

ABSTRACT

SUH, SOO HWAN. Selection and Characterization of DNA Aptamers with Binding Specificity for *Listeria* spp. (Under the direction of Dr. Lee-Ann Jaykus).

As the causative agent of listeriosis, a severe food-borne disease, *Listeria monocytogenes* is a major problem for the food industry. The need for pre-analytical sample processing to facilitate concentration of pathogens from complex sample matrices is a well accepted prerequisite to eventual “real-time” detection. Many ligands (antibodies, peptides, phage binding proteins, nucleic acid aptamers) have been either used or proposed to facilitate this process. Aptamers are small, single-stranded (ss) DNA or RNA molecules that bind with high affinity and specificity to various molecular targets. This dissertation describes the development of (ss) DNA aptamers selected for their binding affinity to *Listeria* species.

In the first study, SELEX (Systematic Evolution of Ligands by EXponential enrichment) was used to select a biotin-labeled ssDNA aptamer specific for *Listeria*. After multiple rounds of selection and counter-selection, aptamers with binding affinity to *L. monocytogenes* were separated, sequenced, and characterized by flow cytometry. Five aptamer candidates were identified, all having binding affinities of 18-24% as evaluated by flow cytometry. Although selected for using *L. monocytogenes*, these aptamers showed similar binding affinity for other members of the *Listeria* genus (13-21%), and low binding affinity for non-*Listeria* species. The dissociation constant (K_d) of aptamer Lbi-17, selected for its high binding affinity for *Listeria* spp. and relatively low binding affinity for non-*Listeria* spp., was $35.7 \pm 8.0 \mu\text{M}$. When Lbi-17 was conjugated to magnetic beads and used in a combined aptamer magnetic capture (AMC)-qPCR assay, the pathogen could be detected at concentrations $<60 \text{ CFU}/500$

μl buffer, with a capture efficiency of 26-77%. Increasing assay volume to 10 and 50 ml resulted in reduced capture efficiency and higher limits of detection, to 2.7 and 4.8 log₁₀ CFU *L. monocytogenes*/sample, respectively, for the AMC-qPCR assay. Based on these preliminary results, the biotinylated ssDNA aptamers developed here appear to be promising ligands for foodborne pathogen concentration prior to detection using molecular methods.

In the second study, carboxyfluorescein (FAM)-labeled aptamers with binding specificity to *L. monocytogenes* cells at different growth phases were produced by SELEX using cells in log phase (6 h cultures) or stationary phase (12 h cultures). Four aptamers (LM6-2 and LM6-116, selected by targeting log phase cultures; and LM12-6 and LM12-13, selected by targeting stationary phase cultures) were chosen for further characterization based on their relatively high binding affinities for *L. monocytogenes* (32-55%) as well as other members of the *Listeria* genus, including *L. innocua*, *L. ivanovii*, *L. grayi*, *L. welshimeri*, and *L. seeligeri*. Aptamer binding exclusivity analysis showed low apparent cross-reactivity with other foodborne bacteria, including *E. coli* O157:H7, *Salmonella enterica* and *Brochothrix thermosphacta*, for which binding efficiencies never exceeded 13%. Minimal cross-reactivity was observed for *Bacillus cereus* (13-18%). Using a sequential binding assay combined with flow cytometry, it was determined that three of the aptamers bound to one apparent cell surface moiety, while a fourth aptamer (LM6-116) appeared to have a different binding site. Dissociation constants (K_d values) of 106.4 ± 43.91 nM and 74.4 ± 52.69 nM were determined for aptamers LM12-6 and LM6-116, respectively. An antibody-based magnetic capture coupled with an aptamer detector (two site binding sandwich assay) was developed as a proof-of-concept assay design. When coupled with qPCR detection of the

detector aptamer, the lower limit of detection for the assay was $0.4 \log_{10}$ CFU *L.*

monocytogenes in 500 μ l buffer. This is juxtaposed to a detection limit of $2.4 \log_{10}$ CFU in 500 μ l for immunomagnetic separation coupled with qPCR detection of the *hlyQ* gene target of *L. monocytogenes*. With further work, the aptamers reported here may be used in assays designed to capture and detect *Listeria* spp. from complex sample matrices such as foods or environmental samples.

Selection and Characterization of DNA Aptamers with Binding Specificity for *Listeria* spp.

by
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DEDICATION

To my parents, Yong Ung Suh and Jung Hee Kim

&

to my wife Soo Jung Choi

BIOGRAPHY

SooHwan Suh, the author of this dissertation was born in Chunchon, eastern part of South Korea. Soon after he was born, his family moved to Seoul and he spent his juvenile years in Seoul. When his family moved to Gongju, he started and finish his elementary education. He attended High school in Seoul and completed his undergraduate studies in Food Science at Konyang University. During his undergraduate studies, he served in Korean Air Force for 2 and half years. After fulfilling his military duties, he completed his undergraduate and continued studies for masters in bioengineering with specific major in food science at Korea University. He got his first industry job at Korean food manufacturing company, LG Ourhome Co. Ltd. He worked at research institute in Seoungnam, South Korea. In 2008, he began working for PhD in Food Sciences program at North Carolina State University, Raleigh under the direction of Dr. Lee-Ann Jaykus.

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CHAPTER 1

Literature Review

Advances in Separation and Concentration of Microorganisms from Food Samples

1.1 Introduction

Foodborne disease is an important public health problem worldwide. In the United States alone, it is estimated that there are 9.4 million episodes of foodborne illness annually, resulting in 55,961 hospitalizations and 1,351 deaths (Scallan et al., 2011).

In addition to other interventions, rapid and accurate identification of pathogens can be an important tool for prevention, control or mitigation of foodborne diseases. Historically, methods for detecting foodborne bacterial pathogens have relied upon cultural enrichment and selective/differential plating, followed by biochemical identification and sometimes, serological characterization. These methods are laborious and it usually takes days to weeks to confirm a positive result, a timeframe that is out of sync with today's rapid food production and distribution networks (Donnelly, 2002, Yang et al., 2007). Standard culture-based pathogen detection methods have been refined in efforts to reduce time to detection, and incremental improvements have been made. Newer detection techniques, including enzyme-linked immunosorbent assay (ELISA), DNA/RNA probes, and polymerase chain

reaction (PCR) have also been developed, but the truly rapid use of these methods has been limited by their relatively high detection limits, necessitating the use of some degree of cultural enrichment prior to their use in detection. These methods can also be limited due to their sensitivity to potential interference from various inhibitory compounds present in food matrices (Yu et al., 2001).

It has been suggested that detection of foodborne pathogens could be made more rapid, and larger sample sizes analyzed, if the target cells were separated, concentrated, and purified from the sample matrix before detection (Brehm-Stecher et al., 2009). Such so-called pre-analytical sample processing could facilitate the removal of residual food matrix components while simultaneously concentrating target pathogen cells. Along with removal of inhibitors, effective pre-analytical sample preparation methods may enable reduction of the initial sample size by 10-1,000 fold (from liter or milliliter volumes down to microliters), making the use of molecular-based methods more realistic with respect to sample size. Ultimately, this could improve our ability to detect low levels of pathogens or sporadic contamination, reducing the time or even the need for cultural enrichment prior to detection. This chapter will focus primarily on cell (or analyte) separation and concentration from food matrices, as these are the key steps from which other elements (i.e. volume reduction, purification, exclusion of inhibitory substances, etc.) are realized.

Research to develop pre-analytical sample processing methods can be divided into two major categories on the basis of target specificity: (i) those that are relatively non-specific (e.g.,

centrifugation, filtration, cationic/anionic exchange resins, aqueous two-phase partitioning, immobilized lectins, and metal hydroxides); and (ii) those that are target-specific (e.g., antibodies, bacteriophage, carbohydrate ligands, nucleic acid aptamers). These two categories are discussed briefly below.

1.2. Candidate pre-analytical sample processing methods

1.2.1. Non-specific approaches

1.2.1.1. Filtration

Filtration is a mechanical or physical separation technique that results in removal of food particles (or organisms) by passing samples through filters of various pore sizes. The pore size of filters has a crucial role in separation efficiency, i.e., microbes will pass through to the filtrate if pore size is large, however if pore size is small, target microorganisms will be concentrated.

Various types of filters have been used for pre-analytical sample processing. For example, Thomas (1988) reported the use of electro-positively charged filters for separation of bacteria from foods. Various AOAC-approved membrane filtration systems (e.g., Neo-Grid™/Iso-Grid™ methods, Neogen Corporation, Lansing, MI) are commercially available for detection of *E. coli* O157: H7 and *Salmonella enterica*, and enumeration of yeasts and molds,

coliforms/*E. coli* and total aerobic bacteria in foods and environmental samples (Dwivedi and Jaykus, 2011). Recently, 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 selective detection of viable (but not inactivated) cells.

The hollow-fiber ultrafiltration (HUF) method has been used for the concentration of microbes from large volume liquid samples (i.e. water). Morales-Morales et al. (2003) reported the simultaneous concentration of enteric bacteria, protozoa, and viruses from water samples using HUF. Similar work was done by Kim et al. (2009), in which case concentrated bacterial and viral microorganisms were further analyzed by either cultivation or TaqMan real-time reverse transcription (RT)-PCR. The methods were fairly efficient for bacterial concentration, demonstrating *E. coli* recovery rates of 65-70% from artificially contaminated ham and lettuce, but less so for virus concentration (1-6% using the murine norovirus in lettuce and ham).

Although filtration can be a reasonable choice for concentration of pathogens from relatively simple sample matrices (such as water or juice), it is not always the ideal choice for pre-analytical sample processing of foods having complex composition. In these cases, combining filtration with other methods is commonly done. For example, several researchers have had success when preceding filtration with pre-treatments like enzymatic digestion or centrifugation (Wang et al., 1992, Starbuck et al., 1992). Two-step filtration has also been

used, usually by following a crude filtration ($> 40 \mu\text{m}$) with a secondary filtration step using a small filter ($0.22 \mu\text{m}$). Using this approach, Wolffs et al. (2006) were able to detect cell numbers as low as 7.5×10^2 CFU per 100 ml sample by quantitative real-time qPCR. However, even after filtration, matrix associated components, sized similar to bacteria or smaller, can be co-concentrated and ultimately interfere with downstream detection methods (Oyofa and Rollins, 1993).

1.2.1.2. Centrifugation

Centrifugation can be applied for the non-specific sedimentation of bacterial cells along with other components of the sample matrix. It is a routine method in most research laboratories and can effectively result in reduced sample volume. The sedimentation of cells by centrifugation depends on various parameters including cell diameter, particle density, solution density, volume, angle and speed of centrifugation (centrifugal force).

There are a number of different centrifugation approaches that have been used for pre-analytical sample processing. Simple high-speed centrifugation is commonly used to concentrate bacterial cells from microbiological media and clinical samples before extracting nucleic acids and detecting by DNA hybridization or PCR. Bacterial cells such as *Clostridium welchii* (Darby et al., 1970); *Lactobacillus* and *Lactococcus* species (Fliss et al., 1991); and *Mycobacterium avium* subsp. paratuberculosis (Özbek et al., 2003) have been

harvested and concentrated by centrifugation in the range of 10,000-23,000 x g for eventual detection by PCR, with high yields of nucleic acids.

Differential centrifugation consists of a step-wise increase in centrifugation speed. In general, the first speed is slower and facilitates the settling of large size high density food components. This is followed in sequence by sedimentation of smaller particles or lower density, including bacterial cells, using higher centrifugation speeds. Differential centrifugation has been successfully applied for the separation and concentration of *E. coli* O157:H7 and other pathogens in ground beef (Cui et al., 2003, Rodrigues-Szulc et al., 1996) in preparation for detection by PCR.

Density gradient centrifugation is based on the use of a suspending solution that forms a density gradient from bottom (highest density) to top (lowest density). Compounds most commonly used to produce the gradient include sucrose, Ficoll, and iodinated media such as Metrzamide, Nycodenz, and Percoll (colloidal suspension of polyvinylpyrrolidone-coated silica particles). Buoyant density gradient centrifugation has been used for the concentration of *E. coli* O157:H7 from beef homogenates (Lindqvist, 1997). Similarly, (Fukushima et al., 2007) used the same approach in conjunction with other physical separation methods to concentrate pathogens from various food homogenates.

Indirectly, coagulation and flocculation are centrifugation methods in that they increase the diameter of matrix-associated particles, and hence can improve the efficiency of centrifugation. Coagulation, facilitated by the removal of electrostatic charges (e.g., usually

done by pH change) allows particles to adhere to one another. Flocculation can be achieved by adding small amounts of high molecular weight, charged materials to bridge oppositely charged particles together and produce a loose aggregate. Both coagulation and flocculation result in particles of higher density that are easier to sediment at lower centrifugal forces. They are commonly used for concentration of viruses and parasitic protozoa from food and environmental samples.

Overall, the major advantage of the centrifugation methods is their ability to handle fairly large sample volumes, while the major disadvantage is the tendency to result in co-concentration of residual matrix components that are of approximately the same size and density as the target microbe(s).

1.2.1.3. Ion-exchange resin/chromatography

Ion exchange resins are comprised of small porous polymer beads that have positively and negatively charged groups. Depending upon the surface charge of bacteria, ion exchange resins can be used to selectively adsorb, and hence concentrate, bacteria. In most instances, it is the positively charged surface of the resin that binds to the bacterial cells that are negatively charged at pH values less than 5.0. The binding capacity of exchange resins is up to 10^{10} CFU bacteria per gram of resin under optimized conditions (Payne and Kroll, 1991).

Bacteria that are adsorbed to ion exchange resins can be released by pH alteration, but certainly not all bacterial species are released under the same pH conditions. Further, the ability of bacterial cells to withstand the extremes of pH needed for their elution from ion exchange columns differs substantially.

Cationic exchange resins have been employed for the separation and concentration of bacterial cells, toxins and DNA from environmental samples such as soils (Jacobsen and Rasmussen, 1992) and foods (Reiser et al., 1974). A beaded anion exchange column, in conjunction with a piperazine-hydrochloric acid buffer (0.02 M, pH 7.0), was used by Liu et al. (2010) for bacterial separation. The absorbed cells were eluted by a linear gradient of NaCl solution (0 to 1.0 M). These investigators observed a distinctive chromatographic profile for key bacteria (*E. coli* O126:H2, O152:H-, K12; *Bifidobacterium adolescentis*) with a high degree of reproducibility and accuracy.

Although ion exchange chromatography can be effective in removing matrix-associated inhibitors with sufficient recovery of target bacteria, it is relatively expensive and prone of non-specific binding problems.

1.2.1.4. 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. Hydroxyl groups of the metal hydroxide bind covalently with amino acids on the cell surface. Once the cell-hydroxide complexes have been established, the complexes can be separated

by centrifugation, resulting in concentration of bacterial cells and removal of portions of the sample matrix.

Immobilization of bacterial cells with metal hydroxides (titanous and zirconium) was first reported by Kennedy et al. (1976) and was later applied in the concentration of cells from culture media, clinical samples, and foods prior to detection by solid-phase immunoassay (Ibrahim et al., 1985). Similarly, zirconium hydroxide has been used for concentration of *L. monocytogenes* and *S. Enteritidis* in reconstituted nonfat dry milk (NFDM) with a recovery rate exceeding 50%. Both microorganisms could be detected by RT-PCR at levels as low as 10^1 – 10^2 CFU/25 ml sample. The method was adapted to more complex dairy products, such as whole milk and ice cream (Lucore et al., 2000), with RT-PCR detection limits of $\geq 10^2$ CFU/ml for whole milk and $\geq 10^1$ CFU/ml for ice cream for both *S. Enteritidis* and *L. monocytogenes*. In 2002, a magnetized form of zirconium hydroxide was used for concentration of *S. Enteritidis* and *L. monocytogenes*, and spores of *Bacillus cereus*, in reconstituted NFDM (Cullison and Jaykus, 2002).

Although metal hydroxide-based bacterial immobilization is rapid, simple and inexpensive, with reportedly high recoveries of viable cells, the method is non-specific meaning that matrix-associated components are readily co-concentrated and interfere with downstream detection (Dwivedi and Jaykus, 2011).

