/g) to each 25 g of ground beef. The second series of eight samples was prepared by adding 10^8 CFU/g dead *E. coli* O157:H7 cells and 10^1 - 10^9 CFU/g viable *E. coli* O157:H7 cells to each of 25 g of ground beef.

Each 25 g sample in series 1 and 2 was added to 225 ml TSBY, followed by 2 min of homogenization by stomaching. One milliliter of homogenized suspension from each sample in series 1 was taken for DNA extraction, following EMA staining. Two milliliters of homogenized ground beef suspension from series 2 were removed and divided into two 1-ml portions, one for DNA extraction without prior EMA staining, and the other for EMA staining followed by DNA extraction, as was optimized above. Ten

microliters of each DNA sample were then taken for conducting the real-time PCR as described above.

Detection of low concentrations of viable *E. coli* O157:H7 from beef by EMA-real-time PCR. Ground beef with four different fat contents (4%, 10%, 20%, 27%) were purchased from a local supermarket and determined to be free of *E. coli* O157:H7. Twenty-five grams of each of four ground beef samples were artificially inoculated with freshly grown *E. coli* O157:H7 at levels of 10, 10^2, 10^3 and 10^4 CFU/g, and dead *E. coli* O157:H7 cells (10^6 CFU/g), massaged to mix and homogenized for 5 min. Each sample was added to 225 ml TSBY broth, and the suspension stomached for 2 min and incubated at 35^{o}C for 24 h. One milliliter of each sample was collected at different enrichment times (0, 8, 12 and 24 h) and centrifuged at 600 × *g* for 1 min to separate out the meat tissues and fat. Cells were collected and washed by centrifuging for 5 min at 12,000 × *g*, and resuspended in 1 ml of distilled water.

EMA staining, DNA extraction and real-time PCR were conducted as described above. At the same time, another 1 ml enrichment broth from each beef suspension was also removed at the different enrichment times and used directly for DNA extraction without prior EMA staining. Primers, probes, reagent concentrations and PCR reaction conditions used for the two sets of DNA samples were the same as described above.

Statistical data analysis. Statistical analyses were conducted using the SAS GLM procedure (SAS 9.1, Copyright 2002-2003, SAS Institute Inc., Cary, NC, USA) to evaluate the effect of EMA staining on dead and viable cells. Fisher's Least Significant Difference (LSD) test was applied to determine differences between various EMA staining, light exposure treatments and their combinations. Different treatments and

treatment combinations were arranged as 7×4 factorials following a randomized complete block design.

RESULTS

Minimum EMA amount required to inhibit DNA amplification from 10^8 CFU dead cells. Heat-killed *E. coli* O157:H7, at a concentration of 10^8 CFU/ml, were stained for 5 min in the dark with 0, 1, 10, 20, 30, 40, 50, or 100 µg/ml EMA, and exposed to a 650 W halogen lamp for 10 min. As shown in Figure 4.1, DNA from dead cells that were not stained with EMA was amplified and could generate false-positive signals, as indicated by a *Ct* value of 31.07. This confirmed the fact that PCR does not distinguish between DNA from dead or live cells. Even though this *Ct* value represented DNA from dead cells, the final PCR result would be "positive" for *E. coli* O157:H7. By staining dead cells with different EMA concentrations, the *Ct* value of the PCR increased to a point where no DNA amplification occurred after 40 real-time PCR cycles. A minimum EMA concentration of 10 µg/ml was necessary for complete binding and inhibition of DNA amplification from all dead cells by real-time PCR (Figure 4.1). The amplification of the IAC (pUC19) in each reaction tube indicated the efficiency of the PCR reaction, thus avoiding any false negative results.

Influence of EMA on DNA amplification from viable cells. Different amounts of EMA were added to 1 ml of freshly grown 10^8 CFU/ml *E. coli* O157:H7 to reach final concentrations of 0, 1, 10, 20, 30, 40, 50, and 100 µg/ml, respectively. The influence of EMA on DNA amplification from viable cells was evaluated by real-time PCR. Figure 2.2 shows that the higher the EMA concentration in the sample, the greater its inhibition

of DNA amplification, as shown by increasing *Ct* values ($P \leq 0.05$). Likewise, EMA staining has also been shown to cause an underestimation of viable cells by up to 4 log CFU for certain cells, such as *Anoxybacillus flavithermus* (Rueckert and others 2005). Further, the length of light exposure time showed the potential to influence the PCR results, especially when the concentration of EMA was higher than 10 μg/ml. When the EMA present in the sample was ≤10 μg/ml, no significant differences between the four light exposure times were found ($P > 0.05$). When the EMA concentration was higher than 10 μg/ml, increasing the light exposure time generally decreased the influence of EMA, as shown by EMA concentrations of 20, 40, and 50 μg/ml. For example, when the EMA concentration was 20 μg/ml and the light exposure time was increased from 1 min to 2, 5 and 10 min, the *Ct* value kept decreasing. This demonstrated that longer light exposure times could decrease the inhibition of DNA amplification by excess EMA (Figure 4.2).

Optimization of the EMA staining procedure. Figure 4.2 shows that light exposure can decrease EMA's inhibition of DNA amplification. On the other hand, it was also found that an extended light exposure time can generate possible inhibition on DNA amplification. For example, at an EMA concentration of 30 μg/ml, the *Ct* value increased if the light exposure time was extended from 5 to 10 min (Figure 4.2). One possible reason is due to the heat generated by the 650-W halogen lamp to which the samples were exposed. This exposure may kill some of the viable cells or damage their cell wall, which allowed the EMA to penetrate and bind intracellular DNA. To avoid an underestimation of *E. coli* O157:H7 count, different ice treatments were applied to the EMA-stained samples. Compared to the *Ct* values from the control that received no ice

treatment, which varied from 32 to 36 (standard deviation 1.54), the standard deviation of the samples with ice treatments decreased from 1.54 to 0.3 (data not shown), indicating a better replication. Although no significant differences were detected between the three ice treatments, the 1-min ice treatment, which was added between the 5 min EMA room temperature dark incubation and the 10-min halogen light exposure, yielded a *Ct* value closest to that of the untreated control and had the smallest standard deviation (data not shown).

Application of EMA real-time PCR to mixed viable and dead cells. One milliliter of 10^8 CFU/ml dead *E. coli* O157:H7 cells was mixed with 1 ml of 10^8 CFU/ml fresh viable cells and the mixture centrifuged at 12,000 × *g* for 5 min. The mixed cell pellets were re-suspended in 1 ml of distilled water, and 10 μl of EMA were added to a final concentration of 10 μg/ml. With the addition of EMA, DNA from dead (heat-treated) cells could not be amplified ($P \leq 0.05$) and DNA from viable cells generated a *Ct* value of 24.93 (sample 2, Figure 4.3). The *Ct* value of the mixed cultures was 26.4, which was higher than that of viable cells, indicating a lower estimation of viable cell numbers ($P \leq 0.0001$). This may be caused by the presence of a high number of dead cells that could have interfered with DNA extraction or amplification from viable cells.

Application of EMA real-time PCR to artificially contaminated beef. Ground beef purchased from a local supermarket was tested by FDA standard cultural-based methods and real-time PCR. Both tests showed that the beef samples for further inoculation was *E. coli* O157:H7-free. EMA real-time PCR with IAC was used to detect only viable *E. coli* O157:H7 cells in ground beef. Ground beef samples were simultaneously inoculated with 10^8 CFU/g dead *E. coli* O157:H7 cells and different

concentrations of viable *E. coli* O157:H7 cells (10^1 CFU/g to 10^9 CFU/g). This set of samples was named the test group. To analyze the efficiency of the EMA real-time PCR protocol optimized in this study, two control experiments were included in the test group. The first control used EMA real-time PCR to detect *E. coli* O157:H7 from ground beef samples which contained only viable cells (10^1 - 10^9 CFU/g in each sample). In the second control, beef samples were prepared exactly as the test group, but DNA extraction from all samples was conducted without prior EMA staining, followed by the real-time PCR. The purpose of adding these two control experiments were to determine if the EMA real-time PCR can sensitively detect only viable cells present in ground beef and to determine if EMA can effectively bind all available dead cells in the presence of meat tissues.

Figure 4.4c shows that the EMA real-time PCR with IAC could detect as low as 10^4 CFU/g of *E. coli* O157:H7 cells from ground beef samples, when both dead and viable cells were present. When the EMA staining step was omitted prior to DNA extraction, the real-time PCR was unable to differentiate DNA from viable cells to that from dead cells (Figure 4.4b), resulting in DNA amplification from both types of cells. Because DNA from dead cells served as the template for PCR, the result was an overestimation of the number of viable cells. For example, when 10^1 CFU/g of viable cells were present in ground beef together with 10^8 CFU/g dead cells, the real-time PCR generated a *Ct* value of 25 (Figure 4.4b). This *Ct* value corresponds to the *Ct* value gained from 10^7 CFU/g viable cells (Figure 4.4a). These results indicated that the addition of the proper EMA staining step prior to DNA extraction, allowed the

subsequent real-time PCR to effectively eliminate false positive results that could be generated by the presence of DNA from dead cells.

Detection of low concentrations of viable *E. coli* O157:H7 in ground beef by EMA real-time PCR with IAC. Ground beef with four different fat contents were artificially inoculated with 10^1, 10^2, 10^3 or 10^4 CFU/g of viable *E. coli* O157:H7 and 10^6 CFU/g dead cells, and enriched in TSBY broth for up to 24 h at 37°C. One milliliter of each beef suspension was subjected to EMA staining and real-time PCR with IAC as described above for identification of viable cells after 0, 8, 12, and 24 h enrichment times. With an 8-h enrichment, viable *E. coli* O157:H7 at initial concentrations of 10^3 and 10^4 CFU/g could be detected in the beef, while lower concentrations of 10^1 and 10^2 CFU/g could not. However, after a 12-h enrichment, the concentration of viable cells, ranging from 10 CFU/g to 10^4 CFU/g could all be detected (Table 4.1). In the meantime, 1 ml from each beef enrichment broth at 0, 8, 12, and 24 h was taken for DNA extraction without prior EMA staining. These DNA samples were used as a control to test the efficiency of EMA staining. By comparing the DNA amplification results from EMA-treated samples to samples that were not stained by EMA, it was clear that without the initial EMA staining, DNA from dead cells could be detected at 0 h. It proves, again, the necessity for adding an extra EMA staining step before DNA extraction for real-time PCR to insure accurate detection of *E. coli* O157:H7 from food samples. One beef sample which was contaminated with only 10^6 CFU/g dead cells was also included as a negative control. After up to 12 h of enrichment, a detection signal was still generated from the PCR if the sample was not stained with EMA before DNA extraction.

DISCUSSION

Real-Time PCR is a highly sensitive technique that can be used for detection of pathogens in food samples by amplifying DNA from target organisms in a real-time manner. Because it can detect more than one target sequence at a time, an IAC, added to the same reaction system as target sequences, would ensure the efficiency of each PCR reaction and prevent the occurrence of false negative results. EMA is a dye that can selectively bind DNA in dead cells, allowing for DNA from only viable cells to be amplified by PCR. The Live/ Dead® *Bac*Light™ bacterial viability kit (Invitrogen) was used in this study to check the dead cells obtained by heat treatment (95^{o}C for 30 min). The staining results showed that, after heat treatment, all cells were dead but most were not destroyed, as shown by a *Ct* value that corresponded to 10^5 CFU/ml from the initial 10^8 CFU/ml dead cells (Figure 4.1). This indicated that EMA can effectively bind to the intracellular DNA in dead cells. By combining EMA staining with real-time PCR, this study, to our knowledge, is the first to use a real-time PCR system with an IAC to detect only viable *E. coli* O157:H7 cells from ground beef.

To optimize the EMA staining procedure, various possible concentrations of EMA and visible light exposure times were tested for staining dead cells so that an optimal concentration that would effectively inhibit DNA amplification from dead cells, without influencing DNA amplification from viable cells, could be determined. Data from all treatments were pooled and illustrated in Figures 4.1 and 4.2. According to Figure 4.1, the concentration of EMA higher than 5 μg/ml was necessary to stain as much as 10^8 CFU dead cells. A concentration of 10 μg/ml could completely prevent the DNA amplification from 10^8 CFU dead cells. After using different amounts of EMA to treat

viable cells, 10 μg/ml EMA was found not to inhibit the amplification of viable cells. Thus, 10 μg/ml EMA was decided to be used for further studies.

Bright visible light can photolyse EMA, resulting in a nitrene that forms a covalent link with DNA. While the light exposure denatures excess unbound EMA in the reaction mixture and lowers its inhibition on PCR amplification, increasing light exposure times can also cause an underestimation of viable cells, by resulting in a higher *Ct* value. For example, when the concentration of EMA was 50 μg/ml, a 1-min light exposure did not denature all of the unbound EMA, resulting in complete inhibition of DNA amplification from 10^8 CFU/ml viable cells (Figure 4.2). After the light exposure time was extended to 2 and 5 min, the inhibition of PCR reaction by 50 μg/ml EMA decreased and the viable cells could be detected. When the length of light exposure time was extended to 10 min, the *Ct* value could not be measured, which indicated that the heat generated from the light could have killed any remaining viable cells. The effect of light exposure is more obvious when the EMA's concentration is higher than 10 μg/ml.

To avoid any possible influence from the light exposure step on DNA amplification, different ice treatments, which could be used to cool down the stained samples, were tested. Among the three ice treatments tested, a 1-min ice cooling step prior to the 10-min light exposure step showed a *Ct* value closest to that of the "no EMA" control, with the smallest standard deviation between two replications. By comparing the ice treatment and no ice treatment control, the addition of ice treatment made the entire EMA staining process replicable with a smaller standard deviation. Compared to a *Ct* value of 20 generated from 10^8 CFU untreated viable cells (data not shown), it should be

noted that the influence of EMA on the amplification of viable cells cannot be completely avoided, even though the ice treatment will minimize the EMA effect.

By applying EMA staining to a mixture of viable and dead cells, the EMA real-time PCR with IAC system developed in this study was shown to be highly sensitive for detecting only viable *E. coli* O157:H7 cells (Figure 4.3). It can effectively differentiate the dead cells from the live cells, bind to the DNA from only dead cells, and prevent its further amplification. After optimizing the staining step and the real-time PCR with IAC protocol, there was still a slight underestimation of viable cell counts (sample 3 compared to sample 2 control, Figure 4.3), which may have been caused by the influence of EMA staining on viable cells and the presence of dead cells. The presence of 10^8 CFU dead cells may have inhibited the release and amplification of DNA from viable cells.

The optimized protocol was then applied to ground beef samples which were inoculated with both dead and live *E. coli* O157:H7 cells. Three experiments were designed to test and compare the optimized protocol. By plotting the *Ct* values and their corresponding viable cell concentrations (CFU/g) together, the results demonstrated the successful application of the developed method to artificially contaminated ground beef. After the addition of EMA to beef samples, the EMA molecules could penetrate the pathogenic bacterial cells without being negatively influenced by meat tissues and background microflora. EMA staining overcame the false positive and overestimation results (Figure 4.4b) generated from dead cells. The detection range of the EMA real-time PCR with IAC was from 10^4 CFU/g to 10^9 CFU/g for viable cells. By comparing the slope and R^2 of standard curve a (Figure 4.4a) to the slope and R^2 of standard curve c (Figure 4.4c), the detection range of the EMA real-time PCR for artificially contaminated

ground beef with both dead and viable cells was found to be the same as that for ground beef contaminated with only viable cells, with the slopes of these two standard curves being similar. The decrease in the R^2 may be due to the presence of a high number of dead cells, which may have inhibited the recovery of viable cells from the ground beef. Even though the DNA from dead cells did not serve as a template for PCR after being bound by EMA, its presence in high concentrations may have influenced the DNA amplification from the viable cells. Viable cells at less than 10^4 CFU/g in ground beef could also be detected by the EMA real-time PCR with IAC. After a 12-h enrichment, this method could successfully detect as low as 10 CFU/g of viable cells, regardless of the fat content of the beef.

Both live and dead pathogenic cells can be present in a food, but only the live ones pose a risk to human health. Effective detection of live cells is of utmost importance to food safety. Inclusion of a sample enrichment step before PCR amplification is currently routinely employed for decreasing DNA amplification influence from dead cells by allowing for the recovery of and increasing the number of viable cells. However, as evidenced in this study, enrichment alone cannot totally prevent DNA amplification from dead cells. This may lead to an overestimation of the original viable cell count in the food. The addition of an EMA staining step prior to DNA extraction could prevent the subsequent PCR from amplifying DNA from dead cells at 0 h and prevent the overestimation of cell numbers following enrichment. An IAC in each PCR system confirmed the reliability of this assay and prevented the reporting of false negative results.

In conclusion, this study demonstrates that EMA real-time PCR can be a new reliable method for detection of viable *E. coli* O157:H7 in beef products. Further studies should include construction of a DNA-based standard curve for direct comparison of DNA target copy numbers recovered by this method with the actual number of cells added to the samples. A pure DNA standard curve will facilitate the evaluation of DNA extraction and amplification efficiency from the viable cells and provide a better understanding of the EMA staining effect. Additionally, recent studies found that propidium monoazide (PMA) may exert less influence on DNA amplification from certain viable cells than EMA. PMA has been used to detect viable *E. sakazakii*, *E. coli* O157:H7, *L. monocytogenes* and *C. jejuni* with success (Nocker and others 2006; Flekna and others 2007; Pan and Breidt 2007; Cawthorn and Witthuhn 2008). Continuation of this work using PMA instead of EMA would be an interesting next step.

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REFERENCES

Cawthorn DM, Witthuhn RC. 2008. Selective PCR detection of viable *Enterobacter sakazakii* cells utilizing propidium monoazide or ethidium bromide monoazide. J Appl Microbiol 105(4): 1178-85.

Chaiyanan S, Chaiyanan S, Huq A, Mangel T, Colwell RR. 2001. Viability of the nonculturable *Vibrio cholerae* O1 and O139. Syst Appl Microbiol 24(3): 331-41.

Coffman GL, Gaubatz JW, Yielding KL, Yielding LW. 1982. Demonstration of specific high affinity binding sites in plasmid DNA by photoaffinity labeling with ethidium analog. J Biol Chem 257(22): 13205-97.

DeTraglia MC, Brand JS, Tometsko AM. 1978. Characterization of azidobenzamidines as photoaffinity labeling for trypsin. J Biol Chem 253(6): 1846-52.

Erickson MC, Doyle MP. 2007. Food as a vehicle for transmission of shiga toxin-producing *Escherichia coli*. J Food Prot 70(10): 2426-49.

FDA. 1995. Bacteriological Analytical Manual, 8th ed. Gaithersburg, MD: AOAC International. 5 p.

Flekna G, Stefanic P, Wagner M, Smulders FJ, Mozina SS, Hein I. 2007. Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. Res Microbiol 158(5): 405-12.

Frenzen PD, Drake A, Angulo FJ,Emerging Infections Program FoodNet Working Group. 2005. Economic cost of illness due to *Escherichia coli* O157 infections in the United States. J Food Prot 68(12): 2623–30.

Fricker M, Messelhäußer U, Busch U, Scherer S, Ehling-Schulz M. 2007. Diagnostic real-time PCR assays for the detection of emetic *Bacillus cereus* strains in foods and recent food-borne outbreaks. Appl Environ Microbiol 73(6): 1892-8.

