ABSTRACT

DWIVEDI, HARI PRAKASH. Development of Molecular-based Methods to Capture and Detect *Salmonella* and *Campylobacter* in Complex Sample Matrices. (Under the direction of Dr. Lee-Ann Jaykus.)

Salmonella and *Campylobacter* are leading causes of food borne bacterial gastroenteritis. Early detection along the farm- to-fork continuum is critical to the success of food safety measures targeting control of these pathogens. It is well recognized that development of methods to concentrate and purify pathogens from complex sample matrices will enhance the application of real-time detection strategies. This dissertation describes the development of novel surface chemistries for pre-analytical sample processing, along with their application to prepare samples for pathogen detection using quantitative real-time PCR (qPCR). The focus is on the food borne pathogens *Salmonella* and *Campylobacter*.

In the first study, a combined immunomagnetic separation (IMS)-qPCR assay for capture and detection of *Salmonella* was developed. This included design and validation of a homologous internal amplification control (IAC) as a signal for amplification failure. In pure culture experiments, the assay demonstrated log linear amplification between 5.19 to 0.19 $\log_{10}$ CFU equivalents *Salmonella* per reaction. Genomic DNA was co-amplified in the presence of $\leq$7.6 ag of IAC with no impact on detection limits at low target concentrations; IAC amplification was out-competed at higher target concentrations ($\leq$2.19 $\log_{10}$ CFU equivalents). The lower limit of detection of the IMS-qPCR method was 10^0-10^1 CFU *Salmonella* per 9 ml artificially contaminated chicken rinsate, with capture efficiency of the IMS step increasing (from 12% to 100%) with

decreasing levels of *Salmonella* (10^5 to 10^0 CFU/sample). The IMS-qPCR approach offers promise in facilitating detection of *Salmonella* at levels anticipated in naturally contaminated products.

Alternative ligands such as nucleic acid aptamers offer advantages over antibodies including ease of synthesis and labeling, lower cost of production, and equal or higher target binding affinity. The purpose of the second study was to select fluorescein (FAM) labeled DNA aptamers with selectivity to *C. jejuni* using a whole-cell SELEX (Systematic Evolution of Ligands by EXponential enrichment) approach. Seven aptamer sequences with binding affinity to *C. jejuni* A9a were identified and the one (aptamer ONS-23) with highest binding efficiency chosen for characterization. Aptamer ONS-23 displayed a dissociation constant of 292.8 nM with 47.3% of *C. jejuni* cells (n = 200,000) bound using 1.48 μM aptamer solution. Inclusivity/exclusivity studies demonstrated a 25-36% binding efficiency for ONS-23 to multiple *C. jejuni* strains and low apparent binding (1-5%) with non-*C. jejuni* strains. The whole-cell SELEX approach was successfully applied and offers the advantage of aptamer selection for microbial cells without prior knowledge of diagnostic markers and the need to purify such markers prior to selection, which may ultimately affect aptamer functionality.

In the third study, biotinylated DNA aptamers with binding affinity to *S.* Typhimurium were identified using the same whole-cell SELEX method. Two of 18 candidate aptamers showed binding efficiency in the range of 13-14% and one of these (S8-7) was further characterized. Aptamer S8-7 displayed a dissociation constant 1.73 μM with 22% of cells bound at a 6.94 μM aptamer concentration. There was low apparent cross-reactivity of this aptamer with *E. coli* O157: H7 and *Citrobacter brkaaii*,

but moderate cross-reactivity with *Bacillus cereus.* In proof-of-concept experiments, the S8-7 aptamer was conjugated to magnetic beads and used for *Salmonella* capture followed by detection using qPCR. The lower limit of detection of the combined aptamer capture-qPCR assay was 10^2-10^3 CFU equivalents of *S.* Typhimurium in 290 µl buffer; capture efficiency ranged from 3-13%. Collectively, this research supports the utility of existing (antibodies) and novel (aptamers) ligands in combination with molecular diagnostics to facilitate the concentration and detection of key food borne pathogens.

Development of Molecular-based Methods to Capture and Detect *Salmonella* and *Campylobacter* in Complex Sample Matrices

by
Hari Prakash Dwivedi

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Comparative Biomedical Sciences

Raleigh, North Carolina

2010

APPROVED BY:

_____

Dr. Lee-Ann Jaykus
(Chair of Advisory Committee)

_____

Dr. Jay F Levine
Committee member

_____

Dr. Vijay K Juneja
Committee member

_____

Dr. Jonathan Olson
Committee member

_____

Dr. Siddhartha Thakur
Committee member

DEDICATION

To My Parents!

(Kasturi Dwivedi and M.L. Dwivedi)

BIOGRAPHY

Hari Prakash Dwivedi, the author of this dissertation was born in the July of 1978 in a town Mahoba of the northern part of India. After completing his school education from Jawahar Navodaya Vidyalaya, he began undergraduate training in Veterinary Sciences and Animal Husbandry (BVSc & AH) at Jawaharlal Nehru Agricultural University (JNKVV), Jabalpur India in 1997. He successfully completed his veterinary training in October, 2002 with prestigious Sanjay Srivastava Memorial award for best outgoing student at the College of Veterinary Sciences and Animal Husbandry, Jabalpur, India. He continued his studies for masters in Veterinary Sciences from January 2003 to January 2005 with major in Veterinary Epidemiology & Preventive Medicine and minor in Veterinary Public Health at GB Pant University of Agriculture and Technology (GBPUAT), Pantnagar, India. He was honored with appreciation award of Indian Society for Veterinary Medicine (ISVM) for the year 2005. Soon after finishing his masters he worked as a Teaching Personnel in Veterinary Clinical Medicine at GBPUAT for a semester to gain teaching experience. Later, he joined as a Senior Research Fellow in the Department of Veterinary Epidemiology and Preventive Medicine for almost a year before starting his PhD training. In August 2006, he began working for PhD in Comparative Biomedical Sciences program of the College of Veterinary Medicine at North Carolina State University, Raleigh with minor in Food Safety under the direction of Dr. Lee-Ann Jaykus. Currently, he is serving as regional secretary (abroad) of Indian Society for Veterinary Medicine.

ACKNOWLEDGEMENTS

First of all I would like to thank Dr Lee-Ann Jaykus, my graduate research advisor and truly a wonderful mentor, for all her support, guidance, constructive criticism and infusing enthusiasm in me throughout the course of my studies. I want to thank my graduate research committee members Dr Jay Levine, Dr Vijay Juneja, Dr Jonathan Olson and Dr Sid Thakur for valuable advises they provided during the entire period of my studies. Thanks is due to all members of Jaykus lab with whom I got chance to interact and learn both on professional and personal front. Specifically, I would like to mention Dr Rawsthorne, Dr Smiley and Dr Blanca for their timely help whenever needed. I would like to thank all funding agencies specifically Food Safety Research Response Network, supported by the USDA CSREES, which provided graduate research assistantship and necessary funds to conduct studies for my PhD research.

I would like to thank my parents, Kasturi and M.L. Dwivedi; parents-in-law, Radha and Devendra Swarup; and all my family members for their love, support and encouragement during the period of study. Last, but not least, special thank is due to Shilpa, my wife, for her endless backup in all moments since she joined me in the journey of life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

NOVEL METHODS FOR SAMPLE PREPARATION AND DETECTION OF FOODBORNE PATHOGENS

1.1. INTRODUCTION

With an estimated 76 million cases annually, resulting in 325,000 hospitalizations, 5,000 deaths, and total medical cost of $37.1 billion, foodborne illness represents a major public health and economic burden in the United States (Dols et al., 2001; Mead et al., 1999). Food is a major vehicle for transmission of illnesses that range in severity from mild diarrheal illness to life-threatening syndromes which are caused by bacteria, viruses, parasites, toxins, and prions. In particular, food borne bacteria such as *Campylobacter*, *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* O157:H7 and other shiga-toxin producing *E. coli* strains (non-O157 STEC), and *Vibrio* spp. are leading causes of foodborne disease. There are many more cases of food borne illnesses than those which are actually reported. For example, there may be as many as 38.6 unreported cases of *Salmonella* infection for each reported case (Jones et al., 2007). In spite of efforts by governmental agencies and industry at preventing and controlling the contamination of foods with pathogens, there has been no significant decrease in the incidence of disease caused by many of the food borne pathogens when comparing U.S. Centers for Disease Control and Prevention (CDC) FoodNet data between the periods 2005-2008 (CDC, 2009b). Food can

become contaminated with pathogens at any stage from production to consumption. Furthermore, new food vehicles continue to be identified, as is evidenced by recent outbreaks linked to *E. coli* O157:H7 contamination in packaged cooking dough (CDC, 2009; CDC, 2009b; CDC, 2009a).

Historically, microbiological testing has played a role in assuring food safety, and the same is true for today. Whether testing is applied at production, processing, or at the retail/consumer level, identification of pathogens at the point of contamination is critical to food producers and processors as they attempt to assure the safety of their products. Microbial testing is also an important part of regulatory compliance. Increasingly, standardized microbial detection and typing methods have become important components of public health surveillance. Microbial testing can also provide much-needed background data on pathogen prevalence and transmission that can inform the setting of regulatory standards and microbial risk assessment.

1.2. DETECTION OF FOODBORNE PATHOGENS

1.2.1. Culture Based Methods

Over the last century, microbiologists have developed techniques to optimize their ability to detect food borne pathogens directly from contaminated foods. These culture-based methods are considered to be the "gold-standard" and are known for their cost effectiveness, sensitivity, ability to confirm cell viability and ease of standardization. By and large, culture-based assays are designed to be able to detect a single target cell in the sample; sample sizes

typically vary from a low of 10 g to 375 g or more. The general approach consists of the sequential steps of cultural enrichment, selective and differential plating, confirmation, and strain typing. Cultural enrichment is frequently divided into two steps, i.e., pre-enrichment and selective enrichment. The purpose of pre-enrichment is to resuscitate injured cells and/or increase the level of the target pathogen in the sample. Pre-enrichment may also be useful for diluting any inhibitory compounds (i.e., preservatives) found in the food sample, as well as rehydrating cells sampled from dried or processed foods. In selective enrichment, specialized media is used to selectively increase the level of the target pathogen while at the same time suppressing the growth of harmless background microflora. Overall, the enrichment process results in exponential amplification of the target pathogen by as much as a million-fold; at this concentration, detection becomes much easier.

The second major step is selective and differential plating, which is facilitated using a combination of selective agents which suppress the growth of competitive microorganisms, and differential agents that allow the organism to be readily distinguished from other microorganisms present. The result of selective and differential plating is the isolation of one or more colonies that fulfill the presumptive positive criteria. In the absence of typical colonies, the analysis is completed and the results reported as negative. In the case of a presumptive positive, additional biochemical and/or serological testing are done to confirm that the isolate is indeed the target pathogen. Further tests (e.g., antibiotic resistance, phage typing, PCR, and molecular typing) may provide additional information about the strain, such as the presence of specific virulence factors or the identification of a particular molecular fingerprint.

Performed in the traditional manner, the combined enrichment and plating steps take 24-48 hours each, which means that presumptive detection of a pathogen can take about 4 days; confirmation of a positive sample can take up to a week or more. Enrichment-based pathogen detection provides qualitative (presence/absence) information; theoretically it could be made quantitative by using a most probable number (MPN) format, or even MPN linked to PCR, although these tend to be labor intensive (Seo et al., 2006). For a few pathogens (*Vibrio* spp.), detection is quantitative through the use of colony lift hybridization (Elvers, 2008). New concepts in culture-based detection such as abbreviated enrichment (Brehm-Stecher et al., 2009) and more recently, low-shear modeled microgravity (LSMMG) may be applied to speed up the enrichment process (Klaus et al., 1997; Nickerson et al., 2004).

1.2.2. First Generation "Rapid" Methods

Over the years, standard culture-based pathogen detection methods have been refined and even improved, with an eye towards reducing time to detection. This is generally done by replacing the selective and differential plating step with more rapid immunological or molecular-based assays. Most notable amongst these are enzyme-linked immunosorbent assay (ELISA), DNA hybridization, and PCR. These "rapid" assays must include a number of features. Firstly, they must exhibit a high degree of assay sensitivity, defined as the ability to detect the pathogen when it is actually present in the sample. This is required to prevent false negative results and hence assure that a contaminated unit is identified accurately. Routinely, detection sensitivity of a single viable cell of contaminating pathogen per sample

unit is considered essential. A high level of test specificity, or the ability to classify a sample as negative if the pathogen is absent, is no less important as it reduces the likelihood of having to spend additional time and resources confirming results on products which do not represent a risk to public health.

Although a full description of all the studies in which these first generation rapid methods have been applied is beyond the scope of this document, they have been reviewed by others (Benoit & Donahue, 2003; Brehm-Stecher et al., 2009; Stevens & Jaykus, 2004). In the next section, we describe some of the recent applications of rapid detection methods, specifically lateral flow immunoassay and nucleic acid amplification. PCR in particular is rapidly becoming the method of choice for food borne pathogen detection.

1.2.2.1. Lateral Flow Immunoassay

The lateral flow immunoassay or dipstick test is based on visual detection of reaction between antibody and target pathogen(s). The capture antibodies are impregnated in a nitrocellulose membrane at a defined distance from the sample application slot. Near to these slots are placed detection antibodies coupled to colloidal latex or gold particles. On applying the sample, the target binds to the detection antibody which moves laterally toward the impregnated capture antibody by capillary action. The moving sample fluid can segregate into two different capture zones, one specific for the target pathogen and other specific for the unbound detection antibodies. The target specific reaction produces different visual signals (such as two visual lines) compared to the control which consists of a different signal

(such as a single line). Time to detection is about 5-10 min after application of the sample (Chapman & Ashton, 2003). Lateral flow immunoassays have been developed and widely applied for the detection of pathogens such as verotoxigenic *E. coli* (VTEC) in matrices such as raw milk, minced beef, apple juice and salami (Aldus et al., 2003), and *Salmonella*, *Campylobacter*, *Listeria*, and *E. coli* O157:H7 in raw and processed meat and poultry products (Bohaychuk et al., 2005). Several lateral flow immunoassays are commercially available (Singlepath®, Duopath®, RapidChek®) for these pathogens from pre-enriched food samples.

The lateral flow assay has relatively high detection limits (10^7-10^9 CFU) so pre-enrichment is required (Banada & Bhunia, 2008); it also has a tendency to produce a relatively higher number of false positive results compared to more traditional ELISA methods (Bohaychuk et al., 2005). Recently automated readers providing chemiluminescent-based detection have been introduced to make the assay more sensitive. Despite issues associated with test sensitivity and specificity, the rapidity of this assay makes it useful for preliminary screening of pathogen contamination of foods.

1.2.2.2. Nucleic acid amplification

Although it could be argued that nucleic acid amplification has already "emerged," the relatively recent introduction of quantitative real-time PCR (qPCR) methods has made the tool all the more attractive. In these automated techniques, the identity of PCR products is determined by the incorporation in the assay of a fluorescently labeled probe that is

complementary to the target amplicon. The probe will bind to the target while PCR is occurring, and such binding results in a fluorescent signal that is read automatically by the PCR thermocycler. Hence, this method results in enrichment of target-specific DNA and at the same time, confirmation that its sequence is consistent with that which is expected. In general, qPCR assays are more reproducible than traditional PCR assays, and less prone to non-specific amplification which is a consistent problem in food borne pathogen assays.

In qPCR, the formation of product can be detected using a double stranded (ds)DNA intercalating dye such as SYBR® Green or using fluorescently labeled hybridization probes such as Taqman™ and Beacons. SYBR® Green binds within minor grooves of dsDNA and fluoresces several times brighter in the bound state than as a free dye. The SYBR® Green signal increases with the increase in double stranded product generated during PCR. SYBR® Green, however, can also bind to PCR generated double stranded byproducts resulting because of primer-dimer or non-specific amplification, so this method lacks some degree of specificity. To account for this, melting curve analysis can be done as the non-specific byproducts will generate melting curve peaks that differ from those of the target amplicon. In addition, optimal primer design can greatly aid in preventing non-specific reactions in SYBR® Green-based PCR. It must be noted that an additional limitation of this chemistry is the difficulty in detecting genes expressed at low levels due to a high degree of background fluorescence.

There are several reports on detection of food borne bacteria using SYBR® Green qPCR. For example, Wolffs et al (2006) developed a SYBR Green qPCR assay targeting the *inv*A gene of *Salmonella* which was reportedly able to detect 7.5 x 10^2 CFU per 100 ml of

chicken rinse and spent irrigation water. The shiga-like toxin genes *stx*1 and/or *stx*2 of *E. coli* O157:H7 were simultaneously detected using melting curve analysis in a single SYBR® Green reaction (Jothikumar & Griffiths, 2002). In a SYBR® Green PCR targeting the *cadF* gene, as little as 1 CFU of *C. jejuni* was detected per 10 g of chicken skin (Oliveira et al., 2005).

The TaqMan™ assay is an alternative to SYBR® Green assays. This chemistry involves the use of a specific hybridization probe that has a reporter fluorophore dye at one end (5') and a quencher dye at other end (3'). In its normal state, the fluorescence of the reporter dye is quenched by the quencher due to the process of fluorescence resonance energy transfer (FRET). During PCR amplification, as the *Taq* polymerase extends the primer and approaches the annealed probe, it uses its 5'-3' exonuclease activity to cleave off the reporter, resulting in fluorescence (Figure 1.1). The TaqMan™ assay is considered by many to have a high degree of specificity, but it can be difficult and costly to design and optimize.

Many investigators have designed TaqMan™ protocols for detection of food borne pathogens (Table 1.1 & 1.2). For example, González-Escalona et al. (2009) reported a TaqMan™ based quantitative reverse transcriptase (RT)-PCR targeting *inv*A mRNA for successful detection of live cells of *Salmonella* spp. in spinach, tomatoes, jalapeno and serrano peppers. A Taqman™ based qPCR targeting gene *prot6e* on the virulence plasmid specific to *S.* Enteritidis was able to detect <3 CFU per 50 ml of whole chicken carcass rinse and 10 ml egg when combined with a pre-enrichment step (Malorny et al., 2007). Hsu et al. (2005) detected from 10^4 to 10^9 CFU per gram (ml) in feces and apple juice, and from 10^5 to

10^9 CFU/ g of beef sample, using a duplex qPCR assay targeting the *rfb* and *stx*2 genes of *E. coli* O157, without a prior cultural enrichment step. The addition of an enrichment step led to improved detection limits of 1 CFU/ml.

Several different organisms can be detected in a single PCR reaction (multiplex qPCR) using multiple Taqman™ probes specific to pathogen marker genes. For example, a multiplex qPCR using three probes targeting the beta-glucuronidase (*uid*A) gene of *E. coli*, the thermonulease (*nuc*) gene of *S. aureus*, and the origin of replication sequence (*oriC*) gene of *Salmonella* spp. was able to detect 10^3 CFU per gram of each pathogen in minimally processed vegetables (Elizaquivel & Aznar, 2008).

Molecular Beacon assays are also probe-based but rely on a different chemistry (Figure 1.2). In this case, the probe portion of the beacon sits in the middle of a DNA strand which is flanked by complementary DNA sequences. One end (5') of the beacon is labeled with a reporter molecule; the other end (3') is labeled with a quencher. The result is a hairpin loop structure which, because of the proximity of the fluorophor and the quencher, allows FRET to occur. When the probe binds with the specific sequence in the amplicon of interest, the hairpin loop comes apart, increasing the distance between the reporter and quencher and allowing the reporter to fluoresce. Molecular beacon-based qPCR has been applied to the detection of *Salmonella* spp. (Hadjinicolaou et al., 2009), *C. Jejuni* and *C. coli* (Churruca et al., 2007), *E. coli* O157: H7, and *L. monocytogenes* (Singh et al., 2009).

Molecular beacons have also been used in the transcription-based isothermal amplification method nucleic acid sequence-based amplification (NASBA), which has been applied to the detection of many food borne pathogens (Cook, 2003; Guatelli et al., 1990).

NASBA is a self-sustained sequence replication system designed for *in vitro* amplification and detection of RNA. The method uses three enzymes, i.e., avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H, and T7 DNA dependent RNA polymerase. The reaction takes place in the presence of two specific oligonucleotide primers: a forward primer with a 5' promoter sequence that is recognized by the T7 RNA polymerase enzyme, and a reverse primer containing nucleotides identical to the target RNA sequence. Candidate NASBA assays include those targeting the 16S rRNA and a variety of mRNA targets of food borne pathogens. These assays can usually detect approximately 10^1-10^2 CFU/ml in pure culture and up to 10^0-10^3 CFU in various food samples if preceded by enrichment (Nadal et al., 2007; Uyttendaele et al., 1995). Real-time NASBA also has been used for the discrimination of viable from non-viable bacterial cells (Churruca et al., 2007; Nadal et al., 2007). Alternative isothermal nucleic acid amplification techniques, including the strand-displacement amplification (SDA) assay (Little et al., 1999) have been reported but standardization of these is complicated, reducing their applicability for routine analyses.

1.2.3. Considerations in Developing Reliable qPCR Assays

1.2.3.1. Internal Amplification Control (IAC)

Besides well known food matrix associated PCR inhibitory compounds such as gelatin and fat, many other factors (such as malfunction of thermal cycler, incorrect PCR mixture, and poor DNA polymerase activity) may inhibit the PCR, resulting in amplification failure or reduced diagnostic sensitivity and specificity (Hoorfar et al., 2004; Wilson, 1997).

For this reason, researchers and commercial clinical diagnostic kit manufacturers have begun using internal amplification controls (IAC) as a marker for amplification failure associated with any number of problems (Hoorfar et al., 2004; Malorny et al., 2003). In point of fact, the European Standardization Committee, in collaboration with the International Standardization Organization (ISO) has specified the requirement of an IAC for PCR-based methods to detect foodborne pathogens. An IAC is a non-target DNA sequence that can be co-amplified simultaneously along with the target (Hoorfar et al., 2004). If the sample matrix is pristine and the PCR reactions prepared and run according to the proper protocol, the IAC should always be amplified. The absence of IAC amplification, whether the target is present or not, signals that there is something wrong with the assay and that it is not reliable. In this way, the analyst can account for potential false positive or false negative results.

Although an IAC can be used in a SYBR® Green assay by applying melting curve analysis to discriminate between target and non-target sequences, the method is best suited to TaqMan™ or molecular beacon (probe-based) qPCR assays (Hoorfar et al., 2004). A number of alternative approaches have been applied to the design of IACs, and these fall roughly into two categories, i.e. IACs which are designed to be amplified using their own set of primers (heterologous IAC), and IACs which can be amplified with the same primers used for the target amplification (homologous IAC). Hoorfar et al. (2004) detailed practical considerations in designing IACs, identifying 10 major recommendations, the most important of which are the following: (i) the target nucleic acid and IAC should share the same primer binding sites; (ii) IAC amplicons should be readily distinguished from target amplicons, preferably by use of a separate sequence-dependent hybridization probe; and (iii) the source

of the IAC should be highly purified nucleic acid. For this reason, the homologous IAC design in a probe-based qPCR is the preferred approach, particularly because it circumvents difficulties associated with design of multiplex amplification, which may result in loss of detection sensitivity. Such chimeric IACs are amplified by the target primers and result in a second amplicon which is distinct from the target, with the two being discriminated from one another using two different Taqman™ probes which fluoresce at different wavelengths (Malorny et al., 2003). For its successful application, careful optimization of IAC concentration in qPCR assays is essential since the target and the IAC are inherently in competition with one another during amplification. The IACs have been designed for the development of real-time PCR assay for detection of various food borne pathogens such as *Salmonella* (Malorny et al., 2004) and *Campylobacter* (Lund & Madsen, 2006); and multiplex real-time PCR for simultaneous detection of food borne viruses such as GI/ GII noroviruses and murine norovirus 1 (Stals et al., 2009).

1.2.3.2. Discrimination of Live and Dead Bacterial Cells

It is now well documented that DNA persists long after cell death (Josephson et al., 1993; Masters et al., 1994). DNA from dead cells amplified during PCR may lead to a false-positive indication of bacterial contamination (Wang & Levin, 2006). Until recently, this was a consistent limitation of PCR-based detection methods. Recently, investigators have used DNA binding dyes such as ethidium bromide monoazide (EMA) and propidium monoazide (PMA) to aid in discriminating live and dead cells in PCR methods. These compounds,

which have previously been applied to viability staining, selectively penetrate the membranes of dead cells but not those of viable cells. Once penetrated, they intercalate the DNA and upon photolysis using visible light, produce nitrene derivatives which covalently cross-link to DNA, rendering it insoluble. Such cross-linked DNA cannot be amplified by PCR (Nocker & Camper, 2006).

This technique has been used to discriminate live from dead food borne pathogens including *E. coli* O157:H7 and *Salmonella enterica* in pure culture (Nocker & Camper, 2006), *Listeria monocytogenes* in cheeses (Rudi et al., 2005), and *Vibrio vulnificus* in seafood (Wang & Levin, 2006). EMA has been also reported to penetrate inside live cells as well thus inhibiting the DNA amplification from cellular DNA of live cells also as reported with *L. monocytogenes* and *C. jejuni* (Flekna et al., 2007). The alternative PMA does not appear to enter intact cell membranes (Nocker et al., 2006), perhaps making PMA a more reliable method to discriminate between live and dead bacterial cells in qPCR assays. To date, PMA has been used in this regard as applied to *L. monocytogenes* cells in the presence of dead cells from planktonic and biofilm sources (Pan & Breidt, 2007) and spores of *Bacillus subtilis* (Rawsthorne et al., 2009).

Although the use of DNA intercalating agents is promising, it must be noted that they are only applicable to treatments that result in the disintegration of the bacterial cell membrane (Nocker et al., 2007). Other methods (UV and ionizing radiation, for instance) result in membrane damage only as a secondary effect, although the primary means of inactivation for these methods may actually occur in the nucleic acids. Clearly, more work is needed to document the efficacy of the DNA intercalating agents in discriminating live from

dead bacterial cells subjected to different inactivation methods and resulting in sublethal damage to bacterial cells.

1.3. PRE-ANALYTICAL SAMPLE PROCESSING FOR MICROBIAL DETECTION

Unfortunately, we have not been able to eliminate the need for cultural enrichment, despite the availability of reliable detection methods such as ELISA and PCR. There are many reasons for this, and they can roughly be described as issues associated with (i) the complexity of the sample matrix; (ii) assay detection limits; (iii) volume constraints; and (iv) and cell viability. Firstly, food samples are a complex and heterogeneous matrix consisting of various components including particulate matter, biochemical and inorganic food components, fats and non-target (harmless) background microflora. Many of these components are incompatible with analytical methods, e.g., fat and particulates can interfere with antibody binding, and complex carbohydrates can inhibit nucleic acid amplification. Also, the harsh environment of the food matrix may sometimes lead to expression of abnormal cell surface signatures and phenotypes that may interfere with detection. Secondly, many of the rapid detection methods can accommodate only a small test sample, much smaller than the volume of food commonly applied in culture-based methods. A third issue is the need to account for sublethally injured cells; traditional cultural enrichment methods were designed in part to aid in the resuscitation of injured cells. Further, as discussed above, a positive detection signal using a DNA-based assay does not always equate to the presence of viable cells. Finally, many of the rapid methods (ELISA and DNA hybridization) have

detection limits of $>10^4$ target cell per test, well above the required detection limit of 1 viable cell per sample. For these reasons combined, cultural enrichment has remained necessary.

With the advent of PCR-based detection methods, food microbiologists were initially intrigued by the potential to replace cultural enrichment with nucleic acid enrichment, but this has not been an achievable goal. This is largely because of volume constraints, the impact of matrix-associated inhibitory compounds, and issues with viability. Although many of the PCR methods may be faster than ELISA and DNA hybridization (Feng, 2007), some degree of cultural enrichment remains essential.

In the last decade, the concept of concentrating and purifying the pathogen in preparation for downstream detection has come into vogue. The general idea would be to process the food sample in a manner that results in concentration of the target cells with removal of matrix-associated components that negatively impact the ability to accurately detect the pathogen. Collectively, this concept has been termed pre-analytical sample processing. Ideally, pre-analytical sample processing should focus on the recovery of intact, viable bacterial cells and result in: (i) isolation and concentration of target cells from a large sample of complex composition; (ii) removal of matrix associated inhibitory compounds from the analytical unit; and (iii) reduction of sample volume and heterogeneity. All of these are done to improve compatibility of the sample matrix with the downstream analytical method. There are two general strategies to pre-analytical sample preparation, i.e., (i) non-specific approaches that mainly depend on physical and/or chemical principles to concentrate the target cell population; and (ii) target-specific approaches which are selective in nature and rely on the use of ligands with binding specificity for the target. In many instances,

combinations of non-specific and specific approaches are used in designing a pre-analytical sample processing scheme for any one food and/or pathogen. In the next sections, the techniques using non-specific and target-specific pre-analytical sample processing have been briefly described.

1.3.1. NON-SPECIFIC APPROACHES

1.3.1.1. Centrifugation

Centrifugation can be applied for the non-specific sedimentation of bacterial cells along with other components of the sample matrix based on centrifugal force. The sedimentation of cells depends on various parameters including cell diameter, particle density, solution density, volume, angle and speed of centrifugation. Centrifugation is a routine method in most research laboratories and can effectively result in reduced sample volume, sometimes in preparation for downstream detection methods such as nucleic acid amplification. For example, centrifugal concentration of *Mycobacterium avium* subsp. paratuberculosis (MAP) from fecal samples was done and, when combined with PCR-based detection, the investigators were able to detect up to 10^2 -10^3 cells (Ozbek et al., 2003). Differential centrifugation provides a slight modification of the technique, and consists of a step-wise increase in centrifugation speeds to settle large size high density food components first, followed by sedimentation of those with smaller size and lower density, as is the case for bacterial cells. Differential centrifugation combined with PCR was successfully applied to the detection of *E. coli* O157:H7 in ground beef (Cui et al., 2003), with detection limits of

10^3 CFU/g product. Differential centrifugation has also been used to remove particulate food matter and concentrate *E. coli*, *Listeria*, *Salmonella* Enteritidis, and *Staphylococcus aureus* from beef samples for subsequent detection and enumeration of cells using direct epifluorescent microscopy (DEFT) and direct plate count (Rodrigues-Szulc et al., 1996).

Density gradient centrifugation is performed using a compound (such as sucrose, Ficoll, and Percoll) which forms a density gradient from bottom (highest density) to top (lowest density). During centrifugation, the free and particle bound cells migrate in the solution and form a band at the location in which their density is in equilibrium with the solution gradient. Buoyant density gradient centrifugation in conjunction with other physical separation methods was applied to the concentration of 12 food borne pathogens including *Salmonella enterica*, and *E. coli* in different food homogenates (Fukushima et al., 2007). The target organisms were concentrated up to 250-fold and detection limits ranging from 10^1 to 10^3 CFU/g were obtained using qPCR; bacteria could also be enumerated by plate counting. To reduce labor and processing time, automated centrifugation systems such as continuous flow centrifugation (CFC) have been evaluated, as applied to the concentration of bacterial spores of *Bacillus subtilis*, *B. atrophaeus*, and *Clostridium sporogenes* from large volumes (3.7 L) of whole milk and skim milk (Agoston et al., 2009). Overall, the major advantage of centrifugation processes is the ability to handle fairly large sample volume but this is usually accompanied by non-specific concentration of residual food components that may interfere with downstream detection assays.

1.3.1.2. Filtration Methods

Filtration results in removal of food particles (or organisms) by passing samples through filters of various pore sizes. If large pore sizes are used, the bacteria will pass through to the filtrate; if filters with small pore sizes are used, the organisms will be concentrated with the residual food matrix that does not pass through the filter. For very clean samples (such as waters), the organisms can be captured on the surface of the filter and eluted off for subsequent detection. Alternatively, detection can occur directly on the membrane. There are many examples of the use of filtration for pre-analytical sample processing. For example, membrane filtration of beef homogenate was used to facilitate direct enumeration of bacterial counts using epifluorescence technology (Walls et al., 1990). Filtration however, is not always the best choice for sample preparation, as it is quite sensitive to the food matrix. For example, in a study on the direct detection of *L. monocytogenes* in cheese and meat samples, it was possible to detect <10 CFU/g of the pathogen in filtered meat samples but no detection was achieved in soft ripened cheese treated in the same manner (Wang et al., 1992).

Various types of filters have been reported and are commercially available. Electro-positively charged filters have been used for separation of bacteria from foods and beverages (Thomas, 1988) and various AOAC-approved membrane filtration systems (e.g., Neo-Grid™/Iso-Grid™ methods, Neogen Corporation, Lansing, MI) are commercially available for detection and quantification of *E. coli* O157:H7, *Salmonella*, yeast and mold, coliforms/*E. coli* and total aerobic plate count. D'Urso et al. (2009) reported a novel

filtration-based method to eliminate dead or severely damaged cells of *S. enterica* and *L. monocytogenes* in food samples which, when combined with qPCR, resulted in detection of viable cells only. Similarly, Wolffs et al. (2006) reported a direct assay that included filtration and when combined with qPCR was able to consistently detect salmonellae in chicken rinse and spent irrigation water at levels of $\geq 7.5 \times 10^2$ CFU/100 ml.

Large size food particles and matrix components frequently clog filters which limits the utility of filter-based separation methods. Filtration techniques separate target cells in a non-specific manner, so matrix-associated components are concentrated along with bacterial cells; this may impact the sensitivity and specificity of nucleic acid amplification assays (Oyofo & Rollins, 1993). The elution of trapped microorganism is another challenge when using filter-based techniques.

1.3.1.3. Dielectrophoresis

Dielectrophoresis capitalizes on the electrical properties of bacteria in an effort to separate the cells other sample components under a non-uniform electric field. The mostly negatively charged bacterial cells are attracted to positive electrodes and are separated from the food matrix (Betts, 1995). Bacteria and other cells/molecules with similar charge cover the electrode and are later eluted off using a conductivity gradient (Wang et al., 2000). Dielectric methods as applied to the separation of different bacterial species have been reported (Markx et al., 1994). For example, in studies to differentiate among microbial populations of *M. lysodeikticus* and yeast (Wang, 1993) and Gram-positive and Gram-

negative bacteria (Markx et al., 1996), each of which have opposite dielectric properties, dielectrophoresis was successfully used.

Dielectrophoresis is a promising technique for bacterial concentration with potential biosensor applications, as evidenced by its use in conjunction with lab-on-a-chip microarray techniques for identification of bacterial species (Cheng et al., 1998). It has been proposed that modifications in existing dielectrophoresis techniques, such as insulation of electrodes using glass beads or use of electrode-"less" methods will further enhance the ability to separate bacterial cells from food and environmental sample matrices (Lapizco-Encinas et al., 2004). However, it must be recognized that the mobility of bacterial cells in food samples is influenced by the components of food, and such components can limit (reduce) the uninterrupted movement of the bacterial cells, thereby diminishing their electrical conductivity. Another limitation for the routine use of dielectrophoresis is the requirement of small sample size and sample fluidity, the latter of which is not possible for all food types (Cheng et al., 1998).

1.3.1.4. Metal Hydroxides

Metal hydroxides are high molecular weight charged particles that work as affinity agents, providing a large surface area upon which to support the immobilization of bacterial cells. Amino acid on the cell surface covalently bind with hydroxyl groups of the metal hydroxide, and these complexes are separated by centrifugation, resulting in concentration of the cells and removal of some of the sample matrix in the discarded supernatant.

Metal hydroxides such as titanous and zirconium hydroxide have been demonstrated to be effective for affinity concentration of bacteria without negatively impacting cell viability (Kennedy et al., 1976). For example, titanous hydroxide was used to immobilize *Salmonella* for subsequent detection using radioimmunometric and enzyme-immunometric assays. These assays were shown to be 100-160 fold more sensitive than assays in which microtiter plates served as the solid support for immobilization of *Salmonella*, as applied to mixed culture samples (Ibrahim et al., 1985). Similarly, zirconium hydroxide was used for immobilization of *L. monocytogenes* and *S.* Enteritidis in reconstituted nonfat dry milk (NFDM). In this case, about 78 to 96% of *S.* Enteritidis and 65 to 96% of *L. monocytogenes* were recovered and when detected using reverse transcription (RT)-PCR, detection limits of 10 to 100 CFU/25 ml of reconstituted NFDM were achieved for both organisms. When applied in more complex dairy products, such as whole milk and ice cream, 60 to 100% of both bacteria were recovered with an RT-PCR detection limit of 100 CFU/ml in whole milk and 10 CFU/ml in ice cream (Lucore et al., 2000). The same group used magnetized carbonyl iron along with insoluble zirconium hydroxide to form a magnetic "complex" which was used for the concentration of *S.* Enteritidis, *L. monocytogenes*, and *Bacillus cereus* spores in reconstituted NFDM (Cullison & Jaykus, 2002). Hydroxyapatite was used to concentrate various spoilage and pathogenic bacteria from enrichment broth and meat slurry with recovery of greater than 92% of the input bacteria from contaminated ground beef (Berry & Siragusa, 1997).

Metal hydroxide-based bacterial immobilization is rapid, simple and inexpensive with reportedly high recoveries of viable cells from food samples. It should be recognized that

metal hydroxides are non-specific in their selectivity, so various food components are also concentrated along with the bound bacterial cell; this may result in interference with the downstream detection assays. The method also requires the use of a centrifugation step, which may limit its practical utility.

1.3.1.5. Ion Exchange Resins

Ion exchange resins are comprised of small porous polymer beads to which are attached positively and negatively charged groups (Stevens & Jaykus, 2004). Ion exchange works on the principle of non-specific adsorption of negatively charged bacterial cells (mostly at pH of $\geq$ 5.0) to the positively charged surface of the resin (Payne, 1991). The adsorbed cells can be eluted from the resin by alteration of pH, depending on the pH tolerance of the bacterial species. Elution performed at a high pH is particularly useful if the detection method does not require the recovery of live cells. Cationic exchange resins have been used in the concentration of bacterial cells, toxins and DNA from environmental samples such as soils (Jacobsen & Rasmussen, 1992) and foods (Reiser et al., 1974). Though an economic approach, ion exchange resins are also non-specific, leading to the same limitations as the methods described above.

1.3.2. TARGET SPECIFIC APPROACHES

The selective separation and concentration bacterial cells rely on the specificity of cell surface moieties. Ligands (including antibodies, bacteriophages, nucleic acid aptamers, and lectins) that recognize and bind to specific cell surface receptor(s), have advantages of high selectivity and binding affinity. However, there are some limitations to the approach and it may not always be the best for all applications. For example, the expression of receptors over the cell surface varies depending upon the sample matrix or phase of growth of the microbe. This in turn can affect the binding affinity of the ligand. Moreover, naturally occurring enzymes in foods may create a harsh physicochemical environment that can result in ligand degradation. Also, the selectivity of ligands may not provide the broad reactivity which is desired for some assays. Therefore, there remains a place for both non-specific and target-specific bacterial separation and concentration approaches as we seek to refine pre-analytical sample processing methods.

1.3.2.1. Flow Cytometry

Flow cytometry is a high throughput technique for identification and sorting of biological cells and particles from liquid food and environmental samples. Inherent physical properties of microorganisms, specifically slow growing organisms such as molds and mycobacteria, modulate the scattered light signals in flow cytometry which aids in their identification and separation. Alternatively, the rapid development of molecular markers and

fluorochrome dyes has provided a powerful tool for bacterial detection and separation using flow cytometry.

Flow cytometry has been applied to the enumeration of total viable bacterial cells (Jepras et al., 1995). Fluorescence-activated cell sorting has also been applied for isolation and concentration of target bacterial cells from complex food and environmental samples. For example fluorescently labeled *E. coli* cells were counted and sorted from water samples using microflow cytometry (Lee SS, 2004).

Flow cytometry can be used for direct assessment of bacterial growth and for monitoring microbial biomass in foods during manufacturing (Laplace-Builhe et al., 1993). The method has also been used for detection of *S.* Typhimurium after labeling with fluorescent monoclonal antibodies. In this case, the assay detection limits were 10 CFU/ml in milk and 1 CFU/ml in eggs amongst a background of 10,000-fold excess of *E. coli* cells (McClelland & Pinder, 1994). The Agilent 2100 bioanalyzer (Agilent Technology, USA) is a commercially available flow cytometry unit which is marketed for the rapid and selective identification and enumeration of bacteria in food samples.

Further advancement in flow cytometry-based techniques has led to development of on-chip flow cytometry for the selective isolation, purification and enumeration of bacteria in various samples. For examples, *E. coli* O157 and *Pseudomonas putida* were detected in culture and environmental samples in 30 min (Sakamoto et al., 2005), and low levels of *Pseudomonas* in milk samples (Yamaguchi et al., 2006) were detected, both using on-chip flow cytometry methods.

The complicated optical alignment process and relatively higher cost of the flow cytometer are some of the practical hindrances of this method to routine analytical use. Furthermore, the technique requires well trained, specialized personnel. Recently, a micro fabricated flow cytometer has provided a portable and cost-effective alternative. Background components of sample matrix may interfere with fluorescence-based detection. A further concern is the potential for loss of cell viability during cell sorting. The complicated process of sample preparation and fluorescence labeling is not alleviated by the miniaturization, thus it is likely that the technique will remain a research tool and not form the basis of routine food microbiology applications.