1.2.2. Target-specific separation and concentration methods

Separation and concentration methods based on naturally-occurring biological interactions between bioaffinity ligands and specific cell surface receptor(s) have the advantages of high selectivity and binding affinity. Antibodies are the most commonly used ligand in this regard, and the method of coupling of antibodies to magnetic beads to facilitate pathogen concentration (called immunomagnetic separation or IMS) was introduced two decades ago (Skjerve et al., 1990). Since that time, this approach has been incorporated into a number of standard food microbiology methods (Bacteriological Analytical Manual: Diarrheagenic *Escherichia coli*, Chapter 4A, FDA, 2011; FSIS Laboratory Guide Book, Method number 5B.01). As a research tool, IMS has been used for separation and concentration of foodborne pathogens such as *Salmonella enterica*, *Listeria monocytogenes*, *E. coli* O157:H7, *Vibrio parahaemolyticus*, *Cryptosporidium parvum* oocysts and enteric viruses in a wide variety of food and environmental samples (Stevens and Jaykus, 2004).

Recently, there has been interest in bypassing, or at least shortening, cultural enrichment, and antibodies are a logical ligand for use in upstream sample processing steps designed to help achieve this goal. For example, Favrin et al. (2001) used antibodies and IMS to isolate cells of *S. enterica*, followed by infection of the captured cells with bacteriophage SJ2 and endpoint detection of released phage progeny by measurement of optical density. The assay detection limit was less than 10^4 CFU/ml with time to detection of 4 to 5 hr. A similar IMS-coupled bacteriophage amplification assay was reported by Madonna et al. (2001, 2003) for

matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF)-based detection of generic *E. coli*, with a detection limit of $\sim 5.0 \times 10^4$ cells/ml.

Although IMS is widely used in sample preparation, antibodies have some limitations, including the requirement of a living host for their production, relatively high cost, and somewhat limited shelf life. It can also be difficult to produce broadly reactive antibodies for some target pathogens. Perhaps the best examples are *Salmonella enterica*, a species comprised of over 2,500 serovars, and human noroviruses. Additionally, antibodies can be sensitive to extremes of pH and can bind non-specifically to food matrix components. Finally, antigen expression can be affected by properties of the sample matrix, enrichment medium used, incubation temperature and bacterial growth phase, making IMS sensitive to these factors as well (Geng et al., 2006, Hahm and Bhunia, 2006, Stancik et al., 2002, Dwivedi and Jaykus, 2011).

1.3. Emerging approaches

Nanotechnology involves manipulation of matter on a molecular scale, an approach that may enable scientists to develop a new generation of portable devices to simplify sample preparation and reduce pathogen detection time. Nanomaterials, which provide the foundation of nanotechnology, are minute materials, structures or devices ranging in size from 1-100 nm. These include nanowires, nanofibers, liposomes, polymeric nanoparticles and magnetic nanoparticles (Gu et al., 2006). Due to their high surface-to-volume ratios,

nanomaterials can theoretically improve target capture efficiency by providing greater surface area for attachment of bioaffinity ligands. Because of their small size, nanoparticles can move more rapidly than larger particles, penetrate inaccessible matrix interstices and interact more effectively with their relatively large cellular targets, resulting in increased capture efficiency, decreased capture time, and faster, more sensitive detection (El-Boubbou et al., 2007, Yang et al., 2008). For example, Varshney et al. (2005) used magnetic nanoparticles conjugated with polyclonal antibodies for detection of *E. coli* O157:H7 in 0.5 ml samples of homogenized ground beef, achieving a minimum capture efficiency of 94% in immunoreactions having starting inoculum levels ranging from 1.6×10^1 to 7.2×10^7 CFU/ml. These experiments were done without prior cultural enrichment and results were obtained in as little as 15 min.

Antibodies are not the only bioaffinity ligands that have potential for use in magnetic particle-based pre-analytical sample processing. Alternative ligands include bacteriophages and their tail fiber proteins, aptamers and carbohydrate moieties, among others. Indeed, magnetic nanoparticles have been functionalized with many different types of biorecognition molecules (Torres-Chavolla and Alocilja, 2009, Johnson et al., 2008, Lin et al., 2002, El-Boubbou et al., 2007). Several of these novel binders are described below.

1.3.1 Bacteriophages

Bacteriophage (bacteria-infecting viruses or “phage”) have long been used in microbial diagnostics, in amplification-based assays, directly as cell capture reagents and even as selective agents in pathogen-specific enrichment media. These applications hinge on the unique binding affinities of phages for their host(s), resulting in highly specific assays (Wolber and Green, 1990, Tanji et al., 2004). For example, the *Salmonella*-specific lytic phage *Sapphire* has been immobilized on polystyrene surfaces and used to capture the organism from mixed bacterial populations, followed by detection using PCR (Bennett et al., 1997). This approach was reportedly able to detect 10^5 CFU *Salmonella* per ml in suspensions containing other *Enterobacteriaceae*; capture efficiency was around 1%. In another application, magnetic particles conjugated to bacteriophage engineered to encode the *lux* operon were used for the selective concentration of *S. enterica* serovar Enteritidis, with subsequent detection based on degree of bioluminescence; this assay demonstrated 20% capture efficiency (Sun et al., 2001).

Although such studies illustrate proof-of-concept, target capture remains relatively inefficient. To address this, investigators have sought to develop methods for phage immobilization that promote optimal target binding (Tolba et al., 2010, Gervais et al., 2007, Singh et al., 2009, Minikh et al., 2010). Most of this work has been done using bacteriophage T4, which binds tail-first to host cells. For example, this phage has been genetically modified by fusing a biotin carboxyl carrier protein gene (*bccp*) or the cellulose binding module gene (*cbm*) with the small outer phage capsid protein gene (*soc*), resulting in

expression of the ligand on the phage head (Tolba et al., 2010). The recombinant phages, when immobilized on solid surfaces such as streptavidin-labeled magnetic beads or microcrystalline cellulose beads, were properly oriented to facilitate capture. Tolba et al. (2010) used this approach to capture *E. coli* B cells at initial cell concentrations of 10^1 - 10^5 CFU/ml with efficiency ranging from 72-90%. The same group immobilized bioengineered phages on nano-aluminum fiber-based filters (DisruptorTM) which provided higher density, achieving detection (based on an ATP bioluminescence assay) of as few as 10^3 *E. coli* cells/ml within 2 h (Minikh et al., 2010). Improved efficiency of bacteriophage immobilization can also be achieved by chemical modification of solid surfaces. For example, Singh and colleagues (2009) immobilized *E. coli* host (EC12)-specific bacteriophages on gold surfaces chemically modified using sugars and amine groups, demonstrating up to a 37-fold improvement in immobilization efficiency with a corresponding nine-fold improvement in *E. coli* capture efficiency.

Biomolecules derived from bacteriophage have also been used for the selective separation of bacterial cells. For example, Kretzer and colleagues (2007) expressed cell wall binding domains (CBDs) of bacteriophage-encoded peptidoglycan hydrolases (endolysins). The CBDs from a *L. monocytogenes* phage endolysin specifically bound to unique peptidoglycans on the surface of *Listeria* cells. Using paramagnetic beads conjugated with the recombinant phage endolysin-derived CBD molecules, members of the *Listeria* genus could be captured and recovered at an efficiency ranging from 86-99% in buffer systems containing target cells at $10^3 \sim 10^5$ CFU/ml.

In addition to cell capture applications, bacteriophages can also be used for target-specific detection, with candidate detection platforms including MALDI- TOF (Madonna et al., 2001, Madonna et al., 2003), competitive ELISA (Guan et al., 2006), and qPCR (Tolba et al., 2010). One particularly interesting method is the phage amplification technique (Favrin et al., 2003). Adapted for *Salmonella*, pre-enriched samples are processed for pathogen-specific capture using antibody-conjugated magnetic beads. The captured cells are then infected with the *Salmonella*-specific phage SJ2, and the efficiency of infection determined using freshly cultured *Salmonella* cells. Optical density (OD) at 600 nm is measured before and after phage infection and lysis, and the ratio between the two OD values is used to determine sample positivity.

Labeling bacteriophages with reporter molecules (e.g., *lux* and *lacZ*) can also be used to facilitate detection of foodborne pathogens (Goodridge and Griffiths, 2002). For example, Goodridge et al. (1999) were able to detect *E. coli* O157:H7 by flow cytometry using a fluorescently labeled bacteriophage, reporting assay detection limits (without prior cultural enrichment) of 10^4 cells/ml. More recently, Oda et al. (2004) engineered phage PPO1 (specific to *E. coli* O157:H7) to express green fluorescent protein (GFP), with positive results visualized by fluorescence microscopy. A similar study was done by Tanji et al. (2004) using GFP-tagged T4 for detection of generic *E. coli*. Chen and Griffiths (1996) developed a reporter containing the *lux* gene and used this for the detection of *Salmonella* in eggs, achieving detection limits as low as 10 CFU/ml after only a 6 h pre-enrichment.

Bacteriophages and their proteins have several advantages over other recognition elements, such as the fact that they are environmentally ubiquitous, easy and inexpensive to culture and highly stable. However, they may be less desirable for applications to pathogens having many serotypes or phage types (e.g. *S. enterica*), or for bacterial species for which phage profiles are poorly characterized (e.g., *Campylobacter* spp.). There are also concerns about undesired consequences of the interactions with target cells, such as the potential for target cell lysis and degradation of DNA (Dwivedi and Jaykus, 2011).

1.3.2 Nucleic Acid Ligands

Nucleic acid aptamers are single-stranded RNA or DNA oligonucleotides that fold into three-dimensional structures having binding affinity and specificity to target molecules and/or organisms (Jayasena, 1999, Wilson and Szostak, 1999). The name ‘aptamer’ originated from the Latin *aptus* meaning ‘fitting’. By analogy with antibodies, the binding sites for aptamers are called ‘apatopes’, although ‘epitope’ or ‘target’ is the most frequently used terminology (Bunka and Stockley, 2006). Naturally-occurring aptamers include nucleic acid sequences capable of binding specific metals or proteins. In the lab, aptamers with binding specificity toward desired biotargets may be selected for using a combinatorial approach called SELEX (Systematic Evolution of Ligands by EXponential enrichment) (Tuerk and Gold, 1990).

The binding characteristics of aptamers are reviewed by Kärkkäinen et al. (2011). It is believed that target-specific aptamer binding occurs by means of non-covalent processes,

such as hydrogen bond formation, electrostatic interaction, Van der Waals and hydrophobic interactions. The strength of aptamer to its target is defined by the three dimensional structure and charge of target molecule. The possible molecular targets for aptamer binding include proteins, peptides, carbohydrates, polysaccharides, glycoproteins, lipids, hormones, receptors, antigens, allergens, antibodies, substrates, metabolites, cofactors, inhibitors, drugs, and vitamins. However, large target molecules, such as proteins, which offer extensive surface area to promote aptamer-target binding, are ideal.

Nucleic acid aptamers have been used in a wide range of applications including therapeutics (Green et al., 1995, Nimjee et al., 2005, Que-Gewirth and Sullenger, 2007, Keefe et al., 2010), as diagnostic tools (Brody and Gold, 2000) and for the development of new drugs (Tombelli et al., 2005). Although aptamers have been created for many different applications, the majority of aptamers are developed for therapeutic purposes. In 2005, U.S. Food and Drug Administration (FDA) approved the use of the first aptamer drug, Macugen (Eyeteck/Pfizer) for the treatment of age-related macular degeneration (AMD) (Nimjee et al., 2005). AMD is a medical condition which usually affects older adults and results in loss of vision in the center of the visual field (the macula) because of damage to the retina.

Macugen is targeted against the angiogenic cytokine vascular endothelial growth factor, and binding prevents choroidal neovascularization.

In recent years, DNA aptamers have been explored for their utility in promoting the detection of microbial agents and their metabolites, including foodborne pathogens and mycotoxins

(Bruno and Kiel, 1999, Cruz-Aguado and Penner, 2008, Jeffrey and Fischer, 2008). Recent studies in which nucleic acid aptamers have been used for pre-analytical sample processing and detection of foodborne pathogens are summarized in Table 1 and 2. As early as 1999, Bruno and Kiel generated DNA aptamers against spores of the *Bacillus anthracis* Stern strain, subsequently developing an aptamer-magnetic bead-electrochemiluminescence (AM-ECL) sandwich assay for detection. This assay was able to detect $< 10^1$ anthrax spores. Jeffrey and Fischer (2008) and Cruz-Aguado and Penner (2008) generated high affinity DNA aptamers specific to *Clostridium botulinum* neurotoxin and ochratoxin A, respectively. Recently, Joshi et al. (2009) immobilized DNA aptamers on magnetic beads which had been selected against purified outer membrane proteins of *S. enterica* serovar Typhimurium, using the beads to capture the organism from whole carcass chicken rinse samples. When their magnetic pull-down assay was combined with qPCR, *S. Typhimurium* could be detected at concentrations as low as 10^1 – 10^2 CFU/9 ml rinsate, while 10^2 – 10^3 CFU/25 ml was the lower detection limit using a recirculating magnetic capture method combined with qPCR. In a similar study, DNA aptamers specific to surface proteins of *C. jejuni* were linked to magnetic beads for capture and subsequent detection using a quantum dot-based fluorescent sandwich assay in which endpoint detection was achieved using fluorometry (Bruno et al., 2009). This assay was able to detect 2.5 CFU equivalents of *C. jejuni* in buffer and 10^1 – 2.5×10^2 CFU/ml in various seeded food matrices without prior cultural enrichment.

There are two different strategies for selecting nucleic acid aptamers by SELEX. In the first, illustrated by the studies described above, the SELEX process is carried out against a purified

target, such as a cell surface protein. Because purified proteins may no longer retain their native conformations, these aptamers can have less than optimal binding affinities for their targets when presented in cell-associated form. To circumvent this problem, whole cells can be used as the target for aptamer development (i.e., whole cell SELEX). Selection of aptamers using whole cells or whole cell lysates may have some inherent advantages. For example, different aptamers having binding affinity to different cell surface targets can be produced, and using them in combination (aptamer “cocktails”) could theoretically increase assay sensitivity, specificity, and capture efficiency, and perhaps even help discriminate between different cellular states (Dwivedi et al., 2010, Shamah et al., 2008). Production of nucleic acid aptamers using whole cell SELEX has been an area of active investigation, as illustrated by their use for capture and/or detection of *Campylobacter jejuni* (Dwivedi et al., 2010), *E. coli* (Lee et al., 2009), *Staphylococcus aureus* (Cao et al., 2009), and *Vibrio* spp. (Zheng et al., 2010).

Aptamers, like antibodies and bacteriophages, can be used for capture, detection, or for both simultaneously. For example, a DNA aptamer specific for the internalin A protein of *L. monocytogenes* was conjugated with a fluorescent dye and used as a reporter in a fiber-optic sensor. The aptamer biosensor was able to detect *L. monocytogenes* at a concentration of approximately 10^3 CFU/ml in mixed culture without enrichment, and in artificially contaminated ready-to-eat meat products having initial concentrations of 10^2 CFU/25 g if preceded by an 18 h enrichment (Ohk et al., 2010). Vivekananda and Kiel (2006) used two different aptamers, one for capture and the other for detection, in a two-site binding sandwich

assay that they called Aptamer-Linked Immobilized Sorbent Assay (ALISA). The capture ligands, which were specific to *Francisella tularensis*, were immobilized on a 96-well microtiter plate; detection was achieved using a biotinylated secondary aptamer, with visualization of a positive binding reaction achieved using streptavidin-conjugated horseradish peroxidase (HRP) with 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) enhancer (ABTS) as the substrate. Detection of *F. tularensis* was achieved at concentrations as low as 1.7×10^3 CFU/ml without prior enrichment.