Guy RA, Kappor A, Holicka J, Shepherd D, Horgen PA. 2006. A rapid molecular-based assay for direct quantification of viable bacteria in slaughterhouses. J Food Prot 69(6): 1265-72.

Hixon SC, White WEJ, Yielding KL. 1975. Selective covalent binding of an ethidium analog to mitochondrial DNA with production of petite mutants in yeast by photoaffinity labeling. J Mol Biol 92(2): 319-29.

Hoorfar J, Cook N, Malorny B, Wagner M, De Medici D, Abdulmawjood A, Fach P. 2004. Diagnostic PCR: making internal amplification control mandatory. J Clin Microbiol 96: 221-2.

Kaur J, Ledward DA, Park RWA, Robson RL. 1998. Factors affecting the heat resistance of *Escherichia coli* O157:H7. Lett Appl Microbiol 26: 325-30.

Lambertz ST, Ballagi-Pordany A, Lindqvist R. 1998. A mimic as internal standard to monitor PCR analysis of food-borne pathogens. Lett Appl Microbiol 26: 9-11.

Lee JL, Levin RE. 2006. Use of ethidium bromide monoazide for quantification of viable and dead mixed bacterial flora from fish fillets by polymerase chain reaction. J Microbiol Methods 67(3): 456-62.

Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV. 1999. Food-related illness and death in the United States. Emerg Infect Dis 5: 607-25.

Murphy NM, McKauchlin J, Ohai O, Grant KA. 2007. Construction and evaluation of a microbiological positive process internal control for PCR-based examination of food samples for *Listeria monocytogenes* and *Salmonella enterica*. Int J Food Microbiol 120: 110-9.

Nocker A, Camper AK. 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. Appl Environ Microbiol 72(3): 1997-2004.

Nocker A, Cheung CY, Camper AK. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods 67(2): 310-20.

Nogva HK, Dromtorp SM, Nissen H, Rudi K. 2003. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR. Biotechniques 34(4): 804-13.

Pan Y, Breidt F. 2007. Enumeration of viable *Listeria monocytogenes* cells by real-time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. Appl Environ Microbiol 73(24): 8028-31.

Roszak DB, Colwell RR. 1987. Survival strategies of bacteria in the natural environment. Microbiol Rev 51(3): 365-79.

Rudi K, Moen B, Dromtorp SM, Holck AL. 2005a. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl Environ Microbiol 71(2): 1018-24.

Rudi K, Naterstad K, Dromtorp SM, Holo H. 2005b. Detection of viable and dead *Listeria monocytogenes* on gouda-like cheeses by real-time PCR. Lett Appl Microbial 40(4): 301-6.

Rueckert A, Ronimus RS, Morgan HW. 2005. Rapid differentiation and enumeration of the total, viable vegetative cell and spore content of thermophilic bacilli in milk powders with reference to *Anoxybacillus flavithermus*. J Appl Microbiol 99(5): 1246-55.

Sharma VK. 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(9): 1371-80.

Smith SE, Maurer JL, Orta-Ramirez A, Ryser ET, Smith DM. 2001. Thermal inactivation of *Salmonella* spp., *Salmonella* Typhimurium DT104 and *Escherichia coli* O157:H7 in ground beef. J Food Sci 66: 1164-8.

Wang L, Li Y, Mustapha A. 2007. Rapid and simultaneous quantitation of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* in ground beef by multiplex real-time PCR and immunomagnetic separation. J Food Prot 70(6): 1366-72.

Wang S, Levin RE. 2006. Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. J Microbial Methods 64(1): 1-8.

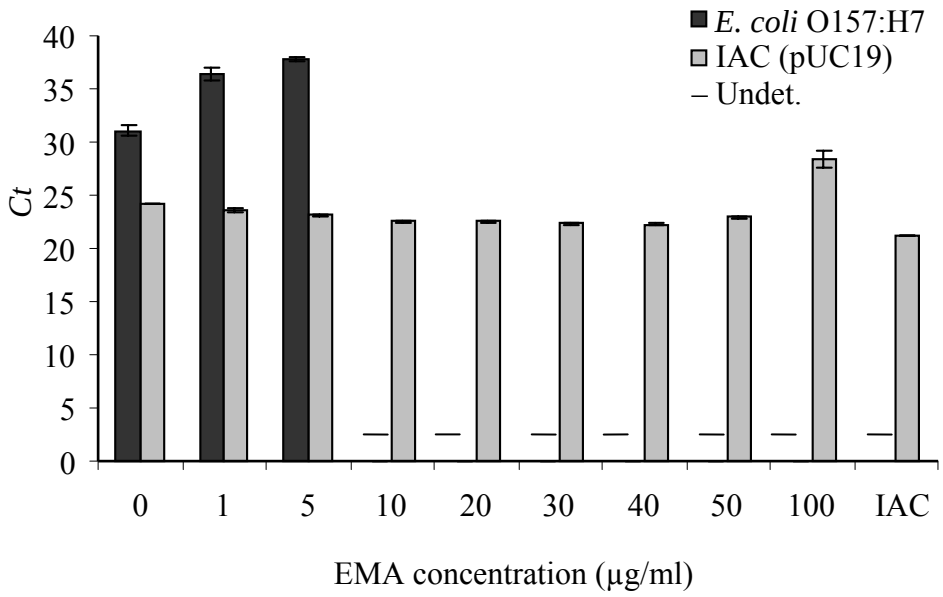


Figure 4.1 Minimum EMA concentration necessary for binding DNA from 10^8 CFU/ml dead *E. coli* O157:H7 cells. Starting at an EMA concentration of 10 µg/ml, no *Ct* value was detected for *E. coli* O157:H7, indicating an "undet." status.

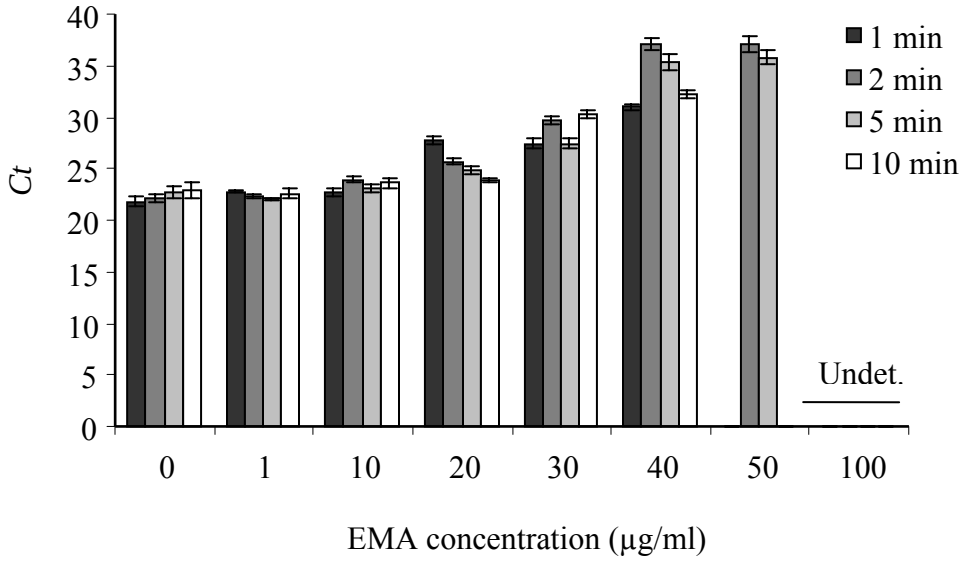


Figure 4.2 Influence of EMA concentration and light exposure time on the amplification of DNA from 10^8 CFU/ml viable *E. coli* O157:H7 cells. Each experiment was replicated twice with each reaction conducted in triplicate.

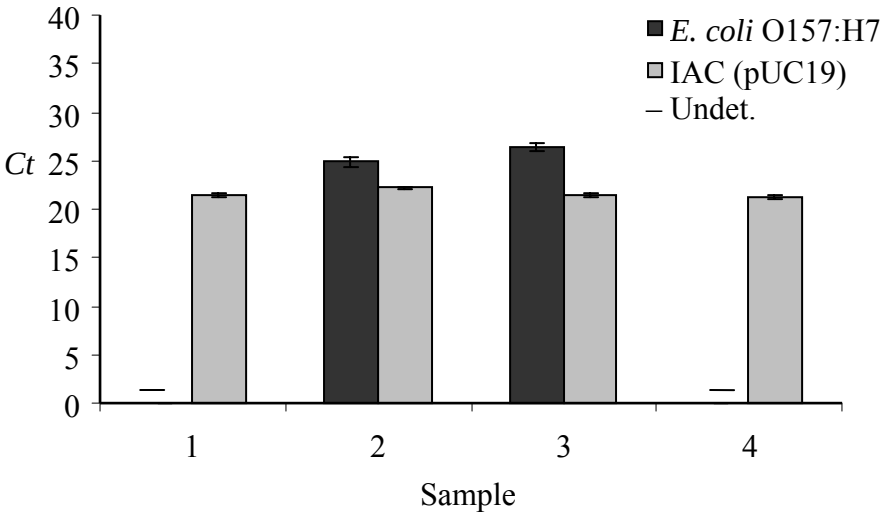
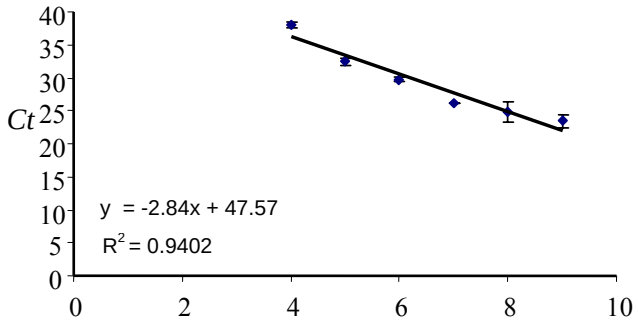
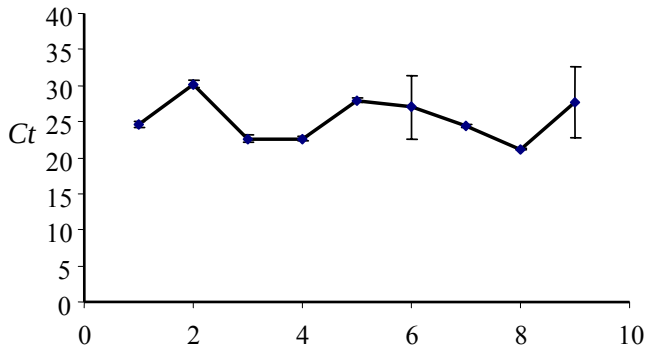


Figure 4.3 Application of the EMA real-time PCR to viable and dead mixed cultures of *E. coli* O157:H7. Sample 1, DNA extracted from 10^8 CFU dead cells after EMA staining; sample 2, DNA extracted after EMA staining from 10^8 CFU viable cells; sample 3, DNA extracted from mixed viable and dead cells after EMA staining (10^8 CFU each); sample 4, water control (with IAC).



a



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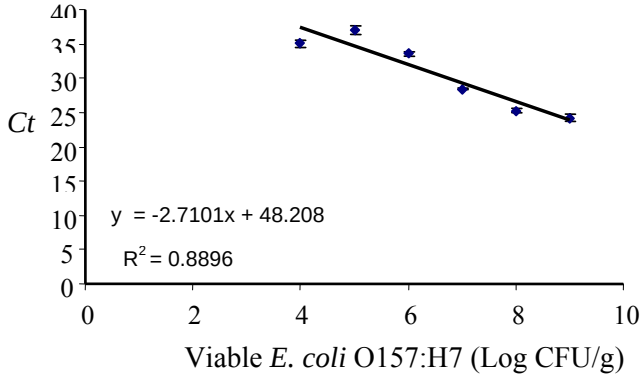

c

Figure 4.4 Application of the EMA real-time PCR with IAC to artificially contaminated beef and its detection range. (a) detection range of EMA real-time PCR for artificially contaminated ground beef contaminated with

only viable *E. coli* O157:H7 cells. (b) amplification results of real-time PCR with IAC, without prior EMA staining, for ground beef contaminated with 10^8 CFU/g dead *E. coli* O157:H7 cells and viable *E. coli* O157:H7 cells. (c) detection range of EMA real-time PCR with IAC for ground beef contaminated with 10^8 CFU/g dead *E. coli* O157:H7 cells and viable *E. coli* O157:H7 cells.

Table 4.1 Real-time PCR results of artificially contaminated ground beef.

Beef sample (Lean:Fat)	Enrichment time (h)	Concentration of viable cells (CFU/g)	Concentration of dead cells (CFU/g)	EMA-treated[1]		No EMA[2]	
				Sample	IAC	Sample	IAC
(73/27)	0 h	1.079	6.079	-	+	+	+
		2.079	6.079	-	+	+	+
		3.079	6.079	-	+	+	+
		4.079	6.079	-	+	+	+
		0	6.079	-	+	+	+
		0	0	-	+	-	+
	12 h	1.079	6.079	+	+	+	+
		2.079	6.079	+	+	+	+
		3.079	6.079	+	+	+	+
		4.079	6.079	+	+	+	+
		0	6.079	-	+	+	+
		0	0	-	+	-	+
(90/10)	0 h	1.079	6.079	-	+	+	+
		2.079	6.079	-	+	+	+
		3.079	6.079	-	+	+	+
		4.079	6.079	-	+	+	+
		0	6.079	-	+	+	+
		0	0	-	+	-	+
	12 h	1.079	6.079	+	+	+	+
		2.079	6.079	+	+	+	+
		3.079	6.079	+	+	+	+
		4.079	6.079	+	+	+	+
		0	6.079	-	+	+	+

	0	0	-	+	-	+
water	0	0	-	-	-	-

[1,2]EMA-treated samples were stained with EMA before DNA extraction, while "No EMA" indicated that samples were used directly for DNA extraction without prior EMA staining for dead cells. Ground beef with fat contents of 20% and 6% were also tested (data not shown) and the results were the same as those for ground beef with 27% and 10% fat content, respectively.

CHAPTER 5

Running heads: *Salmonella* detection from chicken and eggs

EMA REAL-TIME PCR AS A RELIABLE METHOD FOR DETECTION OF VIABLE *SALMONELLA* IN POULTRY PRODUCTS

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ABSTRACT

Cultural-based *Salmonella* detection takes at least four days to complete. With the use of TaqMan® probes, the real-time PCR technique is a rapid and sensitive way to detect foodborne pathogens. However, DNA-based PCR techniques cannot differentiate between DNA from live and dead cells, while RNA-based PCR can. Ethidium bromide monoazide (EMA) is a dye that can bind to DNA of dead cells and prevent its amplification by PCR. An EMA staining step prior to real-time PCR allows for the effective inhibition of DNA contamination from dead cells. The aim of this study was to design a more accurate detection method that can detect only viable *Salmonella* cells from poultry products. The sensitivity of EMA staining coupled with real-time PCR was compared to that of an RNA-based reverse transcription (RT)-real-time PCR. To prevent false negative results, an internal amplification control was added to the same reaction mixture as the target *Salmonella* sequences. With an optimized EMA staining step, the detection range of a subsequent real-time PCR was determined to be 10^3 to 10^9 CFU/ml for pure cultures and 10^5 to 10^9 CFU/ml for food samples, which was a wider detection range than that for RT-real-time PCR. After a 12-h enrichment step, EMA staining combined with real-time PCR (EMA-real-time PCR) could detect as low as 10 CFU/ml *Salmonella* from chicken rinses and egg broth. The use of EMA with a DNA-based real time PCR can successfully prevent false positive results and represent a simple, yet accurate detection tool for enhancing the safety of food.

Key words: Reverse transcription-real-time PCR, EMA, real-time PCR, *Salmonella*, poultry products

INTRODUCTION

Salmonellosis is responsible for about 30% of all food poisoning cases in the United States (Olsen and others 2000; Taitt and others 2004). In poultry, *Salmonella* can persist in critical organs, including the spleen and reproductive tract for a long period of time (Chao and others 2007). *Salmonella* Typhimurium and *Salmonella* Enteritidis can "bind to isthmal secretions, enter the inner side of the egg shell and be protected from antimicrobial factors present in egg white" (Buck and others 2003). According to the CDC's annual outbreak surveillance data (CDC 2006), chicken and chicken-related products are still the main causes of *S.* Typhimurium infections. This serotype has consistently been the most frequently isolated in the U.S. since 1970 (CDC 2008). *S.* Enteritidis accounts for the largest number of outbreaks, illness, and deaths in 2,751 outbreaks from 1993 to 1997, with most of the outbreaks being related to eggs (Olsen and others 2000). This *Salmonella* serotype has been recognized as the only human pathogen that routinely contaminates eggs (Byrd and others 1999*;* Caldwell and others 1995, Gruard-Petter 2001; Soerjadi-Liem and Cumming 1984). Both *S*. Typhimurium and *S*. Enteritidis top the list at about 17% of 20 of the most frequently reported *Salmonella* serotypes from human sources in 2006 (CDC 2008).

In conventional culture-based *Salmonella* detection techniques, up to four days is necessary to achieve negative results and more than five days are needed to confirm positive results. PCR techniques are rapid and sensitive due to their ability to amplify a target DNA 10^7-fold in 2-3 h (Bailey and Cosby 2003). With the use of TaqMan® probes, real-time PCR is much faster than conventional PCR. Unfortunately, although such DNA-based PCR techniques have been widely developed to detect the presence of

Salmonella, they cannot differentiate between DNA from live and DNA from dead cells, which may cause false positive results and unnecessary product recalls and economic losses.

The analysis of RNA via PCR can prevent false positive results that can result from DNA of dead cells in DNA-based PCR methods. However, RNA templates are difficult to extract from foods due to the presence of inhibitors, such as fat, proteins and blood cells.

Ethidium bromide monoazide (EMA) is a dye that can bind to and prevent the amplification of DNA from dead cells. The combination of EMA with a DNA-based real time PCR may overcome the challenges of using RNA templates because the target molecule, DNA, is more stable than RNA. EMA combined with PCR (EMA-PCR) has been used to detect pathogenic *Salmonella* from slaughterhouses (Guy and others 2006), and *Campylobacter jejuni* from chicken breast and leg muscle (Rudi and others 2005) and *Escherichia coli* O157:H7 from ground beef (Wang and Mustapha 2009).

An internal amplification control (IAC) is a sequence that is included in the same reaction tube as the target DNA template during PCR. It can prevent the occurrence of false negative results from the PCR reaction and monitor the efficiency of each reaction, without inhibiting the amplification of the target sequence (Hoorfar and others 2004, Lambertz and others 1998). Plasmid pUC19 has been successfully employed as an IAC in a PCR method for detecting *Bacillus cereus* in foods (Fricker and others 2007). In this study, an EMA staining step was added prior to conducting real-time PCR which includes an IAC and target DNA sequence. By comparing this method to an RNA-based reverse

transcription real-time PCR, EMA combined with real-time PCR can be a simple and accurate way to detect only viable *Salmonella* cells from poultry products.

MATERIALS AND METHODS

Bacterial strains

Salmonella Enteritidis 13076 and *Salmonella* Typhimurium 14028 were procured from ATCC (Rockville, MD). Cultures were grown overnight at 37°C in Tryptic Soy Broth supplemented with 0.5% yeast extract (TSBY).

Primers and probes

Primers and corresponding TaqMan® probes were synthesized according to Wang and others (2007) and Fricker and others (2007). Sequences are shown in Table 1.