1.3.2.2. Immunomagnetic Separation (IMS)

Antibodies attached to paramagnetic particles have been used widely for the selective separation and concentration of target pathogens from complex food and environmental samples. The antibody-labeled particles remain free to bind with epitope(s) on target cells until they are exposed to a magnetic field for subsequent concentration. The recovery efficiency of immunomagnetic separation (IMS) methods varies depending on the target pathogen and its antigenic expression, the affinity of the antibody, and physico-chemical properties of food matrices.

There are many papers which report the use of IMS for upstream separation and concentration of pathogens from foods. For example, IMS has been used in combination with culture based methods for detection of *Salmonella* (Hara-Kudo et al., 2001) and *Listeria*

(Bauwens et al., 2003). IMS in conjunction with PCR has been evaluated for detection of many food borne pathogens including *Salmonella* (Mercanoglu & Griffiths, 2005), *L. monocytogenes* (Amagliani et al., 2006) and *E. coli* O157:H7 (Fu et al., 2005). IMS coupled with multiplex PCR was reported for the simultaneous detection of *L. monocytogenes* and *Salmonella* from food samples, with a reported detection limit of 10^3 CFU of each pathogen (Hsih & Tsen, 2001). Although theoretically IMS could be applied to samples in an effort to forgo the need for cultural enrichment, the reality is that a cultural enrichment step is still required in order to provide sufficient target cells to detect low contamination levels in foods. IMS-PCR with a brief pre-enrichment of samples was reported to detect up to 1 CFU of *L. monocytogenes* (Hudson et al., 2001) and *E. coli* O157 (Chapman & Ashton, 2003).

Magnetic nanoparticle conjugates (MNCs) coated with polyclonal anti-*E. coli* antibodies were recently described and purported to further enhance capture efficiency in IMS assays. For example, Varshney et al. (2005) reported a 94.5% recovery of *E. coli* O157:H7 in ground beef samples using this sort of approach. To enhance fluorescence signal intensity, quantam dot labeled immunomagnetic beads were used for quantitative detection of *L. monocytogenes* (Tully et al., 2006). Many IMS commercial products containing target specific paramagnetic particles are available for food borne pathogens (e.g., Dynabeads® Anti-*Salmonella* and anti-*E. coli* antibodies). To increase the contact between cells and antibodies, automated recirculation filter systems (Pathatrix™, BeadRetriever™) can be used for capture of cells in relatively small (10 ml) and large (225 ml) volumes. These systems facilitate simultaneous processing of several samples using a single platform.

The specificity of antibodies imparts specificity to IMS assays. Monoclonal antibodies raised against a unique epitope on pathogen cells provide a high degree of specificity but monoclonal antibodies are quite expensive. Polyclonal antibodies raised against various epitopes on the same or multiple antigenic agent(s) are cheaper but have a greater likelihood to suffer from cross-reactivity with related targets. Therefore, the balance between false positive and false negative binding reactions is a fine line. Finding a broadly reactive IMS method targeting multiple pathogen serotypes and/or strains can be a particular challenge. *Salmonella*, with its 2500 serotypes, is a good example of this. Also, the expression of the target epitope on a bacterial cell can be affected by various physical and chemical properties associated with the culture medium and/or matrix. Sometimes the metabolic state of the bacterial cell itself can affect antibody binding properties (Geng et al., 2006; Hahm & Bhunia, 2006; Stancik et al., 2002). These factors must be considered when optimizing IMS assays.

1.3.2.3. Bacteriophage

Bacteriophage can be used as a novel biosorbent for selective separation and concentration of target bacteria. The ease of phage production, high affinity of phage-cell interactions, and the ability of phage to infect host cells in heterogeneous environments make bacteriophages a promising means upon which to base separation technologies. For example, the *Salmonella*-specific lytic phage *Sapphire* immobilized on polystyrene surfaces was used to capture *Salmonella* from mixed bacterial populations followed by detection using PCR.

This combined assay was able to detect 10^5 CFU *Salmonella* per ml in suspensions having other members of the *Enterobacteriaceae* family with capture efficiency around 1% (Bennett et al., 1997). A biotinylated *lux* operon containing bacteriophage was conjugated to magnetic particles for the selective concentration of *S.* Enteritidis with subsequent detection by bioluminescence; this assay demonstrated a 20% capture efficiency (Sun et al., 2001).

Bacteriophage-derived biomolecules including lytic enzymes such as endolysins are a new generation of ligands which have been applied to the selective separation of bacterial cells (Kretzer et al., 2007). Kretzer et al. (2007) used cell wall-binding domains (CBDs) of bacteriophage-encoded peptidoglycan hydrolases (endolysins) conjugated to magnetic particles to recover more than 90% of viable *L. monocytogenes* cells in diluted suspensions. It was possible to separate different species and serovars of *Listeria* both in artificially and naturally contaminated food samples. Furthermore, specific phage-encoded CBDs recognizing *B. cereus* and *C. perfringens* cells were also evaluated with successful results. CBD polypeptides represent an innovative tool for separation and concentration of bacterial cells, but there applicability is, unfortunately, limited to Gram-positive bacteria.

Phage-based separation and concentration of bacteria can provide value added with respect to speed and specificity of food borne pathogen detection. Further investigations are needed before the more widespread use of these technologies, including the identification of suitable phage, the orientation of the phage, and the choice of solid support. The bacteriophage binding to target cells is not limited to cell surface receptors only but also involves other interactions and modifications in the host cells which may impair phenotypic

and genotypic characteristics of the target cells such as bacterial cell lysis and degradation of bacterial DNA (Warner et al., 1970).

1.3.2.4. Nucleic Acid Aptamers

Nucleic acid aptamers are single stranded DNA or RNA molecules with sequence-defined secondary structures demonstrating specificity with respect to target binding. Aptamers are selected from a random oligonucleotide library by an iterative process of *in vitro* selection of sequences showing target binding affinity, followed by PCR amplification. This method is termed Systematic Evolution of Ligands by EXponential enrichment (SELEX) (Pai et al., 2005; Tuerk & Gold, 1990). Due to their high affinity and specificity, aptamers have emerged as macromolecules that rival antibodies in their potential for diagnostic applications. Aptamers provide an unlimited source of identical affinity recognition molecules with several characteristics that make them attractive for separation, concentration and, detection of bacteria including smaller size, ease of synthesis and labeling, lower cost than antibodies and high target specificity (Tombelli et al., 2005). Despite all these advantages, only a few aptamers have been developed for microbial targets, including those specific for *C. jejuni* (Bruno et al., 2009), *S. enterica* (Joshi et al., 2009), *S. enterica* serovar Typhi (Hsu et al., 2005), *Francisella tularensis* (Vivekananda & Kiel, 2006), *Bacillus anthracis* vaccine strain A (Zhen et al., 2002), *Mycobacterium tuberculosis* (Chen et al., 2007), and *E. coli* (So et al., 2008).

DNA aptamers specific to outer membrane proteins (OMPs) immobilized on magnetic beads were used in a proof-of-concept study focused on the separation and concentration of *S. enterica* serovar Typhimurium in whole carcass chicken rinse samples (Joshi et al., 2009). When combined with qPCR, one of the aptamers facilitated detection of 10^1-10^2 CFU/9 ml of rinsate in a magnetic pull-down assay and 10^2-10^3 CFU/25 ml in a recirculation assay. A reproducible detection limit of <10 *S.* Typhimurium CFU/g was recorded in spike-and-recovery experiments using bovine feces. More recently, DNA aptamers specific to surface proteins of *C. jejuni* were linked to magnetic beads for capture of the organism on the inner side of a plastic cuvette which was followed by detection using a quantum dot-based sandwich assay (Bruno et al., 2009). Endpoint detection was evaluated using a spectrofluorometer and a commercially available handheld fluorometer. The assays detected up to 2.5 CFU equivalents of *C. jejuni* in buffer and 10-250 CFU in various food matrices without prior cultural enrichment. However, there was substantial cross-reactivity with *C. coli* and *C. lari*.

The primary limitation in the use of aptamers (specifically RNA aptamers) as capture molecules for bacterial targets in food and environmental samples is their nuclease sensitivity. However, chemical modification of the ribose ring can be done to enhance their stability (Pieken et al., 1991). A different approach to stabilize aptamers comes from selection of aptamers binding to stereoisomers of intended target molecules followed by chemical synthesis of the mirror image of those selected aptamer sequences (Klussmann et al., 1996). The use of a combinatorial library having modified bases such as locked nucleic acid (LAN) can also enhance resistance to nuclease degradation.

1.3.2.5. Lectins

Lectins are carbohydrate binding proteins which can be isolated and purified from various biological sources including microorganisms, plants and animals. Lectins recognize various carbohydrate targets on the surface of bacterial cells, specifically the N-acetyl glucosamine residue of the peptidoglycan. It is possible to immobilize lectins on solid supports such as agarose beads, magnetic particles or beads packed into affinity columns, all with capabilities for use in pre-analytical sample processing. For example, lectins immobilized on magnetic microspheres and affinity columns were used to adsorb different strains of *S.* Typhimurium and *L. monocytogenes* (Patchett et al., 1991). The binding affinity of lectins for microbes suspended in food matrices is highly dependent on the nature of the food matrix. Source of lectin and type of solid support are other factors which affect capture efficiency. Lectins isolated from *T. vulgaris* were used in the magnetic separation of *L. monocytogenes* (43% recovery) and *Staphylococcus aureus* (26% recovery) from diluted milk and, and *Salmonella* (31-54% recovery) from raw egg (Payne et al., 1992). Other lectins isolated from *Agaricus bisporus* were used in a magnetic capture assay to remove *L. monocytogenes* from undiluted milk and ground beef, with capture efficiencies ranging from 10-50%. Lectins have been used for concentration of bacteria prior to the application of PCR-based detection. For example, lectins isolated from *A. bisporus* were used to capture *Bronchothrix* from beef homogenate (Grant et al., 1993). In this case, lectin immobilized magnetic beads captured 78% of the *Brochothrix* cells, with 92% of the captured cells eluted

from the lectin-bound magnetic beads using fetuin. Unfortunately, fetuin was inhibitory of PCR.

Lectins can provide an effective means by which to achieve rapid and efficient bacterial separation. The major trouble with lectins is the tedious process necessary to isolate them from their biological sources, making them expensive and not readily available. The elution of lectin-bound cells is another problem as the elution agent may interfere with the downstream detection methods, specifically PCR systems.

In addition to the ligands described above, other natural and synthetic binders such as antibiotics, molecular imprinted polymers (Ngundi et al., 2006) and alternative binding proteins (Skerra, 2008) have demonstrated affinity to bacterial cells. These ligands could be further exploited as potential capture agents for pre-analytical sample processing as applied to complex sample matrices.

1.4. EMERGING DETECTION TECHNIQUES

Given the need for rapid "real-time" detection and the move towards sample preparation strategies to facilitate this process, we are rapidly approaching a time when "culture independent" methods may be a reality. Such methods are widely used in microbial ecology to identify and differentiate microbial communities found in all sorts of samples, including foods. Culture independent methods offer the opportunity for rapid identification of the major components of bacterial communities, but will fail to detect those microbes in low concentration; unfortunately, most pathogens are in low concentrations and hence cultural enrichment will still be necessary (Jany & Barbier, 2008). However, a major advantage of

culture independent methods is their ability to detect microbes not readily culturable (including those in the viable but not culturable, or VBNC state), although the live-dead conundrum has yet to be completely resolved.

1.4.1. DNA Microarrays

DNA microarray is a candidate tool for the detection and characterization of a single or multiple organism(s) based on the presence of one or more marker genes. It can also be used for discriminating sequence variations among isolates, as is the case in molecular typing. In a DNA microarray, thousands of specific PCR products can be simultaneously detected by hybridization with specific probes. DNA microarrays usually consist of a solid support such as glass or silicon to which are attached target specific oligonucleotide probes. High density tiled oligonucleotide arrays synthesized on a wafer surface using photolithographic manufacturing processes provides further high throughput detection capability (Zarrinkar et al., 2001).

DNA microarrays can be used to characterize complex microbial populations or to detect specific microbes. For example, using a 16S ribosomal DNA array, investigators have been able to characterize the complex microbial communities in vegetable salads without any prior cultural enrichment (Rudi et al., 2002). DNA microarrays have also been applied to the detection and characterization of *E. coli* O157:H7 in chicken rinsate (Call et al., 2001); *Enterobacter sakazakii*, *Salmonella*, *E. coli* O157 and many other pathogens from artificially inoculated powdered infant formula (Wang et al., 2009); *Mycoplasma* spp., *Salmonella* spp.,

Bacillus spp., *Campylobacter* spp. and other pathogens from milk samples (Cremonesi et al., 2009); and *Yersinia enterocolitica* from alfalfa, cilantro, mamey sapote, and mung bean (Siddique et al., 2009).

High throughput arrays designed to simultaneously identify and discriminate several pathogenic bacterial species has been reported, some of which are of significance to food safety. For example, Wilson et al. (2002) developed a high throughput Multi-Pathogen Identification (MPID) microarray for identification of 18 pathogens using an array of overlapping 20-mer oligonucleotide probes. Similarly, Hong et al. (2004) developed an oligonucleotide array for simultaneous detection of 14 species of pathogenic bacteria using species (genus)-specific oligonucleotide probes which was reportedly sensitive enough to consistently detect 100 CFU/ml of each pathogen.

The practical application of microarray techniques to the detection of microbes in foods is limited by several factors including difficulties in isolation, concentration, and purification of quality DNA from foods and standardization of optimum hybridization conditions given the variety of array probes necessary to detect multiple pathogens. Together, these lead to a tendency toward non-specific probe hybridization which can cause false positive and/or false negative results. Also, as the limit of detection for DNA hybridization is normally in the range of 10^4-10^5 target gene copies, cultural enrichment will be almost certainly required to obtain detectable levels of pathogens in naturally contaminated foods. Theoretically, PCR amplification prior to hybridization with array probes could enhance the sensitivity and specificity of such methods but as described above, even PCR cannot yet be applied without prior cultural enrichment (Call et al., 2001). The

application of target specific concentration strategies such as immunomagnetic separation or other emerging strategies could further the routine use of microarray technology for food borne pathogen detection (Chandler et al., 2001). However right now, the method is more of a research tool than a diagnostic reality, as evidenced by the development of a "water chip" which never made it to market due to sensitivity, specificity and cost issues (L. Jaykus, personal communication).

1.4.2. Proximity Ligation Assay

By combining the advantages of immunological and molecular techniques, assays with exquisite specificity can be designed. The proximity ligation assay (PLA) offers an excellent platform upon which to base such assays. The method involves the sequential steps of target binding to a specific monoclonal or polyclonal antibody (or other ligand) which is linked to non-specific DNA probes. When the target binds to the antibody, the probes are brought into proximity by exposure to a connecter DNA molecule which ligates the free ends of the probes. A qPCR is then used to amplify the ligated probe sequences, and this amplification reaction serves as an indirect means by which to detect the presence of the target. The PLA reaction can be performed either as a homogeneous assay with all components in liquid phase or the target can be immobilized on a solid support (such as magnetic particles) followed by the use of probe-bound antibodies. For example, a homogenous PLA method could be developed for the detection of bacterial targets in complex sample matrices, theoretically with minimal requirements for sample preparation

prior to assay (Gustafsdottir et al., 2006). Or, a solid phase PLA could be performed in an ELISA format with the advantage of subsequent washing steps for further removal of potentially interfering substances. In fact, the solid phase PLA has been previously found to be a highly sensitive and economic assay for direct detection of protein or whole cell targets in a very small representative samples, such as demonstrated by (Fredriksson et al., 2002) who used this platform to detect *Lawsonia intracellularis* cells in fecal samples with a high degree of sensitivity (1 cell per sample). The PLA has also been applied to the detection of *Bacillus anthracis*, *B. subtilis* and *B. cereus* spores (Pai et al., 2005) and food animal viruses (Nordengrahn et al., 2008).

As is the case for most assays, the PLA has both advantages and disadvantages. For example, in the absence of the target, non-specific ligation of probes is still possible which results in a high level of background noise which can be difficult to distinguish from actual target signal. Thus, appropriate assay optimization is crucial, particularly with respect to antibody and connector concentrations. However, the fact that the same probes and connectors can be used from assay to assay means that once the system is in place, it may be modified with ease in an effort to apply it to a different target. Furthermore, the PLA reaction may be particularly promising in the sense that detection over a live cell surface may provide a more reliable indication of target cell viability.

1.4.3. Phage Based Detection Assays

The selectivity of bacteriophages for their host bacteria makes them ideal candidates for pre-analytical sample processing and subsequent detection (Dubow, 1994). The specificity of phage binding is mediated by unique surface receptors on the host cell's surface. A particularly interesting phage, called the green fluorescent protein (*gfp*) reporter phage, has been widely used. The genome of this phage contains a reporter gene which can transcribe green fluorescent protein (GFP), a protein that produces a discernible fluorescent signal (Tanji et al., 2004). Other reporter phages have been designed to produce proteins for bioluminescence. For example, a reporter phage containing the *lux*$^{+}$ gene has been used for the detection of *Salmonella* in eggs (Chen & Griffiths, 1996) and the a *lac*Z reporter phage has been applied to the detection of generic *E. coli* (Goodridge & Griffiths, 2002). An ice nucleation reporter phage contains the *Ina*W ice nucleation gene which induces ice formation in super-cooled water kept at a low enough temperature, resulting in freezing of water and ice formation. This has been developed into a commercial kit named Bacterial Ice Nucleation Diagnostic or BIND™ (Idetek, Inc., Sunnyvale, CA) which is reportedly able to detect as little as 10 CFU/ml of *Salmonella* in contaminated foods such as raw eggs and milk (Wolber & Green, 1990).

Fluorescently stained phages have also been applied to the detection of target bacterial cells. For example, stained LG1 phage was used to develop a fluorescent bacteriophage assay (FBA) for detection of *E. coli* O157:H7 in ground beef and raw milk (Goodridge et al., 1999). When used in conjunction with flow cytometry, FBA detected up to

2.2 CFU/g of *E. coli* O157:H7 in ground beef and 10^1-10^2 CFU /ml of raw milk following cultural enrichment. Fluorescently labeled host-specific phages could be also used in imaging platforms such as epifluorescence microscopy.

The phage amplification technique is another candidate method for food borne pathogen detection. This is based on the principle that lytic phages produce a large number of progeny upon rupture of the host cell, and these progeny phages can serve as an indirect signal of host cell infection, and hence the presence of a target bacterium. Amplified bacteriophage can be detected using a variety of techniques. For example, live/ dead cell staining such as exogenous fluorochromic staining has been used to detect as little as 10^1 CFU/ml of *Pseudomonas aeruginosa* (Jassim & Griffiths, 2007). Advanced detection techniques such as mass spectrometry for *E. coli* (Madonna et al., 2003) and competitive enzyme-linked immunosorbent assay (PR-cELISA) for *S.* Typhimurium DT104 (Guan et al., 2006) have also been reported.

Phage libraries having indefinite numbers of antigen recognition sites can be generated using the phage display technique. These library clones can be used as probes for the detection of target bacteria in a variety of modalities. In one study, a *S.* Typhimurium-specific phage clone derived from a phage library was used in a megnatoelastic sensor to detect up to 10^3 CFU/ml of *S.* Typhimurium in pure culture (Lakshmanan et al., 2007). The amphoteric changes (ionic fluctuation) which occurred because of host cell lysis were used for detection in this case. Phage-based bacterial sensing techniques such as Sensing of the Phage-Triggered Ion Cascade (SEPTIC) have been used to measure microscopic voltage fluctuations after a phage injects its nucleic acid into host cells (King et al., 2006). Phage

recombinant protein derived from specific bacteriophage tail fibers demonstrating high binding specificity and sensitivity to target bacteria also could be a valuable detection tool. Recently bioMérieux industry has commercialized a phage recombinant protein-based kit, VIDAS UP™ for the detection and confirmation of *E. coli* O157: H7 in food samples.

1.4.4. Carbohydrate Based Detection

This method is based on naturally occurring carbohydrate-protein interactions which occur within living systems. For example, many bacterial strains produce surface lectins (fimbriae or pili) that bind specifically to carbohydrate targets on mammalian cell surfaces, e.g., the mannose-specific type 1 pili of *E. coli* that specifically binds to mannosylated glycoproteins of urinary bladder epithelial cells (Connell et al., 1996; Mammen et al., 1998). Similarly *C. jejuni* cells bind to fucosylated carbohydrate epitopes on intestinal epithelium cells (Ruiz-Palacios et al., 2003). In fact, many specific carbohydrates in animal tissues have been recognized as binding sites for bacterial pathogens (Sharon, 2006). The carbohydrate moiety of these receptors can be artificially synthesized and fluorescently labeled to develop conjugated fluorescent glycopolymers. By way of example, glycopolymers such as glycopolythiophenes were used for calorimetric detection of *E. coli* (Baek et al., 2000). In this study, binding of the glycopolymer with *E. coli* caused a detectable red shift in the visible spectrum of the polymer fluorescence. Glycopolymers such as glycopolythiophenes have mannose residues that bind with clusters of bacteria through multivalent interactions; the fluorescent cluster thus formed results in a red shift in the fluorescent spectra of the

glycopolymer, signaling the presence of the target bacteria. Carbohydrate ligands have also been used in microarray platforms to study carbohydrate–protein interactions at the molecular level (Khan et al., 2004). These microarrays provide high throughput detection capabilities for simultaneous detection of bacterial cells. A carbohydrate array fabricated on a glass slide using different amine-functionalized monosaccharide derivatives (Disney & Seeberger, 2004) was reported to detect at levels of 10^5-10^6 fluorescently labeled *E. coli* cells and to discriminate between two strains, ORN 178 and ORN 209. This array was able to effectively detect the bacteria in complex biological samples containing erythrocytes and serum, but at the expense of detection limit. It has been proposed that specific fabrication approaches such as Diels-Alder-mediated immobilization could be used to fabricate carbohydrate microarray platforms to reduce non-specific adsorption of matrix-associated proteinous components (Houseman & Mrksich, 2002).

Detection based on glycan, which constitutes a major component of the lipopolysaccharide (LPS) of Gram-negative bacterial cells, has also been reported. Specifically, detection of cell-specific glycan can be done using naturally occurring lectins. For example, it has been demonstrated that fluorescently labeled multiple bacterial cells could be detected using an array of lectins spotted over glass slides (Hsu & Mahal, 2006). The lectin arrays not only detected the bacteria but also recorded changes in surface glycosylation in response to environmental stimuli. Lectin arrays may be considered an emerging tool for bacterial strain typing but are limited due to the fact that only a few lectins with glycan specificity are commercially available.

1.4.5. Aptamer Based Detection

Aptamers have been used as capture and/or detection probes in development of assays designed to detect bacterial pathogens. A dot blot assay was developed by spotting bacterial (*F. tularensis*) antigen onto a nitrocellulose membrane followed by using biotin-labeled aptamer(s) which were tagged with streptavidin-conjugated alkaline phosphatase for detection of a positive reaction by visual color change using nitro-blue tetrazolium (Vivekananda & Kiel, 2006). In a similar study, the Aptamer-Linked Immobilized Sorbent Assay (ALISA), a two-site binding or sandwich assay, was developed using aptamers as both capture and detection ligands. In this case, immobilized DNA aptamers were used to capture *Francisella tularensis* on a 96-well microtiter plate and later detected using biotin-labeled aptamers. Detection was achieved by labeling bound aptamers with streptavidin-conjugated horseradish peroxidase (HRP) and observing color development using ABTS as the substrate (Vivekananda & Kiel, 2006).

Aptamers also provide capability as amplifiable template for PCR-based detection of target. For example, aptamer sequences bound to the *E. coli* cell surface were amplified in an indirect detection assay; the assay detected up to 10 cells in a 1 ml sample using RT-qPCR targeting the aptamer sequences. This assay not only bypassed the need for nucleic acid extraction also enhanced the sensitivity of detection due to the high degree of availability of multiple aptamer sequences bound over the cell surface (Lee et al., 2009). Clearly, nucleic acid aptamers are emerging molecules for development of biodiagnostics for bacterial

targets. Further studies are required to examine nuclease sensitivity and stability of aptamers, particularly when applied to detect bacteria in food and environmental samples.

1.4.6. Biosensors

A biosensor is a device designed to detect a target (analyte) using a combination of a recognition element and a detector element. Biosensors have two main components (i) a ligand that acts as a target recognition element, which produces a primary signal upon successful binding to the target; and (ii) a transducer that acts as a detector element, which converts the signal obtained from the recognition element into detectable form. The signals thus obtained are stored, amplified, manipulated, analyzed and displayed in a user-friendly way. Transducers work on one of many principles including fluorescence, optics, and mass detection; signals can be detected either directly or indirectly. Direct detection is based on quantifying the presence of the target using a single ligand such as an antibody. Indirect detection uses two ligands; a primary ligand bound to the surface of the biosensor which acts as a capture molecular, and a secondary labeled ligand, the purpose of which is to generate signals. There have been numerous biosensors developed specifically for the detection of bacterial contaminants in food and environmental samples.

1.4.6.1. Surface Plasmon Resonance Biosensors (SPR)

SPR biosensors are electro-optical instruments used for analyzing ligand-target interactions in real-time. An SPR is a resonant oscillation of electrons on the surface of a metal (e.g., gold). In SPR biosensors, the recognition element (often an antibody) is immobilized on the gold surface. Binding of the target causes a shift in the output signal from the SPR system. Direct SPR biosensors are not very sensitive and can be improved upon by using a secondary ligand that binds to the captured target molecule in a sandwich assay format. The secondary ligand both verifies and amplifies detection signals. The binding of the second ligand also decreases the possibility of a false positive reading due to the redundant specificity of the sandwich assay (Rasooly & Herold, 2006).

The sandwich SPR immunosensor format uses two antibodies and has been applied to the detection of staphylococcal enterotoxin B (SEB) in milk and meat samples (Rasooly, 2001). The primary antibody, which was used to capture SEB, was covalently linked to the biosensor chip, while the secondary antibody was used for signal amplification and subsequent detection. Pure SEB as well as SEB in spiked foods (milk and meat) was detected at concentrations of 10 ng/ ml with little interference from the food matrix.

SPR technology can be combined with DNA hybridization. Peptide nucleic acid (PNA) probes, which are artificial DNA analogs with potential to hybridize with target nucleic acids, have been used as recognition elements in these sorts of assays. For example, the shiga toxin-2 (*stx*2) genes of *E. coli* O157: H7 were amplified by PCR in human fecal sample and using SPR having PNA probes (Kai et al., 2000).

The subtractive inhibition technique has also been applied as a simple and rapid method for detection of *L. monocytogenes* using a polyclonal antibody; the assay supposedly requires minimal sample handling and preparation. In detection using subtractive inhibition, a known quantity of primary antibody (Fab) is bound to *L. monocytogenes* cells and the unbound antibodies are recovered in the supernatant by centrifugation. The unbound antibodies in the supernatant are passed over a sensor chip surface coated with anti-Fab antibodies to generate a response. Based on this response, the concentration of *Listeria* bound to the Fab antibody can be indirectly calculated (Leonard et al., 2004). The assay was able to detect up to 10^5 cells/ ml in less than 30 min and performed well in pure culture but not in contaminated food samples or other media.

SPR technology is limited by a number of constraints. First, the capillaries and sensor channel clog easily due to their small diameters. The shear force that occurs during laminar flow may inhibit the attachment of target bacterial cells to ligands attached to the sensor chip surface. During sample application, the orientation of target cells attaching to the sensor surface is very important as sometimes a particular orientation of is necessary for attachment and alternative orientations prevent close contact with the sensor surface, thus producing faulty signals. As is the case with most biosensors, matrix-associated components may bind non-specifically to the receptor surface resulting in inhibition of target binding and/or the generation of false positive signals. Clearly, the binding specificity of the ligand(s) used in the biosensor is critical to the production of valid results.

1.4.6.2. Multianalyte Array Biosensors (MAAB)/Evanescent Wave

MAAB consists of an array of multiple capture ligands (target specific antibodies) immobilized onto an optical waveguide in a specific pattern (such as parallel strips). Multiple samples are applied simultaneously to the waveguide surface which encounters multiple capture ligands. The target cells present within each sample bind to appropriate capture ligands and are then detected by fluorescent detection elements, usually in the form of antibodies. The evanescent wave, an electromagnetic component of the light, is launched onto the optical waveguide surface, selectively exciting the fluorophores present in the array of surface-bound immuno-complexes. The imaging of the array using mechanisms such as Peltier-cooled charge-coupled devices can be performed and data on mean fluorescence intensity of spots associated with samples and controls subsequently analyzed (Taitt et al., 2004). The change in the refractive index of the excited evanescent wave from target bound and non-target bound optical wave guide surfaces is another means by which to detect the presence of the target.

This sort of assay has been applied to the direct detection (without prior cultural enrichment) of *Campylobacter* and *Shigella* in a variety of food and beverage samples. The limit of detection for *Shigella dysenteriae* in chicken carcass wash samples was 4.9×10^4 CFU/ml, whereas for *C. jejuni*, detection limits were 9.7×10^2 CFU/ ml. The limit of detection was further improved by running the sample over the waveguide surface for a longer duration (Sapsford et al., 2004). Single analyte sandwich immunoassays have been used for MAAB-based detection of *S.* Typhimurium in various spiked foodstuffs including

sausage, cantaloupe, whole liquid egg, alfalfa sprouts, and chicken carcass rinse (Taitt et al., 2004). In the same study, MAAB also detected up to 8 x 10^3 CFU/g of *Salmonella* in chicken excreta (Taitt et al., 2004). Although no false positive results were reported in this study, matrix components such as polyphenols in sprout homogenate were reported to interfere with sensor performance.

A planar waveguide array biosensor which was based on the use of cocktails of fluorescent antibodies was applied to simultaneously detect cholera toxin, *S. aureus* enterotoxin B, ricin toxin, and the pathogens *B. anthracis*, *F. tularensis* and *Brucella abortus*. When a panel of environmental interferents was added to seeded samples, the interferents neither prevented the detection of the target nor caused false-positive responses (Rowe-Taitt et al., 2000). In another study, the investigators reported that a very low concentration (0.5 ng/ ml) of various bacterial and non-bacterial toxins was simultaneously detected in food, clinical and environmental samples on the surface of a single waveguide using sandwich and competitive fluoroimmuno-assay formats in 12 different samples. Binding of a fluorescent target or fluorescent immuno-complex resulted in a specific pattern of fluorescent spots which was detected using automated image analysis which calculated net mean fluorescence for each assay spot (Ligler et al., 2003).

MAAB appears to be able to detect multiple targets on a single sensor surface with a minimum of sample preparation. The limiting factor in using these sensors is their low level of sensitivity. For bacterial food borne pathogens, enrichment could be done to enhance detection sensitivity but the necessity of enrichment may negate the value of such biosensor approaches (Taitt et al., 2004).

1.4.6.3. Quartz Crystal Microbalance

Quartz crystal microbalance (resonant crystal biosensors, piezoelectric and acoustic wave biosensors) detects net changes in acoustic resonance frequency of a quartz crystal that occurs in association with the binding of a target to ligands immobilized on a quartz crystal surface. A quartz disc attached to electrodes is used as a transducer. In the absence of target binding, an acoustic wave due to resonance of piezoelectric crystal is produced by applying a known amount of external oscillating electric potential. The increase in mass caused by the binding of the target bacteria to the crystal surface decreases the resonant frequency of the crystal, with the frequency shift proportional to the concentration of the target. A piezoelectric immunosensor using antibodies immobilized onto a quartz crystal gold electrode surface was developed for the detection of *E. coli* O157:H7. Various sample application techniques and analytical procedures, including immersion, dip-and-dry and flow-through were investigated in the study. The immunosensor detected *E. coli* O157:H7 at concentrations ranging from 10^3-10^8 CFU/ ml within 30-50 min (Su & Li, 2004). In a different study, a piezoelectric biosensor using filamentous phage as the capture ligand was developed for detection of *S.* Typhimurium, with a reported detection limit of 100 cells/ml in less than 3 min. The change in resonance frequency was linear over a range of 10^1-10^7 CFU/ml of *S.* Typhimurium. No adverse impact was observed as a function of sample viscosity and non-specific binding was absent (Olsen et al., 2006).

The change in frequency of a quartz crystal is dependent on the sensor surface location on which the target is deposited. This means that a non-uniform distribution of the

analyte result in sensitivity problems for quartz crystal biosensor assays. The other limitation of QCM is a high degree of background noise which occurs due to the inherent vibrating nature of the quartz crystal microbalance; this can be alleviated by careful calibration of QCM devices.

1.4.6.4. Surface Enhanced Raman Scattering (SERS)

SERS is a surface sensitive phenomenon where light passing through molecules adsorbed on rough metal surface is scattered more than molecules alone. For example, light passing through pyridine adsorbed on rough silver surface is amplified several times more compared to light passing through non-adsorbed pyridine (M. Fleischmanna, 1974).

For SERS to be successful, target labeling motifs (Extrinsic Raman Label or ERL) are required which consist of roughened metal surfaces such as gold nanoparticles which are attached to reporter compounds (such as a raman scatterer) and a target-specific detection element such as an antibody. The scattering of light through ERL enhances the magnitude of light scattering due to an electromagnetic effect developed by formation of a charged (electron) transfer state between the metal surfaces and the raman scatterer (Vo-Dinh, 1998).

For detection of a bacterial target, SERS is performed in a sandwich assay format which consists of three prime components. The first is a capture substrate which is a metal (gold) surface covalently linked to a ligand such as an antibody, the purpose of which is to facilitate primary capture of the target from the sample solution. The second component is an ERL which consists of metal nanoparticles coated with reporter compounds and having a

target-specific detection antibody; the ERL is needed for selective labeling of captured target for detection using spectroscopy. Each metal nanoparticle is coated with several reporter compound molecules (10^3–10^5) which amplify scattering light several folds upon binding with a single target molecule. The third component is a Raman spectroscopy unit which reads the pattern of scattered radiation and obtains the spectra for quantification of target (Yakes et al., 2008).

Yakes et al. (2008) developed a sandwich immunoassay based on SERS that used monoclonal antibodies for capture and ERLs for detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The MAP protein concentration was detected in artificially contaminated whole milk and correlated to microbial count which was equivalent to a detection limit of 1000 MAP cells/ml of spiked whole milk. A SERS-based assay for detection of generic *E. coli* was developed using polyclonal antibodies and silver nanoparticles which produced a signal 20-fold more intense than that produced using a normal Raman spectrum (Naja et al., 2007).

SERS spectroscopy can also be used for the direct detection, quantification and determination of germination kinetics of endospores such as *B. subtilis* and *B. anthracis*. In this regard, Evanoff et al. (2006) fabricated a sandwich-type SERS for detection of a single *B. subtilis* spore using silver particles immobilized on a poly diallyldimethylammonium chloride (PDDA) modified silver mirror film as the capture substrate. The positively charged silver particles adsorbed the negatively charged spores during an overnight incubation. Spectra originated from endospores were collected and analyzed with a sensitivity of detection of one single spore.

Prolonged sample incubation is needed to facilitate both capture and ERL labeling in SERS-based detection assays. The reason for this is that delivery of antigens and ERLs relies on diffusion mass transport. Unfortunately, the prolonged incubation times may also lead to non-specific binding of non-target antigens to capture ligands. Further advancement is thus required to reduce the incubation time to take full advantage of this highly sensitive detection technique (Yakes et al., 2008).

1.5. CONCLUSIONS

For the past several decades, significant advancements in the microbial analysis of foods and environmental samples have been made. All advancements are aimed at achieving sensitive and specific detection of pathogens, but these have neither resulted in quantitative methods nor the ability to limit the need for prior cultural enrichment. In fact, the biggest hurdle in the development of more rapid detection methods is the dependency on culture. Although cultural enrichment amplifies the target in preparation for downstream detection, it remains time consuming, alters the original microbial community of the sample, and prevents the quantitative determination of pathogen load. It appears that the only way around this dilemma is to apply upstream methods to separate and concentrate microbial targets from the sample in preparation for direct downstream detection. Ideally, such methods must be able to selectively recover all target microbial cells in each candidate sample matrix. Further, the method(s) should be simple and broadly applicable to multiple matrices with different

physico-chemical properties. Most of the reported separation methods have some shortcomings in terms of their applicability and/or performance.

If such pre-analytical sample processing methods can be successfully developed, the options for downstream detection are virtually endless. In the near term, techniques such as IMS and alternative bioaffinity ligands such as bacteriophage, carbohydrates and aptamers are logical approaches to explore for improved target capture and subsequent sample preparation. However, it appears that the performance of these ligands in the food matix remain the most significant challenge. For example, antibodies may cross-react with closely related antigens and can lose their binding efficiency in the presence of matrix components. Ligands such as bacteriophages are highly target specific but they may inadvertently manipulate target cell phenotype or cause cell lysis upon binding. Aptamers are particularly promising as they offer tremendous opportunities to simultaneously address various steps in pathogen detection ranging from target separation to detection. For example, the binding of an aptamer to the surface of a target cell could serve as a sample concentration step and at the same time, provide a surface upon which PCR can take place, as would be the case for the proximity ligation assay. This sort of approach could combine a high degree of specificity with ease of detection and at the same time confirming functionality and pathogenic potential of target pathogen cells.

The highly automated real-time methods of the future are in particular need of pre-analytical sample processing. These sorts of methods offer promise for on-line microbial detection from farm-to-fork using simple, integrated platforms contained in miniaturized and portable devices. Advanced real-time PCR technologies, DNA microarrays, and biosensors

all fall into this category of promising future detection approaches. Although these methods need further development and validation, they represent a promising future. As we move forward, it remains important to recognize the variety in microbial targets, sample matrices, detection approaches, and testing needs. Clearly, the field of food microbial diagnostics is and will remain highly dynamic with tremendous potential for development and application of exciting new concepts and techniques.

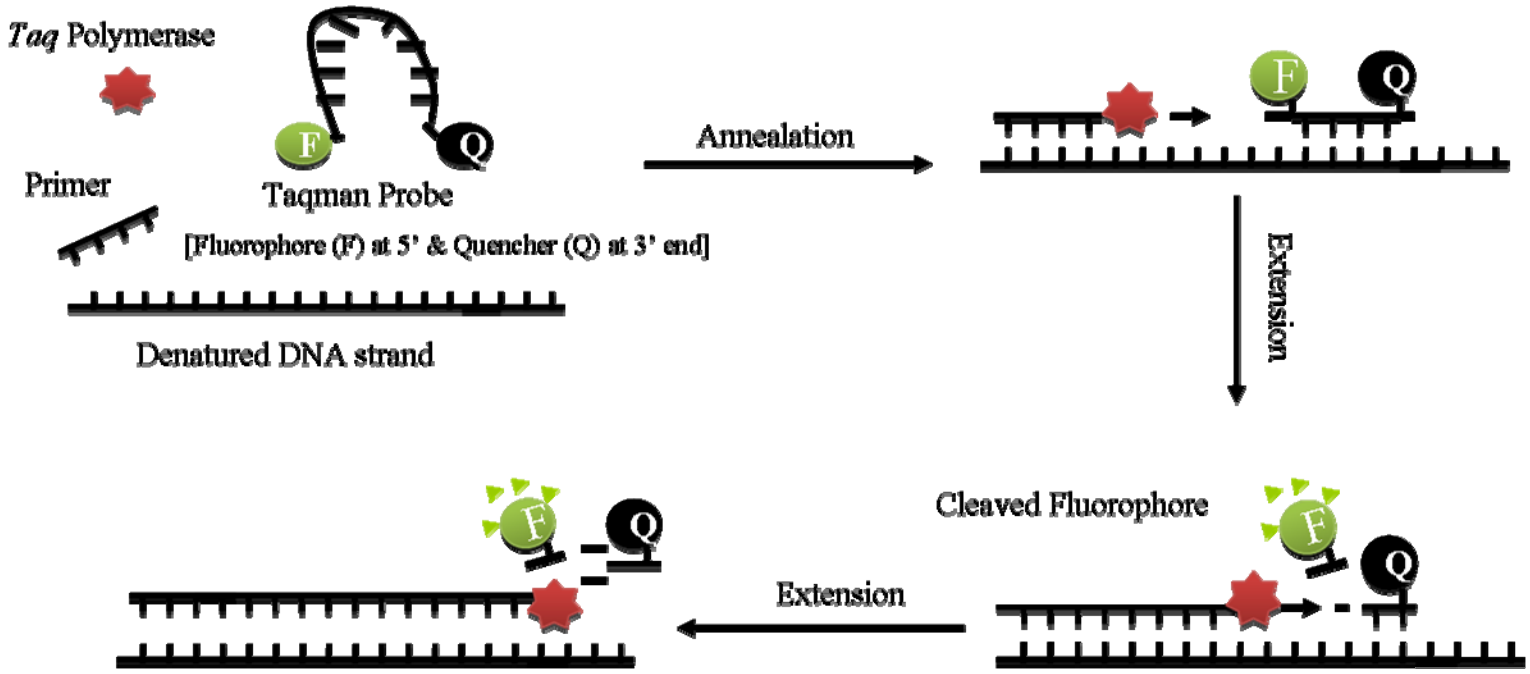

Figure 1.1. Mechanism of Taqman™ probe chemistry. Due to close proximity to fluorophore, the quencher molecule of Taqman probe quenches the fluorescence emitted by the fluorophore on excitement. Taqman probe anneal within a specific site on DNA template that is amplified by primers. During extension, as the Taq Polymerase extend the primer to synthesize new strand, 5'-3' exonuclease activity of Polymerase degrade the annealed Taqman probe and releases the fluorophore from close proximity of quencher thus allowing it to fluoresce.