Aptamers hold great promise as molecular recognition tools for their incorporation into analytical devices and, in particular, they can be used as immobilized ligands in separation technologies such as high-performance liquid chromatography (HPLC). (Tombelli et al., 2005). Currently, in the field of pharmaceutical or biological analysis, aptamers are considered as alternatives to common chiral selectors such as amino acids, crown ethers and antibodies. For example, Michaud et al. (2003) immobilized a biotinylated DNA aptamer specific for a D-peptide (arginine-vasopressin, potential drug with resistance to proteolytic degradation), on a streptavidin chromatographic support (polystyrene-divinylbenzene). With a mobile phase consisting of 5 mM phosphate buffer, 100 mM KCl, and 3.0 mM MgCl₂, pH 7.0, at a temperature of 20°C, the D-peptide was retained by the affinity column, while the L-peptide eluted in the void volume. In another study, the same group used immobilized DNA aptamers to resolve enantiomers of small bioactive molecules (D-adenosine and L-tyrosinamide), by HPLC (Michaud et al., 2004). The use of aptamers in HPLC analysis and other affinity chromatography applications is also very promising in the field of separation

and detection of foodborne chemical hazards. For example, the high target specificity of aptamers can be capitalized on in the monitoring of drug (antibiotics) residues in meat, pesticide residues in fresh produce, and the detection of mycotoxins in various foods. Although there has yet to be a specific project that has used aptamers for monitoring chemical hazards in foods and environmental samples, this is a logical extension in the future.

Nucleic acid aptamers have several advantages over other ligands, especially antibodies. Like antibodies, aptamers specifically bind to a selected target; however, because they are composed of nucleic acid rather than protein, the user has more control over their manipulation. Additionally, aptamers are resistant to protease degradation, inexpensive to manufacture, highly stable, easy to modify chemically, and more consistently produced. The primary limitation of nucleic acid aptamers (particularly RNA aptamers) is their nuclease sensitivity. However, chemical modification of the ribose ring can enhance their stability (Pieken et al., 1991). Another potential drawback is the relatively high dissociation constants of some aptamers, which can limit assay sensitivity (Shlyahovsky et al., 2007). Cross reactivity with non-target substances can also be problematic, particularly for aptamers targeting complex analytes such as intact bacterial cells.

1.3.3. Peptide Ligands

1.3.3.1. Peptide aptamers

Like nucleic acid aptamers, peptide aptamers are combinatorial binders selected for their affinities to target proteins or small molecules. They consist of a variable peptide loop attached at both ends to a protein scaffold, a structure that results in a ligand with binding affinity comparable to that of an antibody (i.e., in the nM range) (Hoppe-Seyler and Butz, 2000, Johnson et al., 2008). The variable loop usually consists of 10-20 amino acids, while the scaffold can consist of any protein that is both compact and soluble. Peptide aptamers have mostly been developed for use in therapeutics (Hoppe-Seyler and Butz, 2000), drug delivery, and drug discovery (Crawford et al., 2003, Baines and Colas, 2006). However, a few antiviral (Butz et al., 2001, Trahtenherts et al., 2008) and antibacterial (Blum et al., 2000) peptide aptamers have been reported in the literature.

Peptide aptamers can be selected in a variety of ways, including the phage display technique, first introduced by G. Smith in 1985. In phage display, random DNA sequences are inserted into gene III or gene VIII, corresponding to coat proteins located on the surface of a filamentous phage, generating a large library ($\sim 10^9$ clones) of fusion phages. A binding-based selection technique (“biopanning”) is then used to screen for and purify phage clones capable of binding to the target analyte.

Over the last decade, peptides produced by phage display technology have been used as biorecognition molecules for selective separation and concentration of target proteins and microorganisms, including some foodborne pathogens. For example, a peptide-mediated magnetic separation technique was developed for the selective isolation of *Mycobacterium avium* subsp. *paratuberculosis* from bulk milk. The candidate peptide was chemically synthesized, biotinylated, and conjugated to streptavidin-coated paramagnetic beads. In artificially contaminated milk, the combined peptide-mediated magnetic separation-PCR method was able to detect *M. paratuberculosis* at concentrations as low as 10 cells per ml. When applied to milk samples derived from naturally infected herds, 7/9 samples classified as serologically positive for *M. paratuberculosis* were also positive by this method (Stratmann et al., 2002). More recently, Foddai et al. (2010, 2011) used magnetic beads coated with a chemically synthesized biotinylated polypeptide specific for *M. avium* subsp. *paratuberculosis*. When applied to broth containing 10^3 to 10^4 CFU/ml, this method demonstrated capture efficiencies ranging from 85-100%.

Due to their complex structures and amphipathic nature, which provides many differently charged regions for potential target binding, peptide aptamers may actually have stronger affinity and selectivity when compared to other ligands. Unlike nucleic acid aptamers, peptide aptamers may have limited abilities to penetrate bacterial cell walls, and may therefore be most appropriate as surface-binding reagents. Drawbacks include the expense of peptide aptamer synthesis and the potential for denaturation from matrix-associated proteases or the extremes of heat and pH that may be encountered in some assays.

1.3.3.2. Antimicrobial peptides

Antimicrobial peptides (AMPs) are part of the innate defense system found in higher eukaryotes and are widely distributed in nature. Hundreds of AMPs have been discovered, and they are classified primarily on the basis of their secondary structures (Boman, 1995). A key step in the action of any antimicrobial is the ability to bind to microbial surfaces, and most AMPs exhibit a relatively broad spectrum of molecular recognition for various Gram-negative and Gram-positive bacteria, fungi and viruses (Nicolas and Mor, 1995, Brogden, 2005). The linear cationic AMPs are of particular interest due to their smaller size and intrinsic stability. These AMPs are attracted to the net negative charges on the outer envelope of Gram-negative bacteria, including anionic phospholipids and phosphate groups on the lipopolysaccharide (LPS).

The ease of synthesis and intrinsic stability of AMPs render them good candidates for use as molecular recognition elements in pathogen separation, concentration, and detection (Arcidiacono et al., 2008) (Table 3). For example, cecropin P1, a porcine homolog of the AMP originally isolated from the moth species *Hyalophora cecropia*, was used to capture pathogenic and non-pathogenic *E. coli* strains when covalently immobilized on maleic anhydride microplates (Gregory and Mello, 2005). Similarly, synthetic magainin I, originally extracted from the skin of the African clawed frog *Xenopus laevis*, was immobilized on glass slides and used as a biosensor for foodborne pathogen detection. In this work, magainin I was used as a capture reagent for fluorescently-labeled target microorganisms, producing

detection limits of 1.6×10^5 and 6.5×10^4 cells/ml of *E. coli* O157:H7 and *Salmonella* Typhimurium, respectively (Kulagina et al., 2005).

The primary limitation of AMPs for use in pre-analytical sampling is the potential for their degradation by proteolytic enzymes, especially trypsin-like proteases. Another consideration is that, because AMPs bind net negative charges that exist on the outer envelope of bacteria, their cell recognition activity tends to be relatively non-specific with respect to genus or species. Some of these drawbacks can be addressed through the use of AMP-like biomimetic polymers, discussed further under the *Additional Binders* heading below.

1.3.4. Carbohydrate Ligands

1.3.4.1. Lectins

Noncovalent interactions between proteins and carbohydrates also occur widely in nature. Prominent examples are carbohydrate-specific enzymes, antibodies directed against carbohydrates, and lectins. This latter group has been used rather extensively in biological research. Lectins are proteins that reversibly bind to mono- and oligosaccharides with relatively high specificity. They are found in most living organisms and even in some viruses. Historically, lectins have been isolated and purified using affinity chromatography; more recently, they have been generated using recombinant DNA techniques (Lis and Sharon, 1998).

The binding efficiency of lectins varies as a function of their source, the target microorganism, and sample matrix. For example, Payne et al. (1992) showed that lectins isolated from wheat (*Triticum vulgare*) showed high binding affinity for *L. monocytogenes* (87-100%) and *S. aureus* (80-100%), and more modest binding efficiency for *Salmonella* spp. (33-45%) and *E. coli* (42-77%). In comparison, lectins isolated from the mushroom species *Agaricus bisporus* bound 31-63% of *L. monocytogenes* cells, 83% of *S. aureus* cells, but only 3-5% of representative *Salmonella* cells. Lectins derived from the Burgundy snail (*Helix pomatia*) bound >92% of *S. aureus* and 64% of *L. monocytogenes*, but showed poor binding affinity for Gram-negative organisms.

Lectins such as these have been used as pathogen recognition agents in microarray-based detection methods. For example, two biotinylated lectins [Agglutinin I, derived from the castor oil plant (*Ricinus communis*) and concanavalin A, extracted from the jack bean (*Canavalia ensiformis*)] were conjugated to gold nanoparticles and used for the detection of *E. coli* and *B. cereus*, detecting as few as 10^3 cells per assay (Gao et al., 2010). Similarly, lectin-based screen-printed gold electrodes have been used for the impedimetric label-free detection of *E. coli* at cell concentrations ranging from 5.0×10^3 to 5.0×10^7 CFU/ml (Gamella et al., 2009).

A major limitation of lectins is the difficulty in isolating them from their natural biological sources, making them both expensive and in short supply. In addition, elution of target cells

from lectins can be inefficient unless stringent elution conditions are used, which can impact cell viability and/or interfere with downstream detection methods like PCR.

1.3.4.2. Histo-blood group antigens (HBGAs)

Over the last decade, human histo-blood group antigens (HBGAs) have been investigated as possible binding ligands. HBGAs are highly polymorphic complex carbohydrates related to the ABO, secretor and Lewis families that are present on key human cells and body fluids. They have been identified as important receptors or co-receptors for the binding of a variety of bacterial and viral agents. For example, bacterial adherence to the human gastric epithelial cell mediated by the fucosylated Lewis b (Le^b) histo-blood group antigen of *Helicobacter pylori* has been reported by Ilver et al. (2003). It has also been reported that *Streptococcus pneumoniae* and *Salmonella typhimurium* have been shown to bind with either precursors of H antigens or the H antigens themselves, while *Staphylococcus aureus* has shown to bind with Lewis a antigen (Marionneau et al., 2001). In addition to the bacterial pathogens, viral pathogen such as norovirus (Cannon and Vinjé, 2008) and protozoan parasite such as *Toxoplasma gondii* (Imberty and Varrot, 2008) have also been reported to bind with HBGAs.

Their use for food-borne pathogen separation and concentration is best illustrated for the human noroviruses (HuNoV), for which the HBGAs demonstrate binding specificity (Harrington et al., 2002, Hutson et al., 2002, Marionneau et al., 2002). The P domain of norovirus capsid protein has been reported as a recognition receptor of both A and B epitopes

of HBGAs. Eight distinct patterns of binding between different HuNoV and HBGA's have been identified to date (Huang et al., 2005).

In 2004, Harrington et al. capitalized on the differential HBGA binding characteristics of HuNoV by developing a capture approach using synthetic, biotinylated HBGA's (H Type 1 and 3 chain histo-blood group antigens) bound to magnetic beads. This approach was later expanded upon by Cannon and Vinjé (2008) who used it to concentrate HuNoV from environmental (water) samples, demonstrating the capability of detecting 30-300 genomic copies of the virus from artificially contaminated samples. A similar approach was used by Morton et al. (2009) to concentrate HuNoV from foods such as strawberries, green onions, lettuce, and deli ham in preparation for detection by RT-qPCR. In this case, a recirculating magnetic capture unit was used in conjunction with synthetic HBGA-conjugated beads, facilitating processing of large sample volumes (225 ml buffer system) and producing a low limit of detection for artificially contaminated lettuce, green onions and strawberry samples of about 100 HuNoV genome copies per 25 g sample.

Unfortunately, synthetic HBGAs are expensive to produce and are not widely available. As an alternative, porcine gastric mucin (PGM), which contains mixed HBGA's, has been used in place of synthetic HBGAs. Specifically, using a PGM containing HBGA type A, H1, and Lewis B conjugated to magnetic beads, Tian et al., (2007, 2008) were able to capture HuNoV from complex samples such as oysters, berries, and lettuce which, when followed by RT-qPCR detection, resulted in a 2 log₁₀ improvement in detection sensitivity.

Although PGM contains multiple types of HBGAs that bind to major genotypes of noroviruses, there are limited studies of binding patterns in between PGM and various bacterial, viral and protozoan pathogens. Thus, identification of additional binding patterns could be provided for better understanding and use of HBGAs in more effective pathogen capture strategy.

1.3.5. Additional Binders: Vancomycin and Biomimetic Polymers

Vancomycin is a glycopeptide antibiotic capable of recognizing cell surface moieties of Gram-positive bacteria. Recognition and binding occurs via hydrogen bonding between vancomycin's heptapeptide backbone and the D-alanyl-D-alanine dipeptide displayed on the bacterial cell wall. Gram-negative bacteria are intrinsically resistant to vancomycin due to the permeability barrier posed by the outer membrane, which restricts access of the antibiotic to its target in the periplasm (Walsh et al., 1996).

Because of its ability to bind bacterial cells, several research groups have used vancomycin-conjugated surfaces for pre-analytical sample processing. For example, vancomycin-modified magnetic nanoparticles have been employed for selective isolation of Gram-positive pathogens from pure cultures (Lin et al., 2005). In this case, the isolated cells were further identified by MALDI-MS, with a detection limit of $\sim 7 \times 10^4$ CFU/ml obtained for both *Staphylococcus saprophyticus* and *S. aureus* in urine. In another study, vancomycin-modified nanoparticles were used for the isolation of *S. aureus* and *Enterococcus faecalis*

from aqueous solutions (Kell et al., 2008). These investigators found that the size of the vancomycin-nanoparticle linker molecule and the size of the nanoparticle were critical variables impacting the efficiency of magnetic capture.

The primary limitation of using vancomycin as a bioaffinity ligand is the relative ease with which important pathogens such as *S. aureus* develop resistance to the antibiotic. A common mode for development of resistance involves chemical modification of the cell wall, which also results in the loss of affinity between vancomycin and the target cell. Apart from natural antimicrobials such as vancomycin, biomimetic antimicrobial polymers, which are fully synthetic compounds with AMP-like functionalities, may also have promise as alternative binders (Tew et al., 2002). Computer-aided modeling and design can be used to guide development of polymers that are at least broadly-selective for specific cell types (e.g. Gram-negative bacteria, yeasts), followed by *in vitro* screening for desired binding (or antimicrobial) activities. Benefits of biomimetic AMPs over their natural counterparts include lower synthesis costs, inherent stability and resistance to proteases, the ability to bind under various salt or cationic conditions, and computer-aided rational design of mimics having desirable performance characteristics. Similar strategies for micro-functionalization of surfaces could be used to create binding or recognition elements within biosensors.

1.4. Conclusions

The area of pre-analytical sample processing is a critical cornerstone in effective microbiological analyses and interest in this topic is great. Despite advances, the identification of sample preparation methods that are both reliable and universally applicable has remained elusive and unfortunately, even the emerging techniques described in this paper are still not considered a substitute for classic methods that are based on cultural enrichment. Additional efforts and resources will be needed to develop methods that can ultimately eliminate the need for enrichment culture, the key rate-limiting step in microbiological detection.