Preparation of viable and dead *Salmonella* cultures samples

Fresh overnight viable *Salmonella* cultures were diluted in 9 ml peptone water to varying concentrations, ranging from 10 CFU/ml to 10^9 CFU/ml. To obtain dead cells, 10^6 CFU/ml viable cells were heat-treated at 95°C for 30 min. Viability of the cells was checked by plating the sample in plate count agar (Difco Labs., BD Diagnostic Systems, Sparks, MD) and by the use of the Live/Dead® *Bac*Light™ bacterial viability kit (Invitrogen™, Eugene, OR). Mixed viable and dead *Salmonella* cultures were made by combining 1 ml each of 10-10^9 CFU/ml viable cells with 1 ml 10^6 CFU/ml dead cells. Nine samples in total were prepared with various viable cell counts and 10^6 CFU dead cells in each.

Ethidium monoazide (EMA) treatment and DNA extraction

Samples prepared from above were stained with 10 μg EMA in the dark for 5 min and chilled by keeping the tubes on ice for 1 min. Following the ice chilling step, the samples were exposed to a 650 W halogen light for 10 min. These treatments were according to a previous study in which we had optimized the EMA amount, light exposure and ice treatment processes and successfully applied the method to artificially inoculated ground beef (Wang and Mustapha 2009). To extract DNA, cells were washed after the EMA staining treatment and resuspended in 100 μl PrepMan® Ultra sample preparation reagent (Applied Biosystems, Foster City, CA). They were incubated at 56°C for 30 min, and placed in boiling water for 8 min. DNA samples were obtained after centrifugation of boiled samples at 10,000 × *g* for 2 min.

RNA extraction from live and dead *Salmonella* mixed cultures

Live and dead *Salmonella* mixed cultures were prepared in the same way as for DNA extraction. Nine samples in total were used for RNA extraction. RNA from mixed *Salmonella* cultures was prepared using the RNeasy® Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. RNA samples were kept in -70°C for the subsequent RT-PCR.

Real-time PCR with internal amplification control

In each 50 μl reaction tube, 1 μl IAC forward primer (0.4 μM), 1 μl IAC reverse primer (0.4 μM), 1 μl IAC probe (0.2 μM) and 5 μl of diluted pUC19 were added, together with *Salmonella*-1 primer, *Salmonella*-2 primer, *Salmonella* probe, and 25 μl of TaqMan™ Universal PCR Master Mix (2×, ABI, Branchburg, NJ). The concentration of *Salmonella* probe was the same as that for the IAC, but the concentration of *Salmonella*

primers (0.5 µM each) was higher than that of the IAC primers. Ten microliters of DNA extracted from each mixed sample were added and nuclease-free water was added to bring each reaction up to 50 µl. A blank (water), as a negative control, was added separately to one reaction tube containing the Master Mix, primers, probes and water. All reactions were processed in triplicate.

The PCR reaction started with holding the sample at 95°C for 2 min, followed by 40 amplification cycles with a melting temperature of 95°C for 1 min, an annealing temperature of 60°C for 1 min, and an extension temperature of 72°C for 1 min. Multiple copies of target DNA were synthesized and fluorescence was read after every cycle. Real Time PCR was carried out in a 96-well ABI Prism® 7700 Sequence Detection System (ABI, Foster City, CA). *Ct* values were calculated using the threshold line determined by the fluorescence of a water sample at the end of 40 cycles.

Reverse transcription (RT) real time PCR

Each 50 µl of RT real time PCR tube, contained 0.5 µM *Salmonella*-1 primer, 0.5 µM *Salmonella*-2 primer, 0.2 µM *Salmonella* probe, 25 µl TaqMan® 2× Universal PCR Master Mix, 1.25 µl 40× Multiscribe™ RNase inhibitor mix, 10 µl RNA and water. The reaction tubes were held at 48°C for 30 min, 95°C for 10 min, and ran through 40 cycles of PCR reactions, which included 95°C for 15 s and 60°C for 1 min. Fluorescence was also read after each cycle.

Comparison between RT-real-time PCR and EMA real-time PCR

The standard curves for the real time PCR and RT-real-time PCR were developed by plotting *Ct* values in the *Ct* value vs. log CFU/ml axis. Detection ranges, regression

coefficients and the standard deviations for the standard curves were compared and analyzed.

Preparation of artificially contaminated chicken rinses

One whole pre-cut chicken was purchased from a local supermarket. It was determined to be *Salmonella*-free by standard methods (FDA 1995) and real time PCR following our previously published protocol (Wang and others 2007). Buffered peptone water (500 ml, 1% [w/v], BPW) was used to wash the chicken carcass and to prepare the chicken rinse. Five hundred milliliters of chicken rinse were aliquoted in 50 ml portions in tubes. Aliquots were inoculated with viable and dead *S.* Typhimurium to generate final concentrations of 10 to 10^9 CFU/ml of viable cells and 10^6 CFU/ml of dead cells. One negative control, containing only 10^6 CFU/ml dead cells, was also prepared.

Preparation of artificially contaminated egg broth

Ten milliliters of homogenized *Salmonella*-free raw consumption eggs were resuspended in 90 ml 1% BPW (Malorny and others 2007). The egg broths were artificially inoculated with *S.* Enteritidis at different final viable cell concentrations (10-10^9 CFU/ml) and 10^6 CFU/ml dead cells.

DNA sample extraction from artificially inoculated poultry samples

A 1 ml sample from each artificially inoculated poultry sample (chicken broth or egg broth) was used. These 1 ml samples were centrifuged at 12,000 × *g* for 5 min to harvest the cells, which were subsequently washed in distilled water and stained with EMA for 5 min to bind the DNA from dead cells. Ice treatment and a 10-min light exposure were conducted as described above. DNA extraction was performed after EMA staining by PrepMan® Ultra sample preparation reagent (ABI).

Application of EMA real time PCR on the detection of viable *Salmonella* cells from artificially contaminated poultry products

DNA samples extracted after EMA staining were used for real time PCR following the protocol described above with an internal amplification control. A standard curve was built based on the *Ct* value generated from real time PCR.

Detection of viable *Salmonella* cells in low concentration from artificially inoculated egg broth and chicken rinse

Artificially contaminated chicken rinses and egg broths with a low inoculation level were prepared. In each sample, 10^6 CFU/ml dead cells were added, together with different concentrations of viable cells (10-10^4 CFU/ml). The inoculated samples were incubated at 37^{o}C for 12 and 24 h before proceeding with EMA staining and DNA extraction. EMA staining, DNA extraction and real-time PCR were conducted as described above.

RESULTS

Efficiency of EMA staining

Equal amounts of 10^9 CFU each of viable and dead *Salmonella* cells were mixed and stained with 10 μg/ml EMA. Conventional multiplex PCR was carried out with the primers and probes shown in Table 5.1. Plasmid pUC19 was added in each PCR reaction as an IAC at a final concentration of 0.005 pg/μl. PCR amplicons were checked by running samples in a 1.8% agarose gel.

DNA amplification results of samples extracted without EMA treatment are shown in lanes 2 to 4 of Figure 5.1. While the *Salmonella*-specific sequence was amplified from the viable culture (lane 2), the same amplicon was also found in lane 3,

which indicated that the DNA from 10^9 CFU dead *Salmonella* cells still effectively served as a template in the PCR reaction. Lanes 5 to 7 demonstrate the amplification results of DNA samples extracted following the EMA treatment. No amplicons were seen when dead cells were treated with EMA before the PCR step (lane 6), as compared to the results with viable cells (lane 5), indicating that EMA can successfully bind DNA in dead cells and stop its amplification in the following PCR reaction. Ten micrograms of EMA effectively prevented amplification from as high as 10^9 CFU dead cells. The amplification of the IAC, pUC19, which showed up as the 118 bp band in all the lanes, demonstrated the efficiency and robustness of each reaction and avoided false negative results.

Detection range of the RT-real time PCR

Samples were prepared by adding 10^6 CFU dead *Salmonella* cells to nine tubes containing different concentrations (10^1 to 10^9 CFU in each tube) of viable *Salmonella* cells. RNA from the nine samples was extracted using the RNeasy® Mini Kit (Qiagen, Valencia, CA) and 10 μl of each RNA extract were applied to RT-real time PCR. As shown in the standard curve (Figure 5.2), the detection range of the RT-real time PCR was from 10^5 CFU/ml to 10^9 CFU/ml for viable cells ($R^2 = 0.8017$), with high standard deviations. Standard deviations of the *Ct* values for 10^5 and 10^7 CFU viable cells were greater than 5 which may caused by the unstable nature of RNA molecules and the additional DNA cleaning step.

Detection range of EMA-real time PCR with IAC

DNA from nine samples containing different concentrations of viable (10^1 to 10^9 CFU) and dead (10^6 CFU) *S.* Typhimurium cells, was extracted after EMA treatment.

The DNA samples were amplified via real time PCR, together with 0.25 pg pUC19 IAC in each reaction tube.

The detection range of the EMA-real time PCR in the presence of 10^6 CFU dead *S*. Typhimurium cells is shown in Figure 5.3b. The detection range of the assay, with the inclusion of pUC19 IAC, ranged from 10^3 to 10^9 CFU/ml for viable *Salmonella* cells (R^2 = 0.9564), compared to 10^2 to 10^9 CFU/ml without the IAC (R^2 = 0.9729) (Figure 5.3a). The inclusion of the IAC resulted in a one log CFU decrease in the detection range and a smaller R^2 than that obtained without the IAC. The decrease in the detection range reflected the inhibition generated from the IAC on the amplification of DNA from viable cells. Although the R^2 of 3b was lower than the one without IAC, a R^2 of 0.9564 still showed a better fit of 3b to the regression line, compared to the R^2 of 0.8017 from the RT-real time PCR (Figure 5.2). In addition to the inhibition generated from the IAC, the influence of the viable cells on the amplification of the IAC is also shown in Figure 5.3b. For example, when the concentration of viable *Salmonella* cells was as high as 10^9 CFU/ml, the *Ct* value of pUC19 was about 35. When the concentration of viable *Salmonella* decreased, the inhibition generated by the *Salmonella* DNA amplification on the IAC also decreased.

Optimization of IAC concentration

The purpose of adding an IAC in a PCR reaction is to prevent the generation of false negative results. There are two requirements for the determination of optimum IAC concentration. First, the amplification of IAC should not inhibit the amplification of target sequences. Secondly, the amount of IAC has to be easily detected and should not be influenced by the target sequences. In this study, the concentration of pUC19 was

adjusted and different concentrations were tested in order to get the best amplification efficiency for both the *Salmonella* and the IAC (data not shown). A final concentration of 0.005 µg/µl pUC19 in a 50 µl PCR reaction system was found to generate the best results (Figure 5.3b). Amounts higher than 0.25 pg inhibited the amplification of the target *Salmonella* sequence and narrowed the detection range. On the other hand, if the concentration is less than 0.25 pg, the IAC cannot be detected when the concentration of viable *Salmonella* is 10^9 CFU/ml.

Detection range of EMA-real time PCR for artificially contaminated poultry samples

The newly designed protocol which includes EMA staining, DNA extraction and real-time PCR was used to detect viable *Salmonella* from artificially contaminated poultry samples. No enrichment was included before extracting DNA from artificially inoculated poultry samples. Two DNA extracting processes were used. Figure 3.4a shows the real time PCR results from DNA samples extracted without prior EMA treatment and the standard curve in Figure 5.4b shows the amplification results generated from DNA samples extracted following EMA treatment. The detection range of the EMA-real-time PCR with IAC was from 10^5 CFU/ml to 10^9 CFU/ml when the method was applied to poultry samples (R^2 = 0.7504). As expected, the detection range is narrower than the detection range for pure cultures and the R^2 is much lower, due to the presence of background microflora and poultry sample tissues. One milliliter from each poultry sample was also used for DNA extraction without prior EMA staining. The results showed that, without an EMA staining step, DNA from dead cells served as templates for PCR and caused the overestimation of viable cell numbers (Figure 5.4a).

Although the detection range of the EMA-real-time PCR was narrower when applied to artificially contaminated poultry samples, it still showed its advantage in preventing DNA amplification from dead cells, thus, avoiding false positive results.

Application of the EMA-real time PCR in poultry samples with low viable *Salmonella* concentrations

According to the detection range of application of EMA real time PCR for artificially contaminated poultry samples, viable *Salmonella* cells with the concentration lower than 10^4 CFU/g can not be detected. Thus, enrichment was used for those poultry samples with low viable *Salmonella* concentrations. Artificially contaminated chicken rinses and egg broths with low (10-10^4 CFU/g) viable *Salmonella* concentrations and 10^6 CFU/g dead cells were enriched at 37°C for 12 and 24 h. One milliliter of the enriched broth from each sample was obtained at 0, 12, and 24 h. Broth samples were centrifuged and cell pellets were washed with distilled water. Pellets were resuspended in distilled water, stained with 10 μg EMA and used for DNA extraction. As shown in Table 5.2, EMA combined with real time PCR can detect as low as 10^1 CFU/ml viable *Salmonella* cells from the artificially contaminated chicken rinses and egg broth after a 12-h enrichment, while samples containing only dead cells generated no signal. In addition, from Table 5.2, it showed that the poultry sample which was artificially inoculated with 10^6 dead cells can still generate positive result after 12 h enrichment, which proved again the importance of adding EMA staining step before real-time PCR.

DISCUSSION

Improperly handled or undercooked poultry or egg products are foods that frequently cause salmonellosis because chickens are a major carrier of *Salmonella*. However, identifying *Salmonella*-infected animals is difficult. As the "infected hens or chickens typically show no signs or symptoms of illness, they might subsequently be used for producing eggs or chicken meat" (Gale Encyclopedia of Children's Health 2006). Infected hens can even pass *Salmonella* into eggs. Food poisoning caused by *Salmonella* is a serious threat to infants, seniors, and people with weakened immune systems because death can ensue if such patients are not treated properly.

Compared to traditional cultural-based detection protocols, the PCR has been recognized as a reliable rapid molecular method. The sensitivity and accuracy of the PCR depend on the specificity of the primers used. In this study, two important pathogenic *Salmonella* serotypes, *S.* Typhimurium and *S.* Enteritidis, were targeted. Because *S.* Typhimurium is the main serotype present in contaminated chicken products (CDC 2006), this strain was used to contaminate chicken rinses. On the other hand, *S.* Enteritidis was used to artificially contaminate egg broths because this serotype has been recognized as a human pathogen that routinely contaminates eggs. The *Salmonella* primers and TaqMan® probes used in this study have been shown in our previous published research (Wang and others 2007) to be highly specific for both serotypes.

RT-PCR is a technique that is used to amplify RNA molecules extracted from target cells. During RT-PCR, RNA is first reversely transcribed into complementary DNA (cDNA), and this molecule was used as the template for further PCR amplification. Because of the short half-life of RNA, it is usually present only in live cells, thus, RT-

PCR is believed to effectively indicate the presence of only live target organisms. DNA-based PCRs, on the other hand, do not separate viable from dead microbial cells, thus, giving way to false positive results. This is because DNA from dead cells can serve as a template during conventional PCR amplification (Chaiyanan and others 2001; Nogva and others 2003; Wang and Levin 2006).

EMA is a dye that has been used for microscopic differentiation of viable cells from dead ones. Recently, it has been found that this dye can bind to DNA in dead cells by penetrating through their impaired cell wall and preventing their DNA amplification during PCR. In other words, by coupling EMA staining with PCR, DNA-based conventional PCR can overcome the contamination from DNA of dead cells and realize the amplification of only *viable* cells.

To compare RT-real time PCR with EMA-real time PCR, the same nine samples containing different concentrations of viable (10^1-10^9 CFU) and 10^6 CFU dead *Salmonella* cells were used for RNA and DNA extractions. The data shows that EMA effectively prevented DNA amplification from dead cells, and the EMA-real time PCR procedure had a higher amplification sensitivity and wider detection range than the RT-real time PCR. Ten micrograms of EMA effectively bound to DNA from 10^9 CFU dead cells, and prevented its further amplification by PCR. Although RT-real time PCR will amplify RNA only from viable cells, pure RNA is difficult to isolate in the presence of high numbers of dead cells. RNA samples often get contaminated by DNA from dead cells, and additional DNA clean-up steps can easily destroy the fragile RNA from live cells. Meanwhile, RNA extraction is more difficult to perform because the molecule is very easily denatured. The results of this study show that EMA staining coupled with

real time PCR can serve as a more efficient and effective way to detect viable cells. It is easy to perform, can effectively prevent false positive results from the presence of dead cells and provide a wider detection range than an RNA-based real time PCR.

In addition, the use of an IAC successfully prevented the occurrence of false negative results, which effectively reflected the result of the EMA staining step. Further, water, which was used as a negative control in this study, illustrated that no DNA contamination occurred from any of the reagents used.

The optimized EMA-real time PCR with IAC was applied to detect *Salmonella* from artificially contaminated chicken rinses and egg products. After a 12-h enrichment, this technique could detect as low as 10^1 CFU/ml of viable *Salmonella* cells in the presence of 10^6 CFU/ml dead *Salmonella* cells (Table 5.2), demonstrating the effective prevention of false negative and false positive results. In addition to the sample enrichment time, the whole process is easily performed in about 2-5 h. To our knowledge, this study is the first to successfully combine EMA staining with real time PCR with the inclusion of an IAC in one reaction, to detect *Salmonella* from chicken and egg products. Hence, this technique has the potential to be employed as a rapid and accurate detection method for the food industry.

REFERENCES

Bailey JS, Cosby DE. 2003. Detection of *Salmonella* from chicken rinses and chicken hot dogs with the automated BAX PCR system. J Food Prot 66(11):2138-40.

Buck JD, Immerseel FV, Meulemans G, Haesebrouck F, Ducatelle R. 2003. Adhesion of *Salmonella* enterica serotype Enteritidis isolates to chicken isthmal glandular secretions. Vet Microbiol 93:223-33.

Byrd JA, DeLoach JR, Corrier DE, Nisbet DJ, Stanker LH. 1999. Evaluation of *Salmonella* serotype distributions from commercial broiler hatcheries and grower houses. Avian Dis 43:39-47.

Caldwell DJ, Hargis BM, Corrier DE, Vidal L, DeLoach JR. 1995. Evaluation of persistence and distribution of *Salmonella* serotype isolation from poultry farms using drag-swab sampling. Avian Dis 39:617-21.

CDC. 2006. Summary Statistics for Foodborne Outbreaks, 2006. http://www.cdc.gov/foodborneoutbreaks/outbreak_data.htm

CDC. 2008. *Salmonella* Surveillance: Annual Summary, 2006. Atlanta, Gerogia: US Department of Health and Human Services. p16.

Chaiyanan S, Chaiyanan S, Huq A, Mangel T, Colwell RR. 2001. Viability of the nonculturable *Vibrio cholerae* O1 and O139. Syst Appl Microbiol 24(3):331-41.

Chao MR, Hsien CH, Yeh CM, Chou SJ, Chu C, Su YC, Yu CY. 2007. Assessing the prevalence of *Salmonella* enterica in poultry hatcheries by using hatched eggshell membranes. Poult Sci 86:1651-5.

FDA. 1995. Bacteriological Analytical Manual, 8th Edition. Gaithersburg, MD: AOAC International. p 530.