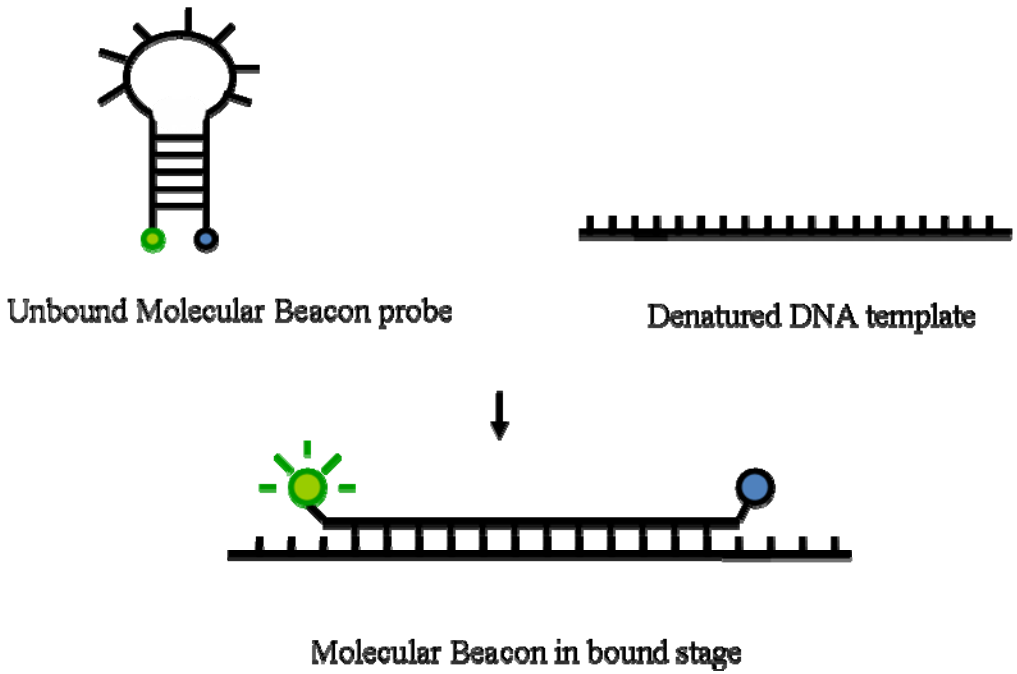

Figure 1.2. Mechanism of molecular beacon chemistry. The molecular beacons are hairpin shaped molecules having a stem and a loop structure. The loop structure has sequences complementary to specific site on the target DNA template. The quencher (blue circle) at 3' end of beacon transfers energy from the fluorophore (green circle) at 5' end and releases it as heat thus quenching the fluorophore. When the complementary sequences of target DNA template comes in contact with the molecular beacon, the molecular beacon undergoes a conformational reorganization and hybridizes with the target strand. As a result quencher is no longer closer enough to accept the energy from the flourophore which make fluorophore to fluoresce.

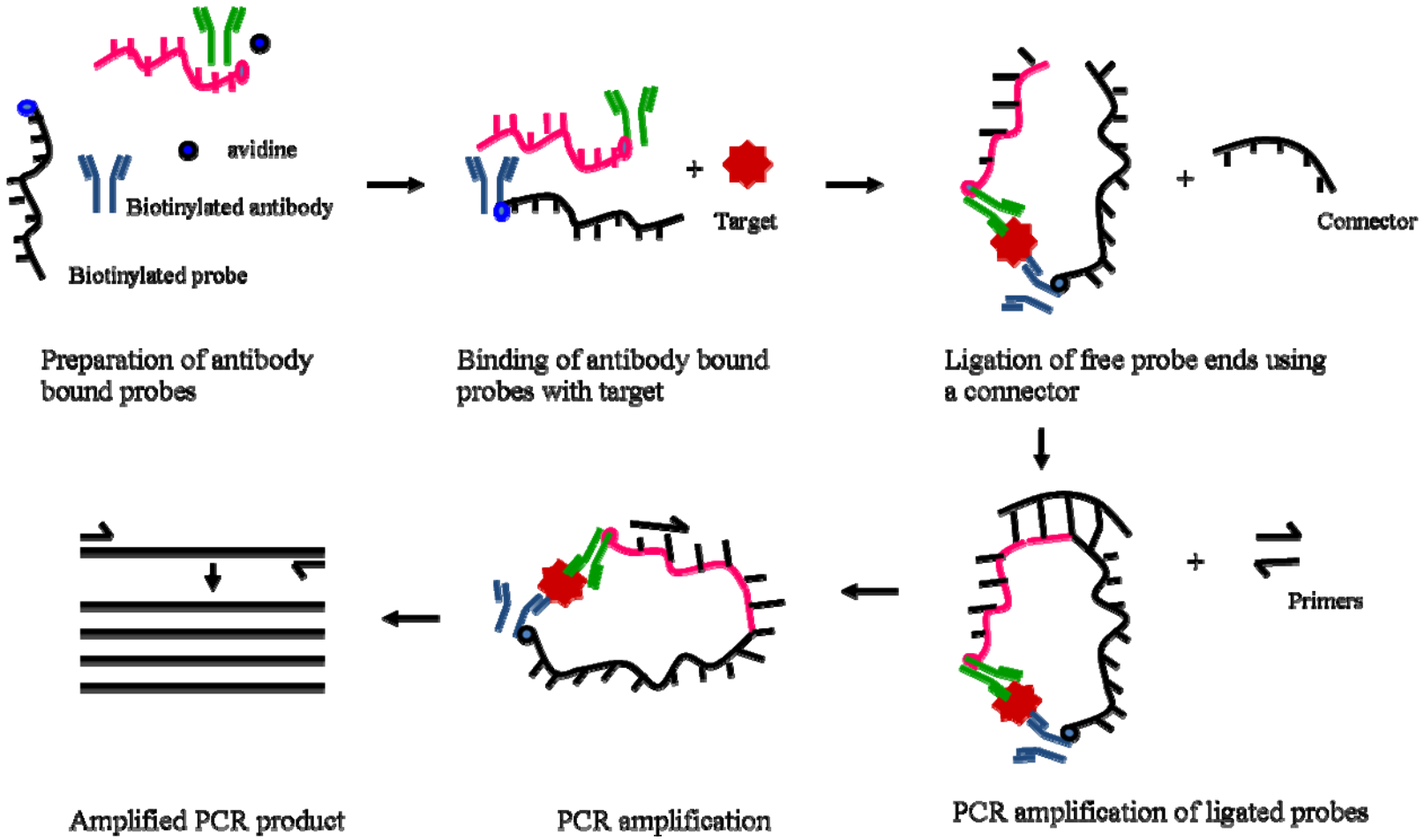

Figure 1.3. Schematic representation of proximal ligation assay. The antibody bound probes are used to bind the target. A connector having complementary sequences to the free ends of probes is used to ligate the probes end. The PCR amplification of ligated probe sequences is used to indirectly detect the presence of the target.

Table 1.1. Detection of *Salmonella* using real-time PCR.

Gene Target	Matrices	Enrichment	Limit of Detection	Primers (F-Forward; R-Reverse) Probe (P)	Reference
fimC	Ice cream	No	10^3 CFU/ml	F: ATA AAT CCG GCG GCC TGA TG R: TGG TAT CGA CGC CTT TAT CTG AGA P: TTA CAC CGG AGT GGA TTA AAC GGC TGG G	Seo et al. 2006
ttrBCA	Chicken rinses, minced meat, fish, raw fish	20 h enrichment	10^2 CFU/ml (no enrichment) <3 CFU/ml (20 h)	F: CTC ACC AGG AGA TTA CAA CAT GG R: AGC TCA GAC CAA AAG TGA CCA TC P: CAC CGA CGG CGA GAC CGA CTT T	Malorny et al. 2004
invA	Raw milk, chicken meat, salmon	16 h enrichment	5 CFU/25 ml (raw milk) 2.5 - 5 CFU/25 g (chicken and salmon)	F: GTG AAA TAA TCG CCA CGT TCG GGC AA R: TCA TCG CAC CGT CAA AGG AAC CGT AA P: TTA TTG GCG ATA GCC TGG CGG TGG GTT TTG TTG	Hein et al. 2006
oriC	Cheddar cheese, raw turkey, and cooked turkey	48 h selective enrichment	6.1 x 10^1 CFU/ml	R: TCACCTGCGACAGCCATGA R: TGAGCATCGCCATCGGCAT P:ATTCCAGCAGTCGGCCATAGCTG (Set I) F: CATTGATGCCATGGGTGACART R: CGTGACGATAATCCGTGTAC P:TACACGAGTCACTAAATCCTTCAGT (Set II)	McCarthy et al. 2009
invA	chili powder and shrimp samples	pre-enrichment 35°C- 24 h Selective enrichment 42°C/24 h	0.04 CFU/g	F: AACGTGTTTCCGTGCGTAAT R: TCCATCAAATTAGCGGAGGC P: TGGAAGCGCTCGCATTGTGG	Cheng et al. 2008
stn	Culture	No	3 CFU/ reaction	F: GCCATGCTGTTCGATGAT R: GTTACCGATAGCGGGAAAGG P: TTTTGCACCACMGCCAGCCC	Moore & Feist 2007
himA (Molecular Beacon)	Culture	No	2 CFU/ ml	F: CGTGCTCTGGAAAACGGTGAG R: CGTGCTGTAATAGGAATATCTTCA P: CGCTATCCGGGGCGTAACC-CGTAGCG	Chen et al. 2000

Table 1.2. Detection of *Campylobacter jejuni* using real-time PCR.

Gene Target	Matrices	Enrichment	Limit of Detection	Primers/ Probe (F-Forward; R-Reverse/ P-Probe)	Reference
hipO	Chicken samples	No	1.18 to 5.30 log CFU/ml	F: CTGCTTCTTTACTTGTTGTGGCTTT R: GCTCCTATGCTTACAACTGCTGAAT P: CATTGCGAGATACTATGCTTTG	Hong et al. 2007
*VS*1 sequence (VS gene)	Whole chicken rinses	No	1CFU/ml (Pure Culture)	F: GAATGAAATTTTAGAATGGGG R: GATATGTATGATT-TTATCCTGC P: TTTAACTTGGCTAAAGGCTAAGGCT	Debretsion et al. 2007
*VS*1 sequence (VS gene)	Poultry, milk and environmental water	No	1 CFU/ml (Pure Culture)	F: GAATGAAATTTTAGAATGGGG R: GATATGTATGATTTTATCCTGC P: TTTAACTTGGCTAAAGGCTAAGGCT	Yang et al. 2003
C. jejuni-specific region of the ORF-C sequence	Raw chicken, offal, shellfish, raw meat, and spiked milk samples	37°C - 24 h 42°C - 24 h	12 genome equivalents per reaction	F:TTGGTATGGCTATAGGAACTCTTATAGCT R: CACACCTGAAGTATGAAGTGGTCTAAGT P: TGGCATATCCTAATTTAAATTATTTACCA GGAC	Sails et al. 2003
*Cad*F gene	Chicken breast	37°C - 42°C (12 - 48 h)	9 x 10^2 to >10^5 CFU/ml (after 12 hrs)	-	Oliveira et al. 2005
An 86 bp region between 381121 - 381206 of *C. jejuni* (NCTC11168) Genome	Pure culture	No	1 CFU/ml	F: CTGAATTTGATACCTTAAGTGCAGC R: AGGCACGCCTAAACCTATAGCT P: TCTCCTTGCTCATCTTTAGGATAAATT CTTTCACA	Nogva et al. 2000
An 86 bp region between 381121 - 381206 of *C. jejuni* (NCTC11168) Genome	Chicken rinse sample	No	2.6 CFU/ml	F: CTGAATTTGATACCTTAAGTGCAGC R: AGGCACGCCTAAACCTATAGCT P: TCTCCTTGCTCATCTTTAGGATAAATT CTTTCACA	Ronner & Lindmark 2007
*gly*A gene	Pure culture and fecal sample	41.5°C - 48 h	-	F: TAATGTTCAGCCTAATTCAGGTTCTC R: R GAAGAACTTACTTTTGCACCATGAGT P: AATCAAAGCCGCATAAACACCTTGATTAGC	Jensen et al. 2005

1.6. REFERENCES

Agoston, R., Soni, K. A., McElhany, K., Cepeda, M. L., Zuckerman, U., Tzipori, S., Mohacsi-Farkas, C. & Pillai, S. D. (2009). Rapid concentration of *Bacillus* and *Clostridium* spores from large volumes of milk, using continuous flow centrifugation. *J Food Prot*, 72(3), 666-8.

Aldus, C. F., Van Amerongen, A., Ariens, R. M., Peck, M. W., Wichers, J. H. & Wyatt, G. M. (2003). Principles of some novel rapid dipstick methods for detection and characterization of verotoxigenic *Escherichia coli*. *J Appl Microbiol*, 95(2), 380-9.

Amagliani, G., Omiccioli, E., Campo, A., Bruce, I. J., Brandi, G. & Magnani, M. (2006). Development of a magnetic capture hybridization-PCR assay for *Listeria monocytogenes* direct detection in milk samples. *J Appl Microbiol*, 100(2), 375-83.

Baek, M. G., Stevens, R. C. & Charych, D. H. (2000). Design and synthesis of novel glycopolythiophene assemblies for colorimetric detection of influenza virus and *E. coli*. *Bioconjug Chem*, 11(6), 777-88.

Banada, P. P. & Bhunia, A. K. (2008). *Antibodies and Immunoassays for Detection of Bacterial Pathogens*. Springer New York.

Bauwens, L., Vercammen, F. & Hertsens, A. (2003). Detection of pathogenic *Listeria* spp. in zoo animal faeces: use of immunomagnetic separation and a chromogenic isolation medium. *Vet Microbiol*, 91(2-3), 115-23.

Bennett, A. R., Davids, F. G., Vlahodimou, S., Banks, J. G. & Betts, R. P. (1997). The use of bacteriophage-based systems for the separation and concentration of *Salmonella*. *J Appl Microbiol*, 83(2), 259-65.

Benoit, P. W. & Donahue, D. W. (2003). Methods for rapid separation and concentration of bacteria in food that bypass time-consuming cultural enrichment. *J Food Prot*, 66(10), 1935-48.

Berry, E. D. & Siragusa, G. R. (1997). Hydroxyapatite adherence as a means to concentrate bacteria. *Appl Environ Microbiol*, 63(10), 4069-74.

Betts, W. B. (1995). The potential of dielectrophoresis for the real-time detection of microorganisms in foods. *Trends in Food Science & Technology*, 6, 51.

Bohaychuk, V. M., Gensler, G. E., King, R. K., Wu, J. T. & McMullen, L. M. (2005). Evaluation of detection methods for screening meat and poultry products for the presence of foodborne pathogens. *J Food Prot*, 68(12), 2637-47.

Brehm-Stecher, B., Young, C., Jaykus, L. A. & Tortorello, M. L. (2009). Sample preparation: the forgotten beginning. *J Food Prot*, 72(8), 1774-89.

Bruno, J. G., Phillips, T., Carrillo, M. P. & Crowell, R. (2009). Plastic-adherent DNA aptamer-magnetic bead and quantum dot sandwich assay for *Campylobacter* detection. *J Fluoresc*, 19(3), 427-35.

Call, D. R., Brockman, F. J. & Chandler, D. P. (2001). Detecting and genotyping *Escherichia coli* O157:H7 using multiplexed PCR and nucleic acid microarrays. *Int J Food Microbiol*, 67(1-2), 71-80.

CDC. (2009). Multistate Outbreak of *E. coli* O157:H7 Infections Linked to Eating Raw Refrigerated, Prepackaged Cookie Dough, Vol. 2009, CDC. Atlanta.

CDC. (2009b). Preliminary FoodNet Data on the incidence of infection with pathogens transmitted commonly through food--10 States, 2008. *MMWR Morb Mortal Wkly Rep*, 58(13), 333-7.

CDC. (2009a). Surveillance for foodborne disease outbreaks - United States, 2006. *MMWR Morb Mortal Wkly Rep*, 58(22), 609-15.

Chandler, D. P., Brown, J., Call, D. R., Wunschel, S., Grate, J. W., Holman, D. A., Olson, L., Stottlemyre, M. S. & Bruckner-Lea, C. J. (2001). Automated immunomagnetic separation and microarray detection of *E. coli* O157:H7 from poultry carcass rinse. *Int J Food Microbiol*, 70(1-2), 143-54.

Chapman, P. A. & Ashton, R. (2003). An evaluation of rapid methods for detecting *Escherichia coli* O157 on beef carcasses. *Int J Food Microbiol*, 87(3), 279-85.

Chen, F., Zhou, J., Luo, F., Mohammed, A. B. & Zhang, X. L. (2007). Aptamer from whole-bacterium SELEX as new therapeutic reagent against virulent *Mycobacterium tuberculosis*. *Biochem Biophys Res Commun*, 357(3), 743-8.

Chen, J. & Griffiths, M. W. (1996). Luminescent *Salmonella* strains as real time reporters of growth and recovery from sublethal injury in food. *Int J Food Microbiol*, 31(1-3), 27-43.

Chen, W., Martinez, G. & Mulchandani, A. (2000). Molecular beacons: a real-time polymerase chain reaction assay for detecting *Salmonella*. *Anal Biochem*, 280(1), 166-72.

Cheng, C. M., Lin, W., Van, K. T., Phan, L., Tran, N. N. & Farmer, D. (2008). Rapid detection of *Salmonella* in foods using real-time PCR. *J Food Prot*, 71(12), 2436-41.

Cheng, J., Sheldon, E. L., Wu, L., Uribe, A., Gerrue, L. O., Carrino, J., Heller, M. J. & O'Connell, J. P. (1998). Preparation and hybridization analysis of DNA/RNA from *E. coli* on microfabricated bioelectronic chips. *Nat Biotechnol*, 16(6), 541-6.

Churruca, E., Girbau, C., Martinez, I., Mateo, E., Alonso, R. & Fernandez-Astorga, A. (2007). Detection of *Campylobacter jejuni* and *Campylobacter coli* in chicken meat samples by real-time nucleic acid sequence-based amplification with molecular beacons. *Int J Food Microbiol*, 117(1), 85-90.

Connell, I., Agace, W., Klemm, P., Schembri, M., Marild, S. & Svanborg, C. (1996). Type 1 fimbrial expression enhances *Escherichia coli* virulence for the urinary tract. *Proc Natl Acad Sci U S A*, 93(18), 9827-32.

Cook, N. (2003). The use of NASBA for the detection of microbial pathogens in food and environmental samples. *J Microbiol Methods*, 53(2), 165-74.

Cremonesi, P., Pisoni, G., Severgnini, M., Consolandi, C., Moroni, P., Raschetti, M. & Castiglioni, B. (2009). Pathogen detection in milk samples by ligation detection reaction-mediated universal array method. *J Dairy Sci*, 92(7), 3027-39.

Cui, S., Schroeder, C. M., Zhang, D. Y. & Meng, J. (2003). Rapid sample preparation method for PCR-based detection of *Escherichia coli* O157:H7 in ground beef. *J Appl Microbiol*, 95(1), 129-34.

Cullison, M. A. & Jaykus, L. A. (2002). Magnetized carbonyl iron and insoluble zirconium hydroxide mixture facilitates bacterial concentration and separation from nonfat dry milk. *J Food Prot*, 65(11), 1806-10.

D'Urso, O. F., Poltronieri, P., Marsigliante, S., Storelli, C., Hernandez, M. & Rodriguez-Lazaro, D. (2009). A filtration-based real-time PCR method for the quantitative detection of viable *Salmonella enterica* and *Listeria monocytogenes* in food samples. *Food Microbiol*, 26(3), 311-6.

Debretsion, A., Habtemariam, T., Wilson, S., Nganwa, D. & Yehualaeshet, T. (2007). Real-time PCR assay for rapid detection and quantification of *Campylobacter jejuni* on chicken rinses from poultry processing plant. *Mol Cell Probes*, 21(3), 177-81.

Disney, M. D. & Seeberger, P. H. (2004). The use of carbohydrate microarrays to study carbohydrate-cell interactions and to detect pathogens. *Chem Biol*, 11(12), 1701-7.

Dols, C. L., Bowers, J. M. & Copfer, A. E. (2001). Preventing food- and water-borne illnesses. *Am J Nurs*, 101(6), 24AA-24HH.

Dubow, M. S. (1994). Bacterial identification- use of bacteriophages. In *Encyclopedia of Virology* eds. R. G. Webster & A. Granoff, Academic Press. San Diego, California, pp. 78-81.

Elizaquivel, P. & Aznar, R. (2008). A multiplex RTi-PCR reaction for simultaneous detection of Escherichia coli O157:H7, *Salmonella* spp. and *Staphylococcus* aureus on fresh, minimally processed vegetables. *Food Microbiol*, 25(5), 705-13.

Elvers, K. T. H., C. R. Wassenaar, T. M. Allen, V. M.Newell, D. G. (2008). Development of a strain-specific molecular method for quantitating individual *Campylobacter* strains in mixed populations. *Appl Environ Microbiol*, 74(8), 2321-31.

Evanoff, D. D., Jr., Heckel, J., Caldwell, T. P., Christensen, K. A. & Chumanov, G. (2006). Monitoring DPA release from a single germinating *Bacillus subtilis* endospore via surface-enhanced Raman scattering microscopy. *J Am Chem Soc*, 128(39), 12618-9.

Feng, P. (2007). Rapid Methods for the Detection of Foodborne Pathogens: Current and Next Generation Technologies. 3 ed. In *Food Microbiology: Fundamentals and Frontiers*, eds. M. P. Doyle & L. R. Beuchat, ASM Press. Washington, D.C., pp. 911-934.

Flekna, G., Stefanic, P., Wagner, M., Smulders, F. J., Mozina, S. S. & 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.

Fredriksson, S., Gullberg, M., Jarvius, J., Olsson, C., Pietras, K., Gustafsdottir, S. M., Ostman, A. & Landegren, U. (2002). Protein detection using proximity-dependent DNA ligation assays. *Nat Biotechnol*, 20(5), 473-7.

Fu, Z., Rogelj, S. & Kieft, T. L. (2005). Rapid detection of *Escherichia coli* O157:H7 by immunomagnetic separation and real-time PCR. *Int J Food Microbiol*, 99(1), 47-57.

Fukushima, H., Katsube, K., Hata, Y., Kishi, R. & Fujiwara, S. (2007). Rapid separation and concentration of food-borne pathogens in food samples prior to quantification by viable-cell counting and real-time PCR. *Appl Environ Microbiol*, 73(1), 92-100.

Geng, T., Hahm, B. K. & Bhunia, A. K. (2006). Selective enrichment media affect the antibody-based detection of stress-exposed *Listeria monocytogenes* due to differential expression of antibody-reactive antigens identified by protein sequencing. *J Food Prot*, 69(8), 1879-86.

Gonzalez-Escalona, N., Hammack, T. S., Russell, M., Jacobson, A. P., De Jesus, A. J., Brown, E. W. & Lampel, K. A. (2009). Detection of live *Salmonella* sp. cells in produce by a

TaqMan-based quantitative reverse transcriptase real-time PCR targeting invA mRNA. *Appl Environ Microbiol*, 75(11), 3714-20.

Goodridge, L., Chen, J. & Griffiths, M. (1999). The use of a fluorescent bacteriophage assay for detection of *Escherichia coli* O157:H7 in inoculated ground beef and raw milk. *Int J Food Microbiol*, 47(1-2), 43-50.

Goodridge, L. & Griffiths, M. (2002). Reporter bacteriophage assays as a means to detect foodborne pathogenic bacteria. *Food Research International*, 35(9), 863-870.

Grant, K. A., Dickinson, J. H., Payne, M. J., Campbell, S., Collins, M. D. & Kroll, R. G. (1993). Use of the polymerase chain reaction and 16S rRNA sequences for the rapid detection of *Brochothrix* spp. in foods. *J Appl Bacteriol*, 74(3), 260-7.

Guan, J., Chan, M., Allain, B., Mandeville, R. & Brooks, B. W. (2006). Detection of multiple antibiotic-resistant *Salmonella enterica* serovar Typhimurium DT104 by phage replication-competitive enzyme-linked immunosorbent assay. *J Food Prot*, 69(4), 739-42.

Guatelli, J. C., Whitfield, K. M., Kwoh, D. Y., Barringer, K. J., Richman, D. D. & Gingeras, T. R. (1990). Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. *Proc Natl Acad Sci U S A*, 87(19), 7797.

Gustafsdottir, S. M., Nordengrahn, A., Fredriksson, S., Wallgren, P., Rivera, E., Schallmeiner, E., Merza, M. & Landegren, U. (2006). Detection of individual microbial pathogens by proximity ligation. *Clin Chem*, 52(6), 1152-60.

Hadjinicolaou, A. V., Demetriou, V. L., Emmanuel, M. A., Kakoyiannis, C. K. & Kostrikis, L. G. (2009). Molecular beacon-based real-time PCR detection of primary isolates of *Salmonella* Typhimurium and *Salmonella* Enteritidis in environmental and clinical samples. *BMC Microbiol*, 9, 97.

Hahm, B. K. & Bhunia, A. K. (2006). Effect of environmental stresses on antibody-based detection of *Escherichia coli* O157:H7, *Salmonella enterica* serotype Enteritidis and Listeria monocytogenes. *J Appl Microbiol*, 100(5), 1017-27.

Hara-Kudo, Y., Kumagai, S., Masuda, T., Goto, K., Ohtsuka, K., Masaki, H., Tanaka, H., Tanno, K., Miyahara, M. & Konuma, H. (2001). Detection of *Salmonella* Enteritidis in shell and liquid eggs using enrichment and plating. *Int J Food Microbiol*, 64(3), 395-9.

Hong, B. X., Jiang, L. F., Hu, Y. S., Fang, D. Y. & Guo, H. Y. (2004). Application of oligonucleotide array technology for the rapid detection of pathogenic bacteria of foodborne infections. *J Microbiol Methods*, 58(3), 403-11.

Hong, J., Jung, W. K., Kim, J. M., Kim, S. H., Koo, H. C., Ser, J. & Park, Y. H. (2007). Quantification and differentiation of *Campylobacter jejuni* and *Campylobacter coli* in raw chicken meats using a real-time PCR method. *J Food Prot*, 70(9), 2015-22.

Hoorfar, J., Cook, N., Malorny, B., Wagner, M., De Medici, D., Abdulmawjood, A. & Fach, P. (2004). Diagnostic PCR: making internal amplification control mandatory. *Lett Appl Microbiol*, 38(2), 79-80.

Houseman, B. T. & Mrksich, M. (2002). Carbohydrate arrays for the evaluation of protein binding and enzymatic modification. *Chem Biol*, 9(4), 443-54.

Hsih, H. Y. & Tsen, H. Y. (2001). Combination of immunomagnetic separation and polymerase chain reaction for the simultaneous detection of *Listeria monocytogenes* and *Salmonella* spp. in food samples. *J Food Prot*, 64(11), 1744-50.

Hsu, C. F., Tsai, T. Y. & Pan, T. M. (2005). Use of the duplex TaqMan PCR system for detection of Shiga-like toxin-producing *Escherichia coli* O157. *J Clin Microbiol*, 43(6), 2668-73.

Hsu, K. L. & Mahal, L. K. (2006). A lectin microarray approach for the rapid analysis of bacterial glycans. *Nat Protoc*, 1(2), 543-9.

Hudson, J. A., Lake, R. J., Savill, M. G., Scholes, P. & McCormick, R. E. (2001). Rapid detection of *Listeria monocytogenes* in ham samples using immunomagnetic separation followed by polymerase chain reaction. *J Appl Microbiol*, 90(4), 614-21.

Ibrahim, G. F., Lyons, M. J., Walker, R. A. & Fleet, G. H. (1985). Rapid detection of salmonellae by immunoassays with titanous hydroxide as the solid phase. *Appl Environ Microbiol*, 50(3), 670-5.

Jacobsen, C. S. & Rasmussen, O. F. (1992). Development and Application of a New Method To Extract Bacterial DNA from Soil Based on Separation of Bacteria from Soil with Cation-Exchange Resin. *Appl Environ Microbiol*, 58(8), 2458-2462.

Jany, J. L. & Barbier, G. (2008). Culture-independent methods for identifying microbial communities in cheese. *Food Microbiol*, 25(7), 839-48.

Jassim, S. A. & Griffiths, M. W. (2007). Evaluation of a rapid microbial detection method via phage lytic amplification assay coupled with Live/Dead fluorochromic stains. *Lett Appl Microbiol*, 44(6), 673-8.

Jensen, A. N., Andersen, M. T., Dalsgaard, A., Baggesen, D. L. & Nielsen, E. M. (2005). Development of real-time PCR and hybridization methods for detection and identification of thermophilic *Campylobacter* spp. in pig faecal samples. *J Appl Microbiol*, 99(2), 292-300.

Jepras, R. I., Carter, J., Pearson, S. C., Paul, F. E. & Wilkinson, M. J. (1995). Development of a Robust Flow Cytometric Assay for Determining Numbers of Viable Bacteria. *Appl Environ Microbiol*, 61(7), 2696-2701.

Jones, T. F., Scallan, E. & Angulo, F. J. (2007). FoodNet: overview of a decade of achievement. *Foodborne Pathog Dis*, 4(1), 60-6.

Josephson, K. L., Gerba, C. P. & Pepper, I. L. (1993). Polymerase chain reaction detection of nonviable bacterial pathogens. *Appl Environ Microbiol*, 59(10), 3513-5.

Joshi, R., Janagama, H., Dwivedi, H. P., Senthil Kumar, T. M., Jaykus, L. A., Schefers, J. & Sreevatsan, S. (2009). Selection, characterization, and application of DNA aptamers for the capture and detection of *Salmonella enterica* serovars. *Mol Cell Probes*, 23(1), 20-8.

Jothikumar, N. & Griffiths, M. W. (2002). Rapid detection of *Escherichia coli* O157:H7 with multiplex real-time PCR assays. *Appl Environ Microbiol*, 68(6), 3169-71.

Kai, E., Ikebukuro, K., Hoshina, S., Watanabe, H. & Karube, I. (2000). Detection of PCR products of *Escherichia coli* O157:H7 in human stool samples using surface plasmon resonance (SPR). *FEMS Immunol Med Microbiol*, 29(4), 283-8.

Kennedy, J. F., Barker, S. A. & Humphreys, J. D. (1976). Microbial cells living immobilised on metal hydroxides. *Nature*, 261(5557), 242-4.

Khan, I., Desai, D. V. & Kumar, A. (2004). Carbochips: a new energy for old biobuilders. *J Biosci Bioeng*, 98(5), 331-7.

King, M. D., Seo, S., Kim, J., Cheng, M., Higgins, S., Young, R., McIntyre, D. H., Thien, B., McFarland, A. R. & Kish, L. B. (2006). Noise for Health: Phage-Based Rapid Bacterial Identification Method. In *Understanding Complex Systems*, Vol. 2006, Springer Berlin. Heidelberg, pp. 171-179.

Klaus, D., Simske, S., Todd, P. & Stodieck, L. (1997). Investigation of space flight effects on *Escherichia coli* and a proposed model of underlying physical mechanisms. *Microbiology*, 143 (Pt 2), 449-55.

Klussmann, S., Nolte, A., Bald, R., Erdmann, V. A. & Furste, J. P. (1996). Mirror-image RNA that binds D-adenosine. *Nat Biotechnol*, 14(9), 1112-5.

Kretzer, J. W., Lehmann, R., Schmelcher, M., Banz, M., Kim, K. P., Korn, C. & Loessner, M. J. (2007). Use of high-affinity cell wall-binding domains of bacteriophage endolysins for immobilization and separation of bacterial cells. *Appl Environ Microbiol*, 73(6), 1992-2000.

Lakshmanan, R. S., Guntupalli, R., Hu, J., Kim, D. J., Petrenko, V. A., Barbaree, J. M. & Chin, B. A. (2007). Phage immobilized magnetoelastic sensor for the detection of *Salmonella* Typhimurium. *J Microbiol Methods*, 71(1), 55-60.

Lapizco-Encinas, B. H., Simmons, B. A., Cummings, E. B. & Fintschenko, Y. (2004). Dielectrophoretic concentration and separation of live and dead bacteria in an array of insulators. *Anal Chem*, 76(6), 1571-9.

Laplace-Builhe, C., Hahne, K., Hunger, W., Tirilly, Y. & Drocourt, J. L. (1993). Application of flow cytometry to rapid microbial analysis in food and drinks industries. *Biol Cell*, 78(1-2), 123-8.

Lee, H. J., Kim, B. C., Kim, K. W., Kim, Y. K., Kim, J. & Oh, M. K. (2009). A sensitive method to detect *Escherichia coli* based on immunomagnetic separation and real-time PCR amplification of aptamers. *Biosens Bioelectron*, 24(12), 3550-5.

Lee SS, I. S. a. I. K. (2004). A CAD study on micro flow cytometer and its application to bacteria detection. *IEEE Sensor*, 1, 308–311.

Leonard, P., Hearty, S., Quinn, J. & O'Kennedy, R. (2004). A generic approach for the detection of whole *Listeria monocytogenes* cells in contaminated samples using surface plasmon resonance. *Biosens Bioelectron*, 19(10), 1331-5.

Ligler, F. S., Taitt, C. R., Shriver-Lake, L. C., Sapsford, K. E., Shubin, Y. & Golden, J. P. (2003). Array biosensor for detection of toxins. *Anal Bioanal Chem*, 377(3), 469-77.

Little, M. C., Andrews, J., Moore, R., Bustos, S., Jones, L., Embres, C., Durmowicz, G., Harris, J., Berger, D., Yanson, K., Rostkowski, C., Yursis, D., Price, J., Fort, T., Walters, A., Collis, M., Llorin, O., Wood, J., Failing, F., O'Keefe, C., Scrivens, B., Pope, B., Hansen, T., Marino, K., Williams, K. & et al. (1999). Strand displacement amplification and homogeneous real-time detection incorporated in a second-generation DNA probe system, BDProbeTecET. *Clin Chem*, 45(6 Pt 1), 777-84.

Lucore, L. A., Cullison, M. A. & Jaykus, L. A. (2000). Immobilization with metal hydroxides as a means to concentrate food-borne bacteria for detection by cultural and molecular methods. *Appl Environ Microbiol*, 66(5), 1769-76.

Lund, M. & Madsen, M. (2006). Strategies for the inclusion of an internal amplification control in conventional and real time PCR detection of *Campylobacter* spp. in chicken fecal samples. *Mol Cell Probes*, 20(2), 92-9.

M. Fleischmanna, P. J. H. a. A. J. M. (1974). Raman spectra of pyridine adsorbed at a silver electrode. *Chemical Physics Letters*, 26 (2), 163-166.

Madonna, A. J., Van Cuyk, S. & Voorhees, K. J. (2003). Detection of *Escherichia coli* using immunomagnetic separation and bacteriophage amplification coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom*, 17(3), 257-63.

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.

Malorny, B., Hoorfar, J., Hugas, M., Heuvelink, A., Fach, P., Ellerbroek, L., Bunge, C., Dorn, C. & Helmuth, R. (2003). Interlaboratory diagnostic accuracy of a *Salmonella* specific PCR-based method. *Int J Food Microbiol*, 89(2-3), 241-9.

Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A. & Helmuth, R. (2004). Diagnostic real-time PCR for detection of *Salmonella* in food. *Appl Environ Microbiol*, 70(12), 7046-52.

Mammen, M., Choi, S. & GM, W. (1998). Polyvalent interactions in biological systems: implications for design and use of multivalent ligands and inhibitors. *Angew. Chem. Int. Ed.*, 37, 2755–2794.

Markx, G. H., Dyda, P. A. & Pethig, R. (1996). Dielectrophoretic separation of bacteria using a conductivity gradient. *J Biotechnol*, 51(2), 175-80.

Markx, G. H., Talary, M. S. & Pethig, R. (1994). Separation of viable and non-viable yeast using dielectrophoresis. *J Biotechnol*, 32(1), 29-37.

Masters, C. I., Shallcross, J. A. & Mackey, B. M. (1994). Effect of stress treatments on the detection of *Listeria monocytogenes* and enterotoxigenic *Escherichia coli* by the polymerase chain reaction. *J Appl Bacteriol*, 77(1), 73-9.

McCarthy, N., Reen, F. J., Buckley, J. F., Frye, J. G., Boyd, E. F. & Gilroy, D. (2009). Sensitive and Rapid Molecular Detection Assays for *Salmonella enterica* Serovars Typhimurium and Heidelberg. *J Food Prot*, 72(11), 2350-7.

McClelland, R. G. & Pinder, A. C. (1994). Detection of *Salmonella* Typhimurium in dairy products with flow cytometry and monoclonal antibodies. *Appl Environ Microbiol*, 60(12), 4255-62.

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M. & Tauxe, R. V. (1999). Food-related illness and death in the United States. *Emerg Infect Dis*, 5(5), 607-25.

Mercanoglu, B. & Griffiths, M. W. (2005). Combination of immunomagnetic separation with real-time PCR for rapid detection of *Salmonella* in milk, ground beef, and alfalfa sprouts. *J Food Prot*, 68(3), 557-61.

Moore, M. M. & Feist, M. D. (2007). Real-time PCR method for *Salmonella* spp. targeting the stn gene. *J Appl Microbiol*, 102(2), 516-30.

Nadal, A., Coll, A., Cook, N. & Pla, M. (2007). A molecular beacon-based real time NASBA assay for detection of *Listeria monocytogenes* in food products: role of target mRNA secondary structure on NASBA design. *J Microbiol Methods*, 68(3), 623-32.

Naja, G., Bouvrette, P., Hrapovic, S. & Luong, J. H. (2007). Raman-based detection of bacteria using silver nanoparticles conjugated with antibodies. *Analyst*, 132(7), 679-86.

Ngundi, M. M., Kulagina, N. V., Anderson, G. P. & Taitt, C. R. (2006). Nonantibody-based recognition: alternative molecules for detection of pathogens. *Expert Rev Proteomics*, 3(5), 511-24.

Nickerson, C. A., Ott, C. M., Wilson, J. W., Ramamurthy, R. & Pierson, D. L. (2004). Microbial responses to microgravity and other low-shear environments. *Microbiol Mol Biol Rev*, 68(2), 345-61.

Nocker, A. & Camper, A. K. (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, C. Y. & Camper, A. K. (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.

Nocker, A., Sossa, K. E. & Camper, A. K. (2007). Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. *J Microbiol Methods*, 70(2), 252-60.

Nogva, H. K., Bergh, A., Holck, A. & Rudi, K. (2000). Application of the 5'-nuclease PCR assay in evaluation and development of methods for quantitative detection of *Campylobacter jejuni*. *Appl Environ Microbiol*, 66(9), 4029-36.

Nordengrahn, A., Gustafsdottir, S. M., Ebert, K., Reid, S. M., King, D. P., Ferris, N. P., Brocchi, E., Grazioli, S., Landegren, U. & Merza, M. (2008). Evaluation of a novel proximity ligation assay for the sensitive and rapid detection of foot-and-mouth disease virus. *Vet Microbiol*, 127(3-4), 227-36.

Oliveira, T. C., Barbut, S. & Griffiths, M. W. (2005). Detection of *Campylobacter jejuni* in naturally contaminated chicken skin by melting peak analysis of amplicons in real-time PCR. *Int J Food Microbiol*, 104(1), 105-11.

Olsen, E. V., Sorokulova, I. B., Petrenko, V. A., Chen, I. H., Barbaree, J. M. & Vodyanoy, V. J. (2006). Affinity-selected filamentous bacteriophage as a probe for acoustic wave biodetectors of *Salmonella* Typhimurium. *Biosens Bioelectron*, 21(8), 1434-42.

Oyofo, B. A. & Rollins, D. M. (1993). Efficacy of filter types for detecting *Campylobacter jejuni* and *Campylobacter coli* in environmental water samples by polymerase chain reaction. *Appl Environ Microbiol*, 59(12), 4090-5.

Ozbek, A., Michel, F. C., Strother, M., Motiwala, A. S., Byrum, B. R., Shulaw, W. P., Thornton, C. G. & Sreevatsan, S. (2003). Evaluation of two recovery methods for detection of *Mycobacterium avium* subsp. paratuberculosis by PCR: direct-dilution--centrifugation and C(18)-carboxypropylbetaine processing. *FEMS Microbiol Lett*, 229(2), 145-51.

Pai, S., Ellington, A. D. & Levy, M. (2005). Proximity ligation assays with peptide conjugate 'burrs' for the sensitive detection of spores. *Nucleic Acids Res*, 33(18), e162.