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Table 1.1. Aptamer based methods for capture and detection of food-borne bacterial pathogens

Target	Type of aptamer	Affinity target	Detection	Reference
<i>Salmonella enterica</i> serovar Typhi	RNA	structural protein of type IVB pili	N/A	Pen et al., (2005)
<i>Salmonella enterica</i> serovar Typhi	RNA	Outer membrane protein	Real-time PCR	Joshi et al., (2009)
<i>Campylobacter jejuni</i>	ssDNA	Membrane protein	Quantum Dot Sandwich assay	Bruno et al., (2009)
<i>Campylobacter jejuni</i>	ssDNA	Whole cell	Flow cytometry	Dwivedi et al., (2010)
<i>Listeria monocytogenes</i>	ssDNA	Internalin A (InIA)	Optic aptamer sensor	Ohk et al., (2010)
<i>E. coli</i>	RNA	Whole cell	Real-time RT PCR	Lee et al., (2009)
<i>Staphylococcus aureus</i>	ssDNA	Whole cell	Flow cytometry	Cao et al., (2009)
<i>Vibrio alginolyticus</i>	ssDNA	Whole cell	ELISA	Zheng (2009)
<i>Francisella tularensis</i>	ssDNA	Protein lysate	ELISA	Vivekananda & Kiel (2006)

Table 1.2. Aptamer based methods for capture and detection of food-borne non-bacterial pathogens

Category	Target	Type of aptamer	Affinity target	Detection/Separation	References
Prions	Prion protein (PrP ^c)	DNA	recombinant human prion protein	N/A	Takemura et al. (2006)
	Prion protein (PrP ^c)	modified RNA	<i>human prion protein</i>	Decrease formation of PrP ^{sc} (abnormal isoform) <i>in vivo</i>	Proske et al. (2002)
	Prion protein (PrP ^c)	RNA	recombinant prion protein	N/A	Weiss et al. (1997)
Mycotoxins	Ochratoxin A	DNA		N/A	Cruz-Aguado and Penner (2008)
	Ochratoxin A	DNA		Aptamer-based affinity column	Girolamo et al. (2011)
Bacterial spore	<i>Bacillus</i> spores	DNA	spores of the <i>Bacillus anthracis</i> Stern strain	aptamer-magnetic bead-electrochemiluminescence sandwich assay	Bruno and Kiel (1999)
Bacterial toxins	botulinum neurotoxin		botulinum neurotoxin	N/A	Jeffrey and Fischer (2008)
	<i>Staphylococcus aureus</i> Enterotoxin B	DNA	<i>Staphylococcus aureus</i> Enterotoxin B	N/A	DeGrasse (2012)

Table 1.3. Antimicrobial peptides for capture of food-borne pathogens

Target	Antimicrobial peptides	Original source of AMPs	Immobilization & Detection	Reference
<i>E. coli</i> O157:H7	ceropin P1,	<i>Hyalophora cecropia</i> (moth), pig intestine	fiber optic biosensor detection	Arcidiacono et al. (2008)
	Cathelicidin (SMAP29)	lysosomes in macrophages and polymorphonuclear leukocytes (PMNs)		
	PGQ	<i>Xenopus laevis</i> (African clawed frog)		
<i>E. coli</i>	ceropin P1	<i>Hyalophora cecropia</i> (moth), pig intestine	Fluorescent microscopic observation	Gregory & Mello (2005)
<i>E. coli</i> O157:H7 <i>Salmonella</i> serovar. Typhimurium	magainin I	<i>Xenopus laevis</i> (African clawed frog)	biosensor detection	Kulagina et al. (2005)
<i>E. coli</i> <i>Salmonella</i>	magainin I	chemical synthesis	biosensor detection (impedance spectroscopy)	Mannor et al. (2010)

CHAPTER 2

Selection and Characterization of DNA Aptamers with Binding Specificity for *Listeria* spp. and the Use of DNA Aptamers for Capture of *Listeria* spp. prior to the Application of qPCR for Detection

2.1. ABSTRACT

The need for sample preparation to facilitate concentration of pathogens from complex sample matrices is a well accepted pre-requisite to eventual “real-time” detection. Many ligands (antibodies, peptides, phage binding proteins, nucleic acid aptamers) have been either used or proposed to facilitate this process. The purpose of this study was to identify biotinylated ssDNA aptamers with binding specificity to *Listeria monocytogenes* and use these for capture and subsequent qPCR detection of the organism. For aptamer selection, SELEX (Systematic Evolution of Ligands by EXponential enrichment) was applied to a biotin-labeled ssDNA combinatorial library. After multiple rounds of selection and counter-selection, aptamers with binding affinity to *L. monocytogenes* were separated, sequenced, and characterized by flow cytometry. Five aptamer candidates were identified, all having binding affinities of 18-24% as evaluated by flow cytometry. Although selected for using *L. monocytogenes*, these aptamers showed similar binding affinity for other members of the

Listeria genus (13-21%), and low binding affinity for non-*Listeria* species. Aptamer Lbi-17 was chosen for further characterization based on its high binding affinity and low cross reactivity with other non-*Listeria* species. The dissociation constant (K_d) of Lbi-17 was $35.7 \pm 8.0 \mu\text{M}$. When Lbi-17 was conjugated to magnetic beads and used in a combined aptamer magnetic capture (AMC)-qPCR assay, the pathogen could be detected at concentrations $<60 \text{ CFU}/500 \mu\text{l}$ buffer, with a capture efficiency of 26-77%. Parallel experiments using immunomagnetic separation (IMS)-qPCR produced the same detection limit but lower capture efficiency (16-21%). Increasing assay volume to 10 and 50 ml resulted in reduced capture efficiency and higher limits of detection, at 2.7 and 4.8 $\log_{10} \text{ CFU}$ *L. monocytogenes*/sample, respectively, for the AMC-qPCR assay. Based on these preliminary results, the biotinylated ssDNA aptamers developed here appear to be promising ligands for foodborne pathogen concentration prior to detection using molecular methods.

2.2. INTRODUCTION

Traditional methods for detecting pathogens in food and environmental samples rely on cultural enrichment and selective/differential plating followed by biochemical identification, and sometimes, serological and/or molecular characterization. These methods are laborious and it takes four days to confirm a negative sample, and up to a week for a positive sample (Donnelly, 2002, Yang et al., 2007a). Newer, highly specific detection techniques including

enzyme-linked immunosorbent assay (ELISA), DNA/RNA probes, and polymerase chain reaction (PCR) have been incorporated into testing schemes over the last several decades, bypassing the selective/differential plating steps and shortening the time to detection of a negative sample from 4 days to 2 days. Positive samples still take up to a week to confirm. Because it remains necessary to perform lengthy (48 h) cultural enrichment to get target cells to a level in which they can be reliably detected by ELISA or DNA hybridization (usually 10^4 - 10^5 CFU/ml), this remains the rate-limiting step. Theoretically, nucleic acid amplification methods such as PCR should be able to amplify much lower numbers of the target pathogen, but because they are highly sensitive to the effects of matrix-associated inhibitory compounds, cultural enrichment remains a necessity even if using a downstream PCR detection method.

Accordingly, it has been suggested that foodborne pathogen detection could be made more rapid if the target pathogens were separated, concentrated, and purified from the sample matrix before detection. Such so-called pre-analytical sample processing would theoretically provide reduction of sample size by 10 to 200-fold (from ml to μ l volumes). It could also facilitate the removal of residual food matrix components with simultaneous concentration of target pathogen cells, hence facilitating the use of PCR detection methods, which have much lower limits of detection when compared to DNA hybridization or ELISA. The immobilization of antibodies to magnetic beads, which forms the basis for immunomagnetic separation (IMS), has been widely used for the concentration and separation of target microorganisms from complex sample matrices (Skjerve and Olsvik, 1991, Uyttendaele et

al., 2000, Hsieh and Tsen, 2001, Hudson et al., 2001, Hibi et al., 2006, Yang et al., 2007a).

Although antibodies are the most commonly used ligands for target separation and purification, they are not terribly robust, are difficult and expensive to produce, have relatively short shelf-lives, and have varying degrees of target specificity and avidity, all of which can impact capture efficiency and subsequent detection sensitivity.

An alternative ligand for pathogen capture is nucleic acid aptamers, which are short (20-80mer) single-stranded (ss)DNA or RNA sequences that are selected *in vitro* based on affinity for a target molecule, virus, or cell. Such single-stranded nucleic acids specifically interact (bind) to their target through their 3-dimensional structure. Aptamers are selected using the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) approach, an *in vitro* method to enrich aptamers with binding specificity to a target using multiple iterations of selection and amplification (Tuerk and Gold, 1990).

The nucleic acid library used in the SELEX process includes random sequences flanked by two constant sequence regions at the 5'- and 3'-ends. The molecular diversity of a library depends on the number of randomized nucleotide positions, but most robust libraries show sequence diversity ranging from 10^{14} to 10^{15} in 1 μ mol scale solid-phase DNA synthesis (Jayasena, 1999). They offer advantages over traditional antibody-based affinity molecules in their ease of production, regeneration and stability, largely due to the chemical properties of nucleic acids versus amino acids (Brody and Gold, 2000, Golden et al., 2000, Murphy et al., 2003, Osborne et al., 1997). The objective of this study was to identify and characterize ssDNA aptamers with high binding affinity and specificity to *Listeria* spp. The selected ligand was used in the development of a prototype capture method that was applied to

serially diluted overnight cultures of *L. monocytogenes* suspended in buffer of varying volumes, from a low of 500 µl and a high of 50 ml.

2.3. MATERIALS & METHODS

2.3.1. Bacterial strains, culture conditions and preparation of cells

The aptamer selection procedure, whole cell SELEX, was performed targeting *Listeria monocytogenes* ATCC19115 obtained from American Type Culture Collection (ATCC). Other *Listeria* spp. used in the study were *L. innocua* ATCC33091, *L. ivanovii* ATCC19119 and *L. grayi* ATCC25401. Naturally-occurring *L. welshimeri* and *L. seeligeri* strains isolated from foods (courtesy of Dr. Sophia Kathariou, NCSU) were used in inclusivity studies. All *Listeria* strains were grown in Trypticase Soy Broth (TSB, Becton, Dickinson and Co., Sparks, MD) with overnight incubation at 37°C. Non-*Listeria* strains including *E. coli* O157:H7 ATCC43895, *Bacillus cereus* ATCC49063, *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC13076, *Staphylococcus aureus* ATCC23235, *Pseudomonas aeruginosa* ATCC23993, *Shigella flexneri* ATCC12022, *Brochothrix thermosphacta* ATCC11509 and *Lactococcus lactis* MG1363 were used for counter SELEX and in select exclusivity studies. Brain Heart Infusion (BHI) broth (Becton, Dickinson and Co., Sparks, MD) with overnight incubation at 37°C was used to cultivate all non-*Listeria* spp. strains except *B. thermosphacta*, which was grown at 26°C.

2.3.2. Preparation of DNA Library for Initial SELEX Screening

For the first round of SELEX, an 81-mer combinatorial DNA library consisting of a 40 nucleotide random region was obtained from Integrated DNA Technologies (IDT, Inc., Coralville, IA). The constant regions of the DNA library, which were located at the 5' and 3' ends, contained restriction endonuclease recognition sites *SnaB I/Pst I* and *EcoR V/Bgl II*, respectively, to facilitate DNA manipulations (Table 1). The DNA library was 5' end-labeled by PCR using a biotinylated reverse constant region primer (Table 1). Briefly, a 50 μ l reaction master mix containing 5 μ l of aptamer library (10 μ M), 1x GoTaq® buffer (Promega Corp., Madison, WI), 0.2 mM GeneAmp® dNTPs Mix (Applied Biosystems, Foster City, CA), 5 U Go Taq® DNA Polymerase (Promega), 500 nM unlabeled Forward Constant Region primer, and 500 nM Biotin labeled Reverse Constant Region primer was amplified using a three-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 a final extension at 72°C for 10 min using a DNA Engine (PTC-200) Peltier Thermal Cycler-200 (MJ Research/Bio-Rad Laboratories, Hercules, CA).

2.3.3. Single strand DNA isolation

The double-stranded PCR product was coupled to Streptavidin MagneSphere® Paramagnetic Particles (SA-PMPs) (Promega Corp., Madison, WI) as per manufacturer recommendations. The forward ssDNA moieties were separated from the biotinylated ssDNA bound to the magnetic particles by alkaline denaturation in 0.15 M NaOH, and the magnetic beads bound

to biotinylated ssDNA were collected using a Dynal MPC®-M magnetic particle concentrator (Dynal A.S, Oslo, Norway). Two additional alkaline denaturations were then performed in 0.15 M NaOH to completely remove the forward strand from the 5' biotinylated complementary strand. The purified 5' biotinylated strands coupled with magnetic particles were washed thrice in 1x Tris-EDTA and transferred into a clean microcentrifuge tube prior to a final alkaline denaturation, the purpose of which was to separate the biotinylated strand from the magnetic particles. The final alkaline denaturation was done in ammonium hydroxide (28% ammonia in water, Sigma-Aldrich, St. Louis, MO) for 10 min at 90°C. The supernatant obtained after magnetic pull-down was mixed with twice the amount of water, then the 5' biotinylated strand was collected by ethanol precipitation with reconstitution of the DNA pellet in 50 µl of nuclease-free water.

2.3.4. *In vitro* selection process (SELEX)

The whole cell SELEX aptamer selection protocol used in this study was adapted from the work of Dwivedi et al. (2010) with modifications. Six rounds of positive-SELEX and two rounds of negative (counter)-SELEX were performed targeting *L. monocytogenes* and a mixture of non-*Listeria* spp., respectively. In brief, approximately 500 pmoles ($1.8\sim 3.0 \times 10^{14}$ of ssDNA) of ssDNA library was denatured by heating at 90°C for 5 min and renatured by flash cooling on ice for 10 min to allow intra-strand base pairing. The ssDNA aptamer library was incubated with *L. monocytogenes* cells at room temperature for 1 h with gentle rotation. The ssDNA-bound cells were recovered by centrifugation and washed to remove

unbound and non-specifically bound ssDNA moieties. The ssDNA (aptamer) bound to the cell was released by heat (90°C) prior to its enrichment by PCR and the double-stranded PCR product was separated and converted to ss DNA for another round of SELEX, as described above. In counter-SELEX, the aptamer pool was incubated with a mixture of non-*Listeria* spp. (i.e., *E. coli* O157:H7, *B. cereus*, *S. enterica*, *S. aureus*, *P. aeruginosa*, *S. flexneri*, *B. thermosphacta*, and *L. lactis*). In the case of counter SELEX, the ssDNA molecules that bound to non-target cells were discarded, while those that remained unbound were recovered for further rounds of selection. Note that the annealing temperature used in aptamer candidate amplification was gradually increased (from 60°C to 67°C) as selection rounds were increased.

2.3.5. Cloning and sequencing

Following the entire combined SELEX and counter-SELEX processes, a gel purification process using the MERmaid® Spin kit (MPbio, La Jolla, CA) was done to clean the aptamer candidates. In brief, the amplified product containing the aptamer candidates was run on a 2% agarose gel, and the single band representing an amplicon size of 81 base pairs was cut and transferred to a filtered tube. The gel was then melted at 55°C and the aptamer candidates were bound to the filter in the tube. After several washing steps, the filter-bound aptamer candidates were eluted in TE buffer and the aptamers were ligated into the pCRTM2.1-TOPO® vector provided by the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). The ligated vectors were transformed into One Shot® Top10 electrocompetent *E. coli*

cells (Invitrogen) and 30-50 μ l of cells were plated on Luria-Bertani (LB) agar plates supplemented with kanamycin (50 μ g/ ml) followed by overnight incubation at 37°C for 16-20 h. White colonies were recovered, recultured, and the plasmids of the individual transformants extracted using the QIAprep®Spin Miniprep Kit (Qiagen, Gaithersburg, MD). Those transformants having an amplicon band size of 81 bases were selected and sent to Genewiz Inc. (South Plainfield, NJ) for sequencing.

2.3.6. Binding characterization of selected aptamers by flow cytometry

The efficiency of binding of selected aptamer candidates for *L. monocytogenes* was quantitatively measured by using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) in preliminary screening studies. These binding assays were performed using 10 nmoles of biotinylated aptamer candidates with 10^8 - 10^9 *L. monocytogenes* cells in a total volume of 1 ml. Each aptamer candidate was denatured by heating at 90°C and renatured by flash cooling on ice prior to mixing with an overnight culture of *L. monocytogenes* for 45 min at room temperature with gentle rotation. The aptamer-exposed cells were then settled by centrifugation (7,000 x g for 10 min) and washed three times prior to resuspending in 200 μ l of PBS. The concentrate was treated with 5 μ g of streptavidin-FITC tags (Invitrogen, Frederick, MD) to provide any cell-bound aptamer with fluorescent signal, prior to subjecting the concentrate to flow cytometry. Flow cytometry (n = 200, 000) was performed to measure the percent fluorescent cells and fluorescence intensity using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA). The data were analyzed using BD CellQuest™

Pro software (BD Biosciences). The rabbit polyclonal anti *L. monocytogenes* antibody (ABcam, Cambridge, MA) was used as control of positive binding using similar methods as described above.