Fricker M, Messelhäußer U, Busch U, Scherer S, Ehling-Schulz M. 2007. Diagnostic real-time PCR assays for the detection of emetic *Bacillus cereus* strains in foods and recent food-borne outbreaks. Appl Environ Microbiol 73(6):1892–8.

Gale Encyclopedia of Children's Health. 2006. *Salmonella* food poisoning. Available at http://www.encyclopedia.com/doc/1G2-3447200497.html. Accessed 1 August 2008.

Gruard-Petter J. 2001. The chicken, the egg and *Salmonella* Enteritidis. Environ Microbiol 3(7):421-30.

Guy RA, Kappor A, Holicka J, Shepherd D, Horgen PA. 2006. A rapid molecular-based assay for direct quantification of viable bacteria in slaughterhouses. J Food Prot 69(6):1265-72.

Hoorfar J, Cook N, Malorny B, Wagner M, De Medici D, Abdulmawjood A, Fach P. 2004. Diagnostic PCR: making internal amplification control mandatory. J Clin Microbiol 96:221-2.

Lambertz ST, Ballagi-Pordany A, Lindqvist R. 1998. A mimic as internal standard to monitor PCR analysis of food-borne pathogens. Lett Appl Microbiol 26:9-11.

Malorny B, Bunge C, Helmuth R. 2007. A real-time PCR for the detection of *Salmonella* Enteritidis in poultry meat and consumption eggs. J Microbiol Methods 70(2):245-51.

Nogva HK, Dromtorp SM, Nissen H, Rudi K. 2003. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR. Biotechniques 34(4):804-13.

Olsen SJ, MacKinon LC, Goulding JS, Bean NH, Slutsker L. 2000. Surveillance for Foodborne Disease Outbreaks --United States, 1993-1997. MMWR 49:1-51.

Rudi K, Moen B, Dromtorp SM, Holck AL. 2005. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl Environ Microbiol 71(2):1018-24.

Sharma VK. 2006. Real-time reverse transcription-multiplex PCR for simultaneous and specific detection of *rfb*E and *eae* genes of *Escherichia coli* O157:H7. Mol Cell Probes 20(5):298-306.

Soerjadi-Liem AS, Cumming RB. 1984. Studies on the incidence of *Salmonella* carriers in broiler flocks entering a poultry processing plant in Australia. Poult Sci 63:892-5.

Taitt CR, Shubin YS, Angel R, Ligler FS. 2004. Detection of *Salmonella enterica* serovar Typhimurium by using a rapid, array-based immunosensor. Appl Environ Microbiol 70(1):152-8.

Wang L, Mustapha A. 2009. Detection of viable *Escherichia coli* O157:H7 by ethidium monoazide real-time PCR. Submitted for publication.

Wang L, Li Y, Mustapha A. 2007. Rapid and simultaneous quantitation of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* in ground beef by multiplex real-time PCR and immunomagnetic separation. J Food Prot 70(6):1366-72.

Wang S, Levin RE. 2006. Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. J Microbial Methods 64(1):1-8.

Table 5.1 Sequences of primers and probes.

	Sequence 5'-3'	Amplicon Size	References
Sal-1	GCGACTATCAGGTTACCGTGGA		
Sal-2	AG TACGGCCTGCTTTTATCG		Wang and others 2007
Sal-probe	FAM-TAGCCAGCGAGGTGAAAACGACAAAGG-TAMRA	261 bp	
IAC-for	GCAGCCACTGGTAACAGGAT		
IAC-rev	GCAGAGCGCAGATACCAAAT		Fricker and others 2007
IAC-probe	VIC-AGAGCGAGGTATGTAGGCGG-TAMRA	118 bp	

Table 5.2 Application of EMA-real-time PCR to artificially contaminated chicken rinses and egg broth.

Sample	Enrichment time (h)	Viable cells (log CFU/g)	Dead cells (log CFU/g)	EMA combined Real time PCR		Real time PCR (No EMA)	
				Salmonella	IAC	*Salmonella*	IAC
Chicken Rinses	0	1.021	6.021	-	+	+	+
		2.021	6.021	-	+	+	+
		3.021	6.021	-	+	+	+
		4.021	6.021	-	+	+	+
		0	6.021	-	+	+	+
		0	0	-	-	-	-
	12	1.021	6.021	+	+	+	+
		2.021	6.021	+	+	+	+
		3.021	6.021	+	+	+	+
		4.021	6.021	+	+	+	+
		0	6.021	-	+	+	+
		0	0	-	-	-	-
Egg Broth	0	1.021	6.021	-	+	+	+
		2.021	6.021	-	+	+	+
		3.021	6.021	-	+	+	+
		4.021	6.021	-	+	+	+
		0	6.021	-	+	+	+
		0	0	-	-	-	-
	12	1.021	6.021	+	+	+	+
		2.021	6.021	+	+	+	+
		3.021	6.021	+	+	+	+
		4.021	6.021	+	+	+	+
		0	6.021	-	+	+	+
		0	0	-	-	-	-

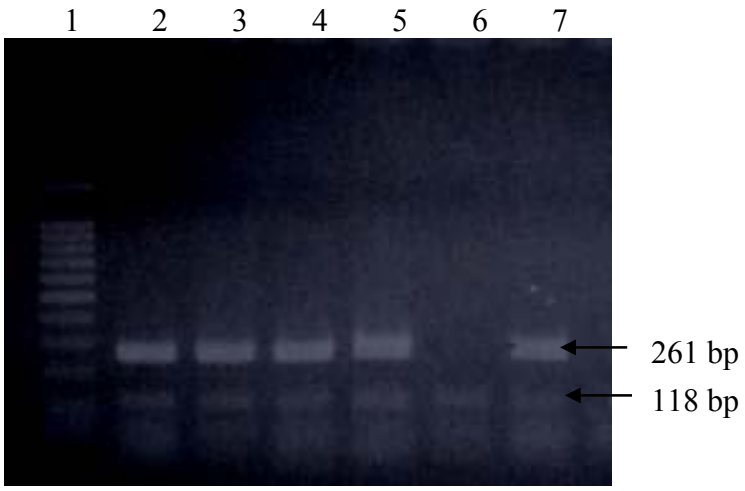

Figure 5.1 Agarose gel electrophoresis of the multiplex PCR from viable and dead *Salmonella*. Lane 1, 100 bp molecular weight marker; lane 2, DNA sample extracted from 10^9 CFU viable *Salmonella* cells without EMA treatment; lane 3, DNA sample extracted from 10^9 CFU dead *Salmonella* cells without EMA treatment; lane 4, DNA sample extracted from a mixture of viable and dead *Salmonella* cultures without EMA treatment; lane 5, DNA sample extracted from 10^9 viable *Salmonella* cells following EMA treatment; lane 6, DNA sample extracted from 10^9 CFU dead *Salmonella* cells after EMA treatment; lane 7, DNA sample extracted from a mixture of viable and dead *Salmonella* cells after EMA treatment. pUC 19 IAC (118 bp amplicon) was added in each reaction mixture.

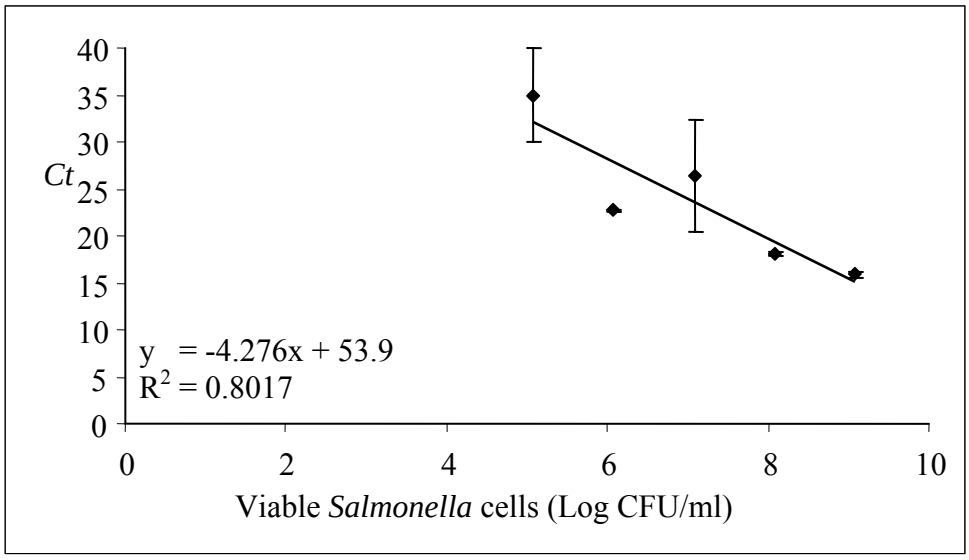

Figure 5.2 Standard curve for RT-real time PCR. RNA samples extracted from mixed cultures of 10^6 CFU dead *Salmonella* cells and different concentrations of viable *Salmonella* cells were used to construct the standard curve.

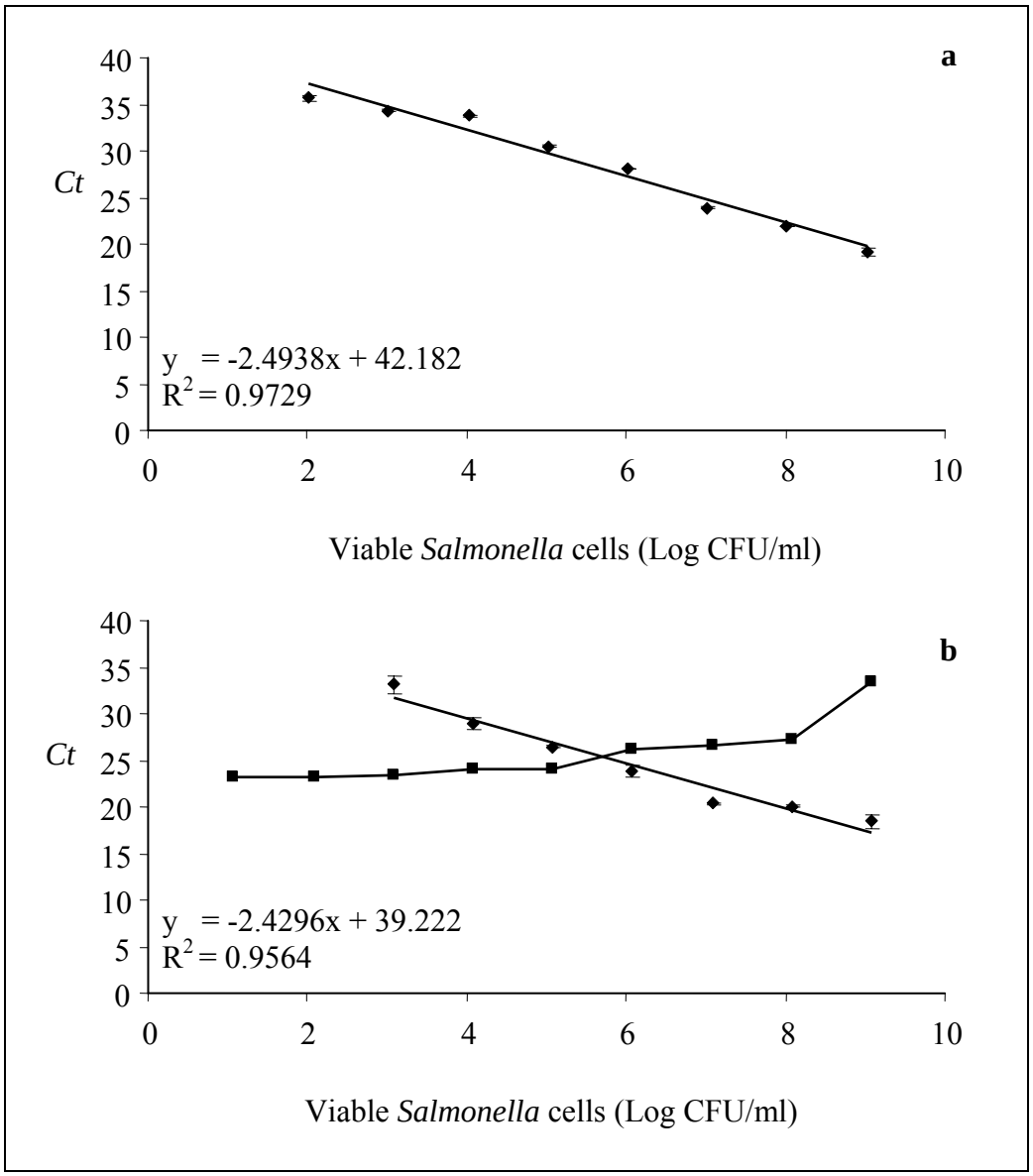

Figure 5.3 Standard curves for the EMA-real time PCR. (◆) *Salmonella* (■) pUC19(IAC). (a) standard curve for EMA-real time PCR without IAC; (b) standard curve for EMA-real time PCR with IAC. DNA samples extracted from mixed cultures of 10^6 CFU dead *Salmonella* cells and different concentrations of viable *Salmonella* cells were used to build these standard curves.

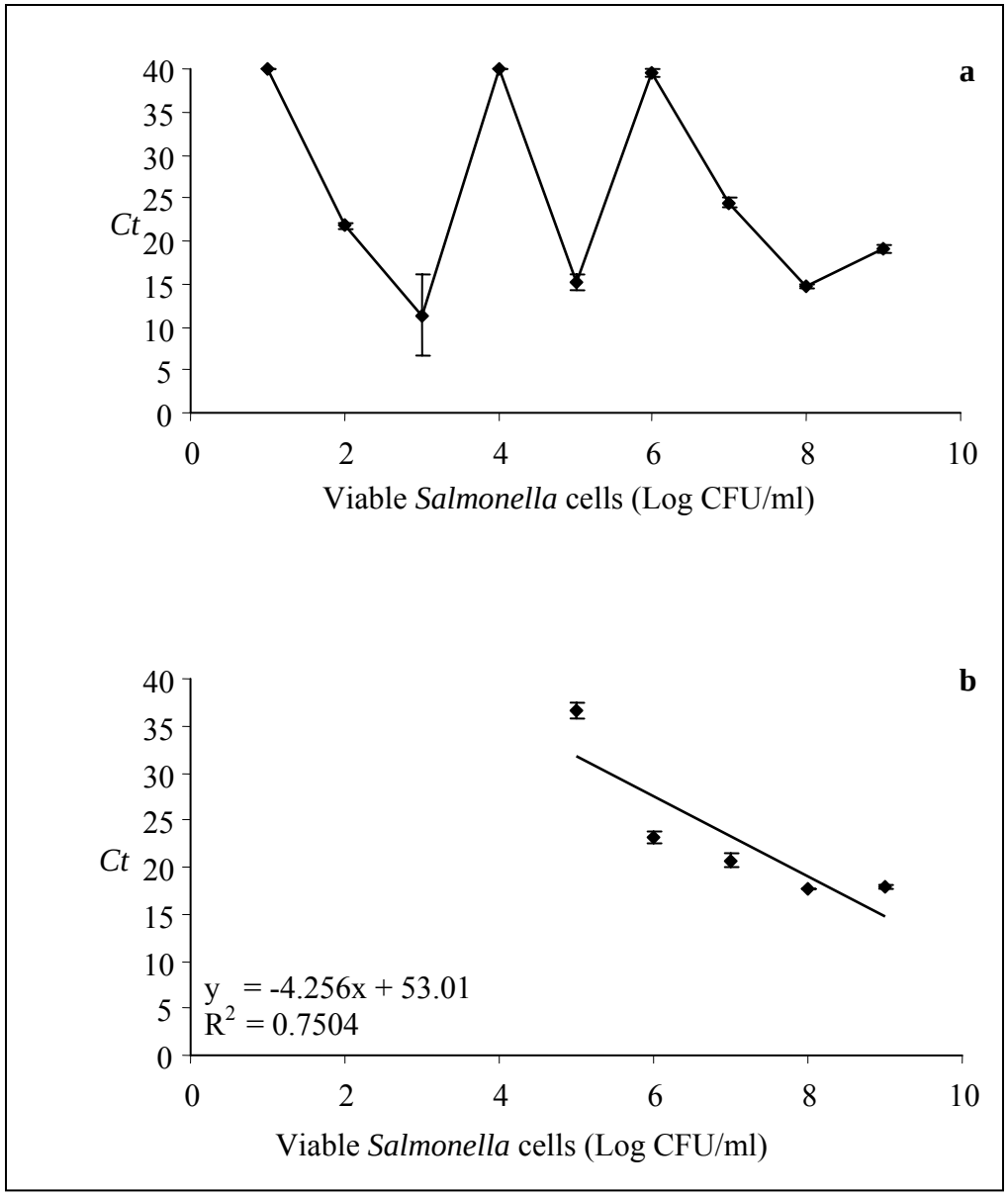

Figure 5.4 Standard curve for the EMA-real time PCR application in artificially contaminated egg broths. (◆) *Salmonella.* (a) detection curve for EMA-real time PCR with IAC of DNA extracted without EMA staining; (b) standard curve for EMA-real-time PCR with IAC of DNA extracted with EMA staining. DNA samples extracted from egg broths artificially contaminated with 10^6 CFU dead *Salmonella* cells and different concentrations of viable *Salmonella* cells.

CHAPTER 6

Running Heads: *E. coli* O157:H7, *Salmonella*, QD antibody conjugates and bead free

DETECTION OF *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA* IN GROUND BEEF BY BEAD-FREE QUANTUM DOT-FACILITATED DETECTION METHOD

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Key words: Quantum dots, Bead-free immunomagnetic separation, *Escherichia coli* O157:H7, *Salmonella*, ground beef

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ABSTRACT

Accurate and fast detection methods for foodborne pathogens from various food samples have always been important goals for scientists from many research areas. Quantum dots (QDs) are a family of nanosized particles with of 1 to 10 nm in radius. It has long-term stable photostability, high quantum yield, broad absorption spectra, narrow emission spectra and high signal-to-noise ratio. QD has been used in cell detection, imaging and DNA hybridization. In this study, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and protein A were used to build crosslinkers for making QD antibody conjugates. In order to minimized the interference generated from magnetic beads, FlowComp™ Dynabeads with DSB-X™ biotin protein labeling kit (D-20655) were used to isolated the cells from food matrix and the beads were removed after isolation. Detection signals were dramatically increased with the usage of bead free isolation method. When the bead-free QD-facilitated detection method was used to detect *Salmonella* and *E. coli* O157:H7 cells from pure cultures, it could detect as low as 10 CFU/ml cells. When it was applied to artificially contaminated ground beef, it could detect 10^6 CFU/g targets. After enrichment, as low as 10 CFU/g *Salmonella* cells were detected from ground beef. The bead-free QD-facilitated detection method developed in this study is the first research that combines a bead-free isolation method and QD labeling technique to detect *E. coli* O157:H7 and *Salmonella* in ground beef.

INTRODUCTION

Escherichia coli O157:H7 is an important cause of foodborne diseases with symptoms of diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (Dune and others 2004; Griffin 1998; Nataro and Raper 1998). *Salmonella* is widely distributed in nature and is a major cause of foodborne illness with symptoms of nausea, vomiting, and diarrhea. "While products such as produce, fruits, and vegetables are increasingly being implicated in *Salmonella* foodborne illnesses, red meat and poultry remain predominant products contributing to foodborne illnesses" (Juneja and others 2009). Contaminated ground beef not only causes diseases, economic losses of processors and distributors are huge. The U.S. Department of Agriculture (USDA) estimated the annual medical costs, productivity losses, and value of premature deaths due to *Campylobacter,* nontyphoidal *Salmonella*, *Escherichia coli* O157:H7, *E. coli* non-O157:H7 STEC, and *Listeria monocytogenes* at $6.9 billion (USDA-ERS 2001). Accurate and fast methods for foodborne pathogen detection from various food samples have always been a desirable and important goal that attracted scientist attention.