Pan, Y. & Breidt, F., Jr. (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.

Patchett, R. A., Kelly, A. F. & Kroll, R. G. (1991). The adsorption of bacteria to immobilized lectins. *J Appl Bacteriol*, 71(3), 277-84.

Payne, M. J., Campbell, S., Patchett, R. A. & Kroll, R. G. (1992). The use of immobilized lectins in the separation of *Staphylococcus aureus*, *Escherichia coli*, *Listeria* and *Salmonella* spp. from pure cultures and foods. *J Appl Bacteriol*, 73(1), 41-52.

Payne, M. J. a. K., R.G. (1991). Methods for the separation and concentration of bacteria from foods. *Trends in Food Science & Technology*, 2, 315.

Pieken, W. A., Olsen, D. B., Benseler, F., Aurup, H. & Eckstein, F. (1991). Kinetic characterization of ribonuclease-resistant 2'-modified hammerhead ribozymes. *Science*, 253(5017), 314-7.

Rasooly, A. (2001). Surface plasmon resonance analysis of staphylococcal enterotoxin B in food. *J Food Prot*, 64(1), 37-43.

Rasooly, A. & Herold, K. E. (2006). Biosensors for the analysis of food- and waterborne pathogens and their toxins. *J AOAC Int*, 89(3), 873-83.

Rawsthorne, H., Dock, C. N. & Jaykus, L. A. (2009). PCR-based method using propidium monoazide to distinguish viable from nonviable *Bacillus subtilis* spores. *Appl Environ Microbiol*, 75(9), 2936-9.

Reiser, R., Conaway, D. & Bergdoll, M. S. (1974). Detection of staphylococcal enterotoxin in foods. *Appl Microbiol*, 27(1), 83-5.

Rodrigues-Szulc, U. M., Ventoura, G., Mackey, B. M. & Payne, M. J. (1996). Rapid physicochemical detachment, separation and concentration of bacteria from beef surfaces. *J Appl Bacteriol*, 80(6), 673-81.

Ronner, A. C. & Lindmark, H. (2007). Quantitative detection of *Campylobacter jejuni* on fresh chicken carcasses by real-time PCR. *J Food Prot*, 70(6), 1373-8.

Rowe-Taitt, C. A., Hazzard, J. W., Hoffman, K. E., Cras, J. J., Golden, J. P. & Ligler, F. S. (2000). Simultaneous detection of six biohazardous agents using a planar waveguide array biosensor. *Biosens Bioelectron*, 15(11-12), 579-89.

Rudi, K., Flateland, S. L., Hanssen, J. F., Bengtsson, G. & Nissen, H. (2002). Development and evaluation of a 16S ribosomal DNA array-based approach for describing complex microbial communities in ready-to-eat vegetable salads packed in a modified atmosphere. *Appl Environ Microbiol*, 68(3), 1146-56.

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

Ruiz-Palacios, G. M., Cervantes, L. E., Ramos, P., Chavez-Munguia, B. & Newburg, D. S. (2003). *Campylobacter jejuni* binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. *J Biol Chem*, 278(16), 14112-20.

Sails, A. D., Fox, A. J., Bolton, F. J., Wareing, D. R. & Greenway, D. L. (2003). A real-time PCR assay for the detection of *Campylobacter jejuni* in foods after enrichment culture. *Appl Environ Microbiol*, 69(3), 1383-90.

Sakamoto, C., Yamaguchi, N. & Nasu, M. (2005). Rapid and simple quantification of bacterial cells by using a microfluidic device. *Appl Environ Microbiol*, 71(2), 1117-21.

Sapsford, K. E., Rasooly, A., Taitt, C. R. & Ligler, F. S. (2004). Detection of *Campylobacter* and *Shigella* species in food samples using an array biosensor. *Anal Chem*, 76(2), 433-40.

Seo, K. H., Valentin-Bon, I. E. & Brackett, R. E. (2006). Detection and enumeration of *Salmonella* Enteritidis in homemade ice cream associated with an outbreak: comparison of conventional and real-time PCR methods. *J Food Prot*, 69(3), 639-43.

Sharon, N. (2006). Carbohydrates as future anti-adhesion drugs for infectious diseases. *Biochim Biophys Acta*, 1760(4), 527-37.

Siddique, N., Sharma, D. & Al-Khaldi, S. F. (2009). Detection of *Yersinia enterocolitica* in alfalfa, mung bean, cilantro, and mamey sapote (Pouteria sapota) food matrices using DNA microarray chip hybridization. *Curr Microbiol*, 59(3), 233-9.

Singh, J., Batish, V. K. & Grover, S. (2009). A Molecular Beacon-Based Duplex Real-Time Polymerase Chain Reaction Assay for Simultaneous Detection of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in Milk and Milk Products. *Foodborne Pathog Dis*.

Skerra, A. (2008). Alternative binding proteins: anticalins - harnessing the structural plasticity of the lipocalin ligand pocket to engineer novel binding activities. *Febs J*, 275(11), 2677-83.

So, H. M., Park, D. W., Jeon, E. K., Kim, Y. H., Kim, B. S., Lee, C. K., Choi, S. Y., Kim, S. C., Chang, H. & Lee, J. O. (2008). Detection and titer estimation of *Escherichia coli* using aptamer-functionalized single-walled carbon-nanotube field-effect transistors. *Small*, 4(2), 197-201.

Stals, A., Baert, L., Botteldoorn, N., Werbrouck, H., Herman, L., Uyttendaele, M. & Van Coillie, E. (2009). Multiplex real-time RT-PCR for simultaneous detection of GI/GII noroviruses and murine norovirus 1. *J Virol Methods*, 161(2), 247-53.

Stancik, L. M., Stancik, D. M., Schmidt, B., Barnhart, D. M., Yoncheva, Y. N. & Slonczewski, J. L. (2002). pH-dependent expression of periplasmic proteins and amino acid catabolism in *Escherichia coli*. *J Bacteriol*, 184(15), 4246-58.

Stevens, K. A. & Jaykus, L. A. (2004). Bacterial separation and concentration from complex sample matrices: a review. *Crit Rev Microbiol*, 30(1), 7-24.

Su, X. L. & Li, Y. (2004). A self-assembled monolayer-based piezoelectric immunosensor for rapid detection of Escherichia coli O157:H7. *Biosens Bioelectron*, 19(6), 563-74.

Sun, W., Brovko, L. & Griffiths, M. (2001). Use of bioluminescent *Salmonella* for assessing the efficiency of constructed phage-based biosorbent. *J Ind Microbiol Biotechnol*, 27(2), 126-8.

Taitt, C. R., Shubin, Y. S., Angel, R. & Ligler, F. S. (2004). Detection of *Salmonella enterica* serovar Typhimurium by using a rapid, array-based immunosensor. *Appl Environ Microbiol*, 70(1), 152-8.

Tanji, Y., Furukawa, C., Na, S. H., Hijikata, T., Miyanaga, K. & Unno, H. (2004). *Escherichia coli* detection by GFP-labeled lysozyme-inactivated T4 bacteriophage. *J Biotechnol*, 114(1-2), 11-20.

Thomas, D. (1988). Electropositively charged filters for the recovery of yeasts and bacteria from beverages. *Journal of Applied Bacteriology*, 65 (1), 35 - 41.

Tombelli, S., Minunni, M. & Mascini, M. (2005). Analytical applications of aptamers. *Biosens Bioelectron*, 20(12), 2424-34.

Tuerk, C. & Gold, L. (1990). Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science*, 249(4968), 505-10.

Tully, E., Hearty, S., Leonard, P. & O'Kennedy, R. (2006). The development of rapid fluorescence-based immunoassays, using quantum dot-labelled antibodies for the detection of *Listeria monocytogenes* cell surface proteins. *Int J Biol Macromol*, 39(1-3), 127-34.

Uyttendaele, M., Schukkink, R., van Gemen, B. & Debevere, J. (1995). Detection of *Campylobacter jejuni* added to foods by using a combined selective enrichment and nucleic acid sequence-based amplification (NASBA). *Appl Environ Microbiol*, 61(4), 1341-7.

Varshney, M., Yang, L., Su, X. L. & Li, Y. (2005). Magnetic nanoparticle-antibody conjugates for the separation of *Escherichia coli* O157:H7 in ground beef. *J Food Prot*, 68(9), 1804-11.

Vivekananda, J. & Kiel, J. L. (2006). Anti-*Francisella tularensis* DNA aptamers detect tularemia antigen from different subspecies by Aptamer-Linked Immobilized Sorbent Assay. *Lab Invest*, 86(6), 610-8.

Vo-Dinh, T. (1998). Surface-enhanced Raman spectroscopy using metallic nanostructures. *TrAC Trends in Analytical Chemistry*, 17(8-9), 557-582.

Walls, I., Sheridan, J. J., Welch, R. W. & McDowell, D. A. (1990). Separation of micro-organisms from meat and their rapid enumeration using a membrane filtration-epifluorescent microscopy technique. *Lett Appl Microbiol*, 10(1), 23-6.

Wang, M., Cao, B., Gao, Q., Sun, Y., Liu, P., Feng, L. & Wang, L. (2009). Detection of *Enterobacter sakazakii* and other pathogens associated with powdered infant formula using a DNA microarray. *J Clin Microbiol*.

Wang, R. F., Cao, W. W. & Johnson, M. G. (1992). 16S rRNA-based probes and polymerase chain reaction method to detect *Listeria monocytogenes* cells added to foods. *Appl Environ Microbiol*, 58(9), 2827-31.

Wang, S. & Levin, R. E. (2006). Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. *J Microbiol Methods*, 64(1), 1-8.

Wang, X.-B., Huang, Y Burt, J P H, Markx, G H and Pethig, R (1993). Selective dielectrophoretic confinement of bioparticles in potential energy wells. *J. Phys. D: Appl. Phys.*, 26 1278-1285.

Wang, X. B., Yang, J., Huang, Y., Vykoukal, J., Becker, F. F. & Gascoyne, P. R. (2000). Cell separation by dielectrophoretic field-flow-fractionation. *Anal Chem*, 72(4), 832-9.

Warner, H. R., Snustad, P., Jorgensen, S. E. & Koerner, J. F. (1970). Isolation of bacteriophage T4 mutants defective in the ability to degrade host deoxyribonucleic acid. *J Virol*, 5(6), 700-8.

Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol*, 63(10), 3741-51.

Wilson, W. J., Strout, C. L., DeSantis, T. Z., Stilwell, J. L., Carrano, A. V. & Andersen, G. L. (2002). Sequence-specific identification of 18 pathogenic microorganisms using microarray technology. *Mol Cell Probes*, 16(2), 119-27.

Wolber, P. K. & Green, R. L. (1990). Detection of bacteria by transduction of ice nucleation genes. *Trends Biotechnol*, 8(10), 276-9.

Wolffs, P. F., Glencross, K., Thibaudeau, R. & Griffiths, M. W. (2006). Direct quantitation and detection of salmonellae in biological samples without enrichment, using two-step filtration and real-time PCR. *Appl Environ Microbiol*, 72(6), 3896-900.

Yakes, B. J., Lipert, R. J., Bannantine, J. P. & Porter, M. D. (2008). Detection of *Mycobacterium avium* subsp. paratuberculosis by a sonicate immunoassay based on surface-enhanced Raman scattering. *Clin Vaccine Immunol*, 15(2), 227-34.

Yamaguchi, N., Ohba, H. & Nasu, M. (2006). Simple detection of small amounts of *Pseudomonas* cells in milk by using a microfluidic device. *Lett Appl Microbiol*, 43(6), 631-6.

Yang, C., Jiang, Y., Huang, K., Zhu, C. & Yin, Y. (2003). Application of real-time PCR for quantitative detection of *Campylobacter jejuni* in poultry, milk and environmental water. *FEMS Immunol Med Microbiol*, 38(3), 265-71.

Zarrinkar, P. P., Mainquist, J. K., Zamora, M., Stern, D., Welsh, J. B., Sapinoso, L. M., Hampton, G. M. & Lockhart, D. J. (2001). Arrays of arrays for high-throughput gene expression profiling. *Genome Res*, 11(7), 1256-61.

Zhen, B., Song, Y. J., Guo, Z. B., Wang, J., Zhang, M. L., Yu, S. Y. & Yang, R. F. (2002). [In vitro selection and affinity function of the aptamers to *Bacillus anthracis* spores by SELEX]. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)*, 34(5), 635-42.

CHAPTER 2

DEVELOPMENT OF A COMBINED IMMUNOMAGNETIC SEPARATION (IMS)-QUANTITATIVE REAL-TIME PCR (QPCR) ASSAY WITH INCLUSION OF A HOMOLOGOUS INTERNAL AMPLIFICATION CONTROL (IAC) FOR DETECTION OF *SALMONELLA ENTERICA*

Hari Prakash Dwivedi and Lee-Ann Jaykus

2.1. ABSTRACT

Despite recent advances, there remains a need to improve the time to result for food borne pathogen testing without sacrificing assay sensitivity and specificity. The purpose of this study was to design and validate a quantitative real-time PCR (qPCR) method targeting the *invA* gene of *Salmonella*, including documentation of its performance when preceded by an immunomagnetic separation (IMS) step. To control for potential matrix-associated interferences, a homologous internal amplification control (IAC) was constructed using the extension overlap method. The qPCR assay was validated both with and without inclusion of the IAC. In pure culture, the assay demonstrated log linear amplification between 5.19 ± 0.15 $\log_{10}$ CFU equivalents and 0.19 ± 0.15 $\log_{10}$ CFU equivalents; the lower limit of detection was 0.19 ± 0.15 $\log_{10}$ CFU equivalents/reaction (1.61 ± 0.52 CFU equivalents/reaction). Genomic DNA was co-amplified in the presence of ≤7.6 ag of IAC at ≤2.19 ± 0.15 $\log_{10}$

Salmonella CFU equivalents per reaction with no impact on detection limits; amplification of the IAC was out-competed at higher target concentrations. The lower limit of detection of the combined IMS-qPCR method, as applied to 9 ml samples of artificially contaminated chicken rinsate, was 10^0-10^1 CFU *Salmonella* per sample without prior cultural enrichment. There was a gradual improvement in the capture efficiency of the IMS step with decreasing levels of *Salmonella,* from a low of 12% to a high of 100% at contamination levels ranging from 10^5 to 10^0 CFU/sample. The technique for designing the homologous IAC is simple and can be adapted for incorporation in other qPCR protocols with minimal manipulation. Careful design and integration of IMS and qPCR components can result in rapid and reliable assays with detection limits approaching those that might be anticipated in naturally contaminated foods, even without prior cultural enrichment.

2.2. INTRODUCTION

Salmonella causes the largest number of bacterial enteric infections in the world (Bell, 2002) and according to the U.S. Centers for Disease Control and Prevention (CDC), accounts for largest percentage of outbreaks (33%) and cases (55%) of all of the food borne pathogens of bacterial etiology (Lynch et al., 2006). The organism naturally colonizes the gastrointestinal tracts of food animals (Garcia-Del Portillo, 2000) and hence animal fecal contamination is the primary means by which foods become contaminated with *Salmonella*. Raw foods including meat and poultry, fresh produce and eggs represent the products at greatest risk for contamination (Bell, 2002; Hackney, 1994). *Salmonella* has more than 2500

serotypes, with serovar. Typhimurium responsible for the largest proportion of human cases of salmonellosis (CDC, 2007).

The detection of *Salmonella* spp. from food and environmental samples is traditionally culture-based and relies on the sequential steps of pre-enrichment, selective enrichment, and selective/differential plating. If presumptively positive growth is identified on selective media, further identification and confirmation of isolates using one or more biochemical, immunological, and/or molecular methods is done. The process of confirming the presence of the pathogen in foods is both time consuming and labor intensive, usually exceeding 4-7 days (Mercanoglu & Griffiths, 2005; Spanova et al., 2000). Over the years, standard culture-based pathogen detection methods have been refined and even improved, with an eye towards reducing time to detection. This is generally done by replacing the selective and differential plating step with more rapid immunological or molecular-based assays. Most notable amongst these detection platforms are enzyme-linked immunosorbent assay (ELISA), DNA hybridization, and PCR.

Despite the reliability of these more recent assays, we have not been able to circumvent the need for cultural enrichment, which is currently the rate limiting step in pathogen detection. There are many reasons for this, including (i) the complexity of the sample matrix; (ii) relatively high assay detection limits; (iii) volume constraints; and (iv) the relationship between positive detection signals and target cell viability. Of particular relevance to this work is the fact that the compositionally complex food matrix can interfere with detection platforms, resulting in non-specific amplification (producing false positive

results), inhibition of amplification (producing false negative results) and/or varying degrees of interference (resulting in a less sensitive assay).

Several approaches have been proposed to deal with matrix-associated interference. For example, the concept of selectively separating, concentrating, and purifying the target microbe from the sample matrix prior to the application of the detection assay, sometimes referred to as pre-analytical sample processing, has gained interest (Stevens & Jaykus, 2004). This sort of approach will provide the combined advantages of increasing target cell number and reducing sample volume with removal of residual matrix components. Immunomagnetic separation (IMS) capitalizes on this principle and is a method widely used for this purpose (Fluit et al., 1993; Skjerve & Olsvik, 1991; Spanova et al., 2000). Numerous IMS-PCR based assays for the detection of *Salmonella* in food have been reported in the last two decades (Fluit et al., 1993; Mercanoglu & Griffiths, 2005; Spanova et al., 2000).

Even with the best pre-analytical sample processing scheme, residual matrix-associated inhibitors may persist and be a source of false negative results that substantially reduce the diagnostic reliability of the assay (Hoorfar et al., 2004). This problem is particularly relevant to PCR-based detection platforms. In an effort to control for this problem, investigators frequently incorporate an internal amplification control (IAC) in diagnostic PCR assays. An IAC is a non-target nucleic acid sequence which is added to the diagnostic assay and is co-amplified with the target sequence. There are a number of ways in which an IAC can be incorporated in an assay. In the heterologous approach, the primer set designed to amplify the IAC differs from that used to amplify the target, requiring a multiplex assay format. A homologous IAC is custom designed to be amplified by the same

primers used for the target amplification, but the internal part of the amplicon differs from the target sequence, an approach which may be more robust as it circumvents difficulties associated with design of multiplex amplification. In this study, we report on the development and characterization of a homologous IAC which was designed for inclusion in a quantitative real-time PCR (qPCR) assay targeting *Salmonella.* We further describe the performance of a combined IMS-qPCR approach applied to the direct (without prior cultural enrichment) detection of *Salmonella enterica* serovar. Typhimurium from a candidate food matrix.

2.3. MATERIAL AND METHODS

2.3.1. Cultures and Nucleic Acid Extraction

A single *Salmonella enterica* serovar. Typhimurium strain previously isolated from a human, which was naturally resistant to ampicillin (courtesy of Dr. W. Gebreyes, Department of Veterinary Preventive Medicine, The Ohio State University, Columbus, OH), was used in all studies. The strain was propagated by overnight culture in BBL™ Trypticase™ Soy Broth (Becton, Dickinson and Company, Sparks, MD) supplemented with 50 μg/ml ampicillin (Sigma-Aldrich Inc., St. Louis, MO) (TSB-A) at 37˚C. Cell counts were determined by direct plating on agar-solidified TSB (TSB-A). Genomic DNA was extracted from 200 μl over night culture using the NucleoSpin® Food genomic DNA extraction kit (Macherey-Nagel, Düren, Germany) in accordance with manufacturer instructions, with minor

modifications. Elution of DNA was done in 35 µl of elution buffer and purified DNA was stored at -20˚C until use.

2.3.2. Quantitative Real-Time PCR (qPCR)

Quantitative real-time PCR amplification of *Salmonella* genomic DNA was carried out using primers and Taqman™ probe corresponding to the invasion A (*inv*A) gene originally described by Hein et al. (2006). All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA) and sequences are detailed in Table 2.1. Amplification reactions of 25 µl volume consisted of 1X PCR buffer (Invitrogen, Carlsbad, CA), 5 mM $MgCl_2$ (Invitrogen), 0.4 mM dNTP Mix (Applied Biosystems, Foster City, CA), 240 nM each of the forward and reverse *inv*A primers, 200 nM Taqman™ probe (*inv*A), 2.5 U Platinum® *Taq* DNA Polymerase (Invitrogen), 2 µg BSA (Promega) and 2.5 µl of target DNA. Amplifications were done in a SmartCycler® (Cepheid, Sunnyvale, CA) using a thermocycling protocol which consisted of initial denaturation at 94˚C for 120 sec followed by 40 cycles of 94˚C for 20 sec and 58˚-60˚C for 30-40 sec. For construction of the standard curve, DNA isolated from an overnight culture of *S.* Typhimurium was ten-fold serially diluted and 2.5 µl of each dilution amplified using the protocol described above. The standard curve was constructed as Ct value (Y axis) vs. CFU equivalent (X axis), where the latter was defined as the estimated CFU corresponding to the input level of DNA per PCR reaction, assuming a DNA extraction efficiency of 100%. The approximate CFU in unknown samples was extrapolated based on resulting Ct values obtained from this standard curve.

2.3.3. Construction of Homologous IAC

The general approach for production of the homologous IAC was reported by Abdulmawjood et al. (2002) and is diagrammed in Figure 2.1. The primers targeting a 191 base pair region of the pUC-19 vector (Gene Bank accession# L09137) located 1056-1246 bp downstream from the origin of replication, along with a corresponding Taqman™ probe (Taqman™ probe-IAC, Table 2.1), were designed using Beacon Designer version 6 software (PREMIER Biosoft International, Palo Alto, CA). Both primers were designed so that the sequence of the 5' overhanging ends corresponded to the diagnostic (*invA*) primers, whereas their 3' ends were complementary to the predetermined (pUC-19) DNA sequence. The 5' overhanging primers (139-IAC forward and 141-IAC reverse primer; Table 2.1) were used to amplify 100 ng of the pUC-19 vector (New England Biolabs, Ipswich, MA) DNA in a 50 µl PCR reaction which consisted of 1X PCR buffer minus Mg (Invitrogen), 5 mM $MgCl_2$ (Invitrogen), 0.4 mM dNTPs mix (Applied Biosystems), 200 nM 139-IAC forward primer, 200 nM 141-IAC reverse primer and 2.5U Platinum® *Taq* DNA Polymerase (Invitrogen). The PCR amplification was performed using a DNA Engine (PTC-200) Peltier Thermal Cycler-200 (MJ Research/ Bio-Rad Laboratories, Hercules, CA) under conditions of initial denaturation at 94˚C for 5 min followed by 30 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec and a final extension at 72˚C for 5 min. The PCR product was separated by 2% agarose gel electrophoresis and the amplicon band was isolated and purified using the QIAquick PCR gel extraction kit (Qiagen Inc., Valencia, CA).

To confirm the identity of the IAC, the purified DNA was amplified as described above but using 200 nM each of forward-*inv*A and reverse-*inv*A primers and a minor modification to amplification conditions (30 cycles of 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 20 sec). The amplified PCR product was again purified with the QIAquick PCR purification kit and concentration (ng/µl) was measured using a Nanodrop™-1000 (Thermo Fisher Scientific Inc., Waltham, MA). This served as the stock solution for subsequent IAC experiments. For quantification purposes, ten fold serially diluted IAC (initial concentration 25 ng) was amplified using the qPCR protocol described above, with minor modifications (160 nM each *inv*A-primer and 160 nM Taqman™ probe-IAC). The concentration of IAC giving amplification was optimized for subsequent co-amplification studies.

2.3.4. Validation of IAC Performance

Initial experiments were undertaken to evaluate the performance of the IAC when amplified in the presence of serial dilutions of DNA extracted from pure cultures of *Salmonella*. These were done using the qPCR protocol described above, with minor modifications [400 nM each *inv*A primers, 200 nM each Taqman™ probe (*inv*A and IAC), 7.6 ag IAC and 2.5 µl of target DNA]. A chicken rinsate matrix was used to evaluate the performance of the combined assay in a model food. Frozen packed whole chicken was procured from a local grocery store and rinsed in 200 ml of buffered peptone water (BPW) in accordance with the laboratory protocols outlined by the U.S. Department of Agriculture (MLG 4.03, USDA, 2004). The rinsate was sterilized by autoclaving at 121˚C for 20 min.

Nine ml of sterilized chicken rinsate was then mixed with 1 ml overnight culture of *S.* Typhimurium (10^7-10^8 CFU/ml), which constituted the stock. Additional 10-fold serial dilutions were made in sterile chicken rinse to achieve desired inoculum levels (between 10^5-10^0 CFU).

Immunomagnetic separation (IMS) was performed using a two-step process (Figure 2.2). Nine ml samples of inoculated chicken rinsate were mixed with 5 µg of goat anti-*Salmonella* polyclonal antibodies (CSA-1) (KPL Laboratories, Gaithersburg, USA) with gentle rotation at 25˚C for 20-25 min. Eight µg of biotin labeled anti-goat antibody (H+L) (KPL Laboratories) bound to 115 µg of Streptavidin MagneSphere® Paramagnetic Particles (SA-PMPs) (Promega) was added to the solution with incubation at room temperature for 20-25 min. The CSA-1 bound salmonellae were then pulled down with a magnetic particle concentrator Dynal MPC®-M (Dynal A.S, Oslo, Norway). Collected magnetic beads were washed four times with 1X PBS-0.05% Tween 20 buffer and suspended in 200 µl 1X PBS. DNA was extracted from this suspension using the NucleoSpin® Food genomic DNA extraction kit as per manufacturer instructions. Target DNA was amplified in the presence of the IAC using the qPCR method described above with minor modifications (400 nM forward and reverse *inv*A primer, 240 nM *inv*A probe, 200 nM IAC probe, 3 U Platinum® *Taq* DNA Polymerase and 7.8 ag IAC). CFU equivalent values were estimated for processed samples using the standard curve; capture efficiency was expressed as the ratio of CFU equivalents to CFU (as determined by plate count of stock culture) multiplied by 100.

2.3.5. Statistical Analysis

All co-amplification studies were performed in three independent replicates. The two-sample equal variance t-test ($p<0.05$) was performed to compare the linear regression equations obtained from Ct values of qPCR amplification using DNA obtained from pure cultures of *S.* Typhimurium amplified with and without the IAC.

2.4. RESULTS

2.4.1. Construction of IAC Using 5' Overhanging Primers

The IAC (size 239 bp) was constructed by amplification of a 191 bp region of pUC19 DNA using 5' overhanging primers. The concentration of purified IAC was approximately 82.67 ng/µl. The qPCR standard curve obtained using 10-fold serial dilutions of the IAC was log linear in the range of 1 to 8 $\log_{10}$ copy number, with the former being the limit of detection of the assay (Figure 2.3).

2.4.2. Performance of Homologous IAC in Pure Culture Experiments

The IAC was readily co-amplified using the same primer set as used for the *inv*A target, but with an alternative probe which corresponded to a pUC19 vector sequence. Because the IAC was labeled with TET, while the *Salmonella*-specific *inv*A probe was

labeled with FAM, the two amplification products could be detected simultaneously using different thermocycler channels.

In the absence of the IAC, the *Salmonella inv*A qPCR assay demonstrated log linear amplification between 5.19 ± 0.15 $\log_{10}$ CFU equivalent and 0.19 ± 0.15 $\log_{10}$ CFU equivalent, with a lower limit of detection of 0.19 ± 0.15 $\log_{10}$ CFU equivalent (1.61 ± 0.52 CFU equivalents/reaction) when amplifications were done without inclusion of the IAC. Co-amplification of *Salmonella* genomic DNA in the presence of ≤7.6 ag (approximately 31 IAC DNA copy number/PCR reaction) of the IAC did not impact the limit of detection of the assay, nor was there a statistically significant difference between the mean slope values of the linear regression equations obtained when comparing target amplification with and without IAC (Figure 2.4). Successful co-amplification of the IAC was observed in the presence of DNA corresponding to ≤2.19 ± 0.15 $\log_{10}$ CFU equivalent of *S.* Typhimurium. Amplification of the IAC was out-competed in PCR reactions with target DNA concentrations exceeding 2.19 ± 0.15 $\log_{10}$ CFU equivalent of *S.* Typhimurium (Figure 2.4).

2.4.3. Performance of Combined IMS-qPCR with IAC Applied to Artificially Contaminate Chicken Rinsate

The lower limit of detection of the combined IMS-qPCR assay was between 10^0-10^1 CFU of *S.* Typhimurium in 9 ml of inoculated chicken rinsate sample. Approximately 7.8 ag of IAC was successfully co-amplified in all the qPCR reactions applied to DNA extracted from captured bacterial cells. Irrespective of initial inoculum level (10^0-10^5 CFU/ml), there

were no statistically significant differences in FAM Ct values when comparing qPCR assays with and without the IAC (Figure 2.5).

Capture efficiency (CE) of the IMS step, as evaluated by extrapolation to the qPCR standard curve, was 12% at an initial inoculum level of 10^5 CFU *S.* Typhimurium/9 ml rinsate sample. A gradual improvement in the CE of IMS was observed at decreasing levels of *S.* Typhimurium contamination, ranging from 54.6% CE at inoculum levels of 10^4 CFU/9 ml rinsate to close to 100% at 10^2 CFU/9 ml (Figure 2.6). At contamination levels of 10^0 CFU/9 ml, CE was 100% (data not shown).

2.5. DISCUSSION

There is a need for continued improvement in the ability to detect bacterial pathogen contamination in food and environmental samples, with a focus on improving time to result without sacrificing specificity and limit of detection. Nucleic acid amplification methods have been particularly appealing in this regard, but even these remain hampered by the need for cultural enrichment, largely dictated by matrix-associated inhibition problems. Immunomagnetic separation is a widely used method for upstream sample processing which can facilitate concentration of the target organism with removal of extraneous matrix components and substantial reduction in sample volume. In fact, IMS in conjunction with PCR has been evaluated for the rapid and sensitive detection of many food borne pathogens including *Salmonella* (Mercanoglu & Griffiths, 2005), *L. monocytogenes* (Amagliani et al., 2006), and *E. coli* O157:H7 (Fu et al., 2005). When preceded by a brief pre-enrichment

period, IMS-PCR was reported to have detection limits as low as 1 CFU of *L. monocytogenes*/g in 25 g ham samples (Hudson et al., 2001). Combined IMS-multiplex PCR assay have also been reported for the simultaneous detection of *L. monocytogenes* and *Salmonella* from food samples (Hsih & Tsen, 2001).

In this work, we developed a combined IMS-qPCR assay and applied it to the direct (without prior cultural enrichment) detection of *Salmonella* in artificially contaminated chicken rinsate samples. The assay had an excellent limit of detection (between 10^0-10^1 CFU/9 ml sample) and demonstrated 100% capture efficiency at contamination levels of $\leq 10^2$ CFU per sample. These results are equivalent to or better than those obtained by other investigators. For example, using a combined IMS-PCR method, Skjerve and Olsvik (1991) reported detection limits of 1-2 CFU/ml and 50% CE in reconstituted milk powder artificially contaminated at a level of 60 CFU *S.* Saintpaul/ ml of sample. The IMS-qPCR method of Notzon et al. (2006) had a detection limit of 10 CFU *Salmonella*/25 g artificially contaminated meat sample; when applied in naturally contaminated samples, the assay sensitivity was 83.7%. Moreira et al. (2008) reported a detection limit of 1-10 CFU/25 g in experimentally contaminated pork and chicken meat samples using a combined IMS-PCR assay targeting the *fim*A gene of *S.* Typhimurium, but only after a brief enrichment step.

There are many reasons for failure of a PCR reaction as applied to the detection of pathogens in complex sample matrices, including matrix-associated inhibition, thermocycler failure, incorrect reagent balance, and poor DNA polymerase activity (Hoorfar et al., 2004; Wilson, 1997). For quality assurance purposes, researchers and commercial clinical diagnostic kit manufacturers have begun using IACs as a marker for amplification failure

associated with any number of problems (Hoorfar et al., 2004; Malorny et al., 2003). In point of fact, the European Standardization Committee, in collaboration with the International Standardization Organization (ISO) has specified the requirement of an IAC for PCR-based methods to detect food borne pathogens. A number of alternative approaches have been applied to the design of IACs, and these fall roughly into two categories, i.e. IACs which are amplified using a separate set of primers (heterologous IAC), and IACs which custom designed to be amplified with the same primers used for the target amplification (homologous IAC). Hoorfar et al. (2004) detailed practical considerations in designing IACs, identifying ten major recommendations, the most important of which are the following: (i) the target nucleic acid and IAC should share the same primer binding sites; (ii) IAC amplicons should be readily distinguished from target amplicons, preferably by use of a separate sequence-dependent hybridization probe; and (iii) the source of the IAC should be highly purified nucleic acid.

The homologous IAC designed in this study meets these criteria. The overlap-extension PCR design, which uses primers with 5' overhanging ends identical to primers used for the diagnostic reaction and 3' ends complementary to a region from the pUC-19 vector, is particularly appealing. For example, the approach is relatively simple and has been noted by others to be better than techniques such as PCR mutagenesis (Hoorfar et al., 2004; Sachadyn & Kur, 1998). An additional advantage is that, once the system is established, it is easy to adapt the method to the construction of IACs for other PCR reactions, and the same Taqman™ probe could be used for these IACs. Additionally, with minor modification of protocol, the method is applicable to the creation of RNA IACs for RT-qPCR. Finally, the

IAC approach used here meets the criterion of size, as it has been suggested that limiting size to less than 500 bases reduces the influence of the IAC on the sensitivity of target amplification (Brightwell et al., 1998; Hoorfar et al., 2004).

In IAC co-amplification assays, optimization of IAC concentration is critical because, even with the homologous IAC design, competition between target and IAC is expected. A low copy number of the IAC reduces the chance of failure to amplify low levels of the target DNA sequence, but unfortunately, at high target concentration, the IAC is frequently out-competed. This phenomenon was evident in our study, where amplification of the IAC was out-competed in PCR reactions that have target DNA concentrations exceeding 2.19 $\log_{10}$ CFU equivalent of *S.* Typhimurium. In instances of high target number in which the IAC fails to be detected, it may be necessary to consider the results as a "confirmed" positive, irrespective of IAC amplification (Hoorfar et al., 2004). This can be justified, as increasing the IAC concentration to favor its amplification at high target copy number would also likely result in failure of target amplification at low target copy number, thereby negating the very purpose of using an IAC.

Altogether, our assay has a number of particularly desirable features. For example, we used a diagnostic primer set and Taqman™ probe specific to the *inv*A gene of *Salmonella*. This is recognized as a highly specific and conserved sequence among the *Salmonella* spp. (Hein et al., 2006) and the primers used in our study have been suggested as an international standard for PCR detection of *Salmonella* spp. (Malorny et al., 2003). The IAC design meets the criteria specified for optimal performance without negatively impacting assay sensitivity or specificity (Hoorfar et al., 2004). Further, this IAC design is

simple and universal, allowing its application to the production of IACs for qPCR and RT-qPCR for virtually any target with only minor modifications to the protocol. Combining the qPCR assay with a prior IMS step resulted in target concentration and purification from the sample matrix, along with substantial volume reduction. The use of ligand-specific capture is appealing because it may be more likely to result in recovery of viable cells rather than inactivated cells, taking into account the live-dead conundrum (Dudak et al., 2009). In addition, sample preparation time was minimal and the assay was highly sensitive. The limit of detection of the combined IMS-qPCR assay in our study was between 10^0-10^1 CFU of *S.* Typhimurium in 9 ml inoculated chicken rinsate sample which remained unaltered on co-amplification with approximately 7.8 ag of homologous IAC. The method results in semiquantitative, if not fully quantitative, estimate of pathogen load. The combined IMS-qPCR assay appears to have applicability to the direct detection of low levels of *Salmonella* in complex sample matrices. With further development and validation, it may prove to be a valuable addition to our arsenal of food borne pathogen detection methods.

Table 2.1. Oligonucleotides used in the design of IAC and real-time PCR amplification of *Salmonella enterica*. The italicized sequences for the Composite Forward Primer and Composite Reverse Primer represent sequences corresponding to the target (*inv*A) primers, while underlined sequences represent the sequences corresponding to the 191 bp region of the pUC-19 vector.

Specification	**Oligonucleotide Sequence**	**Reference**
Composite Forward Primer (139-IAC)	5'-*GTG AAA TTA TCG CCA CGT TCG GGC AA*T TCT CAT AGC TCA CGC TGT AGG-3'	in this study
Composite Reverse Primer (141-IAC)	5'-*TCA TCG CAC CGT CAA AGG AAC C*TC GCT CTG CTA ATC CTG TTA CC-3'	in this study
Forward-*inv*A Primer	5'- GTG AAA TTA TCG CCA CGT TCG GGC AA - 3'	Hein et al. 2006
Reverse-*inv*A Primer	5' - TCA TCG CAC CGT CAA AGG AAC C - 3'	Hein et al. 2006
Taqman™ Probe (*inv*A) (*inv*A Probe)	5' - /56-FAM/TTA TTG GCG ATA GCC TGG CGG TGG GTT TTG TTG /3BHQ_1/ - 3'	Hein et al. 2006
Taqman™ Probe (IAC) (pUC19)	5'-/5TET/ATC TCA GTT CGG TGT AGG TCG TTC GCT CC/3BHQ_2/-3'	in this study

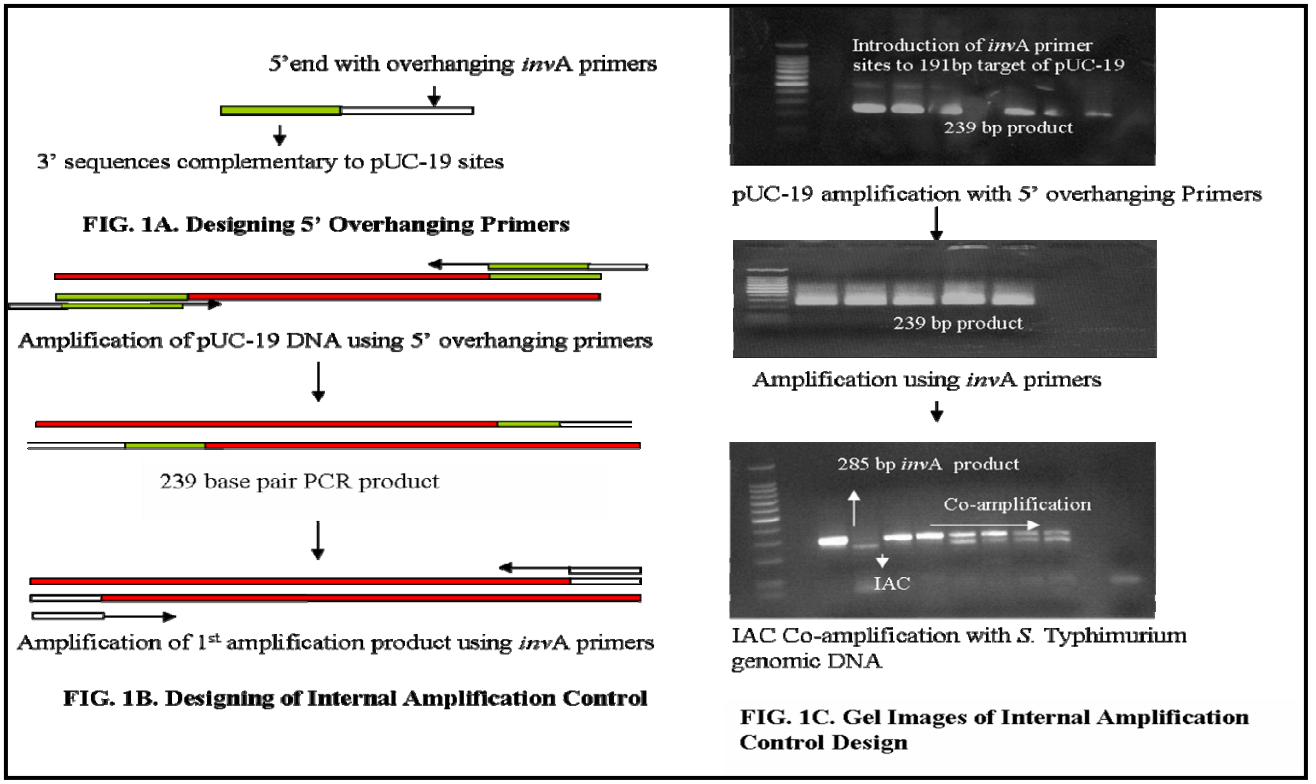

Figure 2.1. Schematic diagram for construction of the homologous IAC. which includes (i) design of 5' overhanging primers having 3' ends corresponding to the 191 bp region of the pUC-19 vector and 5'ends corresponding to the 285 bp region amplified in the *inv*A qPCR assay (Figure 1.A.); (ii) IAC production using the extension overlap method (Figure 1.B.); and (iii) Figure 1.C. shows gel images of PCR product from corresponding to the various steps in the IAC construction and co-amplification with *Salmonella* DNA using *inv*A primers.