2.3.7. Binding inclusivity and exclusivity of selected aptamers

On select aptamers, binding inclusivity and exclusivity studies were done. For inclusivity studies, six *Listeria* spp. (i.e., *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. grayi*, *L. welshimeri* and *L. seeligeri*) were used. Exclusivity studies were done using *E. coli* O157:H7, *S. enterica*, *B. cereus* and *B. thermosphacta*. In all cases, cells were grown overnight (10^8 ~ 10^9 CFU/ml) and incubated with 10 μ M biotinylated aptamer. After discarding unbound and non-specifically bound aptamers by performing sequential washing steps, the cell concentrate was treated with 5 μ g of streptavidin-FITC tags to provide any cell-bound aptamer with fluorescent signal, prior to subjecting the concentrate to flow cytometry (n=200,000) as described above.

2.3.8. Determination of equilibrium dissociation constant

The equilibrium dissociation constant of select aptamer candidates was measured by performing binding assays with *L. monocytogenes* cells using varying concentrations (i.e. 10 nM, 50 nM, 100 nM, 500 nM, 1 μ M, 5 μ M, 10 μ M, and 20 μ M) of the aptamer under conditions described above, followed by flow cytometry. The equilibrium dissociation constant (K_d) was calculated by fitting the average total percent fluorescent bacterial cells

(Y) [due to binding with FITC tagged aptamer] as a function of the concentration of the aptamer (X) using a non-interacting binding sites model in SigmaPlot (Jandel, San Rafael, CA).

2.3.9. Predicted Aptamer Structure of selected aptamers

The structural folding (secondary structure) of aptamer sequences displaying binding affinity to *L. monocytogenes* was predicted using the on-line software DNA Mfold version 3.2 (<http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi>) (Zuker, 2003). The modeling was done assuming ionic conditions of 1 mM of sodium and 0.5 mM of magnesium and a temperature of 25°C.

2.3.10. Aptamer Magnetic Acpture (AMC)-qPCR assay

2.3.10.1. Preparation of aptamer bound magnetic beads

The best aptamer candidate (designated Lbi 17) was selected for these studies due to its high binding affinity and selectivity to the target. To prepare aptamer-bound magnetic beads, biotinylated ssDNA aptamer Lbi-17 (obtained from Integrated DNA Technologies) was denatured at 90°C and flash renatured on ice prior its conjugation to streptavidin- coated magnetic particles (M280, Invitrogen Dynal AS, Oslo, Norway) as per manufacturer instruction using a concentration of 0.5 nmol aptamer per 25 µg beads. For comparison purposes, biotinylated rabbit polyclonal anti-*L. monocytogenes* antibody (Abcam,

Cambridge, MA) was also conjugated to streptavidin-coated magnetic particles (4 µg antibody per 25 µg beads). To minimize non-specific binding, the aptamer and antibody-conjugated magnetic particles were blocked with 5% skim milk suspended in 1x PBS containing 0.05% Tween20 (PBST) for 2 h at RT with gentle rotation. After the blocking, the ligand-conjugated magnetic particles were washed and stored at 4°C until used for the assay.

2.3.10.2. Aptamer magnetic capture (AMC) of *L. monocytogenes*

The aptamer magnetic capture was done using three different sample volumes (500 µl, 10 ml and 50 ml). In initial studies using small sample volumes (500 µl), a fresh overnight *L. monocytogenes* culture was 10-fold serially diluted in PBST to yield concentrations ranging from 10^0 to 10^6 CFU/ml. Each diluted cell suspension was mixed with a cocktail containing *B. thermosphacta* and generic *E. coli* each at a concentration of 10^4 – 10^5 CFU/ml. Four-hundred and seventy-five (475) µl aliquots of the bacterial suspension were mixed with 25 µl of aptamer (1 nmol/25 µl) or antibody (4 µg/25 µl)-conjugated magnetic particles with incubation for 45 min at RT with gentle rotation (Figure 1). The captured *L. monocytogenes* cells were then pulled-down using an external magnetic stand (DynaL MPC®-M magnetic particle concentrator, Dynal A.S, Oslo, Norway). After discarding unbound and non-specifically bound cells by three consecutive washings, the *L. monocytogenes*-bound magnetic particles were recovered and resuspended in 200 µl, and the genomic DNA was extracted using the automated nucleic acid extraction system, NucliSENS® easyMAG®

(bioMérieux, Durham, NC) in accordance with manufacturer instructions. The entire 200 µl of sample suspension obtained after the AMC was used for DNA extraction, with a final reconstitution volume of 50 µl. The extracted DNA was stored at -20°C until use in qPCR.

2.3.10.3. Scaled up assays for capture of *L. monocytogenes*

The first scale-up study was done using 50 µg of aptamer-conjugated magnetic particles in 10 ml PBST containing from 10^2 to 10^7 CFU/10 ml of *L. monocytogenes* pre-mixed with non-*Listeria* spp. strains (*B. thermosphacta* and generic *E. coli* at a concentration of 10^4 – 10^5 CFU/ml). After capture for 45 min, the *L. monocytogenes*- bound Dynabeads were washed and extracted for DNA isolation using the easyMAG[®] system with a final reconstitution volume of 50 µl. Further scale-ups were done using 50 ml PBST to which had been added 10^2 to 10^7 stationary phase *L. monocytogenes* cells amongst a background of non-*Listeria* cells. In this case, 200 µg of aptamer-conjugated Dynabeads were used to capture *L. monocytogenes* cells using a recirculating magnetic capture system, Pathatrix[™] (Matrix Microscience Ltd., Newmarket, United Kingdom). The automated capture process was performed in a 50 ml sample volume for 15 min with 35 ml of washing buffer. The captured Dynabeads were then processed for DNA extraction using the easyMAG[®] system with a final reconstitution volume of 50 µl.

2.3.10.4. Downstream detection by real-time qPCR

The extracted DNA was amplified using a TaqmanTM qPCR protocol targeting a 64-bp fragment associated with the *L. monocytogenes hly* gene (positions 113 to 177) as reported by Rodríguez-Lázaro et al. (2004) (Table 1). The qPCR was carried out in a SmartCycler PCR system (Cepheid, CA, USA) in 25 µl reactions consisting of 1 x PCR Buffer, 5 mM MgCl₂, (Invitrogen Life Technologies, Carlsbad, CA,), 0.3 mM dNTPMix (Applied Biosystems, Foster City, CA), 200 nM of each primer, 120 nM Taqman probe, 1.75 U Platinum Taq DNA Polymerase (Invitrogen Life Technologies) and 5 µl of *L.monocytogenes* DNA. The following two-step temperature protocol was used in the amplifications: initial denaturation of 95°C for 120 sec followed by amplification for 45 cycles of 95°C for 15 sec and 60°C for 60 sec.

For quantification purposes, a standard curve was prepared using DNA isolated from a stationary phase *L. monocytogenes* culture that was serially diluted and simultaneously enumerated by culture-based methods and DNA extraction followed by qPCR. These data were plotted as Ct value (Y axis) vs. CFU equivalent (X axis), where the latter was defined as the initial CFU prior to aptamer (or antibody) capture, DNA extraction, and qPCR. The approximate CFU in unknown samples subjected to magnetic pull-down 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.

2.3.11. Statistical analysis

The results of multiple experiments are presented as mean \pm standard deviation. The data were analyzed by one-way ANOVA, followed by the Tukey-Kramer multiple comparisons test ($p < 0.05$; GraphPad Prism ver. 5.0d, Dan Diego, CA). Comparisons between groups were performed using t-tests when the ANOVA test was statistically significant. Values of $p < 0.05$ were considered significant.

2.4. RESULTS

2.4.1. *In vitro* selection of aptamer candidates

Six rounds of positive-SELEX and two rounds of negative (counter)-SELEX were performed targeting *L. monocytogenes* and a mixture of non-*Listeria* spp., respectively.

A total of 25 transformants obtained from the SELEX process were selected for sequencing. From these transformants, 16 aptamer sequences were sequenced, from which seven unique sequences (i.e. Lbi-13, 14, 16, 17, 118, 200 and 203) were obtained. Among the 7 unique sequences, five sequences (i.e. Lbi-16, Lbi-17, Lbi-118, Lbi-200 and Lbi-203) showed better or similar binding affinities with overnight cultured *L. monocytogenes*, ranged from 20 to 25%, compared to the binding affinity of anti-*L. monocytogenes* antibody (which was about 22%). The highest binding affinity was observed for aptamer Lbi-17, followed by Lbi-16, Lbi-118, Lbi-200 and Lbi-203, respectively (Figure 2).

2.4.2. Binding Specificity (exclusivity) study

Three top-binding aptamer candidates (Lbi-16, 17 and 118) were subjected to binding exclusivity analysis using several non-*Listeria* spp. as evaluated using flow cytometry. Ten μM solutions of biotinylated aptamers (Lbi-16, 17 and 118) were mixed with 10^8 - 10^9 cells of non-*Listeria* spp. (in separate experiments), and the first 200,000 cells were analyzed for fluorescence intensity using flow cytometry. All three aptamers showed low apparent cross-reactivity with other foodborne bacteria, including *S. enterica* and *B. thermosphacta*, for which binding efficiencies never exceeded 10% (Figure 3). Slightly higher binding affinity (approx. 13%) was observed for the Gram positive organism *B. cereus*. The aptamer Lbi-16 also showed relatively high binding affinity (13%) for *P. aeruginosa*. Overall, aptamer Lbi-17 showed relatively low cross-reactivity against all four non-*Listeria* strains, less than 11%. This aptamer was chosen for further characterization.

2.4.3. Binding inclusivity studies against multiple *Listeria* spp.

Aptamer Lbi-17 (10 μM concentration) was separately exposed to 10^8 - 10^9 cells of six *Listeria* spp. including *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. grayi*, *L. welshimeri*, and *L. seeligeri*. Flow cytometry analysis (n = 200, 000) suggested binding efficiencies about 21% for *L. monocytogenes* and 10-23% for other members of the *Listeria* genus (Figure 4). Aptamer Lbi-17 demonstrated the highest binding affinity for *L. grayi* (similar to that of *L. monocytogenes* at 21%), followed by *L. seeligeri*, *L. ivanovii*, *L. innocua*, *L. welshimeri* (16%, 14%, 12% and 10% binding affinity, respectively). Based on these results, it appeared

that aptamer Lbi-17 had relatively strong binding affinity for a variety of species within the *Listeria* genus.

2.4.4. Determination of equilibrium dissociation constant

Data from binding studies done using 10^8 - 10^9 *L. monocytogenes* cells and various concentrations (i.e. 10 nM, 50 nM, 100 nM, 500 nM, 1 μ M, 5 μ M, 10 μ M, and 20 μ M) of aptamer Lbi-17 were fitted to a non-linear regression model for one-site saturation. The percent fluorescence measured by binding analysis of 10^8 - 10^9 *L. monocytogenes* cells with 10 nM, 100 nM, 500 nM, 1 μ M, 2 μ M of aptamer Lbi-17 solution were 16.2 ± 1.37 , 22.1 ± 3.79 , 32.4 ± 0.79 , 43.5 ± 1.79 , $50.4 \pm 5.33\%$, respectively. An equilibrium dissociation constant (K_d) of 35.7 ± 8.015 μ M was obtained for aptamer Lbi-17 with maximum binding of $78.7 \pm 12.38\%$ (Figure 5).

2.4.5. Predicted secondary structure of aptamer candidates

The predicted secondary structure of aptamer Lbi-17 is shown in Figure 6. With minimum free energy of -21.47 kcal/mol, the aptamer structure consists of multiple hairpins and one body loop in the center. The three hairpin structures are located between sequences 2-14, 18-47, and 59-74.

2.4.6. Aptamer magnetic capture (AMC)-qPCR assay

2.4.6.1. Aptamer magnetic capture in small volume (500 µl) mixed culture

When AMC was applied to a mixed bacterial culture containing *L. monocytogenes*, generic *E. coli* and *B. thermosphacta* and followed by qPCR detection, the % CE for *L. monocytogenes* ranged from 26% to 77%, progressively increasing as target cell number decreased. The limit of detection (LOD) of the combined aptamer capture-qPCR method was $1.8 \log_{10}$ CFU *L. monocytogenes* in 500 µl (Figure 7). This is juxtaposed to the combined immunomagnetic separation-qPCR method, for which the % CE was lower, ranging from 16% to 21%, with little relationship to cell number, and a similar limit of detection of $1.8 \log_{10}$ CFU/500 µl. Residual non-specific binding did occur in the negative control (beads lacking ligand conjugation) at all target cell concentrations except the lowest one tested ($1.8 \log_{10}$ CFU/500 µl), but was usually <10% CE.

2.4.6.2. Scale-up AMC-qPCR experiments

Two scale-up AMC studies were done: one in which *L. monocytogenes* cells (along with representative candidate competitive microflora) were suspended in 10 ml PBST and used in an aptamer-based capture using a simple magnetic pull-down device, and the other in which *L. monocytogenes* was suspended in 50 ml PBST and the cells concentrated using an automated magnetic capture system in conjunction with aptamer Lbi-17- bound magnetic beads. When the 10 ml experiments using the bead-bound aptamer was combined with

qPCR, the lower LOD was $2.7 \log_{10}$ CFU *L. monocytogenes*/sample, with little improvement in % CE (which remained at about 10%) as a function of target cell concentration. Non-specific binding of non-conjugated magnetic beads was apparent at high target cell concentration, but disappeared at low target cell number ($<3.7 \log_{10}$ ml) (Figure 8). Similar experiments using antibody-bound beads produced a LOD one \log_{10} higher, at $3.7 \log_{10}$ CFU *L. monocytogenes*/10 ml, and the % CE never exceeded 5%. For the large volume (50 ml) scale-up, the lower LOD using aptamer-bound beads was approximately $4.8 \log_{10}$ CFU *L. monocytogenes*/sample, with a % CE not exceeding 1%.

2.5. DISCUSSION

In this study, a whole-cell SELEX approach was used for the selection of single stranded DNA aptamers, as compared to the more traditional selection method that uses an isolated and/or purified marker/receptor as the target. Because the whole cell SELEX approach theoretically provides for the selection of aptamers targeting any number of cell surface targets, the method should result in aptamers able to recognize multiple cell surface targets. Another advantage of the whole cell SELEX method is that there is no need to isolate or purify a single target protein, the process of which can result in structural changes that might impact the presentation of the target molecule in an intact, live, bacterial cell (Cao et al.,

2009, Dwivedi et al., 2010). In short, the whole cell SELEX method has the potential to result in aptamers with binding affinity for the target in its native conformation.

Nonetheless, there are limitations to the whole cell SELEX approach. For example, limited knowledge about the exact binding site for the aptamer may hinder the ability to obtain accurate information about binding characteristics. Others have sought to resolve this issue using techniques such as the gel mobility shift or Southwestern blot analysis as a means to identify the target cell surface recognition site. For example, in their work developing an aptamer with binding specificity to a cell surface protein of *Salmonella*, Joshi et al. (2009) used both of these methods to confirm binding specificity. Although we attempted similar studies using a membrane extract of *L. monocytogenes*, were unable to identify specific binding sites (data not shown). The most plausible explanation for these negative results is that, as a consequence of membrane extraction, the target molecules lost their native conformation and were no longer presented in the form in which they are presented in a live cell. As such, aptamers selected against the whole cell were not able to bind to the extracted cell surface moieties. It is also possible that the aptamer target might not have been a single molecule, but rather a complex structure of multiple molecules. In this case too, confirmation of binding using a purified membrane extract might not have been possible.