Quantum dots (QDs) are a family of nanosized particles comprised of a few thousands atoms, with typical size of 1 to 10 nm in radius. QD exhibits a size tunable band gap and, hence, fluorescence spectra, allowing different colors to be exhibited. Compared with conventional fluorescent dyes, QDs have long-term stable photostability, high quantum yield, broad absorption spectra and narrow, symmetric emissions (Duong and Rhee 2007; Xue and others 2009). QDs demonstrate high signal-to-noise ratio, thus the sensitivity of detection method using QDs can be improved. QDs, as novel fluorescent markers, have been applied broadly in biological studies, like cell imaging

(Hirschey and others 2006), DNA labeling (Wu and others 2006), and pathogenic bacteria detection (Hahn and others 2005). In 1998, usage of colloidal QDs for biological labeling was first reported and the result suggested that its photochemical stability and its ability to tune broad wavelength make QDs extremely useful for biolabeling (Alivisatos and others 2004). Colloidal method synthesized CdSe/ZnS nanocrystal is a core/shell structure nanoparticle. It has attracted great attention due to its significantly larger linear absorption cross section for excitation (Leatherdale and others 2002). CdSe/ZnS core/shell QDs have been the most commonly used and the best understood QDs.

To apply QDs in biological studies, crosslinkers are built to conjugate them with different biological groups. Interactions between biotin and avidin are among the strongest non-covalent affinities. This interaction has a dissociation constant of about 1.3×10^{-15} M and is one of the most popular crosslinkers used for QD biological molecule conjugation. Immunomagnetic separation (IMS) is a method used for cell isolation from environmental or food samples. Detection of foodborne pathogens by combining QD-labeled antibodies with IMS has been developed in previous studies. Successful applications have been demonstrated for *E. coli* O157:H7 detection with a detection range of 10^3 to 10^7 CFU/ml (Su and Li 2004), *Salmonella* Typhimurium detection from chicken carcass wash water with a detection range of 10^3 to 10^7 CFU/ml (Yang and Li 2005), and *S*. Typhimurium, *Shigella flexneri* and *E. coli* O157:H7 detection from apple juice and milk, with a detection limit of 10^3 CFU/ml (Zhao and others 2009).

In this study, instead of using avidin/biotin crosslinkers, a combination of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and protein A will be used to build crosslinkers between the antibodies and QDs. In addition, a bead-free IMS

method will be applied and evaluated. The purpose of this study is to develop a QD-based bead-free detection method to detect *E. coli* O157:H7 and *Salmonella* in artificially contaminated ground beef products.

MATERIALS AND METHODS

Fabrication of CdSe/ZnS QDs. Four grams of 99% trioctylphosphine oxide (Sigma-Aldrich, St. Louis, MO), 4 g of hexadecylamine (Sigma-Aldrich, St. Louis, MO), 30 mg of cadmium oxide (CdO) (Sigma-Aldrich) and 0.6 g lauric acid were mixed to make QDs. By injecting the element selenium (180 mg in 2 ml trioctylphosphine (TOP)) (Sigma-Aldrich) at different temperatures (240^{o}C for green QDs, 300^{o}C for orange QDs), the core structures of QDs were fabricated in the Biophotonic laboratory of the University of Missouri, Columbia. To increase the fluorescence efficiency and stability of these freshly fabricated QDs, shell structures, consisting of layers of ZnS, were added to the cores. The temperature of the 3-neck flask was set to 80^{o}C, 1.68 ml or 1.06 ml of diethylzinc (Sigma-Aldrich) (1.0 M in hexane) and hexamethyldisilathiane $(TMS)_2S$ (Sigma-Aldrich) stock mixture were added to green or orange QD core structures, respectively. The ZnS stock mixture was prepared by combining 1 M of $(TMS)_2S$ in the TOP solution with 1 M dimethylzinc in a sealed vessel. Due to the concentrated nature of these solutions, the combined ZnS solution cannot be used for more than two days, therefore, it was prepared in the minimum necessary amount right before the fabrication of QD core structures.

Water soluble QDs. The method for making water soluble QDs was adopted from Mattoussi's group (Mattoussi and others 2000; 2001) and modified. QDs in

chloroform were washed with methanol for removing any residual TOPO. The QDs were then transferred into a pre-weighed vial and the chloroform was evaporated, leaving dry QDs as a powder. After drying, 1 ml DHLA and 0.5 ml methanol was added for each 80 mg QDs. The vial was then sealed and heated on an 80°C hotplate for 8 h with vigorous stirring. After 8 h, the QDs were well dissolved in the DHLA/methanol mixture. To this mixture, 0.5 mL of 5 M aqueous sodium and 3 ml dimethylformamide were added to each 80 mg QDs. The QDs were then precipitated by centrifugation, resuspended in 1 mL of pH 7 buffer and filtered through a 0.2 μm syringe filter.

Construction of EDC protein A crosslinker on QD surfaces. The concentration of water soluble QDs was around 120 nmol/L based on the analysis by Instrumental Neutron Activation Analysis (INAA) at the Chemistry Department of the University of Missouri, Columbia. Ten milligrams of EDC (Sigma-Aldrich) was dissolved in 0.5 ml MES (2-N-morpholino) ethanesulfonic acid) (Sigma-Aldrich) solution to make EDC stock solution. Twenty five microliters and 28 μl of EDC stock solution were added to 100 μl green and 100 μl orange QDs, respectively. Various amounts of protein A (0.5 mg/ml) (Sigma-Aldrich) were added right after the EDC. The mixtures were incubated on a rotator (Labquake®, Barnstead International, Dubuque, IA) in a cold room (4^{o}C) for 2 h.

Gel electrophoresis. The gel electrophoresis of QDs with crosslinkers was conducted in a Bio-Rad Mini-Sub® cell GT and Bio-Rad Power Pac 300 (Bio-Rad, Hercules, CA) at 100 V for 50 min. A 1 kb DNA ladder (Promega, Madison, WI) was used as a molecular weight marker. An agarose gel was made by dissolve 0.5 g of Agarose Low EEC (Fisher, Fair Lawn, NJ) in to 1 X TBE buffer. Two microliters of

Amresco® Envision DNA dye loading buffer (Amresco, Solon, OH) was used to load each 10 µl QD sample.

Antibody-QD conjugation. *E. coli* O157:H7 monoclonal antibodies and *Salmonella* monoclonal antibodies were purchased from Genway (San Diego, CA). Antibody QD conjugation process was started with the addition of 25 µl (for green QDs) or 28 µl (for orange QDs) EDC and 1.5 mg protein A to each 100 µl QDs. After that, 100 µg of antibodies were added, followed by an additional 2 h incubation in a 4°C cold room. Antibody-QD conjugates with EDC protein A crosslinker were then ready for usage.

Biotin antibody labeling. A biotin protein labeling kit (DSB-X™, Invitrogen Dynal AS, Oslo, Norway) was used to label antibodies with the unique DSB-X biotin ligand according to the manufacturer's instructions. Two hundred microliters of 0.5 mg/ml antibody solution were added to a 2 ml reaction tube containing a stir bar. Twenty microliters of 1 M sodium bicarbonate solution and 2 µl of DSB-X biotin solution were added to the antibody and reacted with antibody in room temperature for 1.5 h with stirring. The mixture was then centrifuged for purification using the columns provided in the kit at 1100×g for 5 min. The mixture passing through the column was the biotin labeled antibody.

Bead-free pathogen isolation. Eight tubes of 1 ml *E. coli* O157:H7 and nine tubes of 1 ml *Salmonella* with different concentrations, 10^1 to 10^8 CFU/ml for *E. coli* O157:H7 and 10^1 to 10^9 CFU/ml for *Salmonella*, were prepared respectively. The cells were washed twice with isolation buffer, which was made of Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS), 0.1% BSA and 2mM EDTA. The cells were then

resuspended in 1 ml isolation buffer. Twenty five microliters of DSB-X labeled antibodies were added to the cells and mixed well. The mixture was incubated at 4°C for 30 min. Seventy five microliters of FlowComp™ Dynabeads were added, followed by a 30-min incubation at 4°C. Tubes containing cells, DSB-X labeled antibodies and FlowComp™ Dynabeads were placed on a magnetic particle concentrator (Dynal MPC®, Dynal Biotech., Oslo, Norway) for 5 min. Dynabeads with cells were retained and washed on the magnetic separator. One milliliter of FlowComp™ release buffer was added to each tube and mixed by rolling and tilting the tube for 20 min at room temperature. Tubes were placed back on the magnetic separator and the beads were discarded. The solution containing the bead-free target cells was transferred into a new tube for further analysis. After this step, the bead-free *E. coli* O157:H7 or *Salmonella* cells were isolated from the food into release buffers (Invitrogen, Carlsbed, CA).

Detection of bead-free pathogens by QD-labeled antibody. Twenty-five microliters of QD-labeled *E. coli* O157:H7 antibodies and 25 µl of QD-labeled *Salmonella* antibodies were separately added into tubes containing isolated *E. coli* O157:H7 cells and isolated *Salmonella* cells, respectively. Tubes were then placed in 4°C for 30 min to allow the QD-labeled antibodies to attach to the cells. After 30 min, cells will be centrifuged at 12,000×g for 5 min and washed twice in PBS buffer (pH 7.0). Cell pellets were then resuspended in 100 µl of PBS and checked using a FluoroMax-3 spectrophotometer (Jobin Yvon, Longjumeau, France).

Comparison between bead-free detection method with bead-assisted detection method. Two tubes of 1 ml 10^8 CFU/ml *E. coli* O157:H7 cells were washed by isolation buffer twice. One tube of the cells was processed following the procedure described above, while the second tube was processed as follows. Seventy-five microliters of Dynabeads® *E. coli* O157:H7 magnetic beads (Invitrogen) were added to the cells and the mixture was incubated at 4°C for 30 min. Cells caught by the magnetic beads were isolated from the suspension by magnetic concentrator. These bead-cell complexes were washed twice with PBS and resuspended in 1 ml PBS. Twenty-five microliters of QD-labeled antibodies were added and incubated at 4°C for 30 min. After 30 min, the beads, cells and QD-labeled antibody complex were recollected by a magnetic concentrator and resuspended in 100 µl of PBS for fluorescence checking. The fluorescence given out by the cells isolated by the magnetic beads and fluorescence from the cells isolated by bead-free method were compared.

Detection of *E. coli* O157:H7 and *Salmonella* from artificially contaminated ground beef by bead-free QD-facilitated detection method. Ground beef samples were obtained from the Meat Laboratory of the University of Missouri, Columbia. Samples were checked by standard methods according to the Bacteriological Analytical Manual (FDA 1995) and confirmed to be *E. coli* O157:H7- and *Salmonella*-free before usage. Eight samples of 25 g artificially contaminated ground beef were prepared by inoculating the beef with pure *E. coli* O157:H7 cells and *Salmonella* cells at final concentrations of 10^1 to 10^8 CFU/g of both pathogens. Two hundred and twenty five milliliters of 0.1% peptone water (Difco Labs., BD Diagnostic Systems, Sparks, Md. USA) were added to each of 25 g artificially contaminated ground beef samples. The

samples were then homogenized by stomaching for 2 min in stomacher (Tekmar Co., Cincinnati, OH). A 1 ml suspension was pipetted out from each sample and centrifuged at 12,000×g for 5 min. The pellets were washed twice and resuspended in 1 ml isolation buffer. Samples were then processed following the bead-free pathogen isolation and the detection of bead-free pathogens isolated from samples by QD-labeled antibody procedures with some modifications to realize the multiplex detection of *E. coli* O157:H7 and *Salmonella* in one tube. After resuspending the pellets in 1 ml isolation buffer, 25 µl of green QD-labeled *E. coli* O157:H7 antibodies and 25 µl of orange QD-labeled *Salmonella* antibodies were added at the same time. The suspensions were then incubated for 30 min at 4^{o}C. Fluorescence generated by *E. coli* O157:H7 cells and *Salmonella* cells were read simultaneously in the spectrophotometer.

Detection of target cells in low concentrations from ground beef. Five samples of 25 g artificially contaminated ground beef were prepared by inoculating them with pure *Salmonella* cells to result in the final concentration of 10^5 to 10^1 CFU/g. Two hundred and twenty five milliliters of tryptic soy broth supplemented with 0.5% yeast extract (TSBY) (Difco Labs.) were added to the beef samples. Homogenized beef suspensions were enriched at 35^{o}C for 24 h. Enriched broths were used for the pathogen detection following the procedure described above.

Fluorescence measurement. One hundred microliters of each sample were used for fluorescence measurement using a FluoroMax-3® spectrophotometer (Jobin Yvon, Longjumeau, France). The excitation light was 350 nm and the slit sittings were 7 nm for excitation light and 7 nm for emission. The integrations time was 0.5 s.

RESULTS

Antibody QD conjugation. Antibody QD conjugation process started with the addition of various volumes of 0.02 mg/ml EDC and different amounts of 0.5 mg/ml protein A. The minimum amounts of protein A needed for QDs were tested to make sure that all active EDC linkers present on QD surfaces would be used. QD:protein A volume ratios of 1:3, 1:15, 1:30, 1:45 and 1:60 were tested and gel electrophoresis was performed to check the conjugation results. As shown in Figure 6.2(a), the size of the conjugation products increased with the addition of protein A to the QDs. In lanes 3 and 4, there were some unconjugated QDs left by showing fluorescence at the molecular weight level of pure QDs. From Lane 5, no fluorescence were seen at the molecular weight of pure QDs, which indicated that all active EDC crosslinkers had been conjugated with protein A. Thus, a volume ratio of 1:30 was chosen to be the final ratio used for making QD:protein A complexes. Figure 6.2(b) shows electrophoresis results of the green QD:protein A complex at a ratio of 1:30 (v:v).

After making the QD:protein A complex, *E. coli* O157:H7 antibodies or *Salmonella* antibodies (0.5 mg/ml) was added to make the QD-antibody conjugates. The structure of the QD-antibody conjugates using EDC and protein A as crosslinkers is shown in Figure 6.1. The fluorescence emitted by pure QDs and that emitted by QD-antibody conjugates were compared and the emission curve is shown in Figure 6.3. As shown in Figure 6.3, the conjugation process did not change the emission efficiency of the QDs, but it slightly enhanced the emission from the QDs.

Comparison between bead-free detection method with bead-assisted detection method. Two tubes of 1 ml 10^8 CFU/ml *E. coli* O157:H7 cells were used. One tube of cells was processed following the bead-free procedure, while the second tube was processed with the traditional IMS method, with the beads left on the cell surfaces in the final fluorescence detection part. As shown in Figure 6.4, the fluorescence generated from cells using the bead-free isolation method was 2.6 times higher than the fluorescence emitted by the cells isolated with beads left on surfaces. The bead-free isolation method increased the signal dramatically and, thus, have the potential to detect lower pathogen concentrations.

Detection of bead-free pathogens by QD-labeled antibody. Twenty-five microliters of QD-labeled *E. coli* O157:H7 antibodies and 25 µl QD-labeled *Salmonella* antibodies were added into tubes containing bead-free *E. coli* O157:H7 and *Salmonella* cells. Tubes were then placed in a 4°C cold room for 30 min, centrifuged at 12,000×g for 5 min and washed twice in PBS (pH 7.0). Cell pellets were resuspended in 100 µl of PBS for fluorescence detection. It can be seed from Figures 6.5 and 6.6 that the detection limit of the bead-free QD-facilitated detection method is 10 CFU/ml for *E. coli* O157:H7 and 10^3 CFU/ml for *Salmonella*. Fluorescence from PBS was also read to determine the baseline.

To prove the specificity of the QD-labeled antibodies and the efficiency of this bead-free QD-facilitated detection method, one tube containing 10^8 CFU/ml *Salmonella* cells was processed as follows: After isolating the *Salmonella* cells by the bead-free method, 25 µl of QD-labeled *E. coli* O157:H7 antibodies were added into 10^8 CFU *Salmonella* cells and incubated with the cells for 30 min in the cold room (4°C). After 30

min, the cells were washed by PBS and the fluorescence was read by a spectrophotometer. The results proved the high specificity of the bead-free detection method by showing very weak false positive signal, by comparing it to fluorescence emitted by *E. coli* O157:H7 (Figure 6.5a).

Detection of *E. coli* O157:H7 and *Salmonella* from artificially contaminated ground beef by bead-free QD-facilitated detection method. Eight samples of 25 g ground beef were artificially inoculated with pure *E. coli* O157:H7 and *Salmonella* cells to make the final concentration of 10^8 to 10^1 CFU/g for both pathogens. Two hundred and twenty five milliliters of 0.1% peptone water were added to each 25 g of artificially contaminated ground beef and homogenized for 2 min. The suspensions were then used for the bead-free *E. coli* O157:H7 and *Salmonella* isolation. Fluorescence from green QDs on *E. coli* O157:H7 surfaces and orange QDs on *Salmonella* surface were measured simultaneously. The results showed that this method can quantitatively detect the target pathogens at concentrations of 10^8 and 10^6 CFU/g, respectively (Figure 6.7).

Detection of low concentrations of target cells in ground beef. Five samples of 25 g artificially contaminated ground beef were prepared by inoculating the ground beef with pure *Salmonella* cells to make the final concentration of 10^1 to 10^5 CFU/g. Two hundred and twenty five milliliters of TSBY were added to the beef samples. Homogenized beef suspensions were enriched at 35^{o}C for 24 h and used for pathogen detection by the bead-free isolation method. As shown in Figure 4.8, the fluorescence of 10^1 CFU/g is equal to 10^8 CFU/g after a 24 h enrichment which indicated that, after a 24 h enrichment, this bead-free QD-facilitated detection method can detect as low as 10 CFU/g target pathogens in ground beef.

DISCUSSION

Quantum Dots (QDs), due to their size dependent fluorescence properties, have been suggested to be useful dye placers. One of their unique fluorescence properties is that they can emit different colors under one light source. This advantage makes QDs an ideal choice for simultaneous detection of target cells, because it can avoid the excitation light energy fluctuation caused by the changing of light sources. In addition, their stability at various environments makes it available for most biological studies and a wise choice for an on-site biosensor development.

In the food safety field, although many preharvest and postharvest intervention methods have been conducted, good agricultural practices (GAPs) are the keys to ensure food safety. The success of food safety control depends on the effect of GAPs, which put sensitive on-site detection sensors, which can monitor GAP results in real time, in high demand. According to the results of this study, we believe that the successful lab based application of QD antibody conjugations in food pathogen detection is a promising first step for future on-site biosensor development.