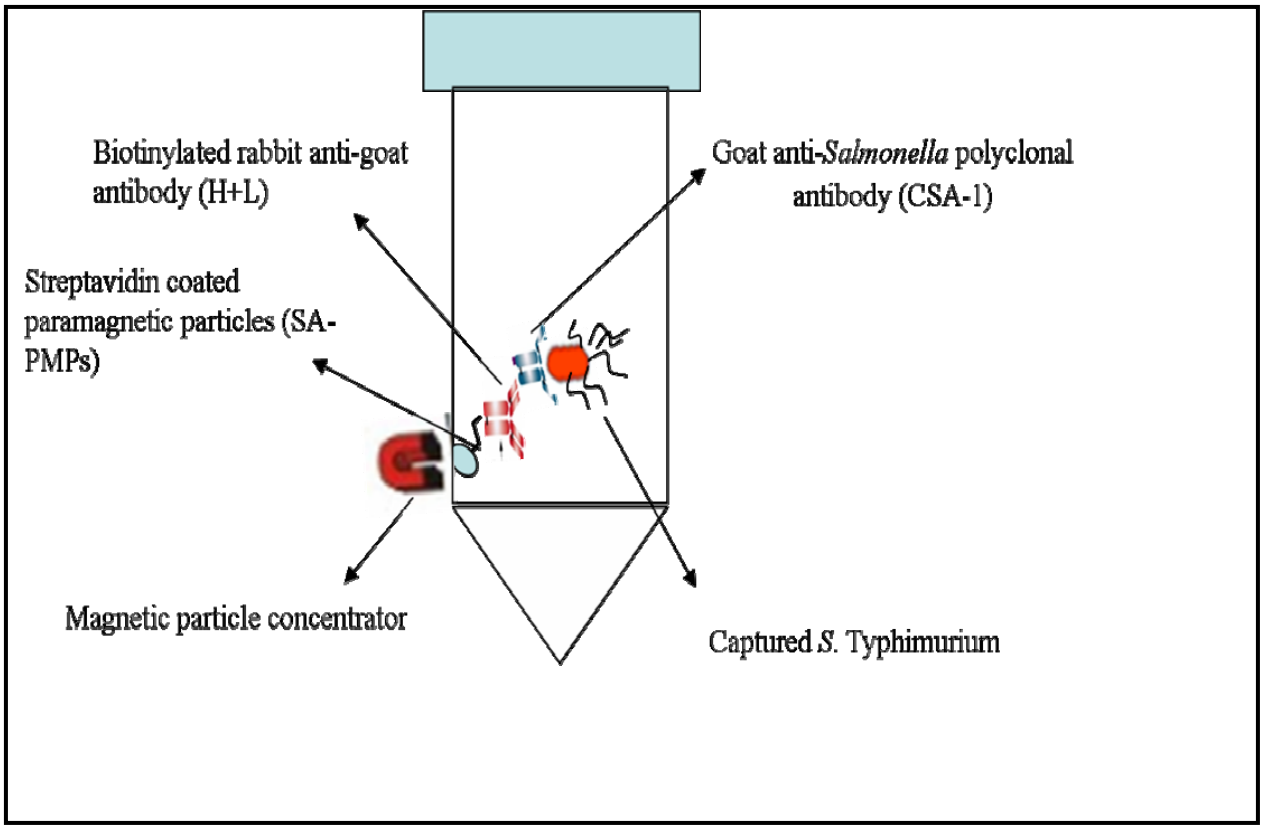

Figure 2.2. Schematic diagram of the IMS approach. Chicken rinsate artificially contaminated with *Salmonella* was first captured using goat anti-*Salmonella* antibody. This complex was then captured and concentrated by magnetic pull down using bead-bound rabbit anti-goat antibodies.

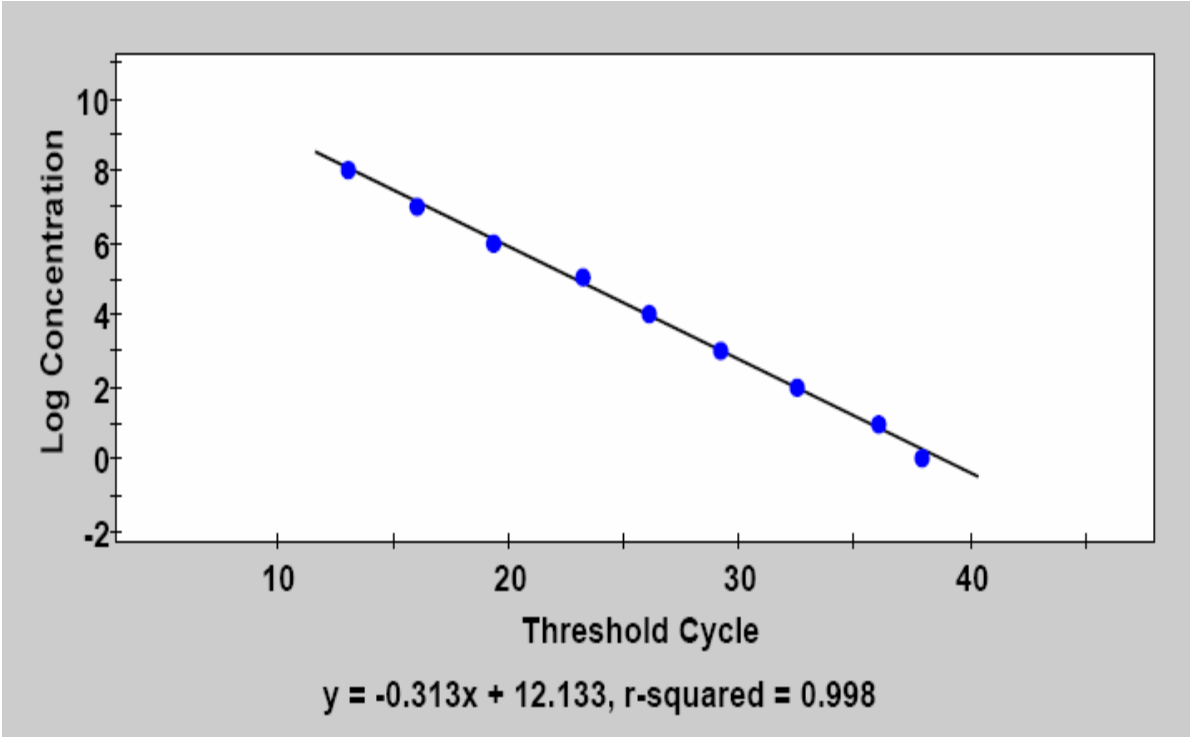

Figure 2.3. Standard curve for IAC qPCR assay. Ct values (X-axis) are plotted against $\log_{10}$ copy number of IAC (Y-axis) on a per amplification reaction basis.

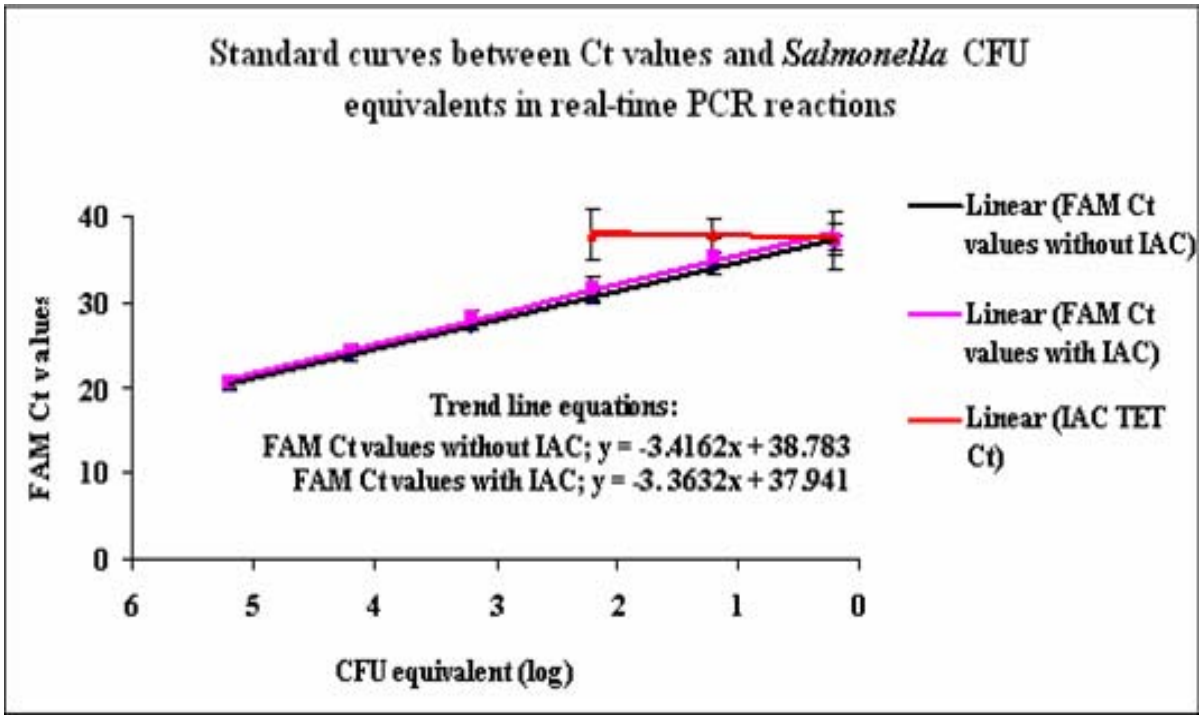

Figure 2.4. Standard curve for *Salmonella inv*A qPCR with and without co-amplification with the IAC. CFU equivalents (X-axis) were plotted against Ct value (Y-axis) for qPCR. 10-fold serial dilutions of *Salmonella* DNA in the absence (pink line) and presence (blue line) of the IAC, expressed as mean $\pm$ standard deviation of three independent experiments. The Ct value of IAC from reactions in which it was successfully co-amplified is plotted in red.

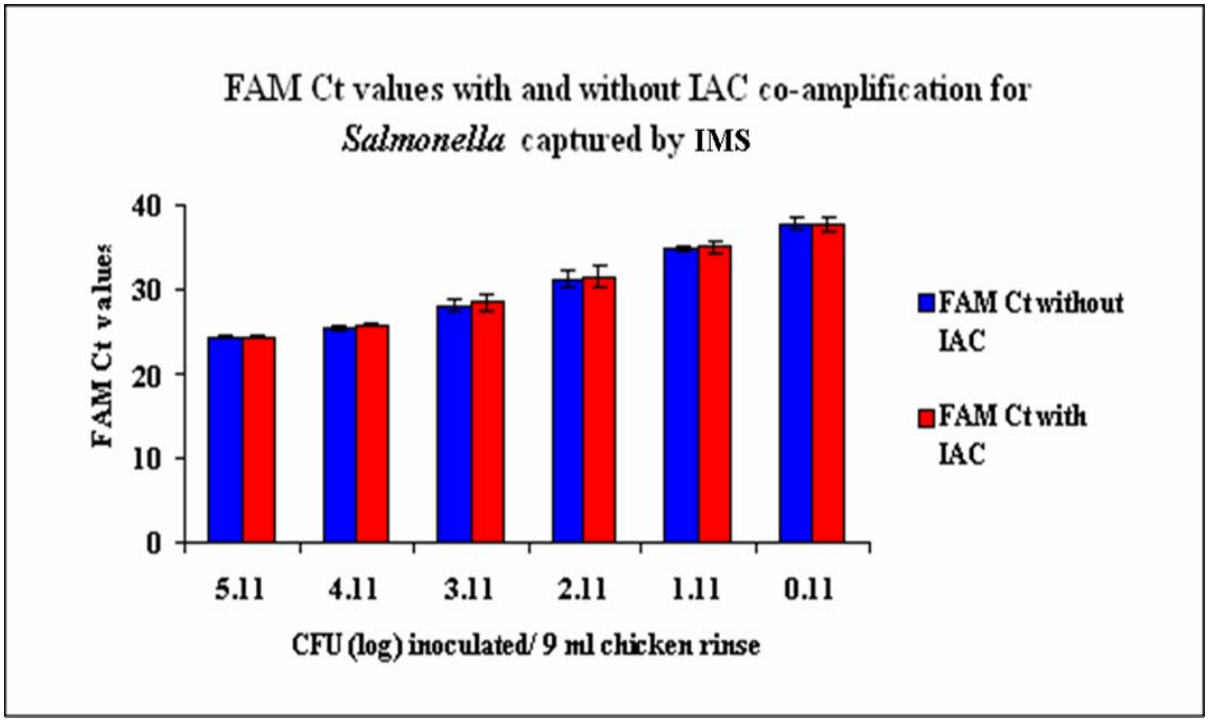

Figure 2.5. Comparison of the performance of the IMS-qPCR assay as applied to artificially contaminated chicken rinsate samples amplified with (red) and without (blue) inclusion of the IAC. Data reflects mean $\pm$ standard deviation of three independent experiments per inoculation level.

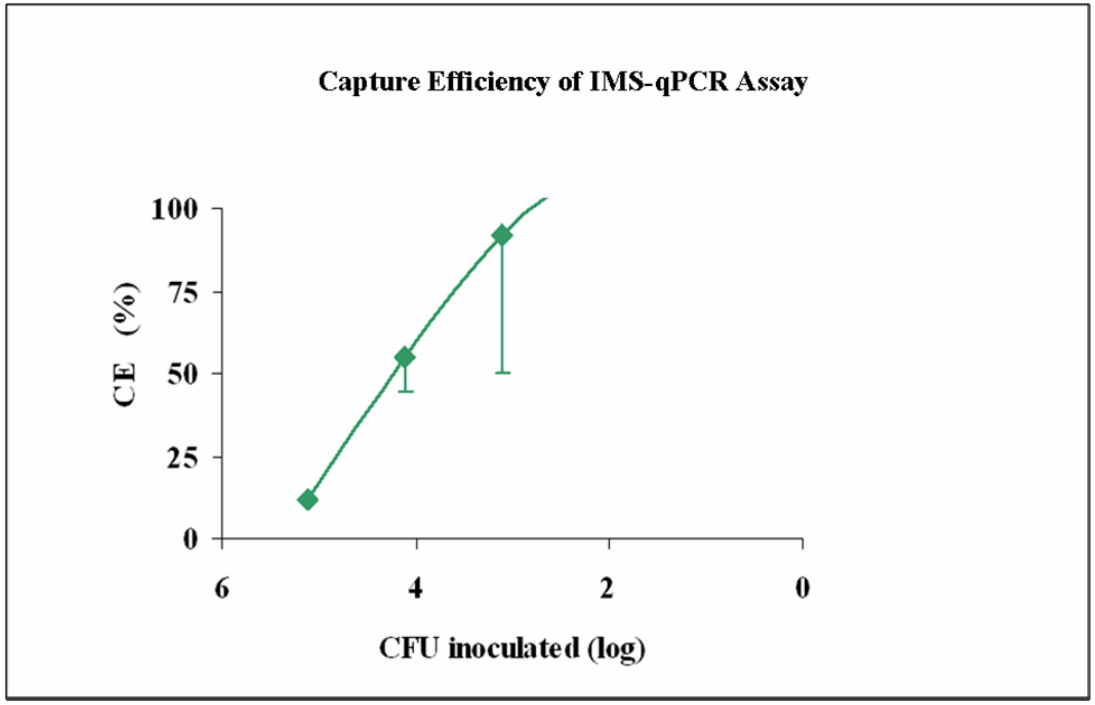

Figure 2.6. Capture efficiency (CE) (%) of IMS-qPCR assay at different inoculation levels of *Salmonella* in chicken rinsate samples obtained from three independent experiments.

2.6. REFERENCES

Abdulmawjood, A., Roth, S. & Bulte, M. (2002). Two methods for construction of internal amplification controls for the detection of *Escherichia coli* O157 by polymerase chain reaction. *Mol Cell Probes*, 16(5), 335-9.

Amagliani, G., Omiccioli, E., Campo, A., Bruce, I. J., Brandi, G. & Magnani, M. (2006). Development of a magnetic capture hybridization-PCR assay for *Listeria monocytogenes* direct detection in milk samples. *J Appl Microbiol*, 100(2), 375-83.

Bell, C., and A. Kyriakides. (2002). *Salmonella: a practical approach to the organism and its control in foods.* . Blackwell Science Ltd., Oxford, United Kingdom

Brightwell, G., Pearce, M. & Leslie, D. (1998). Development of internal controls for PCR detection of *Bacillus anthracis*. *Mol Cell Probes*, 12(6), 367-77.

CDC. (2007). *Salmonella* Surveillance: Annual Summary, 2005. Atlanta, Georgia. US Department of Health and Human Services, CDC, 2007.

Dudak, F. C., Boyaci, I. H., Jurkevica, A., Hossain, M., Aquilar, Z., Halsall, H. B., Seliskar, C. J. & Heineman, W. R. (2009). Determination of viable *Escherichia coli* using antibody-coated paramagnetic beads with fluorescence detection. *Anal Bioanal Chem*, 393(3), 949-56.

Fluit, A. C., Widjojoatmodjo, M. N., Box, A. T., Torensma, R. & Verhoef, J. (1993). Rapid detection of salmonellae in poultry with the magnetic immuno-polymerase chain reaction assay. *Appl Environ Microbiol*, 59(5), 1342-6.

Fu, Z., Rogelj, S. & Kieft, T. L. (2005). Rapid detection of *Escherichia coli* O157:H7 by immunomagnetic separation and real-time PCR. *Int J Food Microbiol*, 99(1), 47-57.

Garcia-Del Portillo, F. (2000). *Molecular and Cellular biology of Salmonella pathogenesis*. Technomic Publishing Company, Lancaster, Pa.

Hackney, C. R., and Potter, M.E. (1994). *Animal-associated and terrestrial baceria pathogens*. Chapman and Hall, New York N.Y.

Hein, I., Flekna, G., Krassnig, M. & Wagner, M. (2006). Real-time PCR for the detection of *Salmonella* spp. in food: An alternative approach to a conventional PCR system suggested by the FOOD-PCR project. *J Microbiol Methods*, 66(3), 538-47.

Hoorfar, J., Cook, N., Malorny, B., Wagner, M., De Medici, D., Abdulmawjood, A. & Fach, P. (2004). Diagnostic PCR: making internal amplification control mandatory. *Lett Appl Microbiol*, 38(2), 79-80.

Hoorfar, J., Malorny, B., Abdulmawjood, A., Cook, N., Wagner, M. & Fach, P. (2004). Practical considerations in design of internal amplification controls for diagnostic PCR assays. *J Clin Microbiol*, 42(5), 1863-8.

Hsih, H. Y. & Tsen, H. Y. (2001). Combination of immunomagnetic separation and polymerase chain reaction for the simultaneous detection of *Listeria monocytogenes* and *Salmonella* spp. in food samples. *J Food Prot*, 64(11), 1744-50.

Hudson, J. A., Lake, R. J., Savill, M. G., Scholes, P. & McCormick, R. E. (2001). Rapid detection of *Listeria monocytogenes* in ham samples using immunomagnetic separation followed by polymerase chain reaction. *J Appl Microbiol*, 90(4), 614-21.

Lynch, M., Painter, J., Woodruff, R. & Braden, C. (2006). Surveillance for foodborne-disease outbreaks--United States, 1998-2002. *MMWR Surveill Summ*, 55(10), 1-42.

Malorny, B., Hoorfar, J., Hugas, M., Heuvelink, A., Fach, P., Ellerbroek, L., Bunge, C., Dorn, C. & Helmuth, R. (2003). Interlaboratory diagnostic accuracy of a *Salmonella* specific PCR-based method. *Int J Food Microbiol*, 89(2-3), 241-9.

Mercanoglu, B. & Griffiths, M. W. (2005). Combination of immunomagnetic separation with real-time PCR for rapid detection of *Salmonella* in milk, ground beef, and alfalfa sprouts. *J Food Prot*, 68(3), 557-61.

Moreira, A. N., Conceicao, F. R., Conceicao Rde, C., Ramos, R. J., Carvalhal, J. B., Dellagostin, O. A. & Aleixo, J. A. (2008). Detection of *Salmonella* Typhimurium in raw meats using in-house prepared monoclonal antibody coated magnetic beads and PCR assay of the fimA gene. *J Immunoassay Immunochem*, 29(1), 58-69.

Notzon, A., Helmuth, R. & Bauer, J. (2006). Evaluation of an immunomagnetic separation-real-time PCR assay for the rapid detection of *Salmonella* in meat. *J Food Prot*, 69(12), 2896-901.

Sachadyn, P. & Kur, J. (1998). The construction and use of a PCR internal control. *Mol Cell Probes*, 12(5), 259-62.

Skjerve, E. & Olsvik, O. (1991). Immunomagnetic separation of *Salmonella* from foods. *Int J Food Microbiol*, 14(1), 11-7.

Spanova, A., Rittich, B., Karpiskova, R., Cechova, L. & Skapova, D. (2000). PCR identification of *Salmonella* cells in food and stool samples after immunomagnetic separation. *Bioseparation*, 9(6), 379-84.

Stevens, K. A. & Jaykus, L. A. (2004). Bacterial separation and concentration from complex sample matrices: a review. *Crit Rev Microbiol*, 30(1), 7-24.

Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. *Appl Environ Microbiol*, 63(10), 3741-51.

CHAPTER 3

SELECTION AND CHARACTERIZATION OF DNA APTAMERS WITH BINDING SELECTIVITY TO *CAMPYLOBACTER JEJUNI* USING WHOLE-CELL SELEX

Hari Prakash Dwivedi, R. Derike Smiley and Lee-Ann Jaykus

3.1. ABSTRACT

The need for pre-analytical sample processing prior to the application of rapid molecular-based detection of pathogens in food and environmental samples is well established. Although immunocapture has been applied in this regard, alternative ligands such as nucleic acid aptamers have advantages over antibodies such as low cost, ease of production and modification, and comparable stability. To identify DNA aptamers demonstrating binding specificity to live *Campylobacer jejuni* cells, a whole-cell SELEX (Systematic Evolution of Ligands by EXponential enrichment) method was applied to a combinatorial library of FAM-labeled single stranded DNA molecules. Seven candidate FAM-labeled aptamer sequences with high binding affinity to *C. jejuni* A9a as determined by flow cytometric analysis were identified. Aptamer ONS-23, which showed particularly high binding affinity in preliminary studies, was chosen for further characterization. This aptamer displayed a dissociation constant (K_d value) of 292.8 ± 53.1 nM with 47.27 ± 5.58% cells fluorescent (bound) in a 1.48 μM aptamer solution. Binding assays to assess the specificity

of aptamer ONS-23 showed high binding affinity (25-36%) for all other *C. jejuni* strains screened (inclusivity) and low apparent binding affinity (1-5%) with non-*C. jejuni* strains (exclusivity). Whole-cell SELEX is a promising technique to design aptamer-based molecular probes for microbial pathogens without tedious isolation and purification of complex markers or targets.

3.2. INTRODUCTION

Campylobacter species are leading causes of acute and sporadic bacterial gastroenteritis worldwide. Campylobacters cause diarrhoeal illness about 2-7 times more frequently than do *Salmonella* spp., *Shigella* spp. or *Escherichia coli* O157: H7, with *C. jejuni*, a zoonotic pathogen with wide host range, being responsible for the majority of these illnesses (Humphrey et al., 2007). *C. jejuni* infection may also trigger autoimmune neurological disorders such as Guillain-Barre´ syndrome and Miller Fisher syndrome (Nachamkin, 2002); less frequently, other complications can result (Gillespie et al., 2002). As few as 500 cells have been reported to cause clinical manifestations in humans (Yu et al., 2001). Contaminated foods (especially raw or partially cooked poultry and raw milk), untreated water, and seafood are common sources linked with infection (Friedman et al., 2004).

Isolation of pathogenic *Campylobacter* cells from food and environmental samples is complicated, requiring precise atmosphere (microaerophilic) and specific temperature conditions to facilitate growth (Humphrey et al., 2007). In fact, the amount of time to

confirm the presence of campylobacters in foods and environmental samples using standard cultural methods frequently exceeds 4-5 days (Moreno et al., 2003). Attempts have been made to reduce the time to detection and confirmation (LaGier et al., 2004; Moreno et al., 2003), but in complex sample matrices with low levels of contamination, this has been difficult. Immunomagnetic separation has shown promise (Lamoureux et al., 1997) in facilitating pathogen concentration and sample clean-up, but cross reactivity (Johansen et al., 1995) and high cost of anti-*Campylobacter* antibodies remain a stumbling block.

The ability of single stranded nucleic acids that fold into unique and stable secondary structures has led to identification of rare nucleic acid sequences with structures that not only bind specifically to selected targets such as proteins (Bibby et al., 2008), cancer cells (Herr et al., 2006), viruses (Li et al., 2008), bacteria (Chen et al., 2007; Vivekananda & Kiel, 2006), and parasites (Ulrich et al., 2002) but also discriminate between subtle molecular differences within the target. These nucleic acid sequences or aptamers are selected from a large random sequence oligonucleotide (10^{13}-10^{15} unique sequences) library by an iterative process of *in vitro* selection of sequences showing binding affinity, followed by PCR amplification in a method termed as Systemic Evolution of Ligands by EXponential enrichment (SELEX) (Ellington & Szostak, 1990; Tuerk & Gold, 1990). Due to their high affinity and specificity, aptamers have emerged as macromolecules that rival antibodies in biodiagnostic and biotherapeutic applications. Aptamers have several characteristics that make them attractive for biodiagnostic assay development including smaller size, ease of synthesis and labeling, lack of immunogenicity, lower cost than antibodies and high target specificity (Tombelli et al., 2005).

First reported in 1990, SELEX has historically been applied to isolated and/ or purified protein and non-protein molecular targets (Shamah et al., 2008). Prior knowledge about the target molecules and specialized biochemical techniques are required for isolation and purification of the particular cell surface target. Moreover, selection against purified membrane-associated targets may not always yield functional aptamer candidates if the targets require the presence of the cell membrane or co-receptor(s) for folding into the stable native conformation (Shamah et al., 2008) necessary for consistent presentation of structural epitopes during aptamer selection. Whole-cell SELEX (also called as complete-target SELEX) strategies (Shangguan et al., 2006; Wang et al., 2003) could be employed to identify aptamers specific to multiple surface membrane targets in their native conformations and physiological environments, leading to candidate ligands with different levels of specificity (genus, species or strain) or even the ability to discriminate between different cellular states (Cerchia et al., 2005). In the present study, whole-cell SELEX was employed to identify DNA aptamers specific to *C. jejuni* which have the potential to be developed into a rapid method to facilitate the detection of *C. jejuni* cells.

3.3. MATERIALS and METHODS

3.3.1. Microbial Strains, Culture Conditions and Preparation of Cells

C. jejuni strain A9a, a naturally-occurring strain isolated from a poultry processing plant was used as the target for whole-cell SELEX. This strain was chosen from among others at our disposal because of the high prevalence of contamination of raw poultry with *C.*

jejuni and the consistent association between human campylobacteriosis and poultry products (Humphrey et al., 2007). Moreover, as a naturally occurring isolate, this strain was assumed to present the cell surface signature typical of field strains. *C. jejuni* cultures of different zoonotic origin were used for inclusivity studies and included *C. jejuni* ATCC33560 (bovine feces), *C. jejuni* 2083 (cattle), *C. jejuni* ATCC33291 (human feces) and *C. jejuni* A14a (poultry processing plant) (all non-ATCC cultures provided courtesy of Dr. Lynn Joens, University of Arizona, Tucson, AZ). All *C. jejuni* strains were cultivated in 10 ml BBL™ brucella broth (Becton, Dickinson and Co., Sparks, MD) under a micro-aerophilic environment generated using the BBL™ CampyPak™ Plus Microaerophilic System (Becton, Dickinson) in a BBL™ GasPak™ jar by incubation at 42°C for 48 h. The cells were observed using Leica DM LB2 bright field microscope with the oil immersion objective (Vashaw Scientific Inc., Norcross, GA) to confirm typical comma and spiral morphology. Cells were harvested by centrifugation, washed 3 times in wash buffer [1X Dulbecco's PBS (pH 7.1) with calcium chloride and magnesium chloride (Invitrogen Corp., Carlsbad, California)] and finally suspended in 100 µl of wash buffer prior to use in experiments.

Other bacterial strains used in this study (for counter-SELEX and exclusivity studies) included the following: *Pseudomonas fluorescens* (ATCC13525), *P. aeruginosa* (ATCC23993), *Shigella flexneri* (ATCC12022), *E. coli* O157: H7, *Bacillus cereus* (ATCC-49063), *Staphylococcus aureus* (ATCC23235), *B. cereus* (strain T), *Listeria monocytogenes* (ATCC19115), *L. monocytogenes* Scott A, *Salmonella enterica* subsp. Enterica (ATCC4931), *E. coli* (ATCC33625), *S. sonnei* (ATCC25931), *Streptococcus gallolyticus* (ATCC9809), *Enterococcus faecium* (ATCC19434), *E. faecalis* (ATCC51299), *S. enterica*

serovar. Enteritidis (ME 46), *Salmonella enterica* serovar. Typhimurium (human isolate), all grown in 10 ml BBL™ BHI broth; and *Lactobacillus plantarum, Leuconostoc mesenteroides (*LA 0268-USDA*),* and *Pediococcus pentosaceus (*LA 0076-USDA*)* grown overnight at 37°C in 10 ml de Man, Rogosa and Sharpe (MRS) broth. One ml culture of each bacterium was pooled, centrifuged, washed three times in wash buffer and finally suspended in 100 µl wash buffer for use in counter-SELEX.

3.3.2. SELEX Process

The whole-cell SELEX process is outlined in Figure 3.1. An 80-base combinatorial DNA aptamer library was obtained from Integrated DNA Technologies (Coralville, IA). The library sequences, location of random and constant regions, fluorescent labels, and attachment chemistry linkers are shown in Table 3.1.

3.3.2.1. Labeling of DNA library

To label with fluorescein (FAM) and biotin, the diluted aptamer library (10 µM initial concentration) was amplified in 50 µl PCR reactions containing 1X Go Taq® Buffer (Promega Corp., Madison, WI), 0.2 mM GeneAmp® dNTPs Mix (Applied Biosystems, Foster City, CA), 5U Go Taq® DNA Polymerase (Promega), 500 nM FAM-Forward Constant Region primer, and 500 nM Biotin- Reverse Constant Region primer (Table 3.1). The PCR was performed in a DNA Engine (PTC-200) Peltier Thermal Cycler-200 (MJ

Research/ Bio-Rad Laboratories, Hercules, CA) using a 3 step thermal protocol of initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min.

3.3.2.2. Separation of the FAM Labeled Single Stranded DNA (FAM-ssDNA)

Streptavidin MagneSphere® Paramagnetic Particles (SA-PMPs) (Promega) were washed 3 times in 0.5X SSC buffer and coupled with the FAM and biotin labeled double stranded DNA library by incubating at room temperature for 30 min with gentle rolling. The library-coupled magnetic beads were washed 3 times in 0.1X SSC buffer. The FAM-ssDNA moieties were separated from the immobilized biotin-labeled strands by alkaline denaturation in 0.15 M NaOH at room temperature for 5-6 min and then recovered by magnetic capture of the beads using a Dynal MPC®-M magnetic particle concentrator (Dynal A.S, Oslo, Norway). Residual NaOH was removed using a Microcon® YM-30 filter device (Millipore, Billerica, MA).

3.3.2.3. SELEX

For enrichment of FAM-ssDNA (aptamer) candidates, a total of 10 rounds of SELEX were performed using *C. jejuni* A9a cells. About 300 pmoles (~1.8×10^{14} sequences) of aptamer pool was dissolved in 400 µl of binding buffer (0.05% Tween 20 in wash buffer), denatured by heating at 95°C for 10 min and renatured by flash cooling on ice for 10 min to

allow intra-strand base pairing. The aptamer pool was incubated with 10^8 - 10^9 *C. jejuni* cells suspended in 100 µl wash buffer for 45 min at room temperature with gentle rotation. Aptamer-bound cells were recovered by centrifugation at 1500 x *g* for 10 min followed by 3 washings with 500 µl binding buffer to remove unbound and non-specifically bound aptamers. Cells were then reconstituted in a final volume of 200 µl using molecular grade water. Aptamer sequences bound to cells were directly enriched by PCR amplification using the FAM-Forward Constant Region primer and Biotin-Reverse Constant Region primer as described above. The FAM-labeled aptamer pool was separated by alkaline denaturation in preparation for the next round of SELEX.

3.3.2.4. Cell Sorting

After the 7th and 10th rounds of SELEX, aptamer-bound *C. jejuni* cells were sorted into different pools based on fluorescence intensity using a Beckman Coulter MoFlo® modular flow cytometer (Beckman Coulter, Inc, Fullerton, CA). Cells with the greatest fluorescence intensity (top 25%) were used for subsequent rounds of PCR-based enrichment of aptamer candidates.

3.3.2.5. Counter-SELEX

To assure the specificity of aptamer candidates, two rounds of counter-SELEX were performed after the 10th round of SELEX (Figure 3.1). Briefly, the aptamer pool (300 p

moles) suspended in 400 µl of binding buffer was incubated with the pooled counter-SELEX bacterial cocktail (non-*C. jejuni* strains described above) suspended in 100 µl wash buffer for 45 min at room temperature with moderate shaking. The aptamer-bound cells were recovered and discarded, while the unbound aptamers in the supernatant were collected for one more round of counter-SELEX.

3.3.3. Identification of *C. jejuni* Specific Aptamers

Following 10 rounds of SELEX and 2 rounds of counter-SELEX, the selected aptamer pool was amplified using PCR with the Forward Constant Region primer and Reverse Constant Region primer. The PCR product was electrophoresed on 1.25% agarose gel in 1X modified TAE electrophoresis buffer and the amplicon band purified using an Amicon® Ultrafree®-DA centrifugal unit for DNA extraction from agarose (Millipore).

3.3.3.1. Cloning

The purified aptamer pool was treated with DNA Polymerase I (Klenow fragment) (Invitrogen) for 20 min on ice to produce blunt-ended aptamer sequences which were then ligated into the pCR®-Blunt vector using T4 DNA ligase provided in the Zero Blunt® PCR cloning kit (Invitrogen). The ligated vectors were transformed into One Shot® Top10 chemically competent *E. coli* cells (Invitrogen) and 30-50 µl of cells were plated on high salt

Luria-Bertani agar (LB agar)plates with kanamycin (50 µg/ ml) and incubated at 37°C for 20-24 h.

Individual colonies of the transformed cells were propagated in 5 ml high salt LB broth with kanamycin (50µg/ml) for 16 h at 37°C. The cells were harvested by centrifugation and plasmid DNA was extracted using the QIAprep® Spin plasmid Miniprep Kit (Qiagen Inc., Valencia, CA). The size of the amplified product was confirmed by PCR and visualized using 2% agarose gel electrophoresis. Transformants with an amplicon band size of 81 bases were selected for sequencing.

3.3.3.2. Sequencing

Sequencing of plasmid DNA of the selected transformants was done using a GenomeLab™ methods development kit (Beckman Coulter, Inc, Fullerton, CA) for dye terminator cycle sequencing. Plasmid DNA (approximately 75 ng) was pre-heated at 96°C for 1 min and sequencing reactions (10 µl total volumes) were performed for dNTP (I) chemistry using 5.5 µl premix (Beckman Coulter) and 0.16 µM M-13 reverse primer (Invitrogen). Thirty cycles consisting of 96°C for 20 sec, 50°C for 20 sec and 60°C for 4 min using a DNA Engine (PTC-200) thermal cycler were performed for all sequencing reactions. Reactions were stopped by mixing freshly prepared stop solution, ethanol precipitated and resuspensed in Sample Loading Solution (Beckman Coulter) as per instructions provided with the sequencing kit. The sequencing was performed using the LFR-1 program for 85 min and sequences were analyzed using the CEQ-8000™ /GenomeLab™ series genetic analysis

system (Beckman Coulter). Some of the plasmid DNA was also sequenced by Davis Sequencing, Inc. (Davis, CA).

3.3.3.3. Flow Cytometric Analysis of Aptamer Binding Affinity

All binding affinity assays using FAM-labeled aptamers were analyzed by flow cytometry using FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA). The mean fluorescence intensity and percentage of fluorescent cells (n = 200,000) due to aptamer binding was determined. Data from the FACSCalibur was analyzed using BD CellQuest™ Pro software (BD Biosciences). Histogram overlays were created with BD CellQuest™ Pro and Microsoft Office Excel 2003.

3.3.3.4. Preliminary Binding Screening and Predicted Structure of Aptamer Candidates

The unique aptamer sequence insert in plasmid DNA, identified by automated fluorescence sequencing, were amplified in the PCR reactions using the FAM-Forward Constant Region primer and Biotin-Reverse Constant Region primer. The FAM-labeled aptamer sequences were separated using alkaline denaturation. Preliminary binding assays using 300 pmol FAM-labeled candidate aptamers were performed on 10^8- 10^9 intact cells of *C. jejuni* strain A9a and analyzed using flow cytometry. In addition, the structural folding (secondary structure) of aptamer sequences displaying binding affinity to *C. jejuni* was predicted using the online software DNA Mfold version 3.2 (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-

form1.cgi) (Zuker, 2003). Common sequence motifs were identified using the online MEME server (http://meme.sdsc.edu), which identifies motifs in groups of related DNA sequences using statistical modeling techniques (Bailey & Elkan, 1994).

3.3.4. Further Characterization of ONS-23

Aptamer ONS-23 was selected for further binding characterization because of its high binding affinity to *C. jejuni* during the preliminary screening. Highly purified (by ion exchange high performance liquid chromatography) aptamer ONS-23 (with 5'FAM) obtained from Integrated DNA Technologies, Inc. was used in these studies.

3.3.4.1. Determination of Equilibrium Dissociation Constant

Varying concentrations of ONS-23 (74 pM, 740 pM, 7.4 nM, 74 nM, 740 nM, 1.48 μM, 2.2 μM) were prepared in binding buffer and incubated with 10^8 -10^9 washed *C. jejuni* A9a cells at room temperature for 45 min with moderate shaking. Aptamer-bound cells were centrifuged at 1500 x *g* and washed with 500 μl of binding buffer. Binding assays for each concentration were performed in three independent trials and analyzed using flow cytometry. The equilibrium dissociation constant (K_d) was calculated by fitting the average total per cent fluorescent bacterial cells (y) due to binding with FAM-labeled aptamer ONS-23 as a function of the concentration of aptamer (x) using a non-interacting binding sites model in SigmaPlot (Jandel, San Rafel, CA).

3.3.4.2. Microscopic Confirmation of Aptamer ONS-23 labeling to *C. jejuni*

Microscopic confirmation of ONS-23 binding to *C. jejuni* A9a cells was done using an Olympus BX51 microscope (100X objectives with 1.3 NA oil immersions) (Olympus America Inc., Center Valley, PA) with FITC-specific Chroma filter (Chroma Technology Corp., Rockingham, VT). The images were recorded using a Hamamatsu (ORCA-ER) digital camera (Hamamatsu Corporation, Bridgewater, NJ) with Metamorph molecular device imaging system (Molecular Devices, Sunnyvale, CA).

3.3.4.3. Confirmation of Aptamer ONS-23 Binding Inclusivity and Exclusivity

Assays to evaluate the binding affinity of aptamer ONS-23 with other *C. jejuni* strains as well as to unrelated bacterial species were undertaken. In these experiments, 10^8 - 10^9 bacterial cells and 1.48 μM aptamer ONS-23 were mixed in solution at 25°C for 45 min with moderate agitation to facilitate aptamer binding. Cells were centrifuged at 1500 x *g* and washed with 500 μl of binding buffer to separate unbound from bound aptamers prior to analysis using flow cytometry. Binding exclusivity studies were done using *B. cereus* strain T, *E. coli* O157:H7 and *L. monocytogenes* (ATCC19115). Similar experiments for evaluation of inclusivity were done using the four *C. jejuni* strains described above.

3.3.4.4. Molecular Confirmation of Aptamer ONS-23 Selectivity to *C. jejuni* in a Mixed Cell Suspension

The selective affinity of aptamer ONS-23 for *C. jejuni* in a mixed bacterial population (10^8-10^9 cells each) comprised of *C. jejuni* A9a, *B. cereus* strain T, *E. coli* O157:H7 and *L. monocytogenes* (ATCC19115) was also assessed. After exposure of aptamer ONS-23 with the mixed cell population, approximately 10^5 cells with highest fluorescence intensity were collected by cell sorting. Total genomic DNA from sorted cells was extracted using a NucleoSpin® Food genomic DNA extraction kit (Macherey-Nagel, Düren, Germany) as per manufacture's instructions. DNA was amplified in a PCR reaction with primers targeting a 176 bp region of hippuricase gene, a *C. jejuni* species-specific target (Marshall et al., 1999). The 20 µl PCR reaction contained 1X Go Taq® PCR buffer, 0.8 mM dNTP mix, 400 nM Primer mix containing 10 µM each of forward primer Hip 1a and reverse primer Hip 2b (Table 3.1), 0.65 U Go Taq® DNA Polymerase. Amplification was performed using DNA Engine (PTC-200) thermal cycler with cycle time/ temperature combinations of 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 1 min followed by final extension at 72°C for 7 min. As a positive control, *C. jejuni* (NCTC11168) DNA was amplified. The PCR product was analyzed by 1.5% agarose gel electrophoresis and bands were visualized following ethidium bromide staining.