Although an aptamer specifically targeting *L. monocytogenes* was identified in this study, the selected aptamer (Lbi-17) showed significant binding affinity to most other *Listeria* spp., suggesting that the binding moieties present on the *L. monocytogenes* cell surface were also

present in these other members of the genus (Figure 4). Because we did not include these other *Listeria* spp. as counter-targets during the SELEX procedure, these results are perhaps not unexpected. Comparatively speaking, two *Listeria* species (*L. welshimeri* and *L. innocua*) had the lowest binding affinities with aptamer Lbi-17, but their binding affinities were still elevated compared to Gram negative bacteria like *S. enterica* or *E. coli*, and were actually similar to binding affinity in exclusivity studies with *B. cereus* (Figure 3). This elevated cross-reactivity with *Bacillus* is also not unforeseen, given that the *Bacillus* genus is closely related to *Listeria*. In fact, the genus *Listeria* belongs to the class, *Bacilli*, and the order, *Bacillales*, which also includes *Bacillus* (den Bakker et al., 2010).

Indeed, there is significant evidence supporting the genetic relatedness of the *Listeria* and *Bacillus* genera. For example, Celandroni et al. (2000) reported that the chemotaxis gene (*cheA*) of *B. cereus* showed the highest homologies with *L. monocytogenes* (55% overall identity), even higher than that for *B. subtilis* (42% identity). In addition, Read et al. (2003) reported that several proteins that contribute to the virulence of *L. monocytogenes* have homology to proteins of the *Bacillus anthracis* Ames strain, including genes for phosphatidyl-inositol-specific and phosphatidyl-choline-preferring phospholipase C, internalin-like proteins, listeriolysin O, sigma factor B and the p60 extracellular protease. High degrees of homology among genes of the *Listeria* and *Bacillus* genera provide some evidence that there may also be similarities in cell surface proteins of these two genera, leading to similar target binding moieties for aptamers.

The binding affinity between an aptamer and its target is expressed by the equilibrium dissociation constant (K_d). The K_d value is inversely proportional to binding strength, such that the lower the K_d , the stronger the binding. A non-interacting binding site (one-site saturation) model with the non-linear least square method was used to calculate the dissociation constant for the *Listeria* aptamers based on the overall change in the number of fluorescently-labeled cells (Davis et al., 1998, Shangguan et al., 2006, Dwivedi et al., 2010). 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. The K_d values previously reported for aptamers have been in the nM to μ M ranges, values similar to those for antibodies, which usually range from 10 to 500 nM (Menezes et al., 2006, Ngundi et al., 2006). For example, an RNA aptamer specific to the type IVB pili of *Salmonella enterica* serovar Typhi had a K_d value of 6.08 nM (Pan et al. (2005). An aptamer specific for *Mycobacterium tuberculosis* (H37Rv) had a K_d value in the range at $18.4 \pm 1.54 \mu$ M (Chen et al., 2007), while a *C. jejuni*-specific DNA aptamer (ONS-23) had a reported K_d of 292.8 ± 53.1 nM (Dwivedi et al., 2010). Two DNA aptamers developed against the food allergen beta-conglutin had K_d values of 515 nM and 360 nM, respectively (Nadal et al., 2012). The K_d value for aptamer Lbi-17 was relatively high at $35.7 \pm 8.02 \mu$ M, suggesting that it may have binding affinity on the lower end of the spectrum compared to similar aptamers developed for foodborne pathogen-related applications.

However, there are many methods that can be used for characterization of K_d values, including surface plasma resonance (Nadal et al., 2012), nitrocellulose filter-binding assays

(Pan et al., 2005), isothermal titration calorimetry (Chen et al., 2007), and flow cytometry (Dwivedi et al., 2010). While no one method is preferred, surface plasma resonance has been used most frequently for the measurement of K_d values by researchers. The only previous study in which flow cytometry was used to estimate an aptamer k_d value was that of Dwivedi et al. (2010). However, this investigator used an aptamer, ONS-23, that was labeled with a fluorescent molecule (5'-FAM), while aptamer Lbi-17 was labeled at the 5' end with biotin. Because the biotin label necessitates that the aptamer be conjugated to a fluorescent molecule (FITC) prior to flow cytometry, it may be that the fluorescent signal intensity generated by this conjugation step differed from that of a FAM-labeled aptamer. Consequently, direct comparison of K_d values determined by flow cytometry may not be possible and the results presented here should be considered a rather rough approximation of binding strength. Although the estimated K_d value of aptamer Lbi-17 was relatively low, the binding affinity of Lbi-17 for *L. monocytogenes* was intense enough for use in AMC with a relatively high recovery rate (%CE) of *L. monocytogenes* and low limit of detection.

As reported by other researchers, immunomagnetic separation (IMS) is the most widely used technique for capturing *Listeria* spp., including *L. monocytogenes* (Skjerve et al., 1990, Hudson et al., 2001, Hibi et al., 2006, Yang et al., 2007b). Skjerve et al. (1990) demonstrated that the combined method of IMS and cultural enumeration produced a detection limit $<1 \times 10^2$ CFU/ml in pure culture, and $<2 \times 10^2$ CFU/ml in enriched food samples. In another study performed without cultural enrichment, *L. monocytogenes* was detected in milk samples with $\geq 10^2$ CFU/0.5 ml when IMS was combined with real-time

PCR (Yang et al., 2007b). Hudson et al. (2001) combined IMS and PCR to achieve a detection limit of as few as 1.1 *L. monocytogenes* cells/g ham sample after a brief enrichment at 14.6°C for 24 hr, while Hibi et al. (2006) reported lower LOD of 10² CFU/ml *L. monocytogenes* using a combination of IMS and flow cytometry without prior enrichment. In addition, Uyttendaele et al. (2000) reported that IMS after a 24 h enrichment procedure was able to detect *L. monocytogenes* in cheese samples at concentrations as low as <10 cfu/g.

In our study, the lower LOD for IMS/qPCR was about 10¹-10² CFU *L. monocytogenes* (1.8 log₁₀) in 500 µl buffer, which is similar to the LODs reported above for non-enriched samples (Skjerve et al., 1990, Hibi et al., 2006, Yang et al., 2007b). A similar LOD was observed for the aptamer magnetic capture assay at the 500 µl sample volume. However, the % CE of the aptamer magnetic capture ranged from 26%-77%, which was statistically significantly higher (better) than the % CE of the IMS/qPCR assay, which ranged from 16% to 21%. In our comparative studies on small volume samples, the aptamer-based magnetic capture-qPCR assay outperformed IMS-qPCR both in %CE and in LOD.

Both IMS-qPCR and AMC-qPCR were less efficient (defined by poorer %CE and higher LOD) as sample volume increased. For example, in 10 ml scale-up experiments, the LOD of the AMC/qPCR and IMS/qPCR assays were 2.7 and 3.7 log₁₀ CFU *L. monocytogenes*/10 ml, respectively; % CE ranged from 9.8-10.8% for AMC-qPCR and from 1.0-2.7% for IMS-qPCR. Despite the poorer performance at higher sample volumes, the AMC-qPCR method still outperformed the IMS-qPCR method in 10 ml samples. At even larger sample volumes

(50 ml), the LOD using aptamer-bound beads was approximately $4.8 \log_{10}$ CFU *L. monocytogenes*/sample, with a % CE not exceeding 1%. This poorer performance is probably related to a lower likelihood of contact between the target *L. monocytogenes* cells and the aptamer, a phenomenon that occurs as a function of increased sample volume. In addition, the 50 ml scale-up experiments were done using a recirculating magnetic capture unit, which relies on liquid flow during capture, rather than a more stationary sort of capture that occurs when using standard magnetic capture stands. It may be that stronger binding affinity (force) between *L. monocytogenes* cells and aptamer-bound beads would be needed to counteract forces acting against capture as a function of liquid flow. In other words, when flow is fast, the chance of binding would be increased, but requires stronger binding affinity. Since the Pathatrix device is optimized for the use of immunomagnetic beads, it may be necessary to optimize flow speed and incubation time in order to maximize the performance of aptamer capture using a recirculating capture device.

The AMC-qPCR assay results were not only comparative to parallel IMS/qPCR results, but they were also similar to what has been observed in other aptamer-based detection/capture assays. For example, when Joshi et al. (2009) immobilized DNA aptamers selected against purified outer membrane proteins of *S. enterica* serovar Typhimurium on magnetic beads, they were able to detect the target organism by qPCR at concentrations as low as 10^1 – 10^2 CFU/9 ml whole carcass chicken rinsate, while 10^2 – 10^3 CFU/25 ml was the LOD using a recirculating magnetic capture method combined with qPCR. No cultural enrichment was involved in this study. In another study, DNA aptamers specific to surface proteins of *C.*

jejuni were linked to magnetic beads for capture and subsequent detection using a quantum dot-based fluorescent sandwich assay, in which endpoint detection was achieved using fluorometry (Bruno et al., 2009). This assay was able to detect 2.5 CFU equivalents of *C. jejuni* in buffer and 10^1 - 2.5×10^2 CFU/ml in various seeded food matrices without prior cultural enrichment. Vivekananda and Kiel (2006) used two different aptamers, one for capture and the other for detection, in a two-site binding sandwich assay that they called Aptamer-Linked Immobilized Sorbent Assay (ALISA) specific for *Francisella tularensis*, achieving detection limits of 1.7×10^3 CFU/ml without prior enrichment.

This study should be considered proof-of-concept only, and further experiments are necessary to better characterize inclusivity, exclusivity, the impact of competitive microflora on capture efficiency and LOD, and the performance of the AMC-qPCR assay when applied to contaminated food matrices. For example, our studies used only a single strain of a single serovar of *L. monocytogenes* (4b), but other serovars and strains are known to cause human disease (Farber and Peterkin, 1991). The ideal assay would detect all of these strains. On the other hand, aptamer Lbi-17 showed some cross-reactivity with other members of the *Listeria* genus. This may not necessarily be a bad feature, as environmental testing frequently targets all members of the genus. However, for that sort of assay design to be useful, it would need to perform equally well with all *Listeria* species and strains, which would require additional confirmation studies. More comprehensive binding characterization studies are also needed, with particular reference to other Gram positive organisms, especially members of the *Bacillus* genus, as preliminary results with *B. cereus* showed a similar level of cross-

reactivity compared to some *Listeria* species (i.e. *L. welshimeri* and *L. innocua*). Although the AMS/qPCR assay was tested using a mixed bacterial culture, additional study will be needed to show the feasibility of the assay and the aptamers in various sample matrices including environmental swabs, waters and various foods (i.e. raw milk, deli meat, and other RTE foods).

2.6. CONCLUSIONS

In this study, biotinylated (ss) nucleic acid aptamers with binding specificity for members of the *Listeria* genus were identified using a whole cell SELEX approach. These same aptamers showed minimal binding affinity for other common foodborne organisms. We provided proof-of-concept that one of these aptamer candidates (Lbi-17) could be used for capture and subsequent qPCR detection of the organism in small (500 µl) and larger (10 ml and 50 ml) volumes of a buffer suspension containing low levels of competitive microflora. The aptamer-based magnetic capture and detection assay appeared to outperform a similar IMS-qPCR method. Although further work is needed to confirm binding specificity, optimize binding as a function of sample volume, and apply the method to more complex sample matrices. Single stranded DNA aptamers appear to be promising ligands for pre-analytical sample processing, as applied to foodborne pathogen concentration and subsequent detection using molecular amplification-based technologies.

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Table 2.1. DNA aptamer library, constructed with a 40 nucleotide random region flanked by two constant regions at the 5' and 3' ends, and primers and probe used in this study

Name	Oligonucleotides
DNA Aptamer Library	5'-GTATACGTATTACCTGCAGC - N ₄₀ - CGATATCTCGGAGATCTTGC-3'
Restriction sites	SnaB I Pst I EcoR V Bgl II
Forward Constant Region Primer	5'- AGTATACGTATTACCTGCAGC -3'
Biotin- Reverse Constant Region Primer	5'-/5Biosg/- GCAAGATCTCCGAGATATCG -3'
<i>hlyQ</i> Forward Primer	5'-CATGGCACCACCAGCATCT-3'
<i>hlyQ</i> Reverse Primer	5'-ATCCGCGTGTTTCTTTTCGA-3'
<i>hlyQ</i> TaqMan probe	5'-/56-FAM/CGCCTGCAAGTCCTAAGACGCCA/BHQ_1/-3'

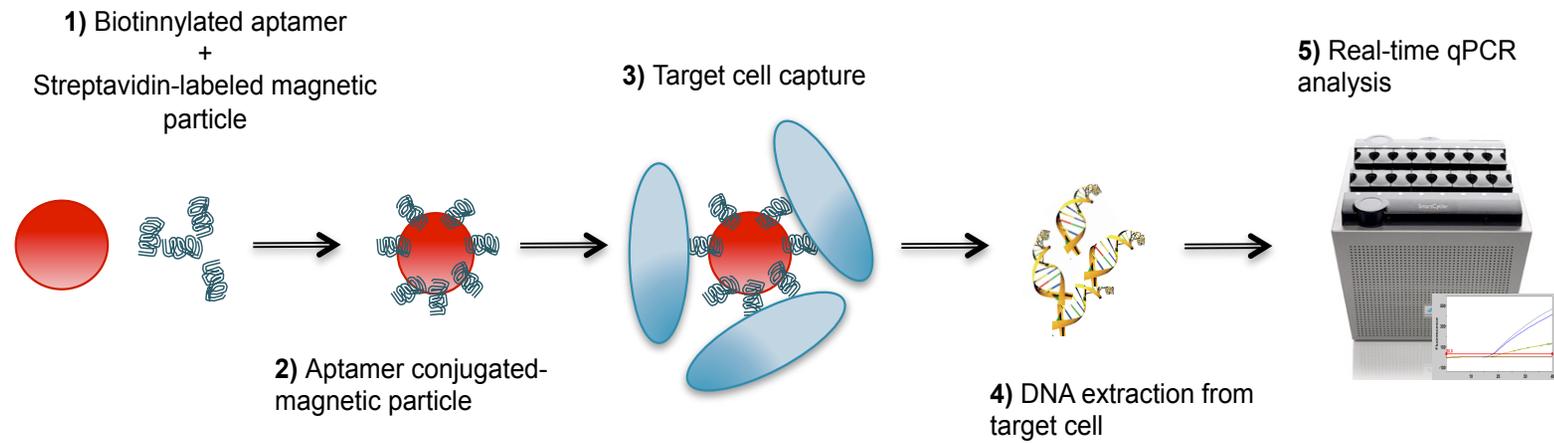


Figure 2.1. Flow diagram of the Aptamer Magnetic Capture (AMC)-qPCR assay.

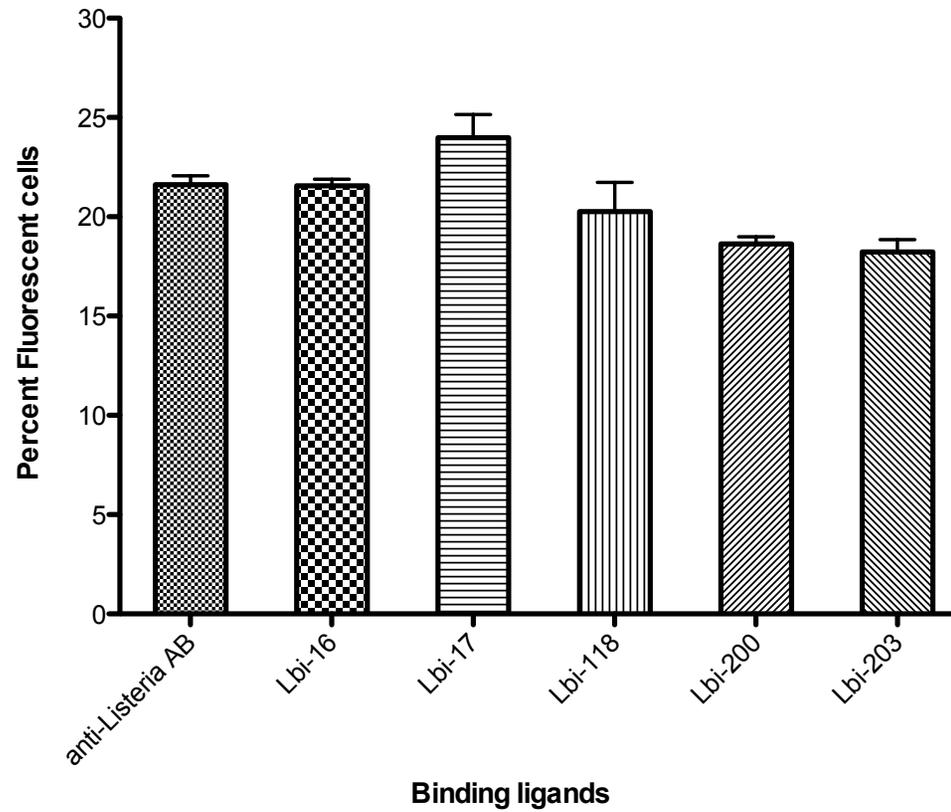


Figure 2.2. Whole-cell SELEX produced DNA aptamers with high binding affinity for *L. monocytogenes*. There were no statistically significant differences between ligand bound cells as expressed by percent fluorescence when comparing the results for any one of the aptamers to that obtained for the anti-*Listeria* antibody ($p < 0.05$).