Instead of using traditional biotin/avidin crosslinkers to construct the QD-antibody conjugates, active EDC and protein A were used. Protein A, a cell wall component of *Staphylococcus aureus*, has a high affinity for the CH_2-CH_3 domains of the Fc regionof IgG, thus blocking the binding to the antigen receptors (Honegger 2008). EDC is a zero-length crosslinker and can decrease cross-reactivity potential by building a direct linkage between two substances (Hermanson 1996). The principle of using EDC as a crosslinker is that the EDC can link two molecules, one containing an amine group

and the other one containing a carboxylate group, together. However, using only an EDC crosslinker has one drawback, which is the presence of both carboxylates and amines on one molecule that can result in self-polymerization (Hermanson 1996). Because water soluble QDs manufactured in this study have carboxyl groups on their surfaces and the antigen binding regions on antibodies are full of amine groups, the application of EDC directly to QDs and antibodies will cause random conjugation and self polymerization between two conjugates [Figure 6.1(a)]. Thus, protein A was added to complete the crosslinkers by building a bridge between the EDC and the antibodies [Figure 6.1(b)]. The conjugates made by EDC and protein A crosslinkers can have uniform structures and, by applying enough protein A for EDC linker on QD surfaces, the EDC protein A crosslinkers can prevent potential self polymerization caused by excess active EDC. In this study a ratio of 1:30 (v:v) between QDs and protein A (0.5mg/ml) showed complete binding of protein A to all active EDC crosslinkers on QD surfaces as demonstrated by agarose gel electrophoresis.

FlowComp™ Dynabeads with DSB-X™ biotin protein labeling kit (D-20655) is a commercial available kit that can isolate target cells from environmental or food samples. Compared to traditional immunomagnetic beads, it can provide bead-free cells for further analyses. FlowComp™ Dynabeads are beads that have active streptavidin linkers on their surfaces and this protein labeling kit can add active biotin linkers to antibodies. Based on streptavidin/biotin binding, antibodies can be bound to magnetic bead surfaces and be separated from the beads by the release buffer when they become unnecessary. As shown in Figure 6.4, the cells isolated by FlowComp™ Dynabeads generated a higher emission signal compared to the cells isolated by immunomagnetic bead method, which

proved that the presence of beads inhibited the fluorescence signal from QDs. Studies have been carried out to figure out the reasons for this inhibition. One possible reason is due to the autofluorescence generated from magnetic beads (Agrawal and others 2007). In Nie's lab, they obtained and compared the emission spectra of magnetic microbeads with QD-605 and QD-525. They found that when a blue excitation source was used (λ_{ex}~488 nm), there was a significant spectral overlap between bead emission and QD-605, and the spectral overlap was reduced when the QD emission spectrum was shifted to 525 nm. In another study conducted by Sathe and others (2006), scientists concluded that the excitation light was strongly absorbed by iron beads, thus attenuating the light intensity reaching the QDs. The interference between QDs and magnetic beads can, thus, be summarized as magnetic beads inhibit the emission efficiency of QDs due to their autofluorescence and strong absorbance. Together with this study's results, the bead-free isolation method is highly recommended for future cell isolation and detection.

The bead-free QD-facilitated detection method was applied to *E. coli* O157:H7 and *Salmonella* pure cultures. As shown in Figures 6.5 and 6.6, the bead-free QD-facilitated method can detect as low as 10 CFU/ml cells for *E. coli* O157:H7 and 10^3 CFU/ml for *Salmonella*. To test the specificity of the QD antibody conjugates, one *Salmonella* sample containing 10^8 CFU cells was prepared and QD-labeled *E. coli* O157:H7 antibodies were added. After a 30 min incubation at 4^{o}C, *Salmonella* cells were recollected and washed by PBS. Fluorescence signal was shown in Figure 4.5. It showed that although *Salmonella* gave out false positive signals, compared to PBS solution, this signal is extremely lower than the signal generated from 10^8 CFU/ml *E. coli* O157:H7 and 10 CFU/ml *E. coli* O157:H7. This demonstrated the successful conjugation of QDs

to *E. coli* O157:H7 antibodies and the specificity of this bead-free detection method. When applying the bead-free detection technique to artificially contaminated ground beef, this method can only detect 10^6 CFU/g *E. coli* O157:H7 and *Salmonella* in ground beef. The most important reason for this low detection limit is the presence of fat and meat tissues in beef suspension. After a 24-h enrichment, the bead-free QD-facilitated detection method can detect as low as 10 CFU/g *Salmonella* cells, the signal of which is equal to that generated from 10^8 CFU/g *Salmonella* in ground beef.

To summarize, this study is the first to use a bead-free isolation method to separate target cells from ground beef samples. By combining this method with QD-labeled antibodies, this newly designed bead-free QD-facilitated detection method can detect as low as 10 CFU/ml target cells from pure cultures and 10 CFU/g target cells from ground beef after a 24-h enrichment. Although false positive signals are generated, especially in artificially contaminated beef samples, the strong fluorescence signal generated from low concentration bead-free cells made it a sensitive detection method. Our ongoing work is to find a better way to minimize the interference generated from meat tissues when applying this detection method in ground beef samples, as well as to apply this new method to other food products, such as fresh vegetables and fruits.

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REFERENCES

Agrawal A, Sathe T, Nie S. 2007. Single-bead immunoassays using magnetic microparticles and spectral-shifting quantum dots. J Agric Food Chem 55: 3778-82.

Alivisatos P. 2004. The use of nanocrystals in biological detection. Nat Biotech 22: 47-52.

Chan WCW, Nie SM. 1998. Quantum dot bioconjugates for ultrasensitive nonisotopic detection. Sci 281: 2016-8.

Committee on the Review of the USDA *E. coli* O157:H7, Farm-to-Table Process Risk Assessment. 2002. *Escherichia coli* O157:H7 in ground beef: Review of a Draft Risk Assessment. Washington, D.C. The National Academies Press.

Dunn JR, Keen JE, Vecchio RD, Wittum TE, Thompson RA. 2004. *Escherichia coli* O157:H7 in a cohort of weaned, preconditioned range beef calves. J Food Prot 67(11): 2391-6.

Duong H, Rhee J. 2007. Use of CdSe/ZnS core-shell quantum dots as energy transfer donors in sensing glucose. Talanta 73:899-905.

FDA. 1995. Bacteriological Analytical Manual, 8th ed. Gaithersburg, MD: AOAC International. p 5.

Griffin PM. 1998. Epidemiology of shiga toxin-producing *E. coli* infections in human in the United States. In: O'Brien AD, Kaper JB, editors. *Escherichia coli* O157:H7 and other shiga toxin-producing *E. coli* strains. American Society for Microbiology, Washington, D. C. p. 15-21.

Hahn MA, Tabb JS, Krauss TD. 2005. Detection of single bacterial pathogens with semiconductor quantum dots. Anal Chem 77(15): 4861-9.

Hermanson GT. 1996. Bioconjugate techniques. San Diego, Calif. Academic Press.

Hirschey MD, Han YJ, Stucky GD, Butler A. 2006. Imaging *Escherichia coli* using functionalized core/shell CdSe/CdS quantum dots. J Biol Inorg Chem 11(5):663-9.

Honegger A. 2008. http://www.bioc.uzh.ch/antibody/ Accessed on May 16th 2009.

Juneja VK, Melendres MV, Huang L, Subbiah J, Thippareddi H. 2009. Mathematical modeling of growth of *Salmonella* in raw ground beef under isothermal conditions from 10 to 45°C. Int J Food Microbiol 131(2-3): 106-11.

Leatherdale CA, Woo WK, Mikulec FV, Bawendi MG. 2002. On the absorption cross section of CdSe nanocrystal quantum dots. J Phys Chem 106: 7619-22.

Mattoussi H, Mauro JM, Goldman ER, Anderson GP, Sundar VC, Mikulec FV, Bawendi MG. 2000. Self-assembly of CdSe-ZnS quantum dot bioconjugates using an engineered recombinant protein. J Am Chem Soc 122(49):12142-50.

Mattoussi H, Mauro JM, Goldman ER, Green TM, Anderson GP, Sundar VC, Bawendi MG. 2001. Bioconjugation of highly luminescent colloidal CdSe-ZnS quantum dots with an engineered two-domain recombinant protein. Physica Status Solidi(b) 224(1):277-83.

Nataro JP, Kaper JB. 1998. Diarrheagenic *Esherichia coli*. Clin Microbiol Rev 11:142-201.

Sathe T, Agrawal A, Nie S. 2006. Mesoporous silica beads embedded with semiconductor quantum dots and iron oxide nanocrystals: dual-function microcarriers for optical encoding and magnetic separation. Anal Chem 78:5627-32.

Su SL, Li Y. 2004. Quantum dot biolabeling coupled with immunomagnetic separation for detection of *Escherichia coli* O157:H7. Anal Chem 76(16):4806-10.

USDA-ERS. 2001. ERS estimates foodborne disease costs at $6.9 billion per year. Economic Research Service-United States Department of Agriculture. Accessed on July 6 2009. Available at: http://www.ers.usda.gov/publications/aer741/aer741.pdf

Wu S, Zhao X, Zhang Z, Xie H, Tian Z, Peng J, Lu Z, Pang D, Xie Z. 2006. Quantum dots labeled DNA probes for fluorescence in site hybridization (FISH) in the microorganism *Escherichia coli*. ChemPhysChem 7(5):1.62-7.

Xue X, Pan J, Xie H, Wang J, Zhang S. 2009. Fluorescence detection of total count of *Escherichia coli* and *Staphylococcus aureus* on water-soluble CdSe quantum dots coupled with bacteria. Talanta 77(5):1808-13.

Yang L, Li Y. 2005. Quantum dots as fluorescent labels for quantitative detection of *Salmonella* Typhimurium in chicken carcass wash water. J Food Prot 68(6): 1241-5.

Zhao Y, Ye M, Chao Q, Jia N, Ge Y, Shen H. 2009. Simultaneous detection of multifoodborne pathogenic bacteria based on functionalized quantum dots coupled with immunomagnetic separation in food samples. J Agric Food Chem 57:517-24.

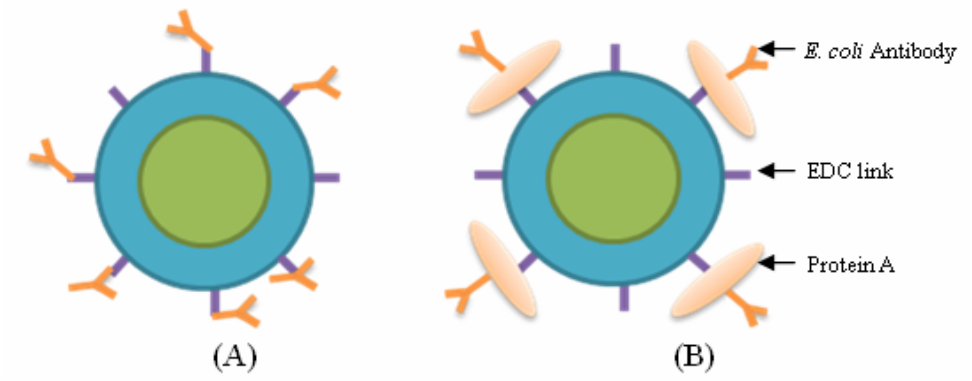

Figure 6.1 Structures comparison of QD antibody conjugates with EDC crosslinkers and QD antibody conjugates with EDC protein A crosslinkers.

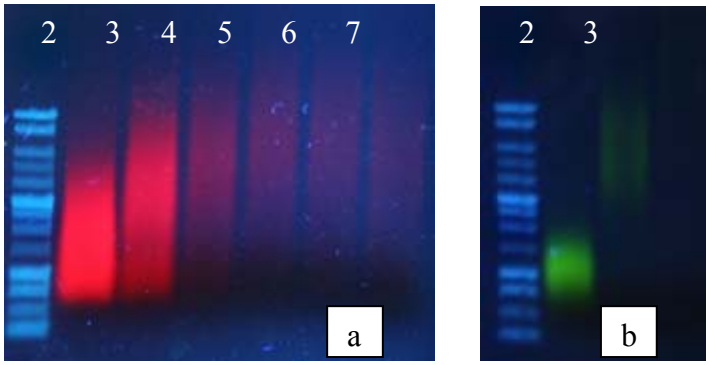

Figure 6.2 Gel electrophoresis of QD protein A complex. (a), gel electrophoresis of QDs (red) with different QDs: protein A volume ratio. Lane 1, 1 kb molecular marker (Promega); Lane 2, pure QDs with EDC crosslinker; Lane 3, QDs with EDC and protein A (QDs:protein A (v:v) = 1:3); Lane 4, QDs with EDC and protein A (QDs:protein A (v:v) = 1:15); Lane 5, QDs with EDC and protein A (QDs:protein A (v:v) = 1:30); Lane 6, QDs with EDC and protein A (QDs:protein A (v:v) = 1:45); Lane 7, QDs with EDC and protein A (QDs:protein A (v:v) = 1:60). (b), gel electrophoresis of QDs (green) with different QDs: protein A volume ratio. Lane 1, 1 kb molecular marker (Promega); Lane 2, pure QDs with EDC crossliner; Lane 3, QDs with EDC and protein A (QDs:protein A (v:v) = 1:30).

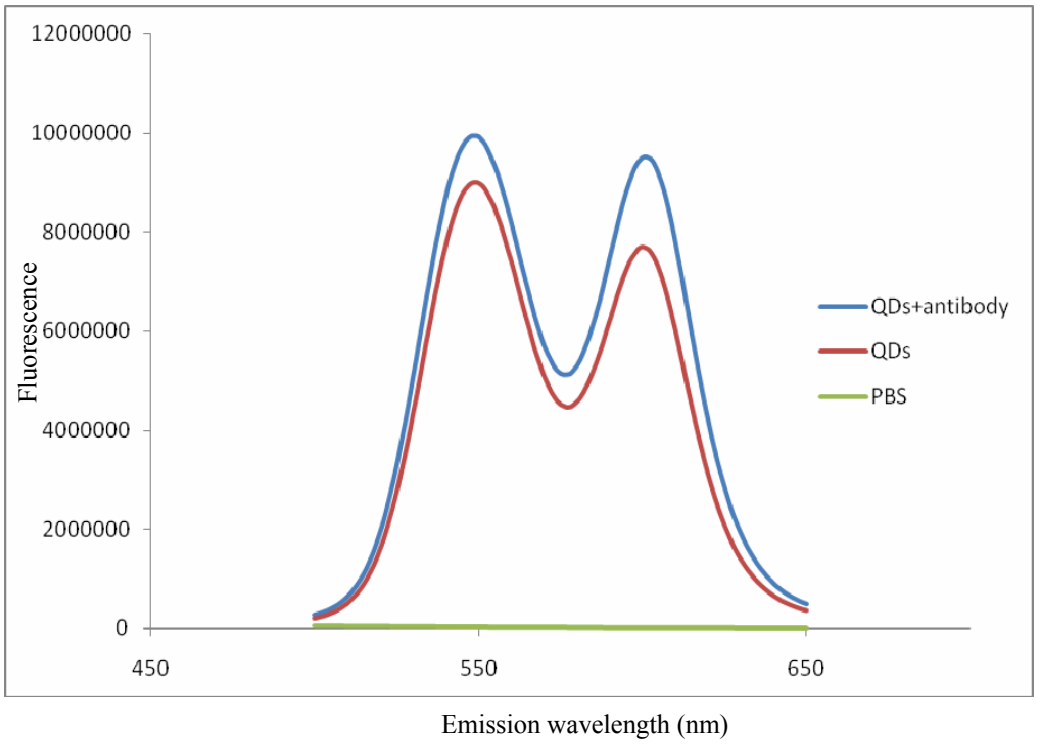

Figure 6.3 Fluoresence comparision between QDs and QD antibody conjugates.

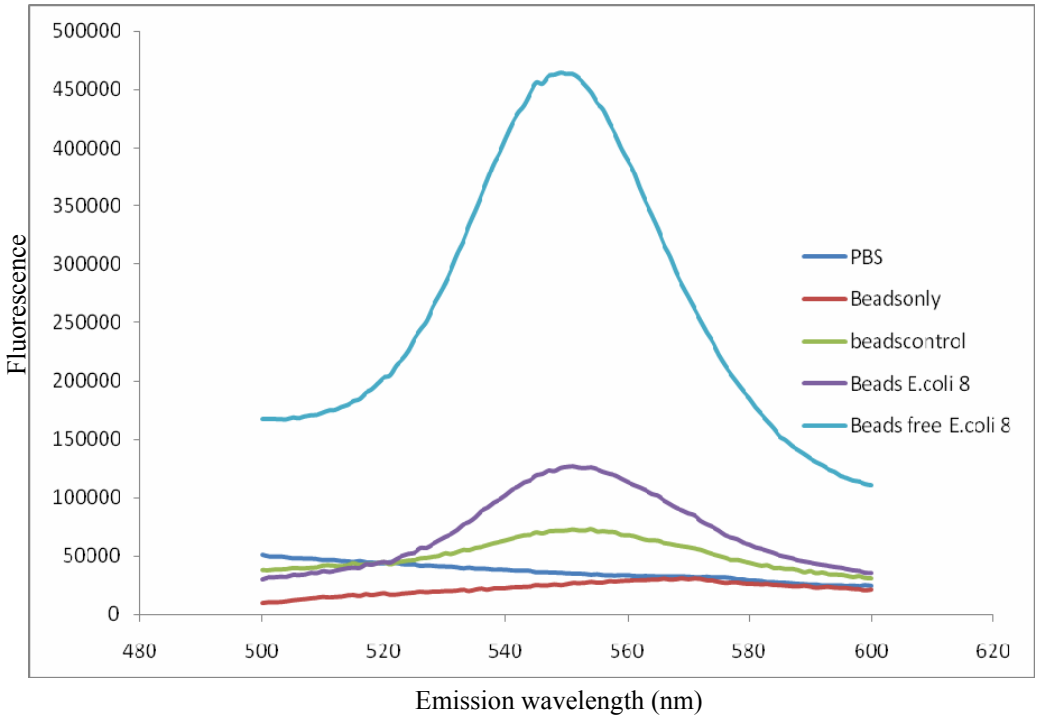

Figure 6.4 Fluoresence comparison between bead free detection method developed in this study and detection method with beads left in the detection system.