3.4. RESULTS

3.4.1. Whole-Cell SELEX for Evolution of *C. jejuni* Specific Aptamers

A whole-cell SELEX (Figure 3.1) approach was used to select DNA aptamers from a large random sequence library demonstrating functional binding specificity to *C. jejuni*. Partitioning of aptamer-bound complexes from unbound aptamer was achieved using a combination of centrifugation and fluorescence automated cell sorting. Ten rounds of selection and two rounds of counter selection against a pooled mixture of unrelated bacteria were used to enrich the pool of functional aptamer sequences. We identified 7 unique aptamer sequences (Table 3.2). Binding affinity [expressed as % total fluorescent cells (n=200,000)] was determined using flow cytometry. The percentage of fluorescent cells for the different aptamer candidates ranged from 19-32% in binding assays using approximately 300 pmol of aptamer sequences with 10^8-10^9 *Campylobacter* cells (Table 3.2). As the concentration of FAM-aptamer was increased, there was an increase in the total number of fluorescent cells, although the average fluorescence intensity per cell remained constant. An approximate 5-8 fold increase in binding affinity was observed for evolved aptamer sequences following 10 rounds of selection and two rounds of counter selection in comparison to the aptamer pool after the 3rd round of SELEX (Figure 3.2). The highest binding affinity was demonstrated by aptamer ONS-23 (31.44%) while comparatively lower affinity was shown by aptamer sequence 22-21 (Table 3.2).

Three sequence motifs were prevalent among the selected aptamers sequences which divided them into three distinct families when analyzed using MEME server (data not

shown). Motif 1 was expressed among all selected candidate aptamers while the other two motifs were not as prevalent. None of the aptamer candidates had all three motifs.

3.4.2. Aptamer ONS-23 Displays High Binding Affinity to *C. jejuni strain A9a*

On the basis of preliminary screening of selected aptamers, aptamer ONS-23 was selected for further characterization. *C. jejuni* A9a cells (10^8-10^9) were titrated with increasing concentrations of aptamer ONS-23 and analyzed by flow cytometry (Figure 3.3.A). Saturation was achieved at higher aptamer concentrations. The non-interacting binding sites model adequately described the binding relationship, yielding a dissociation constant (*Kd* value) of 292.8 ± 53.1 nM (Figure 3.3.B). The aptamer ONS-23 labeling of *C. jejuni* complex could be visualized using fluorescent microscopy (data not shown).

3.4.3. Counter-SELEX Maintains Aptamer Specificity during Whole-Cell SELEX

Counter-SELEX was performed against a cocktail of non-target bacterial strains that are commonly found in foods, or which are common targets in food borne pathogen detection assays. To assess the effectiveness of counter-SELEX for aptamer ONS-23, we performed separate binding assays using *B. cereus* Strain T, *E. coli* O157: H7 and *L. monocytogenes* ATCC-19115; all three of these strains were included in the pooled bacterial cocktail for counter-SELEX. There were no appreciable fluorescently labeled cell counts (n= 200,000)

associated with binding of aptamer ONS-23 for any one of these non-target microorganisms as compared to signals obtained for *C. jejuni* strain A9a (Figure 3.4.A, B, C, D).

3.4.4. Aptamer ONS-23 Binds Selectively with Multiple *C. jejuni* Strains of Different Zoonotic Origins

To assess the sub-species effect of aptamer ONS-23 binding, assays were performed with *C. jejuni* strains ATCC-33291 (human), A14a (poultry processing plant), 2083 (cattle), ATCC-33560 (bovine). The percentage of fluorescent cells (n=200,000) following binding with all four strains ranged from 25-36%. The binding affinity was most similar for strain ATCC-33560 (Figure 3.5.A), ATCC-33291 (Figure 3.5.B), and 2083 (Figure 3.5.C) and somewhat decreased for strain A14a (Figure 3.5.D).

3.4.5. Aptamer ONS-23 Selects for *C. jejuni* in a Mixed Cell Population

We assessed the selectivity of aptamer ONS-23 for *C. jejuni* capture in a mixed cell population consisting of *C. jejuni* A9a and a single strain each of *B. cereus*, *L. monocytogene*s and *E. coli* O157: H7 (10^8-10^9 cells each). After treating the pooled cells with aptamer ONS-23, the top 10^5 cells based on fluorescence intensity were isolated using automated fluorescence cell sorting. Genomic DNA was then isolated and amplified by PCR using primers targeting a 176 bp region of the *C. jejuni* hippuricase gene (*hip*O).

Amplification was confirmed by agarose gel electrophoresis demonstrating that *C. jejuni* cells were captured by aptamer ONS-23 even in the presence of a mixed cell population.

3.4.6. Prediction of Unique Secondary Structure of Aptamer ONS-23

The unique 3 dimensional structural folding of aptamer ONS-23 at 37°C was predicted using DNA Mfold (version 3.2) (Zuker, 2003) (Figure 3.6). With a minimum free energy of -6.36 kcal/ mol**,** the structure of aptamer ONS-23 consisted of an external loop of 30 bases with three closing helices. The first hairpin loop was located between the closing base pair G17-C40 with a 4 base pair helix. The second hairpin loop was located between the closing pair at T56-A61 with a 3 base pair helix. A third hairpin loop with a 4 base pair helix was located between the closing base pair at T69-A73. The first two hairpin loops were partially located in the random region, while the third hairpin loop was located in the constant region of the DNA aptamer library.

3.4.7. ONS-23 Binds with Specific Cell Surface Targets of *C. jejuni*

A reduction of aptamer ONS-23 binding was observed for Proteinase K treated cells (9.94% fluorescent cells) compared with untreated cells (26.14% fluorescent cells) suggesting a proteinous nature for cell surface targets being recognized by ONS-23 (Figure 3.7).

3.5. DISCUSSION

Single-stranded nucleic acid aptamers represent a new generation of macromolecules with applicability to the selective capture and subsequent detection of target molecules for development of sensitive, specific and rapid diagnostics (Chen et al., 2007; Vivekananda & Kiel, 2006). The unique secondary structural elements formed by these single-stranded DNA oligomers can be exploited using multiple rounds of selection and sequence enrichment resulting in target-specific probes. The probes can be labeled for visual detection or tethered to a solid support for target capture and concentration. In this study, a whole-cell SELEX method was used for aptamer selection as an alternative to the more traditional SELEX approach applied to crude or purified extracellular surface targets. Whole-cell SELEX or complete target SELEX is an emerging holistic approach for aptamer selection which has previously been applied to *Mycobacterium tuberculosis* (Chen et al., 2007), Rous sarcoma virus (Pan et al., 1995), *Trypanosoma brucei* (Homann & Goringer, 1999) and *Trypanosoma cruzi* (Ulrich et al., 2002). Whole-cell SELEX has several advantages including the fact that it is not necessary to have prior knowledge of the target and that aptamers are selected against targets in their native conformation and physiological environment (Cerchia et al., 2005; Pestourie et al., 2006). The whole-cell SELEX approach is also amenable to flow cytometry for both selection and binding affinity analyses (Chen et al., 2007; Shangguan et al., 2006). Specifically we used fluorescence based automated cell sorting of aptamer labeled cells for isolation and recovery of the top binding aptamers from the candidate pool. Multicolor flow cytometry in conjunction with cell sorting as a high-throughput screening

technique to separate target bound magnetic bead linked aptamers from non-functional bead linked aptamers was recently reported (Yang et al., 2003), but the real-time application of fluorescence-based automated cell sorting for separation of aptamer-labeled food borne bacterial pathogens is unique to this study.

As has been the case in previous studies (Bibby et al., 2008; Lorger et al., 2003; Shangguan et al., 2006), we also were unable to demonstrate absolute binding affinity (approaching 100% cells labeled) of aptamer sequences to the *C. jejuni* cell population during preliminary binding assays. This could be a function of *C. jejuni* cell death occurring due to prolonged exposure to $O_{2,}$ especially since the binding assays took much longer to complete than did any iteration of the SELEX process. Specifically, the same aptamers that had been selected against viable cells may not effectively bind to inactivated cells. An alternative explanation could be differential binding affinity as a function of cell type. A number of cell types in *C. jejuni* culture have been previously reported to occur in both solid and liquid growth media (Griffiths, 1993; Ng et al., 1985). Furthermore, under sub-optimal conditions, the characteristic curved and spiral morphology of *C. jejuni* cells may undergo transition via several variant types (comma, spiral, S shape, ring or donut shape) before finally adopting a coccoid morphology (Griffiths, 1993; Ng et al., 1985) with associated loss of culturability. If the aptamers are selected against a population of cell variants, they could be cell type or cell stage-specific, a phenomenon which has also been reported during selection of aptamers against live *Trypanosome brucei* (Lorger et al., 2003). Similarly, Shangguan et al. (2006) found their aptamer sequences sgc3, sgc6 and sgd3 obtained by whole-cell SELEX to be specific to only a fraction of target CCRF-CEM cells, hypothesizing

the presence of a specific subset of cells having unique molecular cell surface signatures. This hypothesis is intriguing as it could open up possibilities for stage-specific capture of bacterial cells, for example discrimination of healthy cells from those which are dead or in the viable-but-non-culturable state, or those having undergone some sort of stress conditioning response. Further investigation of our aptamer is merited in this regard.

We chose aptamer ONS-23 for further detailed characterization because of its high binding affinity in initial screens and the presence of a highly conserved sequence motif. More advanced binding studies revealed that the apparent dissociation constant of the aptamer ONS-23 and *C. jejuni* interaction was in the high nM range, confirming the high degree of affinity of aptamer ONS-23 for the *C. jejuni* A9a cell type. Previous studies on aptamers selected using whole-cell SELEX have reported binding affinities of aptamer-target interaction in the higher micromolar [binding constant K_a between selected aptamer (NK2) and target *Mycobacterium tuberculosis*, H37Rv interaction to be K_{1a}: $1.84 \times 10^5(\pm 1.5 \times 10^4)\ M^{-1}$, or K_{2a}: $7.65 \times 10^6(\pm 6.0 \times 10^5)\ M^{-1}$] (Chen et al., 2007) to nanomolar ranges (dissociation constant between selected aptamers sga16 and the target CCRF-CEM Cells to be Kd = 5.0 ± 0.52 nM) (Shangguan et al., 2006), so our results are consistent with those of other studies. Unfortunately, the dissociation constant for ONS-23 was calculated using a non-interacting binding sites model with the non-linear least square method based on the overall change in the number of fluorescently labeled cells (Davis et al., 1998; Shangguan et al., 2006). Consequently, we cannot predict the number of individual aptamer recognition sites residing on the surface of an individual cell.

The aptamer ONS-23 selected against *C. jejnui* strain A9a showed similar binding capabilities with other *C. jejuni* strains, suggesting that conserved cell surface targets are associated with aptamer binding. Similar findings have been reported for an aptamer developed for *Trypanosome brucei* strains (Lorger et al., 2003), for which the authors speculated the convergence of aptamers against invariant surface targets shared by different strains. Strain-specific aptamers could theoretically be selected by targeting strain-specific cell surface markers using negative selection, but this was not the intention of our study. In addition, the counter-SELEX step was successfully utilized to reduce the number of aptamers exhibiting non-specific cell labeling characteristics and this was confirmed by low apparent binding of aptamer ONS-23 for non-*C. jejuni* strains. Counter selection during whole-cell SELEX has been reported to be effective in some but not all applications (Cerchia et al., 2005; Pestourie et al., 2006; Shangguan et al., 2006).

The preliminary result of the Proteinase K assay suggested a proteinous nature of the cell membrane target molecule for specific binding to aptamer ONS-23. The proteinases trypsin and proteinase K has been previously used for the preliminary characterization of the nature of targets over cell surfaces for selected aptamers (Chen et al., 2007; Shangguan et al., 2006). Although there have been reports of isolation of specific targets in the purified forms responsible for binding aptamers during whole-cell SELEX (Mallikaratchy et al., 2007), we did not attempt to isolate the specific protein target responsible for the binding of ONS-23 to *C. jejuni* A9a in this study.

This study is the report on the use of whole-cell SELEX to identify DNA aptamers specific for *C. jejuni.* Based on the results of the present study we anticipate that the DNA

aptamer ONS-23 may be employed for the capture of *C. jejuni* and its subsequent detection, as applied to complex sample matrices such as food. As shown in this study, the FAM-conjugated DNA aptamers could be used in flow cytometric analysis (Chen et al., 2007; Shangguan et al., 2006), fluorescence-based automated sorting of specific cell types, and fluorescent microscopy. We suspect that they may be adapted to other detection platforms such as aptamer-linked immobilized sorbent assay (Drolet et al., 1996; Vivekananda & Kiel, 2006), calorimetric analysis (Chen et al., 2007), dot blot assays (Vivekananda & Kiel, 2006), proximal ligation assays (Fredriksson et al., 2002), and biosensors (Liss et al., 2002). These applications are currently under investigation in our laboratory. This study provides proof-of-concept that aptamers targeting *C. jejuni* can be isolated using a whole-cell SELEX process and that these aptamers demonstrate the degree of highly specific binding affinity required for pre-analytical sample processing and detection methods.

Table 3.1. Oligonucleotides used in the selection and characterization of aptamers. The DNA aptamer library was constructed with a 40 nucleotide random region flanked by two constant regions at 5' and 3' ends having digestion sites for various restriction enzymes for future modifications. Labeled constant region primers were used to amplify and label DNA aptamer library. Primers complementary to hippuricase gene (*hip*O) were used to amplify *C. jejuni* genomic DNA.

Name	**Oligonucleotides**
	SnaB I Pst I EcoR V Bgl II
DNA Aptamer Library	5′-GTATACGTATTACCTGCAGC - N_{40} - CGATATCTCGGAGATCTTGC-3′
FAM-Forward Constant Region Primer	5′-/56FAM/- AGTATACGTATTACCTGCAGC -3′
Biotin- Reverse Constant Region Primer	5′-/5Biosg/GCAAGATCTCCGAGATATCG -3′
Forward Constant Region Primer	5′-AGTATACGTATTACCTGCAGC -3′
Reverse Constant Region Primer	5′-GCAAGATCTCCGAGATATCG -3′
Forward Primer Hip 1a (Marshall et al., 1999)	5'-ATGATGGCTTCTTCGGATAAG-3'
Reverse Primer Hip 2b (Marshall et al., 1999)	5'-GCT CCT ATG CTT ACA ACT GC-3'

Table 3.2. Aptamers selected using whole-cell SELEX with preliminary binding affinity. Aptamer sequences were obtained after 10 rounds of SELEX and 2 rounds of counter-SELEX. For preliminary screening of the selected aptamer sequences, % fluorescent cells were recorded from binding assays with *C. jejuni* cells performed using flow cytometry.

Aptamer name	**% fluorescent cells in binding assay**	**% GC**
ONS-20	28.58	63
ONS-23	31.44	50
ONS-30/ONS-34/22-24	22.83	55
22-4/22-17	24.24	55
22-13	25.19	58
22-19	30.37	63
22-21	19.86	53

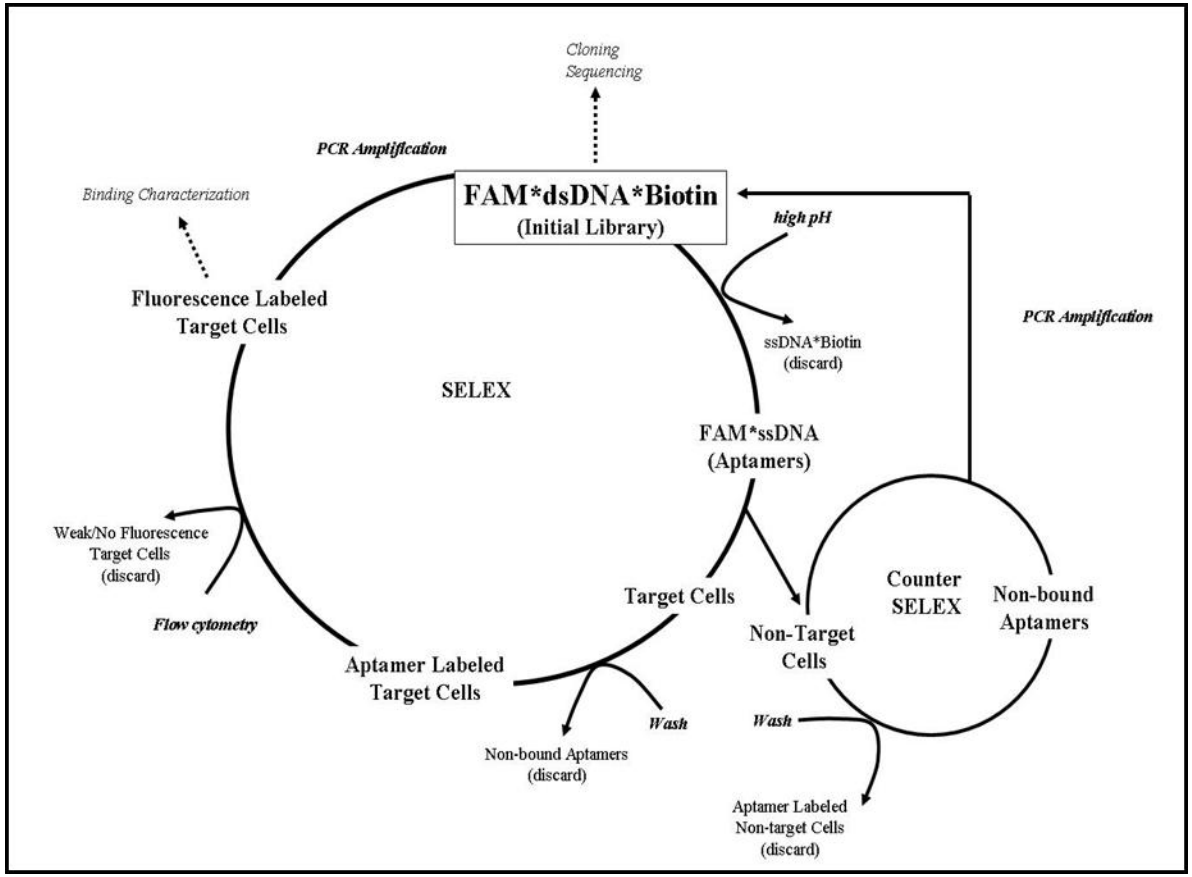

Figure 3.1. Schematic diagram of the whole-cell SELEX process used to isolate DNA aptamers with high binding affinity for *C. jejuni.*

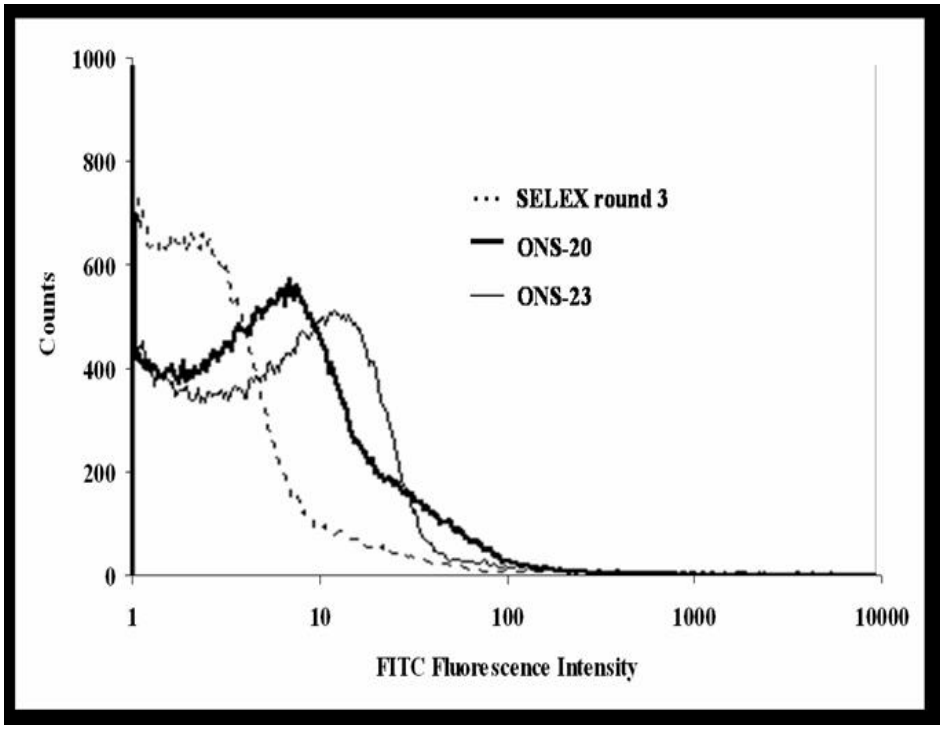

Figure 3.2. Binding affinity of selected aptamer candidates after 10 rounds of SELEX and 2 rounds of counter-SELEX as compared with the aptamer pool selected after the 3rd round of SELEX. Aptamers ONS-20 and ONS-23 showed 28.58 % and 31.44 % total fluorescent cells, respectively, in flow cytometric analysis while the aptamer pool from 3rd round of SELEX showed only 4.16% total fluorescent cells.

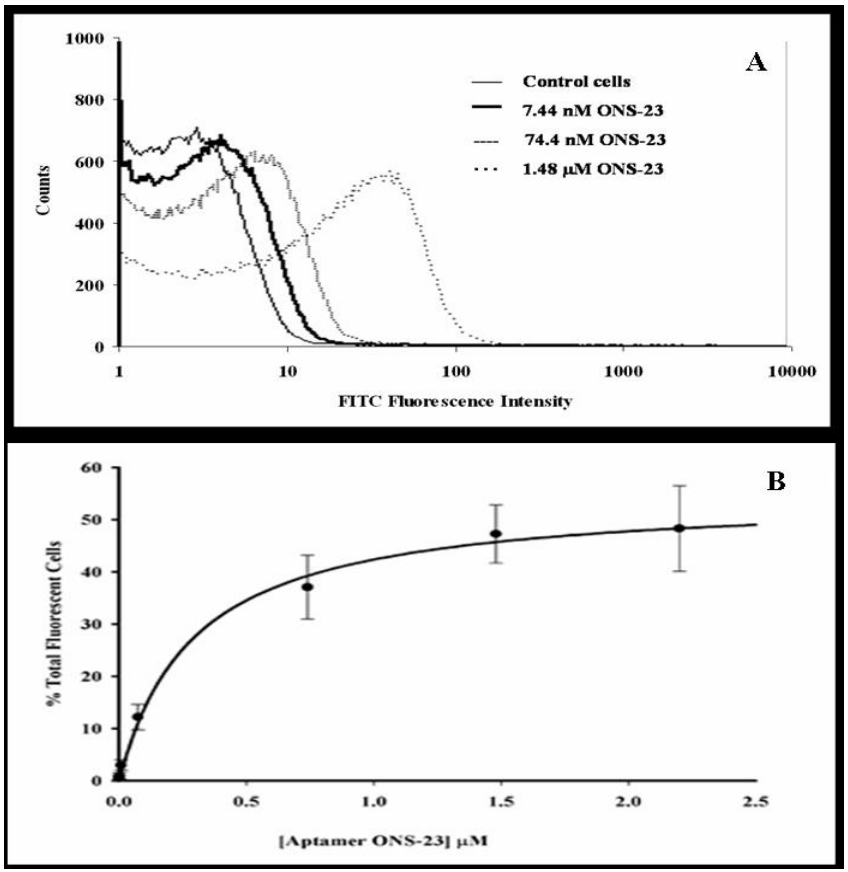

Figure 3.3. Correlation between aptamer ONS-23 concentration and total fluorescence intensity as a measure of target recognition. The percentage of fluorescent cells after incubation with 7.4 nM, 74 nM, 740 nM, 1.48 µM, 2.2 µM aptamer ONS-23 solution with 10^8- 10^9 *C. jejuni (*A9a) cells was 3.00 ± 0.99, 12.21 ± 2.43, 37.07 ± 6.1, 47.27 ± 5.58, 48.31 ± 8.22 respectively (A). The data were fitted to a non-interacting binding sites model y = Bmax*x/ [Kd + x], which yielded a dissociation constant (*Kd value*) of 292.8 ± 53.1 nM (B).

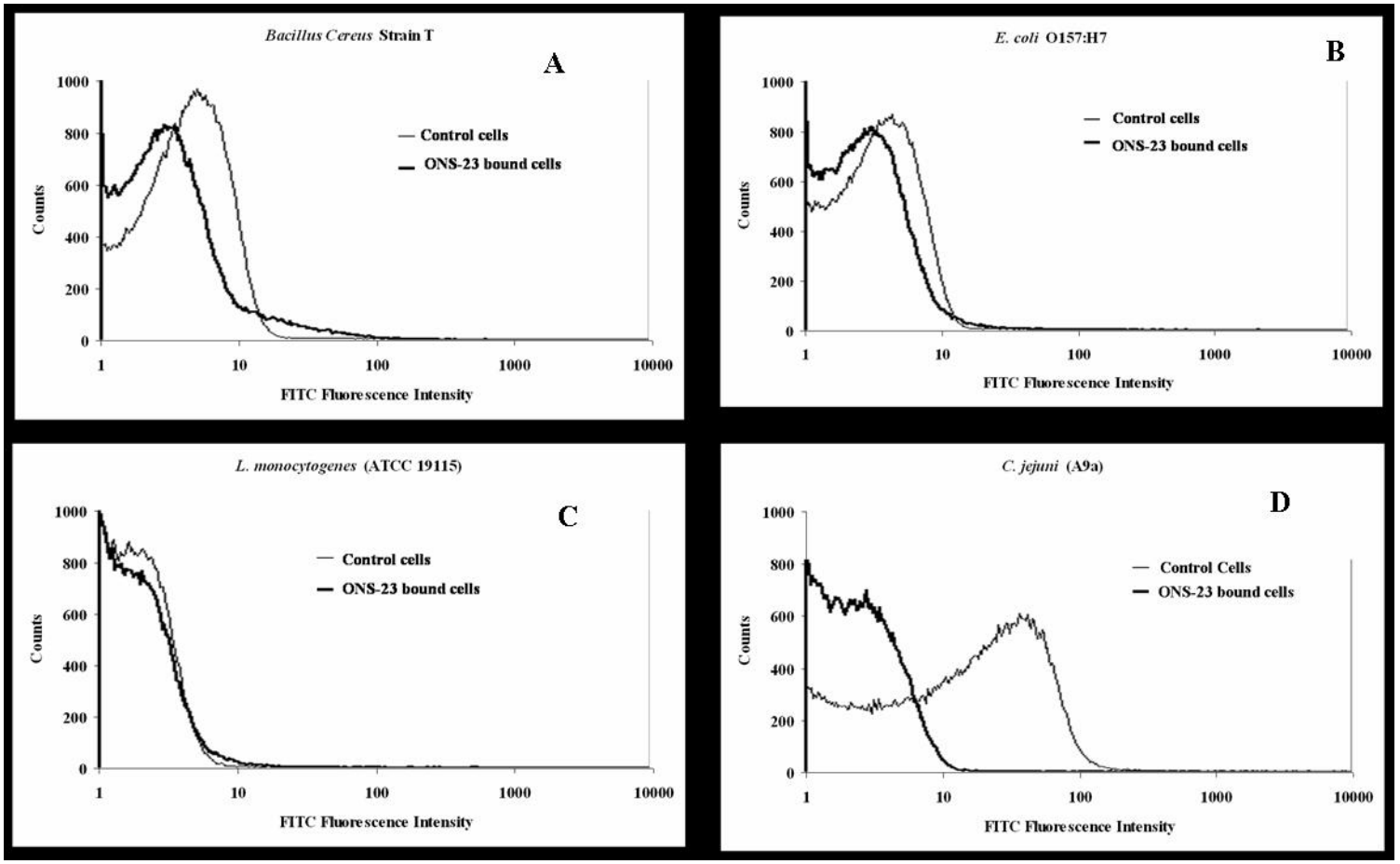

Figure 3.4. Binding specificity for aptamer ONS-23. The percentage of total fluorescent cells was determined for each bacterial species as described in text. Aptamer ONS-23 showed minimal non-specific binding with *B. cereus* strain T (4.71 %) (A), *E. coli* O157: H7 (1.26 %) (B) and *L. monocytogenes* ATCC19115 (1.24 %) (C) when comparing to binding with *C. jejuni* A9a (51.72%, highest binding affinity recorded in an assay) (D).

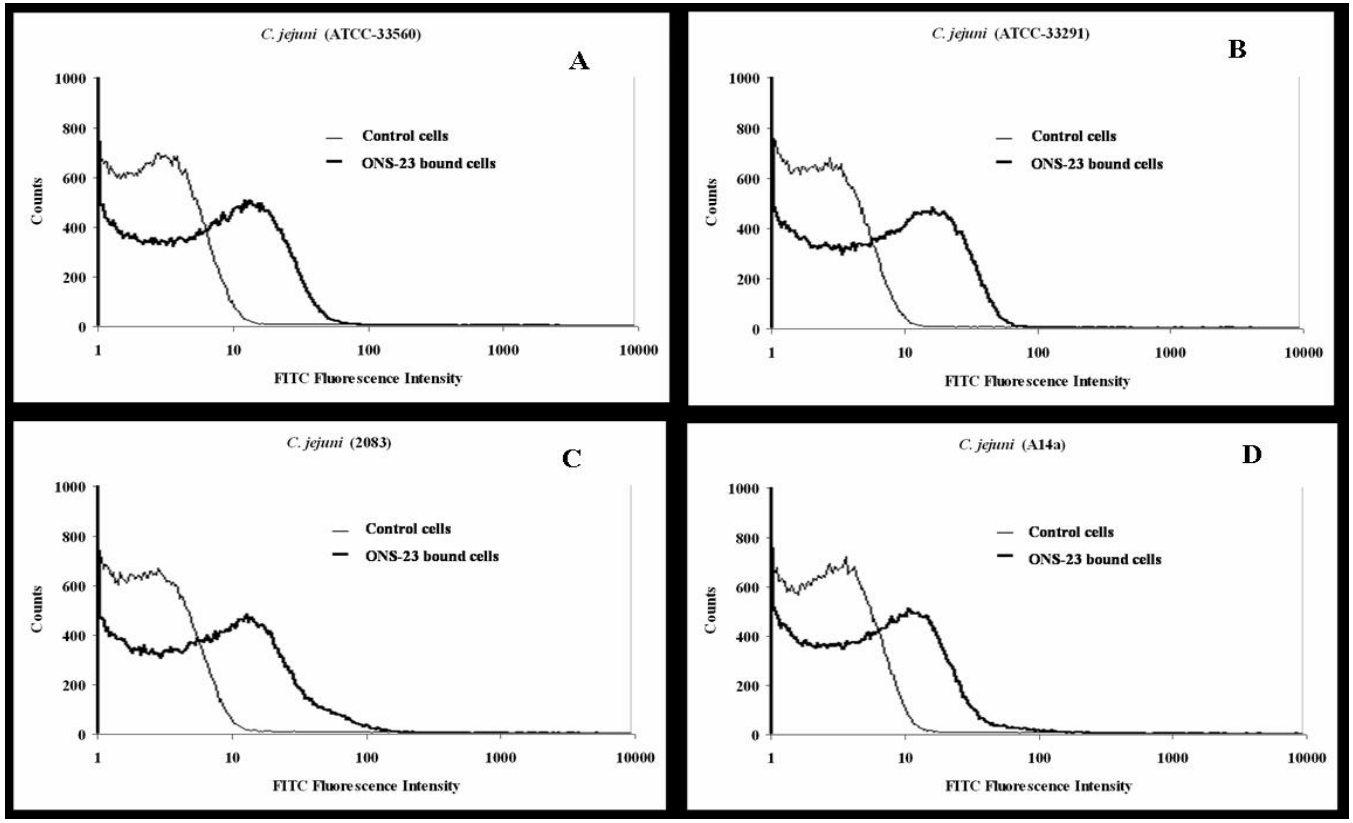

Figure 3.5. Binding inclusivity for aptamer ONS-23. The percentages of fluorescent cells for different strains were determined using flow cytometery after labeling with aptamer ONS-23 as described in text. Similar binding affinity was recorded for *C. jejuni* strain ATCC33560 (32.18 %) (A), ATCC33291 (35.53 %) (B), and 2083 (33.67 %) (C); binding affinity was slight reduced for *C. ejuni* strain A14a (25.31 %) (D) although well above what was observed for non-*C. jejuni* strains .

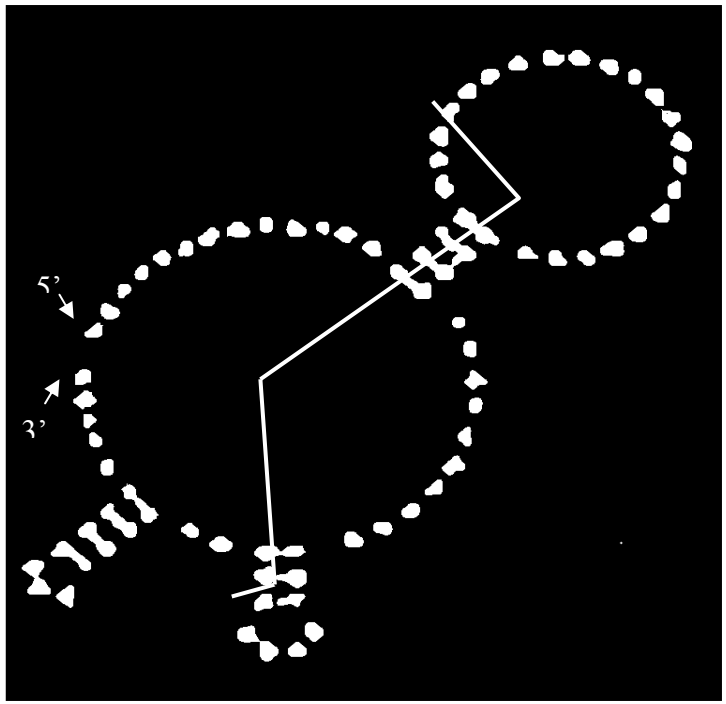

Figure 3.6. Predicted secondary structure of aptamer ONS-23 using DNA Mfold. Each white block represents a base of aptamer sequence. The 4 base pair helix of the first hairpin loop, 3 base pair helix of the second hairpin loop, and 4 base pair helix of the third hairpin loop are shown by white line joining the complementary bases of helix. The region of the aptamer from white dividing lines towards the 5’ and 3’ ends constitute the constant region.

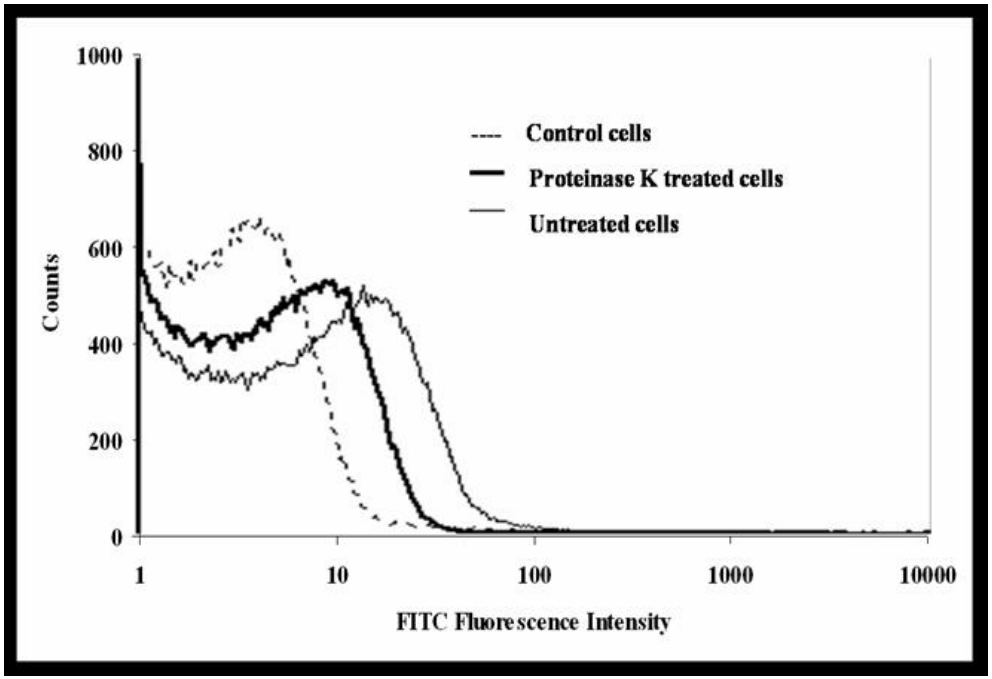

Figure 3.7. Proteinase K digestion of *C. jejuni* prior to labeling with aptamer ONS-23. An approximate one-third reduction in the percentage of fluorescent cells was observed.

3.6. REFERENCES

Bailey, T. L. & Elkan, C. (1994). Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc Int Conf Intell Syst Mol Biol*, 2, 28-36.

Bibby, D. F., Gill, A. C., Kirby, L., Farquhar, C. F., Bruce, M. E. & Garson, J. A. (2008). Application of a novel in vitro selection technique to isolate and characterise high affinity DNA aptamers binding mammalian prion proteins. *J Virol Methods*, 151(1), 107-15.

Cerchia, L., Duconge, F., Pestourie, C., Boulay, J., Aissouni, Y., Gombert, K., Tavitian, B., de Franciscis, V. & Libri, D. (2005). Neutralizing aptamers from whole-cell SELEX inhibit the RET receptor tyrosine kinase. *PLoS Biol*, 3(4), e123.

Chen, F., Zhou, J., Luo, F., Mohammed, A. B. & Zhang, X. L. (2007). Aptamer from whole-bacterium SELEX as new therapeutic reagent against virulent *Mycobacterium tuberculosis*. *Biochem Biophys Res Commun*, 357(3), 743-8.

Davis, K. A., Lin, Y., Abrams, B. & Jayasena, S. D. (1998). Staining of cell surface human CD4 with 2'-F-pyrimidine-containing RNA aptamers for flow cytometry. *Nucleic Acids Res*, 26(17), 3915-24.

Drolet, D. W., Moon-McDermott, L. & Romig, T. S. (1996). An enzyme-linked oligonucleotide assay. *Nat Biotechnol*, 14(8), 1021-5.

Ellington, A. D. & Szostak, J. W. (1990). In vitro selection of RNA molecules that bind specific ligands. *Nature*, 346(6287), 818-22.

Fredriksson, S., Gullberg, M., Jarvius, J., Olsson, C., Pietras, K., Gustafsdottir, S. M., Ostman, A. & Landegren, U. (2002). Protein detection using proximity-dependent DNA ligation assays. *Nat Biotechnol*, 20(5), 473-7.

Friedman, C. R., Hoekstra, R. M., Samuel, M., Marcus, R., Bender, J., Shiferaw, B., Reddy, S., Ahuja, S. D., Helfrick, D. L., Hardnett, F., Carter, M., Anderson, B. & Tauxe, R. V. (2004). Risk factors for sporadic *Campylobacter* infection in the United States: A case-control study in FoodNet sites. *Clin Infect Dis*, 38 Suppl 3, S285-96.

Gillespie, I. A., O'Brien, S. J., Frost, J. A., Adak, G. K., Horby, P., Swan, A. V., Painter, M. J. & Neal, K. R. (2002). A case-case comparison of *Campylobacter coli* and *Campylobacter jejuni* infection: a tool for generating hypotheses. *Emerg Infect Dis*, 8(9), 937-42.

Griffiths, P. L. (1993). Morphological changes of *Campylobacter jejuni* growing in liquid culture. *Lett Appl Microbiol*, 17(4), 152-5.

Herr, J. K., Smith, J. E., Medley, C. D., Shangguan, D. & Tan, W. (2006). Aptamer-conjugated nanoparticles for selective collection and detection of cancer cells. *Anal Chem*, 78(9), 2918-24.

Homann, M. & Goringer, H. U. (1999). Combinatorial selection of high affinity RNA ligands to live African trypanosomes. *Nucleic Acids Res*, 27(9), 2006-14.

Humphrey, T., O'Brien, S. & Madsen, M. (2007). *Campylobacters* as zoonotic pathogens: a food production perspective. *Int J Food Microbiol*, 117(3), 237-57.

Johansen, H. K., Norgaard, A., Andersen, L. P., Jensen, P., Nielsen, H. & Hoiby, N. (1995). Cross-reactive antigens shared by *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Campylobacter jejuni*, and *Haemophilus influenzae* may cause false-positive titers of antibody to H. pylori. *Clin Diagn Lab Immunol*, 2(2), 149-55.

LaGier, M. J., Joseph, L. A., Passaretti, T. V., Musser, K. A. & Cirino, N. M. (2004). A real-time multiplexed PCR assay for rapid detection and differentiation of *Campylobacter jejuni* and *Campylobacter coli*. *Mol Cell Probes*, 18(4), 275-82.

Lamoureux, M., MacKay, A., Messier, S., Fliss, I., Blais, B. W., Holley, R. A. & Simard, R. E. (1997). Detection of *Campylobacter jejuni* in food and poultry viscera using immunomagnetic separation and microtitre hybridization. *J Appl Microbiol*, 83(5), 641-51.