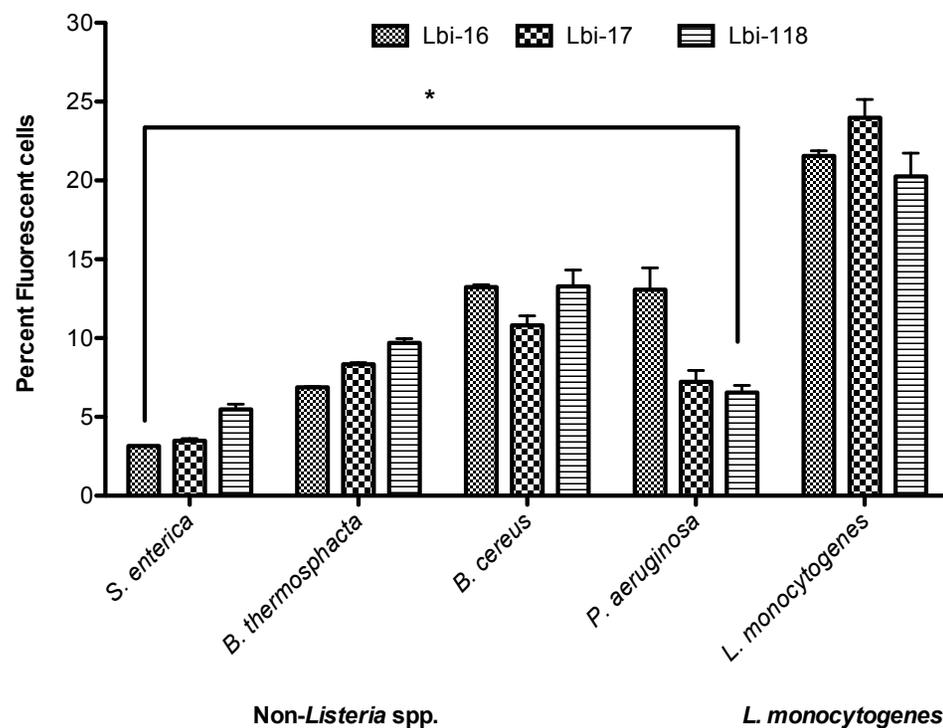


Figure 2.3. Binding exclusivity test for non-*Listeria* spp. Aptamer candidates (Lbi-16, 17 and 118) were subjected to binding exclusivity analysis using different *Listeria* spp. as evaluated using flow cytometry. Ten μM solutions of biotinylated aptamers (Lbi-16, 17 and 118) were mixed with 10^8 - 10^9 cells of non-*Listeria* spp. (in separate experiments), and the first 200,000 cells were analyzed for the fluorescence intensity using flow cytometry. For statistical purposes, % fluorescence of aptamer-bound *L. monocytogenes* was compared with % fluorescence of aptamer-bound non-*Listeria* strains (i.e. *S. enterica*, *B. thermosphacta*, and *B. cereus*). Statistically significant differences are designated with an asterisk ($p < 0.05$).

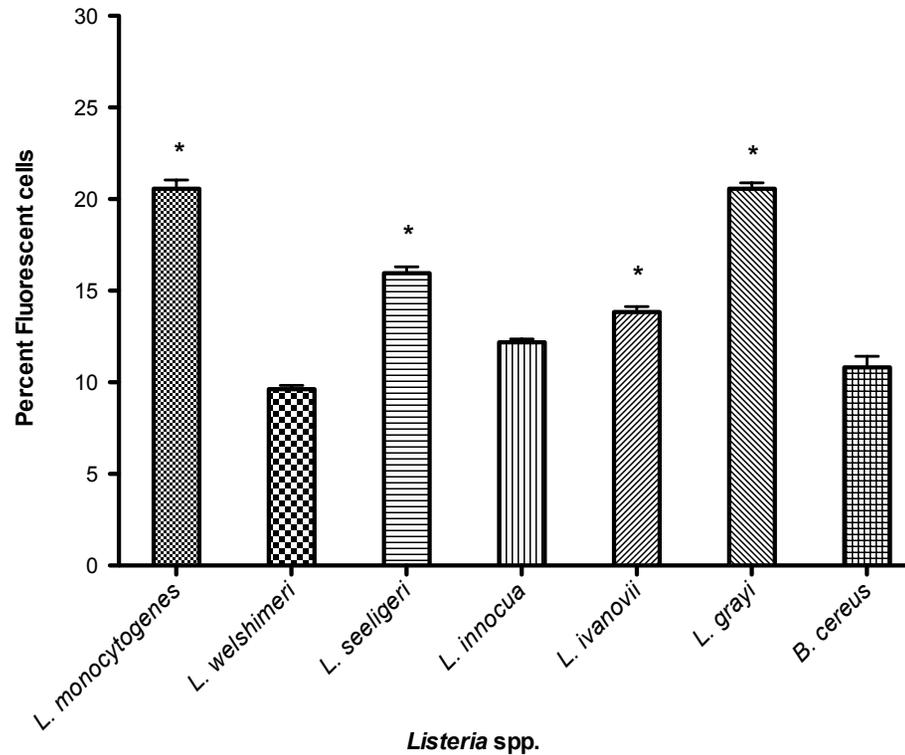


Figure 2.4. Binding inclusivity against multiple *Listeria* spp. Aptamer Lbi-17 (10 μ M concentration) was separately exposed to 10^8 - 10^9 cells of six *Listeria* spp. including *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. grayi*, *L. welshimeri*, and *L. seeligeri*. Flow cytometry analysis (n = 200, 000) was performed to measure the percent binding efficiency. For statistical purposes, % fluorescence of aptamer-bound *L. monocytogenes* was compared with % fluorescence of aptamer-bound *Listeria* spp. (i.e. *L. welshimeri*, *S. seeligeri*, *L. innocua*, *L. ivanovii*, *L. grayi*, and *B. cereus*). Statistically significant differences are designated with an asterisk ($p < 0.05$).

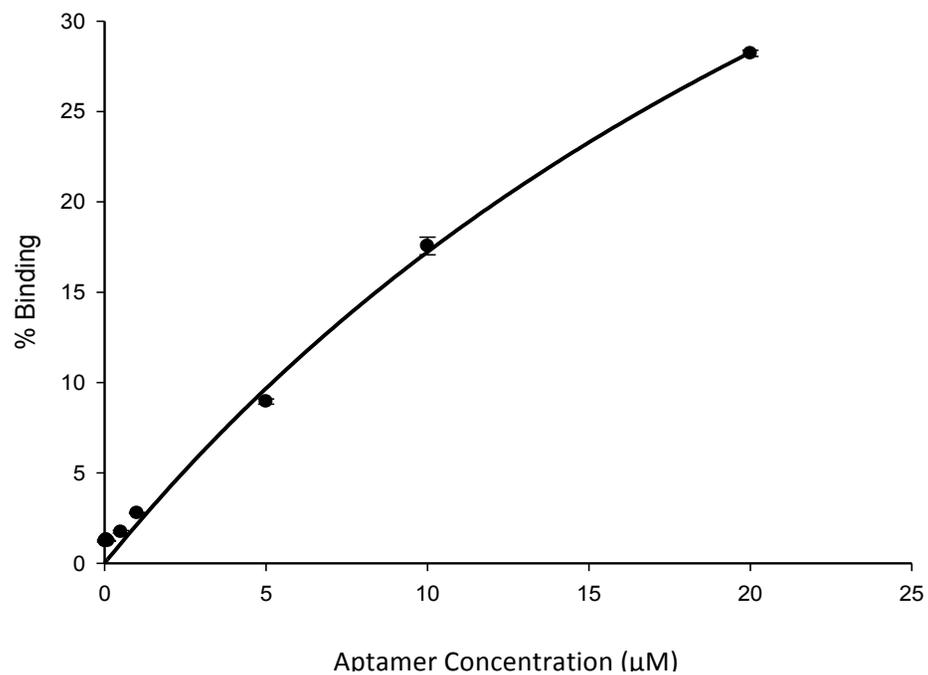


Figure 2.5. Determination of equilibrium dissociation constants. The results from a binding study done using 10^8 - 10^9 *L. monocytogenes* cells and various concentrations (i.e. 10 nM, 50 nM, 100 nM, 500 nM, 1 µM, 5 µM, 10 µM, and 20 µM) of aptamer Lbi-17 was fit to a non-linear regression model for one-site saturation using SigmaPlot (Jandel, San Rafael, CA).

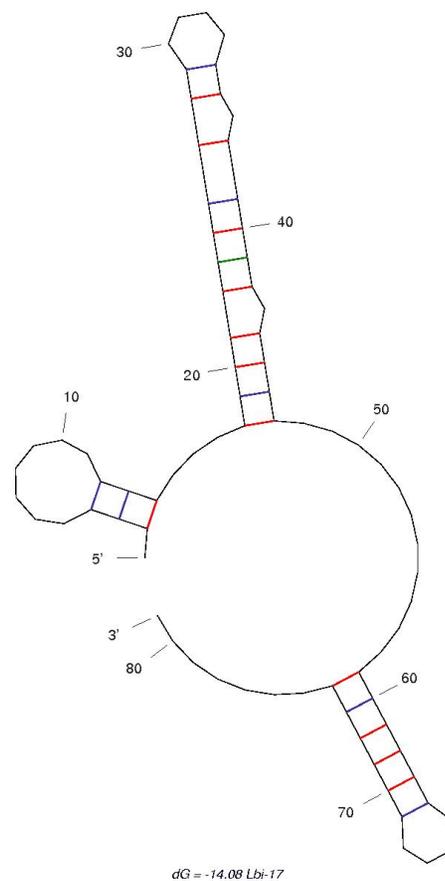


Figure 2.6. Predicted secondary structure of aptamer Lbi-17. The modeling was done assuming ionic condition of 1 mM of sodium and 0.5 mM of magnesium and a temperature of 25°C. With minimum free energy of -14.08 kcal/mol, aptamer Lbi-17 showed multiple structural loops.

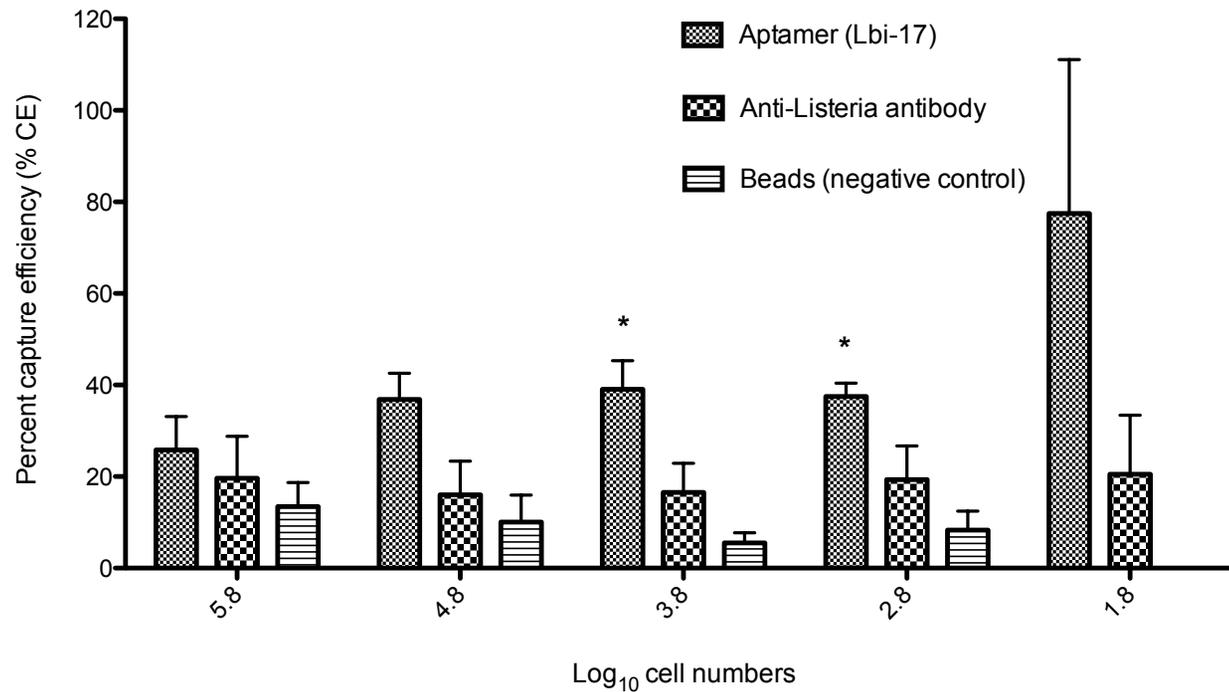


Figure 2.7. AMC-qPCR and IMS-qPCR assays applied to small volumes of mixed cultures. Percent capture efficiency (% CE) of aptamer and antibody–conjugated magnetic beads followed by qPCR as applied to serially diluted overnight culture of *L. monocytogenes* culture suspended in 500 μ l of a bacterial cocktail (mixture of serially diluted *L. monocytogenes* with generic *E. coli* and *B. thermosphacta* at concentration of 10^5 - 10^6 CFU/ml). The data were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparisons test. Values of $p < 0.05$ were considered to be statistically significant when comparing aptamer, IMS, and control beads (*= $p < 0.05$ for aptamer vs. antibody and bead control).

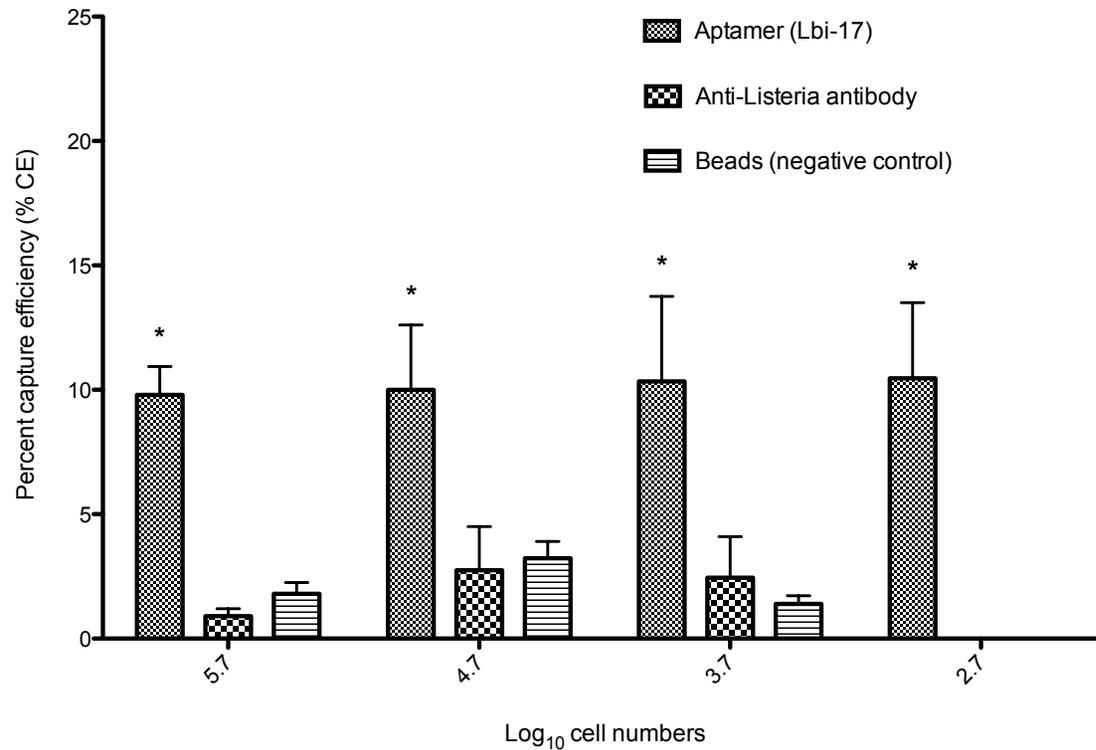


Figure 2.8. AMC-qPCR and IMS-qPCR assays applied to larger volumes of mixed cultures. Percent capture efficiency (% CE) of aptamer and antibody-conjugated magnetic beads followed by qPCR as applied to serially diluted overnight culture of *L. monocytogenes* culture suspended in 10 ml of a bacterial cocktail (mixture of serially diluted *L. monocytogenes* with generic *E. coli* and *B. thermosphacta* at concentration of 10^5 - 10^6 CFU/ml). The data were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparisons test. Values of $p < 0.05$ were considered to be statistically significant when comparing aptamer, IMS, and control beads (*= $p < 0.05$ for aptamer vs. antibody and bead control).