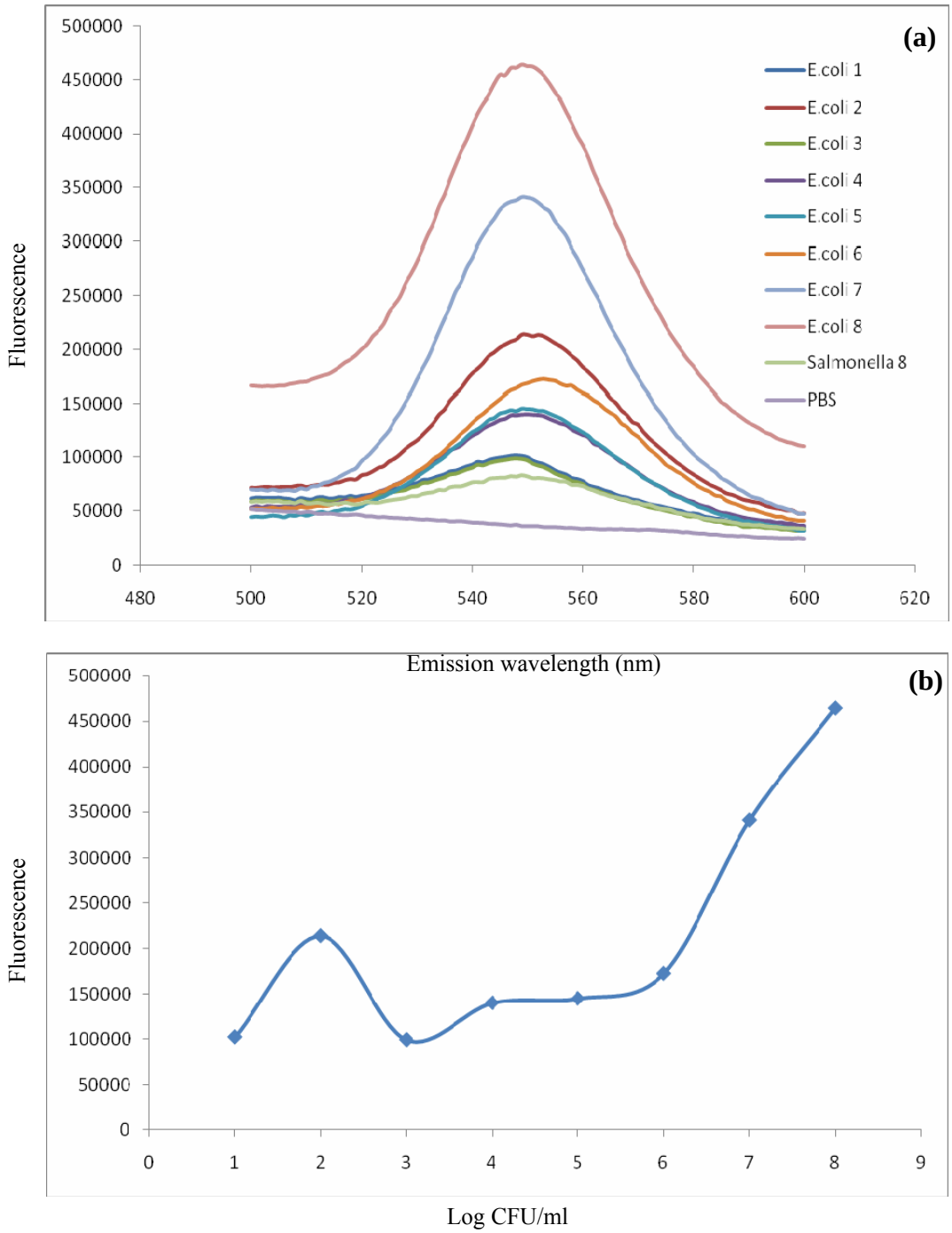

Figure 6.5 Detection range of bead free green QD facilitated detection method for *E. coli* O157:H7. (a) Fluoresence from *E. coli* O157:H7 cells with various concentration (10^8 CFU/ml to 10^1 CFU/ml). (b) Detection curve for *E. coli* O157:H7.

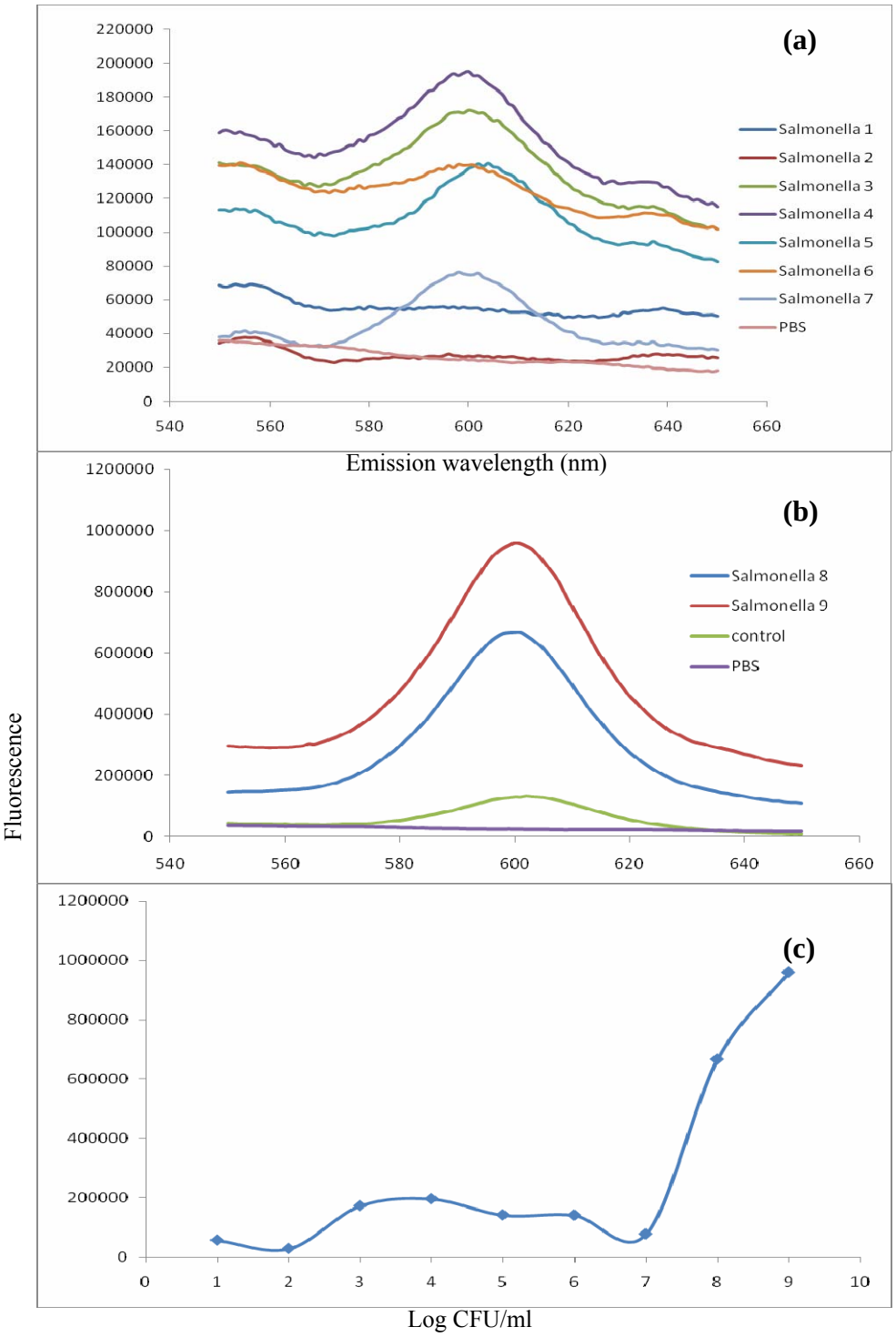

Figure 6.6 Detection range of bead-free orange QD-facilitated detection method for *Salmonella*. (a) and (b) are fluorescence generated from cells with different concentrations. (c) is the detection curve for *Salmonella*.

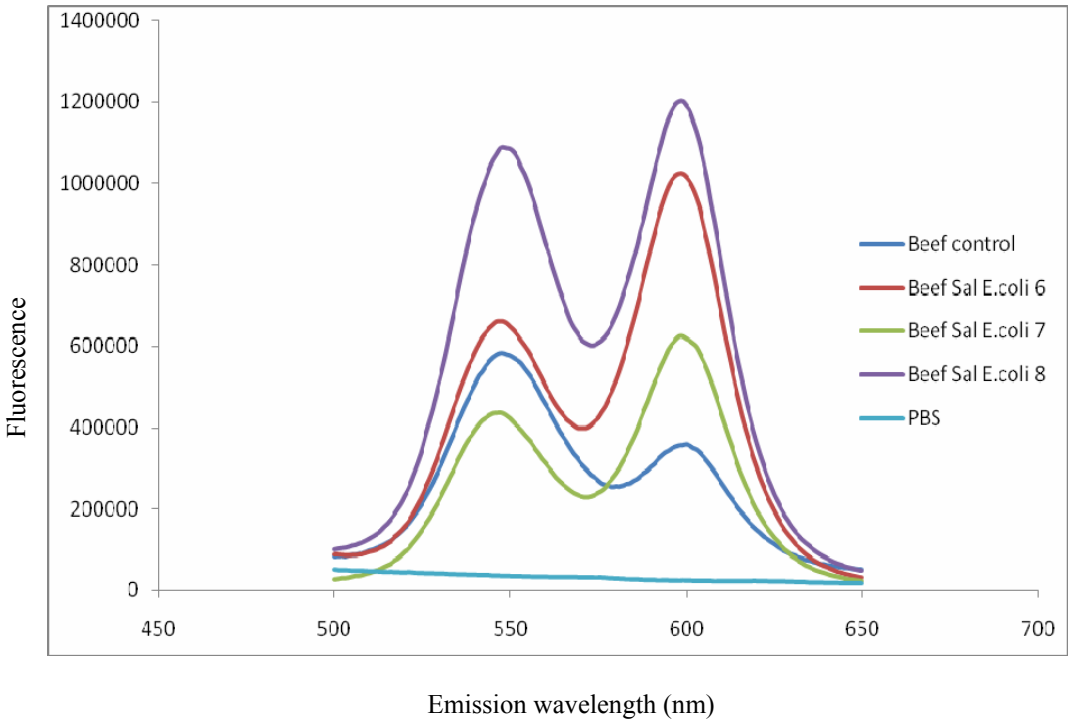

Figure 6.7 Simultanous detection of *E. coli* O157:H7 and *Salmonella* from artifically contaminated ground beef products. Beef control represents the non-inoculated beef sample. "Beef Sal. E. coli 6" represents the ground beef samples artifically contaminated with 10^6 CFU/g *Salmonella* and 10^6 CFU/g *E. coli* O157:H7. "Beef Sal. E. coli 7" represents the ground beef samples artifically contaminated with 10^7 CFU/g *Salmonella* and 10^7 CFU/g *E. coli* O157:H7. "Beef Sal. E. coli 8" represents the ground beef samples artifically contaminated with 10^8 CFU/g *Salmonella* and 10^8 CFU/g *E. coli* O157:H7.

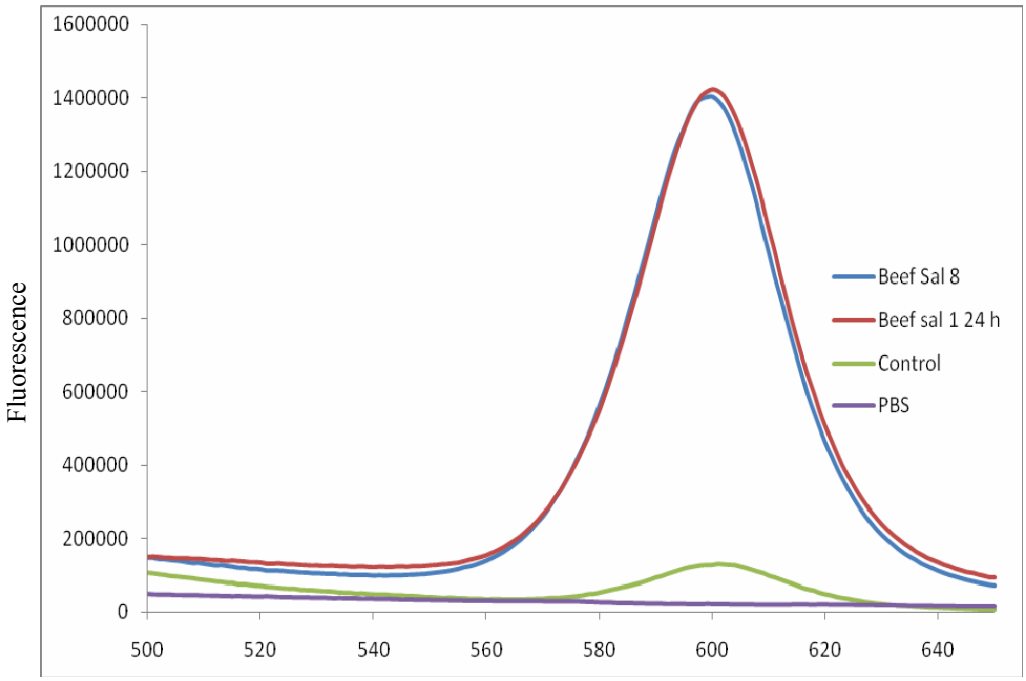

Figure 6.8 Detection of artificially contaminated ground beef at an inoculation level of 10 CFU/g of *Salmonella* with a 24-h enrichment. "Beef Sal 8" represents the ground beef sample contaminated with 10^8 CFU/g *Salmonella* without enrichment. "Beef Sal 1 24 h" represents the ground beef sample contaminated with 10 CFU/g *Salmonella* with 24 h enrichment.

CHAPTER 7

CONCLUSIONS AND FUTURE WORK

The increase in foodborne infections became an important public health concern. Due to changes in people's life style and overall health conditions, the epidemiology of foodborne diseases changed (Meng and Doyle 2002). During the last five years, most of my work focused on the optimization of pathogen detection methods. In Chapter 7, these three projects will be summarized, together with a discussion of some food safety research trends.

7.1 Research project summary

From the first two studies, an ethidium monoazide (EMA) real-time PCR system was successfully developed and was compared with reverse transcription (RT) real-time PCR. The overall EMA staining process takes about 20 min. It starts with a 5 min dark staining, followed by a 1 min ice treatment and a 10 min 650 W light exposure. Ten micrograms of EMA can stain as many as 10^8 CFU dead cells and prevent their further amplification. After staining, the detection range of EMA-real-time PCR is from 10^2 CFU/ml to 10^9 CFU/ml with R^2 of 0.9729, while the detection range of RT-real-time PCR is from 10^5 CFU/ml to 10^9 CFU/ml with R^2 of 0.8017. The addition of 0.25 pg of IAC in each PCR system didn't generate big inhibition on the PCR detection range, although there is a 1 log reduction and small decrease in R^2. The results showed that the EMA real-time PCR can serve as a better detection method for viable cells than RT-real-time

PCR, because EMA real-time PCR showed wider detection range and higher sensitivity. In addition, the DNA templates EMA real-time PCR used are much easier to handle than RNA samples.

The EMA combined real-time PCR was also applied to check artificially contaminated ground beef, chicken carcasses and eggs. This novel detection method can detect 10^4 CFU/g of *E. coli* O157:H7 from ground beef and 10^5 CFU/ml of *Salmonella* from chicken carcass rinses and egg broths. After 12 h enrichment, EMA real-time PCR can detect as low as 10 CFU/g of *E. coli* O157:H7 from ground beef and 10 CFU/ml of *Salmonella* from poultry samples.

In the third project, a nanoparticle based biosensor was invested. After optimizing the conjugation processes and conjugation crosslinkers, the QD-antibody conjugates are in uniform shapes, and all the antigen binding sites on antibodies are facing outside. The crosslinker used in this study was a combination of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and protein A. This crosslinker not only can build uniform conjugates, but also can prevent self aggregation that may happen between two conjugates. By using commercially available bead-free cell isolation kits, QD-antibody facilitated detection method can detect as low as 10 CFU/ml *E. coli* O157:H7 from pure cultures. When this bead-free QD-facilitated detection method was applied to artificially contaminated ground beef products, it could only detect 10^6 CFU/g of *E. coli* O157:H7 and *Salmonella* Typhimurium. After 24 h enrichment, this novel nanoparticle biosensor can detect 10 CFU/g of *Salmonella* Typhimurium from ground beef samples.

7.2 Food safety research trends

7.2.1 Antibiotic-resistant pathogens

Foodborne bacterial infections cause self-limiting diarrhea, thus, appropriate antimicrobial therapy is needed when infection spreads beyond the intestinal tract. Unfortunately, as many foodborne pathogens have developed resistance to antimicrobials, the treatment of severe foodborne infections may be compromised. The reason for these is not only due to the inappropriate usage of antimicrobials in humans, but also due to the routinely application of antimicrobials as a growth factor in animals, for efficiency improvement of feed conversion into meat. This routine practice may have speed up the emergency of antibiotic-resistant bacteria. Antibiotic resistant *Salmonella*, *Campylobacter*, Shiga toxin-producing *Escherichia coli*, *Listeria monocytogenes* and *Yersinia enterocolitica* have already been found (Meng and Doyle 2002).

Although banning the use of antibiotics appears to have resulted in decreases in prevalence of some drug resistant bacteria, subsequent increases in animal morbidity and mortality, especially in young animals, might result in higher use of therapeutic antibiotics.

A considerable amount of information regarding antibiotic resistant foodborne pathogens is available; however, the mechanisms of antibiotic resistant development remain unclear. "As livestock producers, animal health experts, the medical community, and government agencies consider effective strategies for control, it is critical that science-based information provide the basis for such considerations, and that the risks, benefits, and feasibility of such strategies are fully considered, so that human and animal

health can be maintained while at the same time limiting the risks from antibiotic-resistant bacteria" (Mathew and others 2006).

7.2.2 Microbial safety of fresh produce

During the last two decades, more and more people started to pay attention to the nutrition balance of their diet. The 2005 Dietary Guidelines for Americans recommended eating at least four and a half cups of fruits and vegetables a day. A diet, rich in fruit and vegetables, promotes good health and reduces the risk of certain diseases (Meng and Doyle 2002). The annual fresh fruit and vegetable consumption in the United States increased 13% from 1980 to 1990. As fruits and vegetables are eaten raw, outbreaks regarding produce consumption increased dramatically. A comprehensive quality assurance plan is urgent needed to control the pathogenic microbes in produce from the preharvest stages to consumers' plates. In addition, the amount of produce purchased at farmer's markets, road-side stands and neighborhood markets increased, because consumers feel that the direct contact with growers at these markets makes them better able to assess the quality and safety of the produce (Brady and Morris 2009). These facts again highlighted the importance of applying Good Agricultural Practices during growing stage for both big plants and small farms.

Good Agricultural Practices (GAPs) are scientifically based guidelines to eliminate microbial contamination of fresh produce in field during preharvest stages. It starts with the appropriate selection of productive farms. Places that were used for animal production or human activities have bigger contamination risk. However, the contamination risk from the land is related to the time that has asses since the land was used for other activities. Water sources used for irrigation, fertilizer application, together

with human and animal waste management are also parts of GAPs and are important to prevent potential contamination. Personal hygiene of the workers and proper training of produce handling techniques, especially good handling practices (GHPs), are critical to assure produce safety. Cooling, produce cleaning, as well as produce packing and storages need to be well designed in order to prevent postharvest contamination (Brady and Morris 2009).

Pathogenic bacteria, parasites and viruses have been isolated from fresh produce, although spoilage bacteria, yeast and molds are the dominate microflora in them. As fresh produce will continue to be involved in foodborne illness, particularly most of them are imported from countries where pathogen contamination of produce frequently occurs (Meng and Doyle 2002), the application of GAPs, as well as efficient detection methods for imported fresh produce, are needed for food safety and food defense purpose.

7.2.3 Inactivation of pathogenic organisms

The use of preservation technologies to inactivate microorganisms in foods without applicating heat, or with less heat, attracted the attention from food industries who want to avoid undesirable sensory changes and loss of nutrients. Novel preservation methods are available and are under intensive investigation. For example, high-temperature short-time (HTST) pasteurization and ultra-high temperature (UHT) sterilization have been proved that they can minimize the vitamin losses in milk compared with batch pasteurization and conventional commercial sterilization (Lado and Yousef 2002).

Satisfactory evaluation of a new preservation technology depends on its efficacy against pathogenic and spoilage foodborne microorganisms. Novel food-preservation

technologies, like ionizing radiation, high pressure processing, pulse electric field, pulsed white light and ultraviolet radiation are techniques that can inactivate foodborne microorganisms without substantially heating the food. These technologies can produce safe food with high sensory and nutritional values. Cold plasma is another new preservation method. Plasma is a high-energy gas; it is created when an electrical current passes through a gas. Recently, cold plasma could be made under atmospheric conditions. It is now possible to apply the plasma technology in the food industry as novel surface treatment technologies for decontamination.