Li, N., Wang, Y., Pothukuchy, A., Syrett, A., Husain, N., Gopalakrisha, S., Kosaraju, P. & Ellington, A. D. (2008). Aptamers that recognize drug-resistant HIV-1 reverse transcriptase. *Nucleic Acids Res*.

Liss, M., Petersen, B., Wolf, H. & Prohaska, E. (2002). An aptamer-based quartz crystal protein biosensor. *Anal Chem*, 74(17), 4488-95.

Lorger, M., Engstler, M., Homann, M. & Goringer, H. U. (2003). Targeting the variable surface of African trypanosomes with variant surface glycoprotein-specific, serum-stable RNA aptamers. *Eukaryot Cell*, 2(1), 84-94.

Mallikaratchy, P., Tang, Z., Kwame, S., Meng, L., Shangguan, D. & Tan, W. (2007). Aptamer directly evolved from live cells recognizes membrane bound immunoglobin heavy mu chain in Burkitt's lymphoma cells. *Mol Cell Proteomics*, 6(12), 2230-8.

Marshall, S. M., Melito, P. L., Woodward, D. L., Johnson, W. M., Rodgers, F. G. & Mulvey, M. R. (1999). Rapid identification of *Campylobacter*, *Arcobacter*, and *Helicobacter* isolates by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene. *J Clin Microbiol*, 37(12), 4158-60.

Moreno, Y., Botella, S., Alonso, J. L., Ferrus, M. A., Hernandez, M. & Hernandez, J. (2003). Specific detection of *Arcobacter* and *Campylobacter* strains in water and sewage by PCR and fluorescent in situ hybridization. *Appl Environ Microbiol*, 69(2), 1181-6.

Nachamkin, I. (2002). Chronic effects of *Campylobacter* infection. *Microbes Infect*, 4(4), 399-403.

Ng, L. K., Sherburne, R., Taylor, D. E. & Stiles, M. E. (1985). Morphological forms and viability of *Campylobacter* species studied by electron microscopy. *J Bacteriol*, 164(1), 338-43.

Pan, W., Craven, R. C., Qiu, Q., Wilson, C. B., Wills, J. W., Golovine, S. & Wang, J. F. (1995). Isolation of virus-neutralizing RNAs from a large pool of random sequences. *Proc Natl Acad Sci U S A*, 92(25), 11509-13.

Pestourie, C., Cerchia, L., Gombert, K., Aissouni, Y., Boulay, J., De Franciscis, V., Libri, D., Tavitian, B. & Duconge, F. (2006). Comparison of different strategies to select aptamers against a transmembrane protein target. *OLIGONUCLEOTIDES*, 16(4), 323-35.

Shamah, S. M., Healy, J. M. & Cload, S. T. (2008). Complex target SELEX. *Acc Chem Res*, 41(1), 130-8.

Shangguan, D., Li, Y., Tang, Z., Cao, Z. C., Chen, H. W., Mallikaratchy, P., Sefah, K., Yang, C. J. & Tan, W. (2006). Aptamers evolved from live cells as effective molecular probes for cancer study. *Proc Natl Acad Sci U S A*, 103(32), 11838-43.

Tombelli, S., Minunni, M. & Mascini, M. (2005). Analytical applications of aptamers. *Biosens Bioelectron*, 20(12), 2424-34.

Tuerk, C. & Gold, L. (1990). Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science*, 249(4968), 505-10.

Ulrich, H., Magdesian, M. H., Alves, M. J. & Colli, W. (2002). In vitro selection of RNA aptamers that bind to cell adhesion receptors of *Trypanosoma cruzi* and inhibit cell invasion. *J Biol Chem*, 277(23), 20756-62.

Vivekananda, J. & Kiel, J. L. (2006). Anti-*Francisella tularensis* DNA aptamers detect tularemia antigen from different subspecies by Aptamer-Linked Immobilized Sorbent Assay. *Lab Invest*, 86(6), 610-8.

Wang, C., Zhang, M., Yang, G., Zhang, D., Ding, H., Wang, H., Fan, M., Shen, B. & Shao, N. (2003). Single-stranded DNA aptamers that bind differentiated but not parental cells: subtractive systematic evolution of ligands by exponential enrichment. *J Biotechnol*, 102(1), 15-22.

Yang, X., Li, X., Prow, T. W., Reece, L. M., Bassett, S. E., Luxon, B. A., Herzog, N. K., Aronson, J., Shope, R. E., Leary, J. F. & Gorenstein, D. G. (2003). Immunofluorescence assay and flow-cytometry selection of bead-bound aptamers. *Nucleic Acids Res*, 31(10), e54.

Yu, L. S., Uknalis, J. & Tu, S. I. (2001). Immunomagnetic separation methods for the isolation of *Campylobacter jejuni* from ground poultry meats. *J Immunol Methods*, 256(1-2), 11-8.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*, 31(13), 3406-15.

CHAPTER 4

SELECTION AND CHARACTERIZATION OF BIOTINYLATED DNA APTAMERS FOR CAPTURE AND DETECTION OF *SALMONELLA* TYPHIMURIUM USING QUANTITATIVE REAL-TIME PCR

Hari Prakash Dwivedi and Lee-Ann Jaykus

4.1. ABSTRACT

Alternative ligands such as nucleic acid aptamers can be used for pathogen capture and detection and offer advantages over antibodies, including reduced cost, ease of production and modification, and improved stability. In an effort to identify DNA aptamers demonstrating binding specificity to *Salmonella enterica* serovar. Typhimurium, whole cell-SELEX (Systematic Evolution of Ligands by EXponential enrichment) was applied to a combinatorial library of biotin-labeled single stranded DNA molecules. Specificity of the aptamers was enhanced using counter-SELEX against select non-*Salmonella* genera. Aptamers selectively binding to *Salmonella* were sorted, cloned, sequenced and characterized for binding efficacy. Out of 18 candidate aptamer sequences, two (S8-7 and S8-46) showed relatively high binding efficiency [13-14% of target cells (n = 200, 000) fluorescent with 300 pmoles of each aptamer]. One of these (aptamer S8-7) was further characterized, demonstrating a dissociation constant (K_d value) of 1.73 ± 0.54 μM, selectively binding 22.0

± 3.4 % of 200, 000 cells at a 6.94 μM concentration. Binding exclusivity analysis of S8-7 showed low apparent cross-reactivity with other food borne bacteria including *E. coli* O157: H7 and *Citrobacter braakii*, and moderate cross-reactivity with *Bacillus cereus*. Aptamer S8-7 was successfully applied to the magnetic capture of serially diluted *S.* Typhimurium cultures for subsequent downstream detection by qPCR. The lower limit of detection of the magnetic capture-qPCR assay was 10^2-10^3 CFU equivalents of *S.* Typhimurium in a 290 μl sample volume. Capture efficiency ranged from 3-13 %. This study provides proof-of-concept that biotinylated aptamers can be used in a qPCR-based capture-detection platform for *S.* Typhimurium.

4.2. INTRODUCTION

Salmonella is a leading cause of food borne bacterial gastroenteritis, with approximately 1.4 million cases per year in the United States, resulting in 16,000 hospitalizations and 550 deaths (Joshi et al., 2009; Taitt et al., 2004). In the U.S., salmonellosis is the cause of approximately 30% of all reported cases of food borne disease of known etiology and is associated with estimated annual economic losses of $1.3 to $4.0 billion (Taitt et al., 2004; Todd, 1989). *Salmonella enterica* serovar. Typhimurium is one of the most frequently documented serovars associated with human infections since 1997 (CDC, 2007). Salmonellae are native to the gastrointestinal tracts of food animals and are shed in their feces. Food borne transmission of *Salmonella* is predominantly due to the consumption of undercooked poultry, meat, milk, eggs and water, and via cross- contamination (Bell, 2002).

The standard method for detection of *Salmonella* in foods includes the sequential steps of pre-enrichment, selective enrichment, and selective-differential plating; presumptively positive isolates are further confirmed by biochemical, serological, and/or molecular methods. These methods are time-consuming, tedious, and not practical for real-time applications. Over the past few decades, several modifications and refinements have addressed time to detection by replacing the selective-differential plating step with ELISA, DNA hybridization or PCR (Feng, 2007). Despite these advancements, some degree of cultural enrichment remains essential to assure that the required low detection limit of 1 CFU per 25 g sample of food is met (Eriksson & Aspan, 2007; Kumar et al., 2008; Prusak-Sochaczewski & Luong, 1989).

It has been suggested that pre-analytical sample processing which seeks to concentrate the target pathogen while simultaneously reducing the volume of the sample matrix, may aid in reducing enrichment times. Non-specific methods such as metal ions and ion exchange resins, and specific methods such as antibodies have been used in this capacity (Cullison & Jaykus, 2002; Jacobsen & Rasmussen, 1992; Mercanoglu & Griffiths, 2005). Currently, immunomagnetic separation (IMS) employing *Salmonella* specific antibodies is the most commonly used method for capture and concentration of the pathogen prior to the application of molecular detection methods such as PCR (Hsih & Tsen, 2001; Mercanoglu & Griffiths, 2005).

But antibodies have several disadvantages, including the requirement of a living host for their production; high cost of production and the need to verify of activity; batch-to-batch

variation in functionality; and limited shelf life (Jayasena, 1999). For this reason, alternative ligands have been investigated for their application to pre-analytical sample processing. Nucleic acid aptamers are single stranded DNA or RNA molecules which form sequence-defined unique structural forms with binding affinity to a specific target. Several characteristics of aptamers make them attractive for pre-analytical sample processing and biodiagnostic assay development including their small size, ease of synthesis and labeling, lack of immunogenicity, low cost of production, and target binding affinity and specificity equal to or better than antibodies (Tombelli et al., 2005).

Aptamers specific to a wide range of protein and non-protein targets have been selected using a method called SELEX (Systematic Evolution of Ligands by EXponential enrichment) (Farokhzad et al., 2004; Joshi et al., 2009; Stanlis & McIntosh, 2003). Historically, knowledge of the relevant diagnostic marker on the target cell membrane has been required for successful application of SELEX, which has been applied to these markers in isolated, purified forms (Pestourie et al., 2006). Alternatively, live pathogen cells can be targeted in the SELEX, in which case the entirety of the cell surface target(s) in their native three dimensional conformation(s) will be available for aptamer selection (Fang & Tan, 2009; Shamah et al., 2008). Some studies have suggested that using whole cell targets in the SELEX process can actually be faster, easier, and more reproducible than using purified target molecules, with greater success in generating functional aptamers with binding affinity to live cells (Guo et al., 2008). In this study we selected and characterized biotinylated DNA aptamers with binding affinity to *S.* Typhimurium using a whole-cell SELEX approach. As

proof-of-concept, these aptamers were then used to capture and concentrate *S.* Typhimurium cells for direct detection using qPCR.

4.3. MATERIALS and METHODS

4.3.1. Microbial Strains, Culture Conditions and Preparation of Cells

A strain of *Salmonella enterica* serovar. Typhimurium, which was isolated from an infected human and naturally resistant to ampicillin (courtesy of Dr. W. Gebreyes, Department of Veterinary Preventive Medicine, The Ohio State University, Columbus, OH) was used as the target in all experiments. The strain was grown overnight at 37°C in Trypticase™ Soy Broth (Becton, Dickinson and Company, Sparks, MD) supplemented with ampicillin to a final concentration of 50 µg/ml (Sigma-Aldrich Inc., St. Louis, MO) (TSB-A). The bacterial population was determined by direct plating of 10-fold serial dilutions on Trypticase™ Soy Agar (Becton-Dickinson) supplemented with 50 µg/ml ampicillin (TSA-A). For SELEX, bacterial cells from 10 ml overnight broth cultures were washed three times in 1X phosphate buffered saline (PBS) and finally suspended in 500 µl PBS. A 10 µl volume of this solution (approximately 10^6-10^7 CFU) was used for each round of SELEX.

Other bacterial strains used in this study were *Listeria monocytogenes* Scott A, *E. coli* O157:H7 ATCC43895, *Bacillus cereus* ATCC49063, *Enterococcus faecalis* ATCC29212 and *Citrobacter braakii* ATCC29219. All bacterial strains were grown overnight in Brain Heart Infusion broth (BHI) at 37°C. For counter-SELEX, 2 ml aliquots of overnight cultures of *L. monocytogenes* Scott A, *E. coli* O157: H7 ATCC43895, *B. cereus* ATCC49063, and *E.*

faecalis ATCC29212 were pooled, washed in PBS, and the pellet resuspended in 1 ml PBS. A volume of 100 µl of this solution was used in the counter-SELEX.

4.3.2. SELEX Process

The whole-cell SELEX process is outlined in Figure 4.1. An 81-base combinatorial DNA aptamer library was obtained from Integrated DNA Technologies (Coralville, IA). The library sequences, location of random and constant regions, fluorescent labels, and attachment chemistry linkers are shown in Table 4.1.

4.3.2.1. Biotin Labeling of DNA library

The diluted aptamer library (10 µM initial concentration) was amplified in 25 µl PCR reactions containing 1X Go Taq® Buffer (Promega Corp., Madison, WI), 0.3 mM GeneAmp® dNTPs Mix (Applied Biosystems, Foster City, CA), 1.25U Go Taq® DNA Polymerase (Promega), 300 nM Forward Constant Region primer, and 300 nM Biotin- Reverse Constant Region primer (Table 4.1). The PCR was performed in a DNA Engine (PTC-200) Peltier Thermal Cycler-200 (MJ Research/ Bio-Rad Laboratories, Hercules, CA) using a 3 step thermal protocol consisting of initial denaturation at 95°C for 5 min followed by 15-20 cycles of 95°C for 1 min, 55-68°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

4.3.2.2. Separation of Biotin Labeled Single Stranded DNA (biotin-ssDNA)

Biotin-labeled double stranded DNA was coupled with Streptavidin MagneSphere® Paramagnetic Particles (SA-PMPs) (Promega) by incubating at room temperature for 30 min with gentle rolling. The library-coupled magnetic beads were washed 3 times in 0.1X SSC buffer. The unlabeled ssDNA moieties were separated from the immobilized biotin-labeled strands by alkaline denaturation in 0.2 M NaOH at room temperature for 5-6 min and then recovered by magnetic capture of the beads using a Dynal MPC®-M magnetic particle concentrator (Dynal A.S, Oslo, Norway). The remaining biotin-ssDNA coupled magnetic beads were washed thrice in 1X Tris-EDTA. A second alkaline denaturation was performed in tightly packed microcentrifuge tubes using ammonium hydroxide (28% ammonia in water) (Sigma Aldrich) at 85°C to recover the biotin-labeled ssDNA molecules which were washed an additional 3-4 times using molecular grade water and concentrated using a Microcon® YM-30 filter device (Millipore, Billerica, MA).

4.3.2.3. Aptamer selection

For enrichment of biotinylated-ssDNA (aptamer) candidates, a total of 8 rounds of SELEX were performed. About 300-500 pmoles of the aptamer pool was dissolved in 200 µl of 0.025% Tween 20-PBS (binding buffer), denatured by heating at 95°C for 10 min and renatured by flash cooling on ice for 10 min to allow intra-strand base pairing. The aptamer

pool was incubated with $10^6 – 10^7$ *S.* Typhimurium cells suspended in 10 µl PBS for 45 min at room temperature with gentle rotation. Aptamer-bound cells were recovered by centrifugation at 1500 x g for 10 min followed by washing 2-3 times in 500-1000 µl binding buffer to remove unbound and non-specifically bound aptamers. Cells were then reconstituted in a final volume of 100-120 µl using molecular grade water. Aptamer sequences bound to cells were directly enriched by PCR amplification using the Forward Constant Region primer and Biotin-Reverse Constant Region primer as described above. The biotin-labeled aptamer pool was separated by alkaline denaturation in preparation for the next round of SELEX.

To assure the specificity of aptamer candidates, two rounds of counter-SELEX were performed (Figure 4.1). Briefly, the selected aptamer pool was incubated with the pooled counter-SELEX bacterial cocktail (non-*S.* Typhimurium strains described above) in binding buffer for 45 min at room temperature with moderate shaking. The aptamer-bound cells were recovered and discarded, while the unbound aptamers in the supernatant were collected for further rounds of selection. After the 8^{th} round of SELEX, aptamer-bound *S.* Typhimurium cells were sorted into different pools based on fluorescence intensity using a Beckman Coulter MoFlo® modular flow cytometer (Beckman Coulter, Inc, Fullerton, CA). Cells with the greatest fluorescence intensity (total 1.5 x 10^5 cells) were sorted and bound aptamer sequences were subjected to further identification and characterization.

4.3.3. Identification and Characterization of *Salmonella* Specific Aptamers

4.3.3.1. Identification of Aptamer Sequences

The sorted aptamer pool was subjected to cloning and sequencing. Briefly, the pool was amplified by PCR using the Forward Constant Region primer and Reverse Constant Region primer as described above. The PCR product was electrophoresed on 1.25% agarose gel in 1X modified TAE buffer and the amplicon band purified using an Amicon® Ultrafree®-DA centrifugal unit for DNA extraction from agarose (Millipore). The purified aptamer pool was treated with DNA Polymerase I (Klenow fragment) (Invitrogen, Carlsbad, CA) for 20 min on ice to produce blunt-ended aptamer sequences which were then ligated into the pCR®-Blunt vector using T4 DNA ligase provided in the Zero Blunt® PCR cloning kit (Invitrogen). The ligated vectors were transformed into One Shot® Top10 chemically competent *E. coli* cells (Invitrogen) and 30-50 µl of cells were plated and incubated overnight at 37°C for 20-24 h on high salt Luria-Bertani (LB) agar plates supplemented with kanamycin (50 µg/ ml). Plasmid DNA from individual transformants was extracted using the QIAprep® Spin plasmid Miniprep Kit (Qiagen Inc., Valencia, CA). The size of the amplified product was confirmed by PCR and visualized using 2% agarose gel electrophoresis. Transformants with an amplicon band size of 81 bases were selected for sequencing, which was performed at the Genomic Sciences Laboratory (GSL), North Carolina State University, Raleigh, North Carolina. Sequencing reactions were performed for plasmid DNA (200-500 ng) in 10 µl total volume containing 2 µl BigDye® v3.1 (Applied Biosystems, Foster City,

CA), 0.5X sequencing buffer (Applied Biosystems) and 0.32 pmoles M-13 universal reverse primer. After initial denaturation at 96°C for 1 min, 25 cycles consisting of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min were performed using a DNA Engine (PTC-200) thermal cycler for all sequencing reactions. The sequences were analyzed using Sequence Scanner Software V1.0 (Applied Biosystems).

4.3.3.2. Flow Cytometric Analysis of Aptamer Binding Affinity

All binding affinity assays of FAM-labeled aptamers were performed by flow cytometry using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA). The mean fluorescence intensity and percentage of fluorescent cells (n = 200, 000) occurring as a consequence of aptamer binding was determined in these assays. Data from the FACSCalibur™ was analyzed using BD CellQuest™ Pro software (BD Biosciences) and histogram overlays were created with BD CellQuest™ Pro and Microsoft Office Excel 2003.

4.3.3.3. Preliminary Screening and Prediction of Candidate Aptamer Structure

The unique aptamer sequence inserts in plasmid DNA characterized as described above were amplified by PCR using the Forward Constant Region primer and the Biotin-Reverse Constant Region primer. The biotin-labeled aptamer sequences were separated by alkaline denaturation. Preliminary binding assays using 300 pmoles biotin-labeled candidate aptamers were performed on 10^6- 10^7 intact cells of *S.* Typhimurium. The binding analysis

using the FACSCalibur™ flow cytometer was performed on biotin-aptamer bound cells after treating with 5-8 µg streptavidin-FITC tags (Invitrogen) as labels. In addition, the structural folding (secondary structure) of aptamer sequences displaying binding affinity to *S.* Typhimurium was predicted using the online software DNA Mfold version 3.2 (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi) (Zuker, 2003).

4.3.3.4. Aptamer Binding Characterization

Aptamers S8-7 and S8-46 were selected for further characterization because of their high binding efficiency to *S.* Typhimurium during preliminary screening. Highly purified and desalted aptamer S8-7 and S8-46 (5'-labeled with biotin) were obtained from Integrated DNA Technologies, Inc. Varying concentrations of biotinylated aptamers S8-7 and S8-46 (0.37, 0.74, 1.47, 2.9, 4.28, 6.94 µM) were prepared in binding buffer and incubated with 10^6 -10^7 washed *S.* Typhimurium cells at room temperature for 45 min with moderate shaking. The bacterial cells were concentrated by centrifugation at 1500 x g and washed with 500 µl of binding buffer. Prior to subjecting the concentrate to flow cytometry, the solution was treated with 5-8 µg of streptavidin-FITC tags which bind to the aptamer-bound pool but not to the unbound *Salmonella* cells. Two independent trials for this binding analysis were performed at each concentration. The equilibrium dissociation constant (K_d) was calculated by plotting the average total per cent fluorescent bacterial cells (Y), which corresponded to aptamer-bound *Salmonella*, against the concentration of aptamer (X) using a non-interacting binding sites model in SigmaPlot (Jandel, San Rafel, CA).

4.3.3.5 Confirmation of Aptamer S8-7 Binding Exclusivity

The performance of aptamer S8-7 exceeded that of S8-46 in binding studies, so the former was chosen for further characterization. Specifically, aptamer S8-7 was evaluated for cross reactivity with related and unrelated bacterial genera. Binding exclusivity studies were done with *B. cereus* (ATCC49063), *E. coli* O157:H7 (ATCC43895), and *Citrobacter braakii* (ATCC29219) in separate experiments. In these experiments, 10^6 -10^7 bacterial cells of each genus were mixed with a 6.94 μM solution of aptamer S8-7 and held at 25°C for 45 min with moderate agitation to facilitate aptamer binding. Cells were centrifuged at 1500 x g, washed with 500 μl of binding buffer, and prepared for analysis using flow cytometry.

4.3.4 Proof-of-Concept that Biotinylated Aptamers Can be Used in a qPCR-Based Capture-Detection Platform

4.3.4.1 Capture of *S.* Typhimurium

An overnight culture of *S.* Typhimurium was 10-fold serially diluted (10^8-10^2 CFU) in a total of 90 μl PBS buffer, out of which 40 μl of this cell suspension was exposed to a 3.45 μM solution of aptamer S8-7 suspended in binding buffer. After incubation at room temperature for 30 min, the cells were centrifuged and washed 2 times in 500 μl binding buffer and then resuspended in 450 μl binding buffer. Immobilization of suspended aptamer bound cells was performed using 50 μl of streptavidin-coated magnetic beads (Promega) for

10 min at room temperature using a roller. The bead-bound cells were washed twice using 500 µl binding buffer and recovered using a Dynal MPC®-M magnetic particle concentrator. Genomic DNA was extracted from the bead-bound cells using a NucleoSpin® Food genomic DNA extraction kit (Macherey-Nagel, Duren, Germany) with a minor modification in protocol. In brief, the 400 µl solution of lysed cells from step 2 was mixed with equal volume of proprietary buffer C4 and ethanol in step 3 of the protocol. Out of this 1200 µl mixed solution, 1000 µl was passed through the silica membrane column and final elution of DNA was done using 35 µl of proprietary elution buffer.

4.3.4.2. qPCR Detection of Captured *S.* Typhimurium

Quantitative real-time PCR amplification of extracted DNA was done using primers and TaqMan™ probe targeting the invasion A (*inv*A) gene as initially reported by Hein et al. (2006) (Table 4.1). The 25 µl of reaction mixture contained 1 X buffer (Promega), 0.3 mM dNPT mix (Applied Biosystems), 300 pmoles forward *inv*A primer and 300 pmoles reverse *inv*A primer, 0.1U Go *Taq* polymerase (Promega), 200 pmoles *inv*A Taqman™ probe (Table 4.1), 2 µg BSA (Promega) and 2.5 µl of extracted target DNA. Amplification was performed in a SmartCycler (Cepheid, Sunnyvale, CA) using a thermal protocol that consisted of initial denaturation at 94˚C for 120 sec followed by 40 cycles of 94˚C for 20 sec and 60˚C for 30 sec. For construction of the standard curve, DNA isolated from an overnight culture of *S.* Typhimurium was ten-fold serially diluted and 2.5 µl of each dilution amplified using the protocol described above. The standard curve was constructed as Ct value (Y axis) vs. CFU

equivalent (X axis), where the latter was defined as the estimated CFU corresponding to the input level of DNA per PCR reaction, assuming DNA extraction efficiency of 100%. The approximate CFU in unknown samples was extrapolated from the standard curve based on Ct values obtained by qPCR. Capture efficiency was expressed as the ratio of CFU equivalents to CFU (as determined by plate count of stock culture) multiplied by 100. To compare the mean capture efficiency at various inoculum levels, one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison Test ($p<0.05$) was performed using GraphPad Prism® version 4.0 for Windows (GraphPad Software, San Diego, CA).

4.4. RESULTS

4.4.1. Selection of Aptamers with Binding Affinity to *S.* Typhimurium

A total of 8 rounds of SELEX and two rounds of counter SELEX were performed to select for aptamers with binding affinity to *S.* Typhimurium. The separation of cell-bound aptamers was done using a combination of centrifugal washing and cell sorting by flow cytometry. The selected aptamers were cloned and a total of 46 transformants were selected for aptamer sequencing. From these, 18 unique aptamer sequences were obtained (including maximum repeated sequences) and these were pre-screened for binding interaction with *S.* Typhimurium cells (10^6 -10^7) using 300 pmoles of each sequence. Flow cytometric analysis of binding interaction as applied to the first 200,000 cells revealed differential binding efficiency for each of the candidates, with a low percent binding of 2% and a high of 14% (Figure 4.2). Fluorescent pools corresponding to aptamer sequences S8-46 and S8-7 had the

highest percentage of fluorescent cells (13-14%) and these were selected for further binding characterization studies.

4.4.2. Dissociation Constants for Aptamers S8-46 and S8-7

The percent binding efficiency as a function of aptamer concentration upon interaction with 10^6-10^7 *S.* Typhimurium cells was evaluated in two independent flow cytometry runs. Aptamer S8-46 bound 5.9 ± 1.2, 7.8 ± 1.7, 8.9 ± 0.5, 10.4 ± 0.4, 12.8 ± 0.2 and 14.6 ± 0.8 percent cells at concentrations of 0.37, 0.74, 1.47, 2.9, 4.28, 6.94 μM, respectively (Figure 4.3); binding for S8-7 at the same concentrations was 6.8 ± 0.6, 8.7 ± 0.2, 10.0 ± 0.5, 15.3 ± 1.1, 17.1 ± 2.4, 22.0 ± 3.4 percent fluorescent cells, respectively (Figure 4.4). When these binding assay data were fitted to a non-interacting binding site model, the dissociation constant for aptamer S8-46 was 0.74 ± 0.20 μM and that for aptamer S8-7 was 1.73 ± 0.54 μM. Although aptamer S8-46 had better binding affinity as per its dissociation constant, the percent fluorescent cells was greater for aptamer S8-7. Prior to selection of the leading aptamer candidate, performance of the two aptamers was also compared using qPCR applied to *Salmonella* cells captured by both aptamers at three different cell concentrations (10^8, 10^5, 10^2 CFU). The resulting Ct values for aptamer S8-7 (22.34, 31.56, no signal) were lower than those for aptamer S8-46 (23.76, no signal, no signal), suggesting better performance for S8-7. Hence, aptamer S8-7 was selected for further study.

4.4.3. Binding Exclusivity of Aptamer S8-7

Individual binding assays with 10^6-10^7 CFU of non-*Salmonella* cells of representative genera were performed using a 6.94 μM aptamer S8-7 solution as analyzed by flow cytometry. A low degree of cross-reactivity was observed for *E. coli* O157:H7 (ATCC43895) (3.16 % fluorescent cells) (Figure 4.5.A) and *Citrobacter braakii* (ATCC29219) (1.84 % fluorescent cells) (Figure 4.5.B) when compared to the target *S. Typhimurium* cells (17.43 %) (Figure 4.5.C); however, moderate cross-reactivity was recorded for *B. cereus* (ATCC49063) (9.2 % fluorescent cells) (data not shown).

4.4.4. Prediction of 3-Dimensional Folding of Aptamer S8-7

Based on minimum free energy ΔG^0 = - 4.1kcal/ mol at 37 °C, the structural folding of aptamer S8-7 was predicted to consist of an external loop of 45 bases and 2 closing helices (Figure 4.6). The first helix consists of a hairpin loop with closing pair between N54-N65 and the second helix consists of an internal loop with external closing pair between N8-N23.

4.4.5. *S.* Typhimurium Can be Detected Using qPCR After Prior Capture with Aptamer S8-7 Capture Assay

The lower limit of detection (LOD) of the combined aptamer capture-qPCR assay was 10^2-10^3 CFU equivalents in 290 μl buffer sample (Figure 4.7). The mean capture

efficiency (%) of aptamer S8-7 as applied to a pure culture of *S.* Typhimurium serially diluted in buffer was 3.6 ± 2.6% at inoculum levels of 8.4 ± 0.2 $\log_{10}$ CFU/290 µl sample; 6.9 ± 3.2% at 7.4 ± 0.2 $\log_{10}$ CFU; 10.9 ± 5.5% at 6.4 ± 0.2 $\log_{10}$ CFU; 11.3 ± 5.8% at 5.4 ± 0.2 $\log_{10}$ CFU; 12.6 ± 5.0% at 4.4 ± 0.2 $\log_{10}$ CFU; and 11.4 ± 7.2% at 3.4 ± 0.2 $\log_{10}$ CFU. The mean capture efficiency increased slightly when comparing high *S.* Typhimurium inoculum levels to low inoculum levels but this increase was not statistically significant. *S.* Typhimurium could not be detected by qPCR when captured with aptamer S8-7 at inoculum levels of $<10^2$-10^3 CFU/290 µl sample (Figure 4.8).

4.5. DISCUSSION

Nucleic acid aptamers are novel ligands with unique structural folding that have been reported to have a high degree of binding affinity and specificity to cell surface targets of pathogens such as *Campylobacter jejuni* (Bruno et al., 2009), *S. enterica* (Joshi et al., 2009), *S. enterica* serovar. Typhi (Hsu et al., 2005), *Francisella tularensis* (Vivekananda & Kiel, 2006), *Bacillus anthracis* vaccine strain A (Zhen et al., 2002), *Mycobacterium tuberculosis* (Chen et al., 2007), *E. coli* (So et al., 2008) and *Staphylococcus aureus* (Cao et al., 2009). Aptamers with pathogen specificity are traditionally selected using the enrichment process of SELEX as applied to crude or purified extracellular surface targets. Multiple iterations of aptamer enrichment consisting of binding with the purified target(s) followed by separation and amplification of the aptamer sequences showing a high degree of binding results into enrichment of "pure" solutions of candidate aptamers.

In this study, we applied a whole-cell SELEX as an alternative to traditional SELEX approaches that are based on the use of purified cell components. Whole-cell SELEX has previously been applied for the identification of aptamers specific to microbial pathogens such as *Mycobacterium tuberculosis* (Chen et al., 2007), *Staphylococcus aureus* (Cao et al., 2009), *Trypanosoma brucei* (Homann & Goringer, 1999), and *Trypanosoma cruzi* (Ulrich et al., 2002). The whole-cell SELEX has advantages over the more traditional approach because aptamers selected against isolated and purified cell membrane protein targets may not be functional when applied to the surface of live host cells (Fang & Tan, 2009; Pestourie et al., 2006; Shamah et al., 2008). This occurs because a key component to aptamer functionality is the necessity to bind to the natural 3-dimensional epitopic presentation of the target.

From the total of 46 sequenced transformants, 18 unique aptamer sequences were selected for preliminary screening of binding affinity. As has been reported by others, none of the sequences demonstrated absolute (100%) binding affinity (Bibby et al., 2008; Lorger et al., 2003; Shangguan et al., 2006). Aptamers S8-46 and S8-7 demonstrated the highest binding efficiency in terms of percent fluorescent cells. Further characterization of binding revealed an apparent dissociation constant for both candidate aptamers (S8-46 = 0.74 ± 0.20 μM and S8-7 = 1.73 ± 0.54 μM) in the lower μM to higher nM range. These binding affinities are consistent with those previously reported for aptamers generated by whole-cell SELEX. For example, previous studies have reported binding at high μM [binding constant K_{1a} between aptamer NK2 and *Mycobacterium tuberculosis* (H37Rv) interaction = $1.84 \times 10^5 (\pm 1.5 \times 10^4)\ M^{-1}$] (Chen et al., 2007) to nM [dissociation constant K_d between aptamers sga16 and the target CCRF-CEM Cell interaction = 5.0 ± 0.52 nM] ranges

(Shangguan et al., 2006). The inability of aptamers to bind 100% of available target cells can be explained by variation in antigenic expression in bacterial cell populations as a function of growth phase. This phenomenon has been documented when whole cell SELEX was used to generate DNA aptamers specific for *Staphylococcus aureus* (Cao et al., 2009).

The dissociation constant for the *Salmonella* aptamers was calculated using a non-interacting binding site (one-site saturation) model with the non-linear least square method based on the overall change in the number of fluorescently labeled cells (Davis et al., 1998; Shangguan et al., 2006). This model was chosen because of our lack of knowledge about the number of individual aptamer recognition sites residing on the surface of an individual cell. Further experiments are necessary to ascertain if polyvalent interactions exist between aptamers and target cell binding.

To facilitate their use as diagnostic probes in *in-vitro* assays, aptamers are oftentimes labeled with dyes such as fluorescein (FAM) and Cy5 (Cao et al., 2009; Shangguan et al., 2006; Stanlis & McIntosh, 2003; Ulrich et al., 2004; Vivekananda & Kiel, 2006). Multiple arrays of biotinylated aptamer-functionalized aptasensors have been constructed and conjugated with avidin-linked gold surfaces for detection of the HIV- transactivating regulatory protein (HIV-1 Tat) (Tombelli et al., 2005). We, too, produced biotinylated aptamers but in this case, used them for conjugation to streptavidin-coated magnetic beads. The approach allowed us to use the aptamers as capture ligands, providing proof-of-concept of their applicability to pre-analytical sample processing. A similar approach was reported by Joshi et al. (2009) who used aptamer-labeled magnetic beads to capture *Salmonella* cells from artificially contaminated fecal and chicken rinsate samples for downstream detection

using qPCR. The interest in aptamer technology as applied to detection of food borne pathogens is indeed growing. For instance, a recent study demonstrated that DNA aptamers could be used for the capture and detection of *C. jejuni* in an assay format which consisted of a spectrofluorometer and a handheld fluorometer. This assay was able to detect as few as 2.5 CFU equivalents of *C. jejuni* per ml of aptamer binding buffer and 10-250 CFU per ml of diluted food matrices without prior cultural enrichment (Bruno et al., 2009).

The candidate aptamers reported in our study are a step in the right direction but not necessarily ideal in their performance. Greater binding efficiency can be achieved by modifying the SELEX process. For example, increasing the stringency during selection by using a greater number of washes or increased volume during washing may be of help in this regard. We could also expand the total number of SELEX iterations for improved aptamer binding efficiency (sensitivity). And although aptamer S8-7 showed minimal cross-reactivity with *E. coli* O157:H7 (ATCC43895) and *Citrobacter braakii* (ATCC29219), moderate cross-reactivity was observed for *B. cereus* (ATCC49063). Counter selection during whole-cell SELEX has been reported to be effective in some but not all applications (Cerchia et al., 2005; Pestourie et al., 2006; Shangguan et al., 2006). In particular, counter-SELEX performed against a mixed bacterial pool may be less effective than that applied to individual non-target bacterial cultures. Additional iterations of counter-SELEX with pure non-target cultures and/or perhaps with inclusion of additional non-target strains, may improve the specificity of our aptamers. Clearly, further studies are also required to validate the inclusivity of candidate aptamers with respect to their ability to bind to various field strains of serovar Typhimurium, as well as their specificity to other non-Typhimurium *Salmonella*

serovars. Further scale-up studies are necessary to demonstrate equivalent capture as applied to much larger and more realistic sample volumes and to a wider variety of sample matrices.

To our knowledge this is the first report on the use of whole-cell SELEX to identify DNA aptamers specific for *S.* Typhimurium. With further optimization, we anticipate that these DNA aptamers or their derivatives can be employed for direct capture of *S.* Typhimurium from complex food matrices in preparation for subsequent detection by qPCR. In addition to pre-analytical sample preparation, DNA aptamers could also be used for simultaneous capture and detection of a pathogen in complex sample matrices (Bruno et al., 2009). For example, aptamers can be labeled with different dyes and chemistry linkers for application in detection platforms such as flow cytometry (Chen et al., 2007; Shangguan et al., 2006), aptamer-linked immobilized sorbent assay (Drolet et al., 1996; Vivekananda & Kiel, 2006), calorimetric analysis (Chen et al., 2007), dot blot assay (Vivekananda & Kiel, 2006), proximal ligation assay (Fredriksson et al., 2002), and for biosensor analysis (Liss et al., 2002). The whole-cell SELEX process is a simple, straightforward and reproducible approach for identification of DNA aptamers specific to any number of microbial targets. The combined aptamer capture-qPCR approach has potential for development into a direct detection assay for *Salmonella* in real world food samples.

Table 4.1. Oligonucleotides used in the selection and characterization of aptamers with binding affinity to *S. enterica* serovar. Typhimurium.

Name	**Oligonucleotides**
	SnaB I Pst I EcoR V Bgl II
DNA Aptamer Library	5′-GTATACGTATTACCTGCAGC - N_{40} - CGATATCTCGGAGATCTTGC-3′
Biotin- Reverse Constant Region Primer	5′-/5Biosg/GCAAGATCTCCGAGATATCG -3′
Forward Constant Region Primer	5′-AGTATACGTATTACCTGCAGC -3′
Reverse Constant Region Primer	5′-GCAAGATCTCCGAGATATCG -3′
Forward-*inv*A Primer (Hein et al., 2006)	5’- GTG AAA TTA TCG CCA CGT TCG GGC AA - 3'
Reverse-*inv*A Primer (Hein et al., 2006)	5' - TCA TCG CAC CGT CAA AGG AAC C - 3'
Taqman™ Probe (*inv*A) (Hein et al., 2006)	5' - /56-FAM/TTA TTG GCG ATA GCC TGG CGG TGG GTT TTG TTG /3BHQ_1/ - 3'

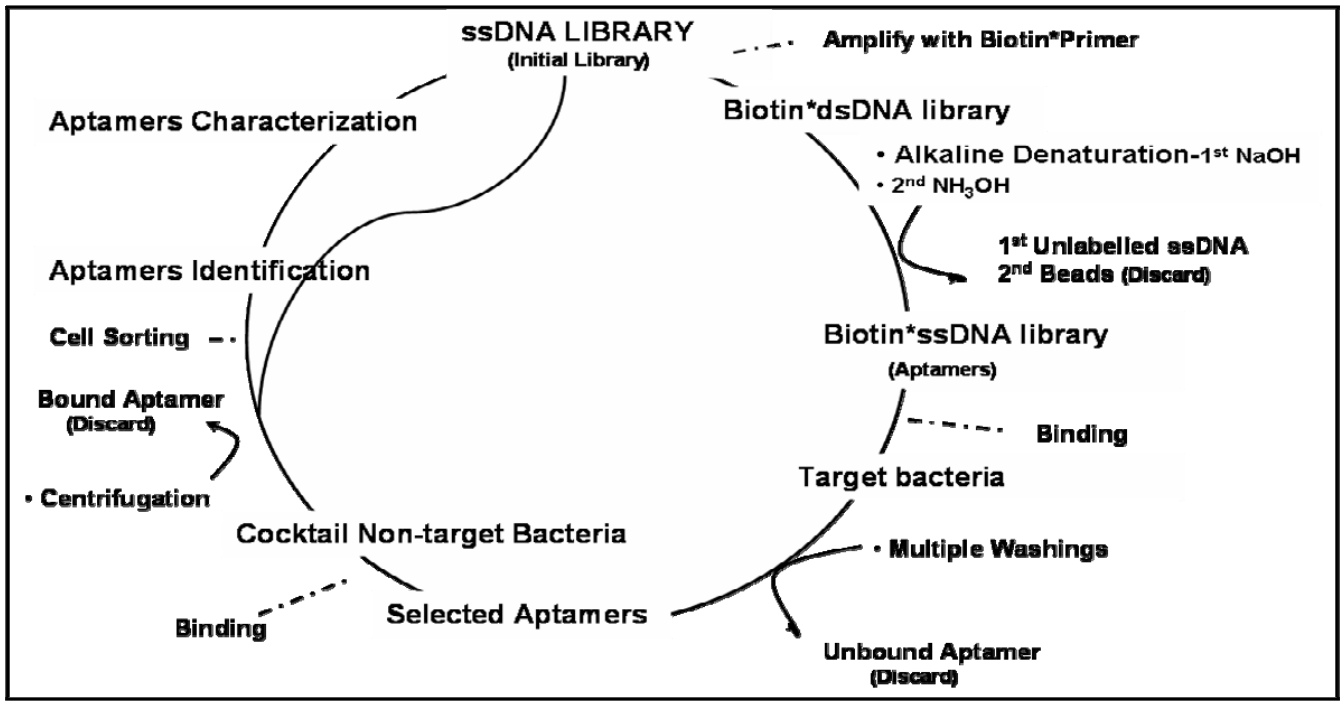

Figure 4.1. Schematic representation of whole-cell SELEX process for aptamer selection against *S.* Typhimurium. The whole-cell SELEX consisted of 8 rounds of selection using *S.* Typhimurium cells as the target and 2 rounds of counter-selection against a pooled cocktail of non-target bacteria, as applied to a combinatorial library of biotin-labeled oligonucleotide sequences.

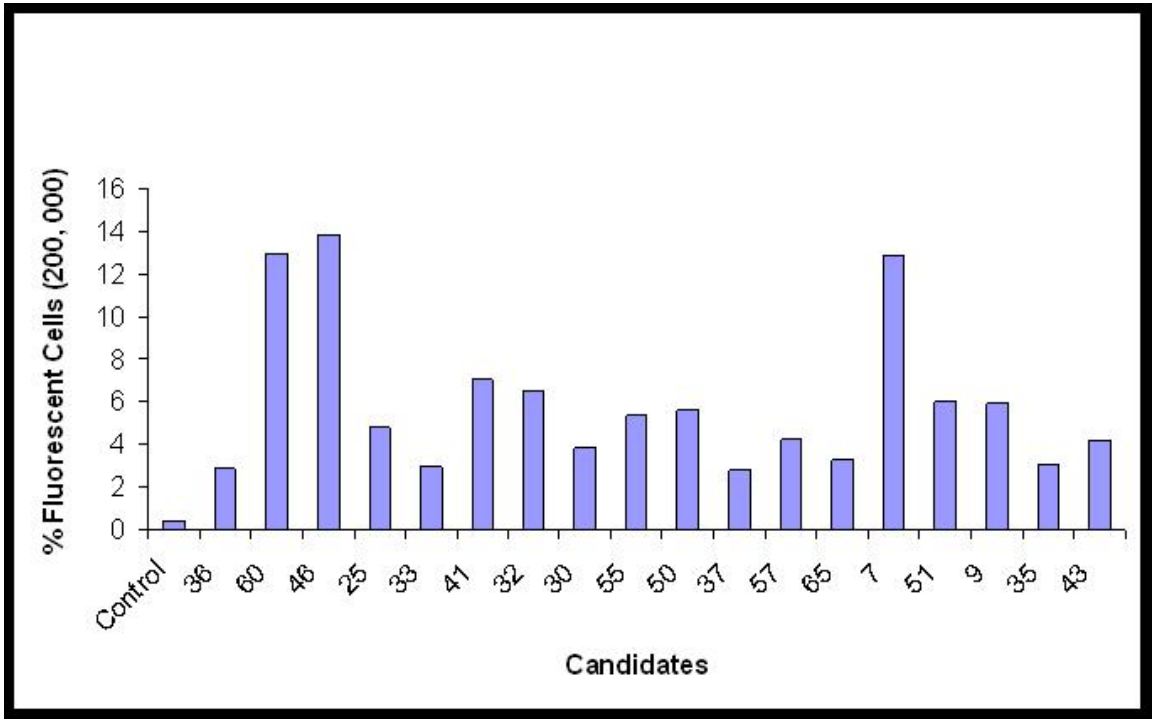

Figure 4.2. Preliminary screen of binding efficiency of panel of 18 candidate aptamer sequences against *S.* Typhimurium. Binding assays using 300 pmoles biotin-labeled candidate aptamers were performed on 10^6- 10^7 intact cells of *S.* Typhimurium and aptamer bound cells (n = 200, 000) were analyzed using FACSCalibur™ flow cytometry.

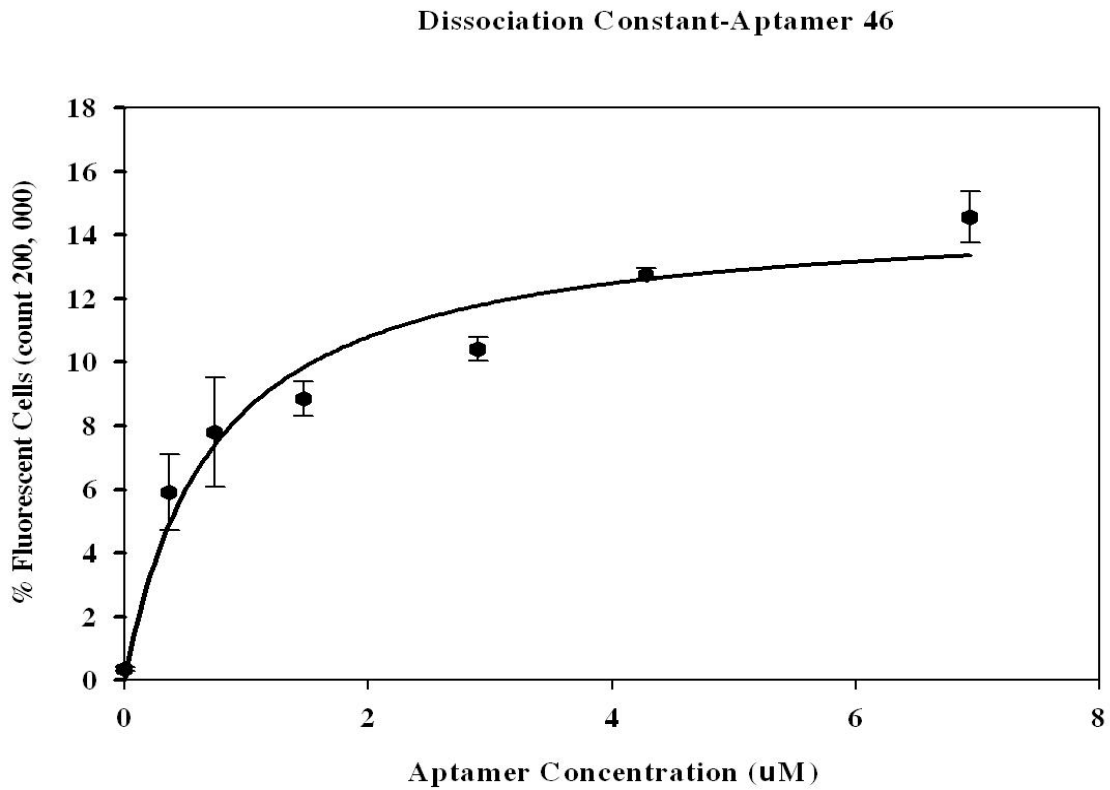

Figure 4.3. Binding affinity analysis of aptamer S8-46. Solutions with varying concentrations of biotinylated aptamer S8-46 were incubated with 10^6-10^7 *S.* Typhimurium cells and analyzed using flow cytometry (n = 200, 000). The equilibrium dissociation constant (K_d) was calculated by plotting the average total per cent fluorescent bacterial cells (Y) against the concentration of aptamer (X) using a non-interacting binding sites model. The resulting K_d value = 0.74 ± 0.20 μM.

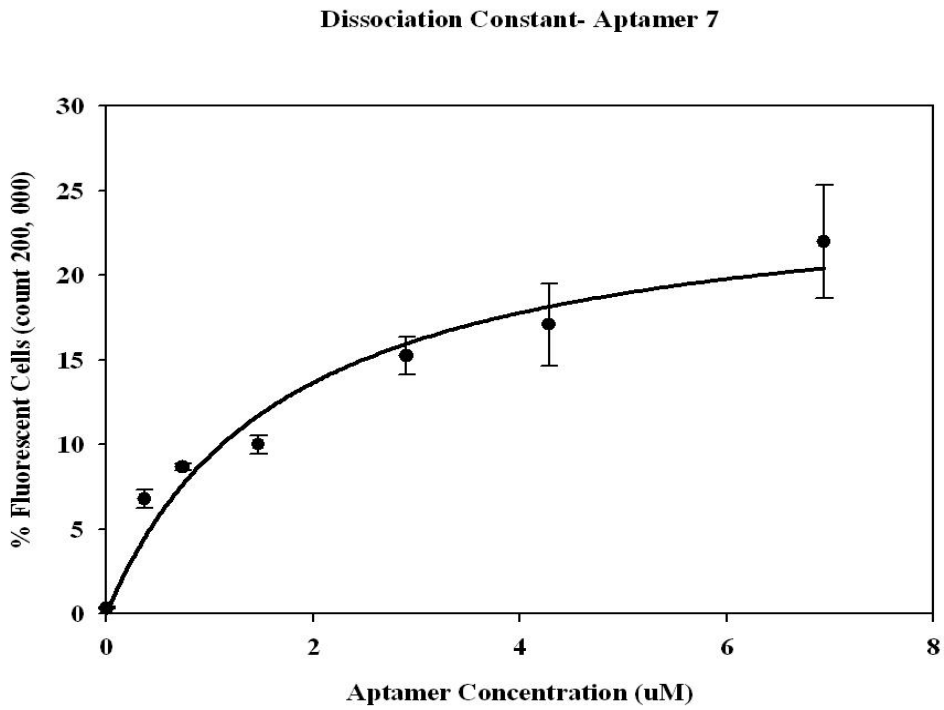

Figure 4.4. Binding affinity analysis of aptamer S8-7. Solutions with varying concentrations of biotinylated aptamer S8-7 were incubated with 10^6 -10^7 *S.* Typhimurium cells and analyzed using flow cytometry (n = 200, 000). The equilibrium dissociation constant (K_d) was calculated by plotting the average total per cent fluorescent bacterial cells (Y axis) against the concentration of aptamer S8-7 (X axis) using a non-interacting binding sites model. The resulting K_d value = 1.73 ± 0.54 μM.

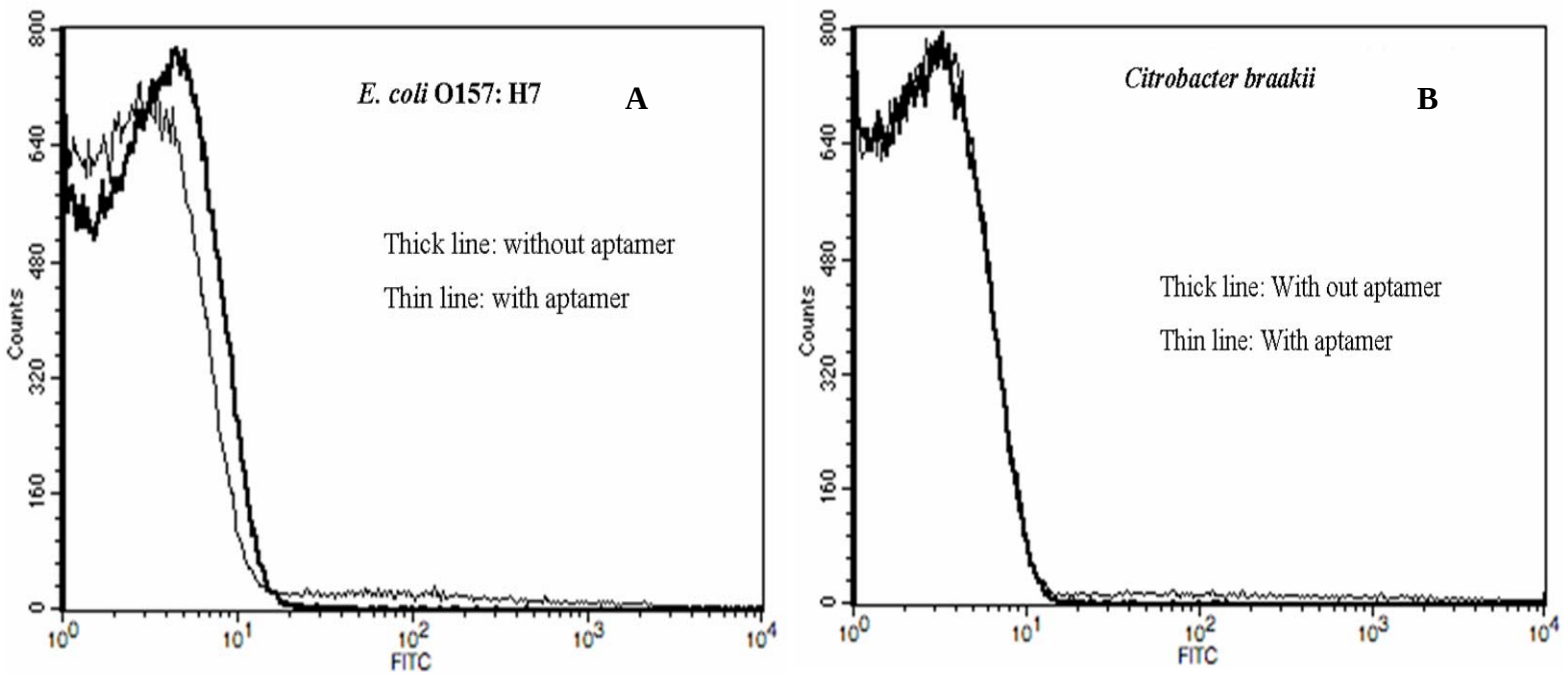

Figure 4.5. Binding exclusivity analysis of aptamer S8-7. Approximately 10^6 -10^7 *E. coli* O157:H7 (A) or *Citrobacter braakii* (B) cells were mixed with a 6.94 µM solution of biotinylated S8-7 and first 200, 000 cells were analyzed for the fluorescence intensity after mixing with streptavidin-FITC labels using flow cytometry. For *E. coli* O157:H7, 3.16 % cells (Figure 4.5.A.); for *Citrobacter braakii*, 1.84 % cells turned fluorescent (Figure 4.5.B.).

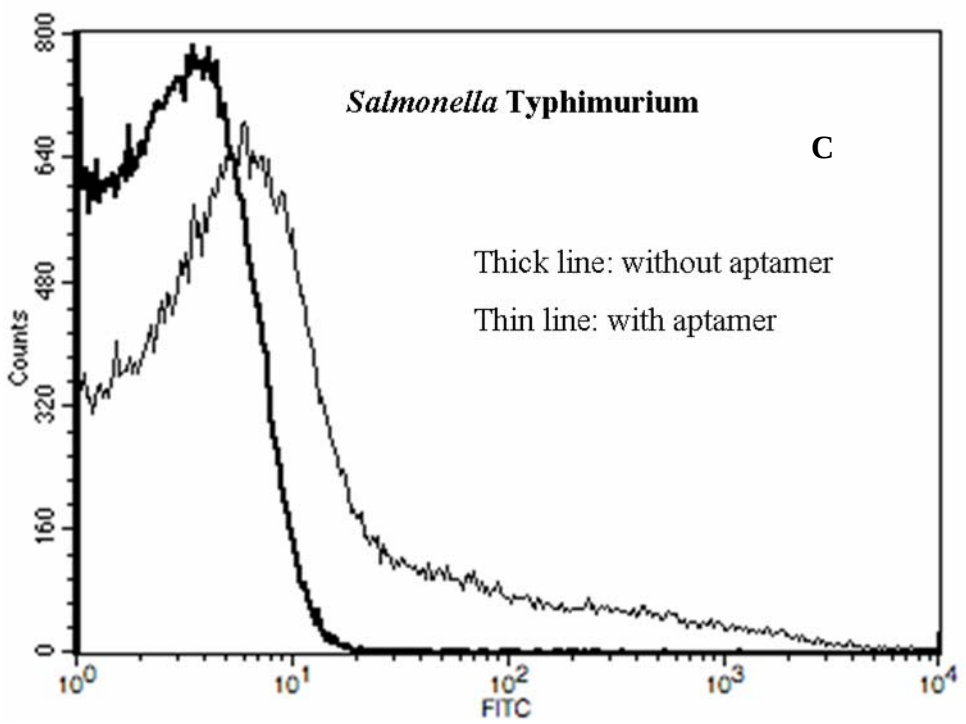

Figure 4.5.C. Binding analysis of aptamer S8-7 as allied to *S.* Typhimurium (positive control). Approximately 10^6 -10^7 *S.* Typhimurium cells were mixed with a 6.94 μM solution of S8-7 and analyzed using flow cytometry (n = 200, 000) which resulted in 17.43 % fluorescent cells.

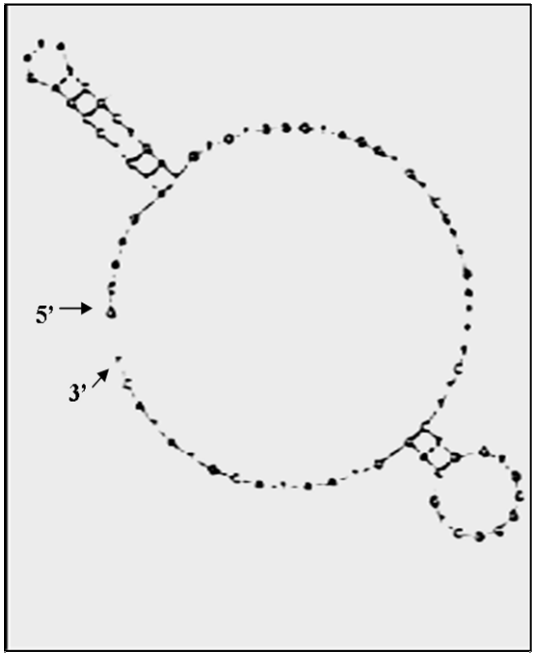

Figure 4.6. Predicted structural folding of DNA aptamer Sal8-7 at 37°C. Predicted structure consists of an external loop of 45 bases and 2 closing helices.

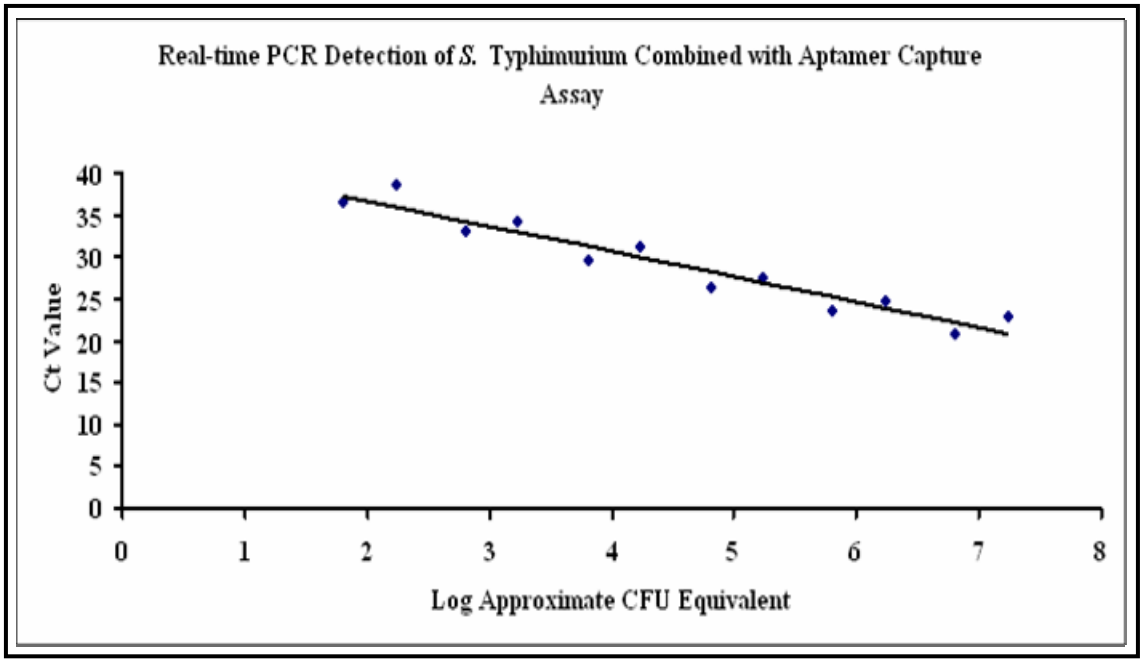

Figure 4.7. Capture of *S.* Typhimurium using aptamer S8-7 followed by detection using qPCR. The lower limit of detection (LOD) of the combined aptamer capture-qPCR assay was 10^2-10^3 CFU equivalents in serially diluted pure cultures of *S.* Typhimurium.

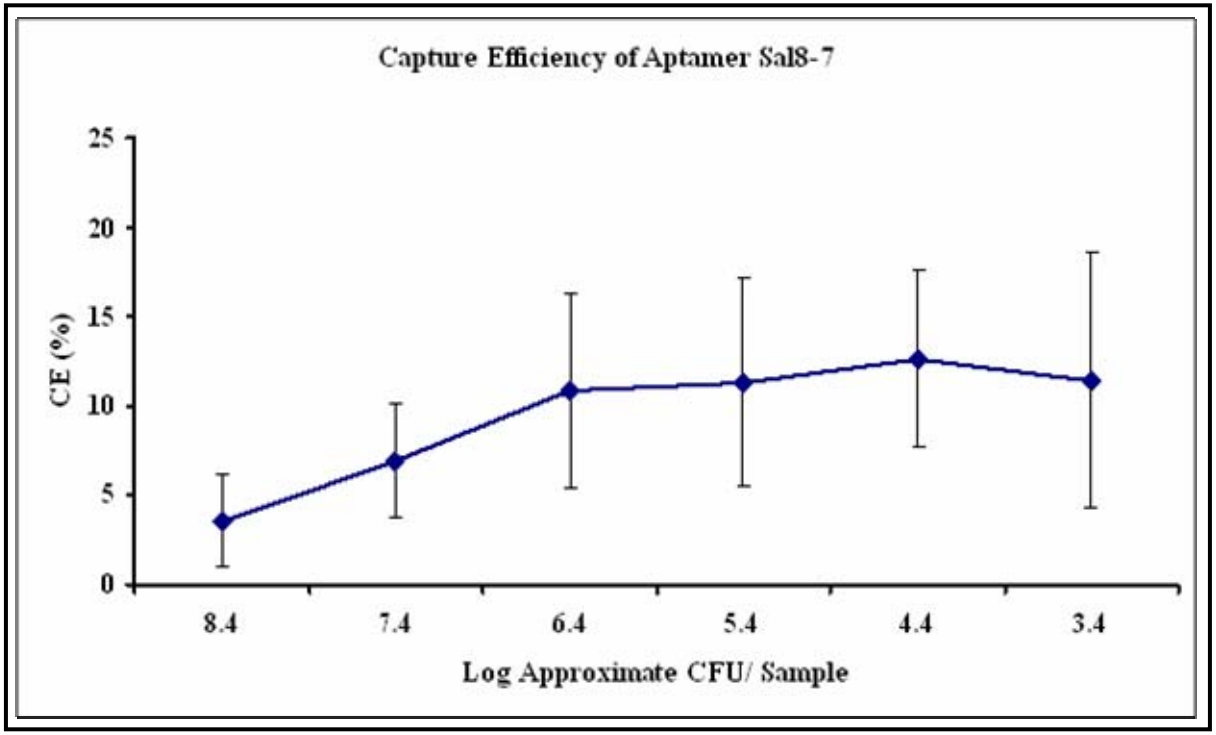

Figure 4.8. Mean capture efficiency of aptamer S8-7 as a function of initial level of *S.* Typhimurium suspended in PBS.

4.6. REFERENCES

Bell, C., and A. Kyriakides. (2002). *Salmonella: a practical approach to the organism and its control in foods.* . Blackwell Science Ltd., Oxford, United Kingdom

Bibby, D. F., Gill, A. C., Kirby, L., Farquhar, C. F., Bruce, M. E. & Garson, J. A. (2008). Application of a novel in vitro selection technique to isolate and characterise high affinity DNA aptamers binding mammalian prion proteins. *J Virol Methods*, 151(1), 107-15.

Bruno, J. G., Phillips, T., Carrillo, M. P. & Crowell, R. (2009). Plastic-adherent DNA aptamer-magnetic bead and quantum dot sandwich assay for *Campylobacter* detection. *J Fluoresc*, 19(3), 427-35.

Cao, X., Li, S., Chen, L., Ding, H., Xu, H., Huang, Y., Li, J., Liu, N., Cao, W., Zhu, Y., Shen, B. & Shao, N. (2009). Combining use of a panel of ssDNA aptamers in the detection of *Staphylococcus aureus*. *Nucleic Acids Res*, 37(14), 4621-8.

CDC. (2007). *Salmonella* Surveillance: Annual Summary, 2005. Atlanta, Georgia. US Department of Health and Human Services, CDC, 2007.

Cerchia, L., Duconge, F., Pestourie, C., Boulay, J., Aissouni, Y., Gombert, K., Tavitian, B., de Franciscis, V. & Libri, D. (2005). Neutralizing aptamers from whole-cell SELEX inhibit the RET receptor tyrosine kinase. *PLoS Biol*, 3(4), e123.

Chen, F., Zhou, J., Luo, F., Mohammed, A. B. & Zhang, X. L. (2007). Aptamer from whole-bacterium SELEX as new therapeutic reagent against virulent *Mycobacterium tuberculosis*. *Biochem Biophys Res Commun*, 357(3), 743-8.

Cullison, M. A. & Jaykus, L. A. (2002). Magnetized carbonyl iron and insoluble zirconium hydroxide mixture facilitates bacterial concentration and separation from nonfat dry milk. *J Food Prot*, 65(11), 1806-10.

Davis, K. A., Lin, Y., Abrams, B. & Jayasena, S. D. (1998). Staining of cell surface human CD4 with 2'-F-pyrimidine-containing RNA aptamers for flow cytometry. *Nucleic Acids Res*, 26(17), 3915-24.

Drolet, D. W., Moon-McDermott, L. & Romig, T. S. (1996). An enzyme-linked oligonucleotide assay. *Nat Biotechnol*, 14(8), 1021-5.

Eriksson, E. & Aspan, A. (2007). Comparison of culture, ELISA and PCR techniques for salmonella detection in faecal samples for cattle, pig and poultry. *BMC Vet Res*, 3, 21.

Fang, X. & Tan, W. (2009). Aptamers Generated from Cell-SELEX for Molecular Medicine: A Chemical Biology Approach. *Acc Chem Res*.

Farokhzad, O. C., Jon, S., Khademhosseini, A., Tran, T. N., Lavan, D. A. & Langer, R. (2004). Nanoparticle-aptamer bioconjugates: a new approach for targeting prostate cancer cells. *Cancer Res*, 64(21), 7668-72.

Favrin, S. J., Jassim, S. A. & Griffiths, M. W. (2001). Development and optimization of a novel immunomagnetic separation- bacteriophage assay for detection of *Salmonella enterica* serovar enteritidis in broth. *Appl Environ Microbiol*, 67(1), 217-24.

Feng, P. (2007). Rapid Methods for the Detection of Foodborne Pathogens: Current and Next Generation Technologies. 3ed. In *Food Microbiology: Fundamentals and Frontiers*, eds. M. P. Doyle & L. R. Beuchat, ASM Press. Washington, D.C., pp. 911-934.

Fredriksson, S., Gullberg, M., Jarvius, J., Olsson, C., Pietras, K., Gustafsdottir, S. M., Ostman, A. & Landegren, U. (2002). Protein detection using proximity-dependent DNA ligation assays. *Nat Biotechnol*, 20(5), 473-7.

Guo, K. T., Paul, A., Schichor, C., Ziemer, G. & Wendel, H. P. (2008). CELL-SELEX: Novel Perspectives of Aptamer-Based Therapeutics. *Int J Mol Sci*, 9(4), 668-78.

Hein, I., Flekna, G., Krassnig, M. & Wagner, M. (2006). Real-time PCR for the detection of *Salmonella* spp. in food: An alternative approach to a conventional PCR system suggested by the FOOD-PCR project. *J Microbiol Methods*, 66(3), 538-47.

Homann, M. & Goringer, H. U. (1999). Combinatorial selection of high affinity RNA ligands to live African trypanosomes. *Nucleic Acids Res*, 27(9), 2006-14.

Hsih, H. Y. & Tsen, H. Y. (2001). Combination of immunomagnetic separation and polymerase chain reaction for the simultaneous detection of *Listeria monocytogenes* and *Salmonella* spp. in food samples. *J Food Prot*, 64(11), 1744-50.

Hsu, C. F., Tsai, T. Y. & Pan, T. M. (2005). Use of the duplex TaqMan PCR system for detection of Shiga-like toxin-producing *Escherichia coli* O157. *J Clin Microbiol*, 43(6), 2668-73.

Jacobsen, C. S. & Rasmussen, O. F. (1992). Development and Application of a New Method To Extract Bacterial DNA from Soil Based on Separation of Bacteria from Soil with Cation-Exchange Resin. *Appl Environ Microbiol*, 58(8), 2458-2462.

Jayasena, S. D. (1999). Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clin Chem*, 45(9), 1628-50.

Joshi, R., Janagama, H., Dwivedi, H. P., Senthil Kumar, T. M., Jaykus, L. A., Schefers, J. & Sreevatsan, S. (2009). Selection, characterization, and application of DNA aptamers for the capture and detection of *Salmonella enterica* serovars. *Mol Cell Probes*, 23(1), 20-8.

Kumar, S., Balakrishna, K. & Batra, H. V. (2008). Enrichment-ELISA for detection of *Salmonella typhi* from food and water samples. *Biomed Environ Sci*, 21(2), 137-43.

Liss, M., Petersen, B., Wolf, H. & Prohaska, E. (2002). An aptamer-based quartz crystal protein biosensor. *Anal Chem*, 74(17), 4488-95.

Lorger, M., Engstler, M., Homann, M. & Goringer, H. U. (2003). Targeting the variable surface of African trypanosomes with variant surface glycoprotein-specific, serum-stable RNA aptamers. *Eukaryot Cell*, 2(1), 84-94.

Mercanoglu, B. & Griffiths, M. W. (2005). Combination of immunomagnetic separation with real-time PCR for rapid detection of Salmonella in milk, ground beef, and alfalfa sprouts. *J Food Prot*, 68(3), 557-61.

Pestourie, C., Cerchia, L., Gombert, K., Aissouni, Y., Boulay, J., De Franciscis, V., Libri, D., Tavitian, B. & Duconge, F. (2006). Comparison of different strategies to select aptamers against a transmembrane protein target. *OLIGONUCLEOTIDES*, 16(4), 323-35.

Prusak-Sochaczewski, E. & Luong, J. H. (1989). An improved ELISA method for the detection of *Salmonella* Typhimurium. *J Appl Bacteriol*, 66(2), 127-35.

Shamah, S. M., Healy, J. M. & Cload, S. T. (2008). Complex target SELEX. *Acc Chem Res*, 41(1), 130-8.

Shangguan, D., Li, Y., Tang, Z., Cao, Z. C., Chen, H. W., Mallikaratchy, P., Sefah, K., Yang, C. J. & Tan, W. (2006). Aptamers evolved from live cells as effective molecular probes for cancer study. *Proc Natl Acad Sci U S A*, 103(32), 11838-43.

So, H. M., Park, D. W., Jeon, E. K., Kim, Y. H., Kim, B. S., Lee, C. K., Choi, S. Y., Kim, S. C., Chang, H. & Lee, J. O. (2008). Detection and titer estimation of *Escherichia coli* using aptamer-functionalized single-walled carbon-nanotube field-effect transistors. *Small*, 4(2), 197-201.

Stanlis, K. K. & McIntosh, J. R. (2003). Single-strand DNA aptamers as probes for protein localization in cells. *J Histochem Cytochem*, 51(6), 797-808.

Taitt, C. R., Shubin, Y. S., Angel, R. & Ligler, F. S. (2004). Detection of *Salmonella enterica* serovar typhimurium by using a rapid, array-based immunosensor. *Appl Environ Microbiol*, 70(1), 152-8.

Todd, E. C. (1989). Costs of acute bacterial foodborne disease in Canada and the United States. *Int J Food Microbiol*, 9(4), 313-26.

Tombelli, S., Minunni, M., Luzi, E. & Mascini, M. (2005). Aptamer-based biosensors for the detection of HIV-1 Tat protein. *Bioelectrochemistry*, 67(2), 135-41.

Tombelli, S., Minunni, M. & Mascini, M. (2005). Analytical applications of aptamers. *Biosens Bioelectron*, 20(12), 2424-34.

Ulrich, H., Magdesian, M. H., Alves, M. J. & Colli, W. (2002). In vitro selection of RNA aptamers that bind to cell adhesion receptors of *Trypanosoma cruzi* and inhibit cell invasion. *J Biol Chem*, 277(23), 20756-62.

Ulrich, H., Martins, A. H. & Pesquero, J. B. (2004). RNA and DNA aptamers in cytomics analysis. *Cytometry A*, 59(2), 220-31.

Vivekananda, J. & Kiel, J. L. (2006). Anti-*Francisella tularensis* DNA aptamers detect tularemia antigen from different subspecies by Aptamer-Linked Immobilized Sorbent Assay. *Lab Invest*, 86(6), 610-8.

Zhen, B., Song, Y. J., Guo, Z. B., Wang, J., Zhang, M. L., Yu, S. Y. & Yang, R. F. (2002). [In vitro selection and affinity function of the aptamers to *Bacillus anthracis* spores by SELEX]. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)*, 34(5), 635-42.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*, 31(13), 3406-15.

CHAPTER 5

CONCLUSIONS

Campylobacter and *Salmonella* spp. are among the leading causes of food borne infections in the United States. Food can become contaminated with both of these pathogens at any stage from production to consumption. Identification of these organisms at the point of contamination is critical to improved food safety. Traditionally, culture-based methods consisting of the sequential steps of enrichment, selective and differential plating, and confirmation are the "gold standard" for identification of pathogens in foods. Although cultural enrichment amplifies the target in preparation for downstream detection, it remains time consuming, alters the original microbial community of the sample, and prevents the quantitative determination of pathogen load. Thus there is a continuing need for improvement in our ability to detect bacterial pathogen contamination in food and environmental samples, with a focus on improving time to result without sacrificing specificity and limit of detection.

This need has, in part, been addressed with the introduction of immunological or molecular-based assays which are designed to replace the selective and differential plating step. Nucleic acid amplification methods have been particularly appealing in this regard, but even these remain hampered by the need for cultural enrichment, largely dictated by matrix-associated interference resulting in non-specific amplification (producing false positive results), inhibition of amplification (producing false negative results), and/or varying degrees

of interference (resulting in a less sensitive assay). A small assay volume (several microliters) relative to a large sample size (25 or more g) is an additional problem.

It is clear that in order to further improve time to detection, it will be necessary to reduce and/or eliminate the cultural enrichment step(s). In this regard, the selective separation, concentration, and purification of the target microbe from the sample matrix prior to application of detection assays have been advocated. This has been collectively referred to as pre-analytical sample processing and can theoretically provide the combined advantages of increasing target cell number and reducing sample volume with removal of residual matrix components. This study was undertaken in support of this concept and to explore alternatives in pre-analytical sample processing, as well as other assay manipulations necessary to move toward this ultimate goal of near real-time detection of food borne pathogens.

The purpose of the first study was to design and validate a quantitative qPCR method targeting the *invA* gene of *Salmonella*, including documentation of its performance when preceded by an immunomagnetic separation (IMS) step. The IMS-qPCR assay was applied to the direct detection of *Salmonella* in artificially contaminated chicken rinsate samples. The assay had an excellent limit of detection (10^0-10^1 CFU *Salmonella* per 9 ml sample) with the antibody capture step being virtually 100% efficient at low levels of *Salmonella* contamination, as applied to a model sample matrix (chicken rinsate). To control for potential matrix-associated interference, a homologous internal amplification control (IAC) was designed using the extension overlap method. The qPCR assay was validated both with and without inclusion of the IAC. The inclusion of the IAC did not impact the efficiency of target amplification at low levels of *Salmonella* contamination, although the IAC was out-

competed at higher target concentrations. This was considered insignificant from a practical standpoint since at these higher target concentrations (>2.19 $\log_{10}$ CFU equivalents *Salmonella*), amplification of the target itself would be considered an indicator of a successful PCR reaction. The approach we used to design the homologous IAC is simple and can be adapted for incorporation in other qPCR protocols with minimal manipulation. Additionally, with minor modification of protocol, the method is adaptable to the creation of RNA IACs for RT-qPCR.

Over the years, IMS has become the leading method for pre-analytical sample processing. However, this method has its limitations, including high costs associated with antibody production, batch-wise variation in antibody functionality, and their relatively limited shelf-life. Further, IMS methods are usually applied to sample volumes $\leq$ 1 ml, meaning that cultural enrichment steps are still necessary to amplify the target prior to capture. Alternative ligands have been suggested as alternatives to antibody-based pre-analytical sample processing methods. The purpose of the second portion of this dissertation research was to identify nucleic acid aptamers with binding specificity to candidate food borne pathogens, with the ultimate goal of using these as an alternative to antibody-based ligands in pre-analytical sample processing. Specifically, DNA aptamers with binding specificity to *Campylobacter jejuni* and *Salmonella* Typhimurium were selected using a modification of the SELEX (Systematic Evolution of Ligands by EXponential enrichment) process as applied to viable, intact bacterial cells. This approach allow us to target the entirety of the cell surface markers in their native three dimensional conformation(s), unlike traditional SELEX which requires purification of the target cell marker, which may be

impractical if the marker is unknown, or alternatively, may result in presentation of the target in a non-native conformational state.

In the second study, fluorescein (FAM)-labeled DNA aptamers with binding affinity and specificity to *C. jejuni* were identified. The aptamer-bound complexes were separated from unbound aptamers in whole-cell SELEX using a combination of centrifugation and fluorescence automated cell sorting (FACS); binding affinity analyses were also done using flow cytometry. A counter-SELEX step was performed to reduce the number of non-specific binders during selection. Seven unique FAM-labeled DNA aptamer sequences with binding affinity to *C. jejuni* were identified, out of which one aptamer (ONS-23) was further characterized. Aptamer ONS-23 showed high binding affinity to *C. jejuni* cells of different strains and low apparent cross-reactivity with bacterial cells from non-*Campylobacter* genera. Overall, the whole-cell SELEX process was found to be fast, easy, and reproducible, generating functional aptamers with a high degree of binding affinity to *C. jejuni*. The FAM-conjugated DNA aptamers appear to have promise for pre-analytical sample processing and could potentially be used as fluorescently labeled detection probes in a myriad of platforms including flow cytometric analysis, fluorescence-based automated sorting of specific cell types, and fluorescence microscopy. They could also be used as capture and/or detection ligands in support of biosensor detection technology.

In the third study, biotinylated DNA aptamers demonstrating binding affinity to *Salmonella* Typhimurium were selected, also using the whole-cell SELEX method. A combinatorial library of biotin-labeled single stranded DNA molecules served as the basis for this work. Out of 18 candidate aptamers screened, two (S8-7 and S8-46) showed high

binding affinity to *S.* Typhimurium. On further characterization, aptamer S8-7 demonstrated low to moderate cross-reactivity with various non-*Salmonella* genera. When conjugated with streptavidin-labeled magnetic beads, aptamer S8-7 was successfully applied for the capture and subsequent detection of *S.* Typhimurium cells in pure culture. The lower limit of detection of the magnetic capture-qPCR assay was 10^2-10^3 CFU equivalents of *S.* Typhimurium in 290 µl buffer, with capture efficiency ranged from 3-13 %. This study provided proof-of-concept that biotinylated aptamers could be used in a qPCR-based capture-detection platform as applied to a significant food borne pathogen.

As should be evident from the results of the second and third studies, aptamer technology offers tremendous opportunities to simultaneously address various steps in pathogen detection, ranging from target separation/concentration to detection. Further, the binding of an aptamer to the surface of a target cell could serve as a sample concentration step and at the same time, provide a surface upon which subsequent nucleic acid amplification can take place, as is the case for the proximity ligation assay. These sorts of approaches might be particularly appealing from a commercial perspective as they are the combination of a high degree of specificity with ease of detection, and at the same time confirmation of functionality and pathogenic potential of target pathogen cells.

To make aptamer technology practically feasible for sample preparation and detection, further studies are needed. For example, once candidate ligands are identified, it is necessary to assure that they meet the desired degree of inclusivity (broad reactivity) in the context of multiple strains of target serovar/ species at the same time maintaining target specificity; exclusivity (failure to bind to non-target cells); and sensitivity (limit of

detection). It is also important to demonstrate that the aptamers have a high degree of binding affinity and are able to maintain functionality over various ranges of physical and chemical parameters (temperature, pH, salt concentration, etc) and with a broad range of sample matrices. A particularly important consideration is to assure that aptamers remain resistant to nuclease attack, a likely problem for real-world samples. Strategies for modification of aptamers to assure nuclease resistance are warranted. For example, chemical modification of the ribose ring can be done to enhance aptamer stability. Alternatively, the use of a combinatorial library having modified bases such as locked nucleic acid (LAN) can also enhance resistance to nuclease degradation. Finally, scale-up studies will be necessary to demonstrate equivalent capture as applied to larger, more realistic sample volumes and to a wider variety of sample matrices.

Overall, our studies on existing (antibodies) and novel (aptamers) ligands provide promising evidence that the future of rapid pathogen detection lies in linking pre-analytical sample processing approaches to rapid molecular-based detection strategies. Although there is much work yet ahead, these new concepts in sample preparation and detection offer hope that we may one day be able to circumvent the need for cultural enrichment we seek to develop rapid and sensitive real-time food borne pathogen detection strategies.