In addition, researchers have found that nonthermal treatments combined with biopreservatives can enhance the efficiency of these mild preservation technologies. Examples are like the use of essential oils (e.g. carvacrol or thymol) and bacteriocins (e.g. nisin). Nisin is the only bacteriocin that has been approved by the WHO to be used as food preservative. Research demonstrated that pulsed electric field treatment acts synergistically with nisin in reducing the viable count of vegetative cells of *Bacillus cereus* (Pol and others 2000). Moreover, high pressure and nisin act synergistically to effectively inactivate both Gram-positive and Gram-negative bacteria in milk (The SAFE consortium, 2004).

Inactivating pathogenic organisms with mild preservation techniques is the trend in food industry. Scientists are putting their efforts in this area and receiving exciting results. The choice of a technique for individual industrial application depends on food properties and process design (Lado and Yousef 2002).

7.2.4 Foodborne pathogen detection methods

Microbiological hazards can enter foods at any point during production, creating dynamic and highly complex environments. Systematic programs, such as good manufacturing practices, hazard analysis, critical control point, good agricultural practices and the food code are approaches used to reduce pathogens in food. Traditional techniques of microbiological culture, isolation and identification methods have played essential roles when foodboren disease outbreak happens. Due to the high requirement of the detection method efficiency and the complexity of food matrices, new detection technologies became more and more important.

Biosensors are analytical devices which combine biospecific recognition systems with physical or electrochemical signaling. There are mainly three components in a biosensor, the biospecific interaction part, the signal emitting part and the platform, which translates the binding reaction into a machine readable output signal. Specific information about biosensor is summarized in Table 7.1.

Technologies advance the development and knowledge of food hazards. Biosensors, which can transfer changes in biological compounds to detectable signals, can be powerful tools to evaluate GAP or GMP effects on site and in time. In addition, the optimization of existing molecular detection methods can improve the detection efficiency, sensitivity and accuracy of foodborne pathogens.

In summary, to realize the better understanding of “antibiotic resistant pathogens”, “microbial safety of fresh produce” and to develop more efficient microbial inactivation methods, continues investigation of novel detection/sensoring methods will play an important role in the future.

Table7.1 Biosensor systems (Hall 2002).

Biosensor architecture	Categories	Properties/mechanism
Providing specificity	Ag/Ab recognition	Advantages: chemical stability, variable affinity from low to high, variable specificity from multiple to unispecific, well-understood dynamics and low cost
	Enzyme/substrate	Advantages: rapidly eliminate the target, release the product and auto-regenerate the binding sites with minimal loss of affinity and specificity
	Thermal sensors	Almost all enzyme reaction release energy and generate detectable heat.
	Translocating sensors	Translocation of an ion or electron in space can be measured electrochemically, like ion channel switch.
	Artificial olfaction	Volatile organic metabolites indicate contaminated cultures, spoiled food or bacterial infection. Analysis of olfactory recognition components can be used for detection purpose.
	Electromechanical devices	Acoustic and optical analysis can identify increased density or refractive index changes
Signaling specific binding	Piezoelectric devices	When mechanically stressed, PZ materials create an electrical charge. The change in oscillating frequency of a PZ crystal is proportionate to the mass variation on the metallic electrodes.
	Nanoelectromechanical devices	Devices are machined from silicon wafers using microfabrication techniques. Cantilevers on the micrometer and nanometer scales naturally oscillate in ambient conditions under thermal energy and the resonant frequency can be determined using HeNe laser based optoelectornics package. With the binding of the targets, the resonant frequency of the device shifts in proportion to the change in attached mass.
	Flexural-plate-wave sensor	Devices can estimate the mass of a vibrating element. FPW sensors have a thin membrane which can propagate an acoustic wave along its surface. When the FPW sensor is in contact with a liquid, a thin film of the liquid is forced to vibrate with the membrane. Any changes happened to the density of the vibrating thin liquid film modifies the acoustic wave and can be detected with high resolution.
Optical system	Surface Plasmon resonance	Changes in the refractive index of the medium above the gold film are associated with the changes of the mass attached to the gold film.
	Colorimetric interference	A specific nucleic acid detection system which yields a visual color signal in response to nucleic acid hybridization
	Optical interferometry	The usage of evanescent field.

REFERENCE

Brady PL, Morris JR. 2009. Good agricultural and handling practices for grapes and other fresh produce. AAES Res Rep 984: 5-18.

Hall RH. 2002. Biosensor technologies for detecting microbiological foodborne hazards. Microbes Infect 4: 425-32.

Lado BH, Yousef AE. 2002. Alternative food-preservation technologies: efficacy and mechanisms. Microbes Infect 4(4): 433-40.

Mathew AG, Cissell R, Liamthong S. 2006. Antibiotic resistance in bacteria associated with food animals: A United Sates perspective of livestock product. Foodborne Pathog Dis 4(2):115-33.

Meng J, Doyle MP. 2002. Introduction. Microbiological food safety. Microbes Infect 4:395-7.

Pol IE, Mastwijk HC, Bartels PV, Smid EJ. 2000. Pulsed electric field treatment enhances the bactericidal action of nisin against *Bacillus cereus*. Appl Environ Microbiol 66: 428-30.

The SAFE consortium. 2004. Novel preservation technologies in relation to food safety. Accessed on July 11 2009. Available at: http://www.safeconsortium.org/uploads/file/Novel_food_preserv_seminar4.pdf

CHAPTER 8

APPENDIX

Appendix 1: Detection range of conventional PCR.

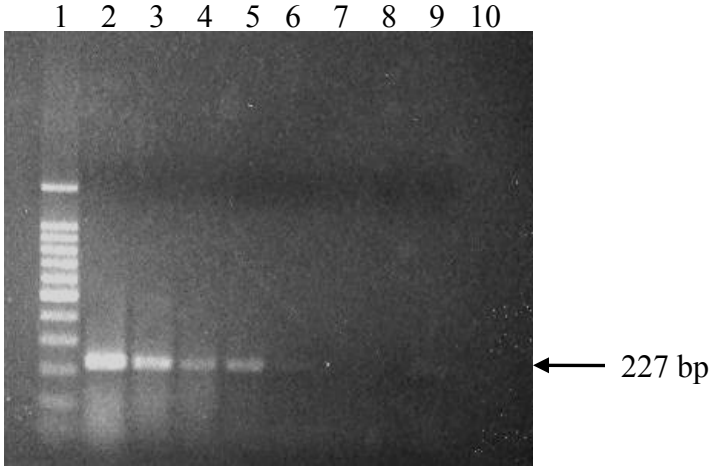

Figure 8.1 Products of conventional PCR from viable *E. coli* O157:H7 cells of different concentrations. Lane 1, marker; lane 2, 10^8 CFU/ml; lane 3, 10^7 CFU/ml; lane 4, 10^6 CFU/ml; lane 5, 10^5 CFU/ml; lane 6, 10^4 CFU/ml; lane 7, 10^3 CFU/ml; lane 8, 10^2 CFU/ml; lane 9, 10^1 CFU/ml; lane 10, water control.

Appendix 2: Detection range of real-time PCR.

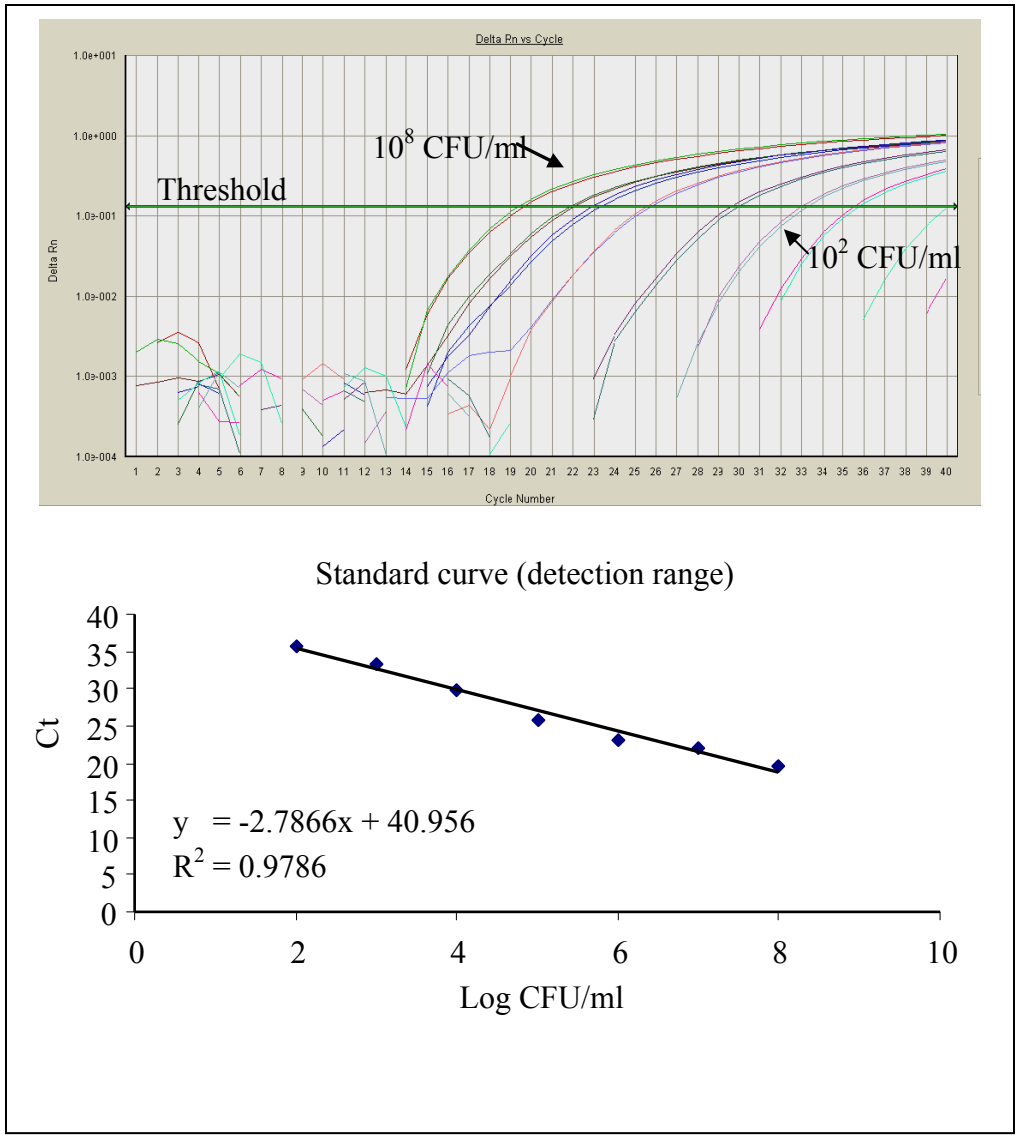

Figure 8.2 Real-Time PCR amplification of DNA from viable *E. coli* O157:H7 cells of 10^1 to 10^8 CFU/ml. The higher the cycle number (Ct), the lower the concentration of cells. The detection range of real-time PCR is from 10^2 to 10^8 CFU/ml.

Appendix 3: Evaluation of ice treatments.

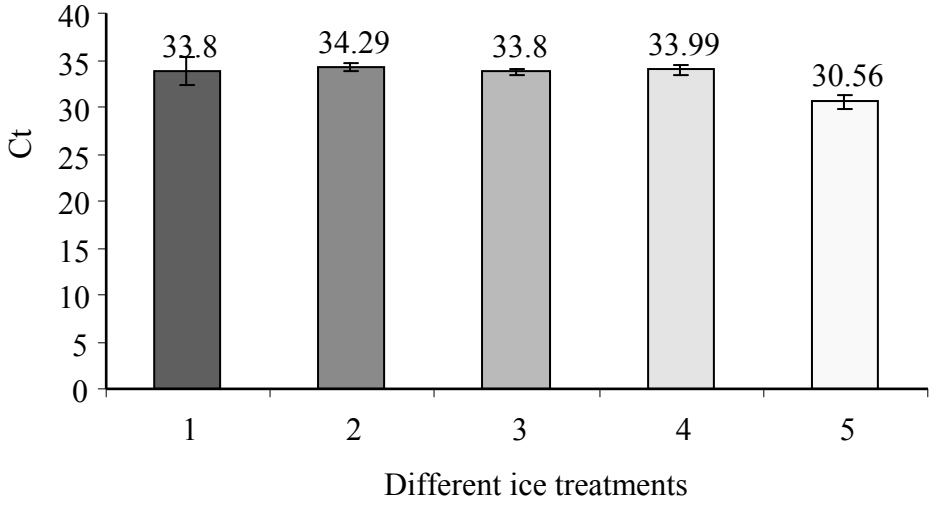

Figure 8.3 Influence of ice treatment on the amplification of DNA from viable *E. coli* O157:H7 cells. Sample 1, 5-min EMA staining, no ice treatment control; sample 2, 5-min EMA staining on ice; sample 3, 5-min EMA staining followed by 1-min ice treatment; sample 4, 5-min EMA staining followed by 10-min light exposure on ice; sample 5, no EMA staining control. Real-time PCR was applied after DNA extraction to compare various ice treatments.

Appendix 4: Application of EMA real-time PCR to artificially contaminated beef samples.

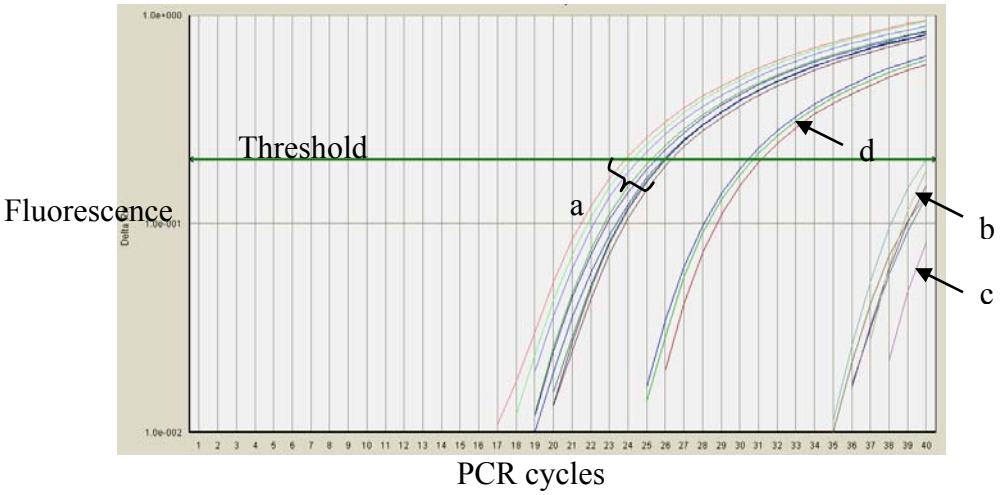

Figure 8.4 Application of the EMA-real-time PCR to ground beef artificially contaminated with 10^1 to 10^4 CFU/g of *E. coli* O157:H7. Following a 24-h enrichment in TSBY broth, the pathogen can be detected by real-time PCR after EMA staining with Ct values from 24 to 26 for cell concentrations of 10^2 to 10^4 CFU/g (a). (b) water control, (c) ground beef contaminated with 10^7 CFU/g of dead cells and (d) ground beef contaminated with 10^1 CFU/g of viable cells.

Appendix 5. Detection of low concentrated *E. coli* O157:H7 from artificially contaminated ground beef samples with an internal amplification control in each PCR reaction.

Beef fat content	Enrichment Time (hours)	Concentration of viable cells (log CFU/g)	Concentration of dead cells (log CFU/g)	EMA Real-time PCR	
				Sample	IAC
73/27	0	1.079	4.043	-	+
		2.079	4.043	-	+
		3.079	4.043	-	+
		4.079	4.043	-	+
		0	4.043	-	+
		0	0	-	+
	8	1.079	4.043	-	+
		2.079	4.043	-	+
		3.079	4.043	+	+
		4.079	4.043	+	+
		0	4.043	-	+
		0	0	-	+
	12	1.079	4.043	+	+
		2.079	4.043	+	+
		3.079	4.043	+	+
		4.079	4.043	+	+
		0	4.043	-	+
		0	0	-	+
	24	1.079	4.043	+	+
		2.079	4.043	+	+
		3.079	4.043	+	+
		4.079	4.043	+	+
		0	4.043	-	+
		0	0	-	+
80/20	0	1.079	4.043	-	+
		2.079	4.043	-	+
		3.079	4.043	-	+
		4.079	4.043	-	+
		0	4.043	-	+
		0	0	-	+
	8	1.079	4.043	-	+
		2.079	4.043	+	+
		3.079	4.043	+	+
		4.079	4.043	+	+
		0	4.043	-	+
		0	0	-	+
	12	1.079	4.043	+	+
		2.079	4.043	+	+
		3.079	4.043	+	+
		4.079	4.043	+	+
		0	4.043	-	+
		0	0	-	+
	24	1.079	4.043	+	+
		2.079	4.043	+	+
		3.079	4.043	+	+

		4.079	4.043	+	+
		0	4.043	-	+
		0	0	-	+
	0	1.079	4.043	-	+
		2.079	4.043	-	+
		3.079	4.043	-	+
		4.079	4.043	-	+
		0	4.043	-	+
		0	0	-	+
	8	1.079	4.043	-	+
		2.079	4.043	+	+
		3.079	4.043	+	+
		4.079	4.043	+	+
		0	4.043	-	+
		0	0	-	+
90/10	12	1.079	4.043	+	+
		2.079	4.043	+	+
		3.079	4.043	+	+
		4.079	4.043	+	+
		0	4.043	-	+
		0	0	-	+
	24	1.079	4.043	+	+
		2.079	4.043	+	+
		3.079	4.043	+	+
		4.079	4.043	+	+
		0	4.043	-	+
		0	0	-	+
	0	1.079	4.043	-	+
		2.079	4.043	-	+
		3.079	4.043	-	+
		4.079	4.043	-	+
		0	4.043	-	+
		0	0	-	+
	8	1.079	4.043	-	+
94/6		2.079	4.043	-	+
		3.079	4.043	+	+
		4.079	4.043	+	+
		0	4.043	-	+
		0	0	-	+
	12	1.079	4.043	+	+
		2.079	4.043	+	+
		3.079	4.043	+	+
		4.079	4.043	+	+
		0	4.043	-	+
		0	0	-	+
	24	1.079	4.043	+	+
94/6		2.079	4.043	+	+
		3.079	4.043	+	+
		4.079	4.043	+	+
		0	4.043	-	+
		0	0	-	+
Water (EAC) (No pUC19 added)		0	0	-	-

VITA

Luxin Wang was born on June 15th, 1983 in Laiwu city in Shandong province on the east coast of China. She grew up in Taian City where her parents worked. She spent 21 years in this small town which is famous for the Mountain Tai, a Mountain that is about 1545 metres (5069 ft) tall and well known for its association with "sunrise, birth, and renewal". After she received her bachelor's degree in Microbiology, she came to University of Missouri to pursue her Master's degree in Food Science, which she got it in 2006. She continued her study at Mizzou and became a dual degree student in Food Science (FS) and Biological Engineering (BE). She defended in the summer of 2009 and received a Master of Science degree in BE and a Ph.D. degree in FS.