CHAPTER 3

Selection and Characterization of Fluorescent labeled single strand DNA Aptamers Specific for *Listeria monocytogenes* in two different growth phases and the Use of Aptamers for Detection of *Listeria* spp.

3.1. ABSTRACT

Aptamers are small, single-stranded (ss) DNA or RNA molecules that bind with high affinity and specificity to various molecular targets. In this study, aptamers with binding specificity to members of the *Listeria* genus were produced. A carboxyfluorescein (FAM)-labeled single stranded DNA library was exposed to *L. monocytogenes* cells in log phase (6 h cultures) or stationary phase (12 h cultures) and aptamers with binding specificity were selected using the SELEX (Systematic Evolution of Ligands by EXponential enrichment) method. Six SELEX rounds targeting *L. monocytogenes* were followed by two counter-SELEX rounds against a cocktail of non-*Listeria* spp. Aptamers with binding selectivity to *L. monocytogenes* were separated, sequenced, and characterized by flow cytometry. Aptamer candidates LM6-2 and LM6-116 (selected by targeting log phase cultures); and LM12-6 and LM12-13 (selected by targeting lag phase cultures) were chosen for further

characterization based on their relatively high binding affinities for *L. monocytogenes* (32-55%) as well as other members of the *Listeria* genus, including *L. innocua*, *L. ivanovii*, *L. grayi*, *L. welshimeri*, and *L. seeligeri*. Aptamer binding exclusivity analysis showed low apparent cross-reactivity with other foodborne bacteria, including *E. coli* O157:H7, *Salmonella enterica* and *Brochothrix thermosphacta*, for which binding efficiencies never exceeded 13%. Minimal cross-reactivity was observed for *Bacillus cereus* (13-18%). Using a sequential binding assay combined with flow cytometry, it was determined that three of the aptamers bound to one apparent cell surface moiety, while a fourth aptamer (LM6-116) appeared to have a different binding site. Dissociation constants (K_d values) of 106.4 ± 43.91 nM and 74.4 ± 52.69 nM were determined for aptamers LM12-6 and LM-6-116, respectively. An antibody-based magnetic capture coupled with an aptamer detector was developed as a proof-of-concept assay design. When coupled with qPCR detection of the detector aptamer, the lower limit of detection for the assay was $0.4 \log_{10}$ CFU *L. monocytogenes* in 500 μ l buffer. This is juxtaposed to a detection limit of $2.4 \log_{10}$ CFU in 500 μ l for immunomagnetic separation coupled with qPCR detection of the *hly* gene target of *L. monocytogenes*. With further work, the aptamers reported here may be used in assays designed to capture and detect *Listeria* spp. from complex sample matrices such as foods or environmental samples.

3.2. INTRODUCTION

Foodborne disease is an important public health problem worldwide. In the United States alone, it is estimated that there are 9.4 million episodes of foodborne illness of known etiology annually, resulting in 55,961 hospitalizations and 1,351 deaths. (Scallan et al., 2011). Although rare, *Listeria monocytogenes* infection (also called human listeriosis) is of considerable concern simply by virtue of disease severity, including high hospitalization and mortality rates. Several large outbreaks of listeriosis have been documented over the last 30 years, many of which were caused by contaminated commercial foods, including meats, vegetables and dairy products (Bubert et al., 1999, Hibi et al., 2006, Gottlieb et al., 2006). In response to several notable outbreaks associated with ready-to-eat (RTE) meat and poultry products occurring in the decades between 1990-2009, the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS) has issued new regulations which include testing mandates for the RTE meat and poultry industries (Gottlieb et al., 2006). Depending on the sample type, this can include testing for the pathogen *L. monocytogenes*, and/or testing for the *Listeria* genus, which is frequently used as an indicator for the potential presence of *L. monocytogenes* (Vazquez-Boland et al., 2001). In fact, *Listeria* testing comprises about one-third of the foodborne pathogen testing market, the other two important pathogens being *Salmonella* and *E. coli* O157:H7.

Historically, methods for detecting foodborne pathogens have relied on cultural enrichment and selective/differential plating, followed by biochemical identification and sometimes,

serological characterization. These methods are laborious and usually take several days to confirm a positive result (Donnelly, 2002, Yang et al., 2007). Over the years, standard culture-based pathogen detection methods have been refined and even improved, with an eye toward reducing time to detection. Newer, highly specific detection techniques including enzyme-linked immunosorbent assay (ELISA), DNA/RNA probes, and polymerase chain reaction (PCR) assays have been developed, but their sensitivity can be problematic, particularly in light of the potential for residual food-associated inhibitory compounds (Yu et al., 2001).

It has been suggested that detection of foodborne pathogens could be made more rapid, and larger sample sizes analyzed, if the target cells were separated, concentrated, and purified from the sample matrix before detection (Brehm-Stecher et al., 2009). Such so-called pre-analytical sample processing or “sample prep” could facilitate the removal of residual food matrix components while simultaneously concentrating target pathogen cells. Sample prep methods can be classified into two major categories: non-specific and specific methods. Non-specific methods enable concentration of a wide variety of microorganisms, regardless of unique features associated with their antigenicity, surface, and/or genetic characteristics. Traditional non-specific methods include filtration, centrifugation, cationic/anionic exchange resins, aqueous two-phase partitioning, and metal hydroxides. These methods promote separation based on particle size, density, and/or charge. They are frequently used in conjunction with one another. Overall, the major advantages of non-specific methods are their cost, simplicity and ability to handle fairly large sample volumes. The major

disadvantage is the tendency to co-concentrate non-target microflora or residual matrix components that are of approximately the same size, density, and/or charge as the target microbe(s). These methods are described in greater detail elsewhere (Stevens and Jaykus, 2004, Dwivedi and Jaykus, 2011).

Target-specific methods are based upon naturally-occurring biological interactions between bioaffinity ligands and specific cell surface receptor(s) (Wright et al., 1994, Hsih and Tsen, 2001, Brody and Gold, 2000, Bruno et al., 2009). Antibodies are the most commonly used ligand in this regard, and the method of coupling of antibodies to magnetic beads (called immunomagnetic separation or IMS) to facilitate pathogen concentration was introduced two decades ago (Skjerve et al., 1990, Chapman et al., 1994). Although IMS is widely used, antibodies may not be the best ligands for this application, as they can lack robustness, are difficult and expensive to produce, have relatively short shelf-lives, and have varying degrees of target specificity and avidity, all of which can impact capture and subsequent detection sensitivity. For this reason, the investigation of alternative ligands has been of great interest.

Nucleic acid aptamers are one such ligand. Aptamers are single stranded oligonucleotides that naturally fold into three-dimensional structures having binding affinity and specificity to target molecules and/or organisms. Aptamers specific for a defined target are generated using an iterative approach called SELEX (Systematic Evolution of Ligands by EXponential enrichment)(Tuerk and Gold, 1990). The SELEX process begins with a library that includes

random nucleic acid sequences flanked by two constant sequence regions at the 5' - and 3' ends, usually with diversity of about 10^{14} to 10^{15} unique nucleic acid sequences in 1 μmol scale solid-phase DNA synthesis (Jayasena, 1999). Aptamer technologies have been used in numerous applications, for example as therapeutics (Green et al., 1995, Nimjee et al., 2005, Que-Gewirth and Sullenger, 2007), diagnostic tools (Brody and Gold, 2000), and analytical agents (Tombelli et al., 2005). In recent years, aptamer technology has been applied to the detection of microbial agents including spores, vegetative cells, and metabolic byproducts (e.g., mycotoxins), including foodborne pathogens (Dwivedi and Jaykus, 2011).

Although this field is still in its infancy, recent research suggests the feasibility of nucleic acid aptamers for sample prep. In this study, a whole-cell SELEX approach was used to produce biotinylated ss DNA aptamers with binding specificity to *L. monocytogenes* cells in the exponential and stationary growth phases. The candidate aptamers were further analyzed for binding efficiency, and binding inclusivity and exclusivity. Finally, we provide proof-of-concept that they can be used as detection probes in a novel sandwich-type assay.

3.3. MATERIALS & METHODS

1.3.1. Bacterial Strains, Culture Conditions, and Preparation of Cells

The bacterial strain used as the target for aptamer selection was *Listeria monocytogenes* ATCC19115, obtained from the American Type Culture Collection (**Manassas, VA**). Other

Listeria spp. used in inclusivity studies included *L. innocua* ATCC33091, *L. ivanovii* ATCC19119 and *L. grayi* ATCC25401, as well naturally-occurring *L. welshimeri* and *L. seeligeri* strains isolated from foods (courtesy of Dr. Sophia Kathariou, NCSU). The non-*Listeria* strains used for counter-SELEX and exclusivity studies included *E. coli* O157:H7 ATCC43895, *Bacillus cereus* ATCC49063, *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC13076, *Staphylococcus aureus* ATCC23235, *Pseudomonas aeruginosa* ATCC23993, *Shigella flexneri* ATCC12022, *Brochothrix thermosphacta* ATCC11509 and *Lactococcus lactis* MG1363. The *L. monocytogenes* used in positive SELEX was separately cultured in Tryptic Soy Broth (TSB, Becton, Dickinson and Company, Sparks, MD) for 6 h and 12 h, and used in two distinct SELEX procedures for developing aptamers targeting cells in different growth phases. The strains of other *Listeria* spp. and non-*Listeria* spp. were grown separately in TSB and Brain Heart Infusion (BHI) broth (Becton, Dickinson and Company), respectively, by overnight incubation at 37°C except for *B. thermosphacta*, which was grown at 26°C. The enumeration of cells was performed by serial dilution and plating on TSB or BHI agar plates, as appropriate. Approximately $10^7 \sim 10^8$ cells of each bacterial culture were used as the target in SELEX or counter-SELEX iterations for aptamer selection. Standard growth curves (Buchanan et al., 1989) were done to identify cells in log and stationary growth phases.

3.3.3. Preparation of DNA Library for Initial SELEX Screening

An 81-mer combinatorial DNA library consisting of a 40 nucleotide random region (Table 1) was obtained from Integrated DNA Technologies (IDT, Coralville, IA). This DNA library was designed with *Sna*B I and *Pst* I restriction endonuclease recognition sites on the 5'-end, and *Eco*R V and *Bgl* II restriction sites on the 3'-end, to aid in DNA manipulations in subsequent experiments.

The DNA library was 5' end-labeled with carboxyfluorescein (FAM) on the forward strand and with biotin on the complementary strand by PCR amplification. The diluted aptamer library (10 μ M initial concentration) was amplified using the labeled constant primers (Table 1) in 50 μ l PCR reactions containing 1x GoTaq buffer (Promega Corp., Madison, WI), 0.2 mM GeneAmp® dNTPs Mix (Applied Biosystems, Foster City, CA), 5 U Go Taq® DNA Polymerase (Promega), 500 nM FAM-labeled Forward Constant Region primer, and 500 nM Biotin labeled Reverse Constant Region primer. The PCR was performed in a DNA Engine (PTC-200) Peltier Thermal Cycler-200 (MJ Research/ Bio-Rad Laboratories, Hercules, CA) using a three-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 a final extension at 72°C for 10 min.

To separate the 5' FAM-labeled forward strand from its complimentary 5' biotinylated strand, the double-stranded PCR product was coupled to Streptavidin MagneSphere® Paramagnetic Particles (SA-PMPs) (Promega) as per manufacturer instructions. The FAM-

labeled ssDNA moieties were separated from the biotin-labeled ssDNA bound to the magnetic particles using alkaline denaturation in 0.15 M NaOH, and the magnetic beads bound to biotin-labeled ssDNA were collected using a Dynal MPC®-M magnetic particle concentrator (Invitrogen Dynal A.S, Oslo, Norway). The remaining supernatant containing the FAM-labeled ssDNA was subsequently recovered and the DNA concentrated using the Vivaspin500 DNA purification/ concentration system (Satorious, Piscataway, NJ) as per manufacturer instructions.

3.3.4. Aptamer Selection Process (SELEX)

A whole-cell SELEX approach was used to select FAM-labeled ssDNA aptamers with high binding affinity and specificity to *L. monocytogenes* cells as applied to cells in the exponential (6 h cultures) and stationary (12 h cultures) growth phases. The entire SELEX scheme is shown in Figure 1. Six rounds of positive-SELEX and two rounds of counter-SELEX were done. In brief, approximately 300~500 pmoles ($1.8\sim 3.0 \times 10^{14}$ sequences) of aptamer pool was denatured by heating at 90°C and renatured by flash cooling on ice to allow intra-strand base pairing. The ssDNA aptamer pool was separately incubated with 6 h or 12 h *L. monocytogenes* ATCC19115 cells at room temperature (RT) for 1 h with gentle rotation. Aptamer-bound cells were recovered by centrifugation and washed to remove unbound and non-specifically bound ssDNA (aptamers). Aptamers bound to the cells were then enriched by PCR amplification and the double-stranded PCR product separated and converted to ssDNA for another round of SELEX, as described above. In counter SELEX, the aptamer

pool was incubated with a cocktail of non-target bacterial cells (i.e., *E. coli* O157:H7, *B. cereus*, *S. enterica*, *S. aureus*, *P. aeruginosa*, *S. flexneri*, *B. thermosphacta*, and *L. lactis*). In this case, ssDNA molecules binding to the non-target cells were discarded and the unbound aptamer pool was recovered. Following the entire combined SELEX and counter-SELEX processes, the aptamer candidates were purified (MERmaid® Spin kit, MPbio, La Jolla, CA), cloned (TOPO TA Cloning kit, Invitrogen, Carlsbad, CA) and the plasmid DNA extracted from the clones (QIAprep®Spin Miniprep Kit, Qiagen, Gaithersburg, MD) prior to sequencing (Genewiz Inc., South Plainfield, NJ)..

3.3.5. Analysis of Aptamer Binding Affinity by Flow Cytometry

Preliminary screening of binding efficiency of selected candidate aptamer sequences against *L. monocytogenes* was performed using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA). Binding assays were performed using 1 nmoles of each FAM-labeled aptamer candidate with 10^8 - 10^9 *L. monocytogenes* cells in a total volume of 1 ml. *Listeria monocytogenes* cultures were separately incubated with each aptamer candidate which was first denatured by heat (90°C) and renatured by flash cooling on ice. Centrifugation (7,000 x g for 10 min) was used to concentrate the cells, and the unbound aptamers present in the supernatant were discarded. After resuspending in 200 µl of Phosphate Buffered Saline (PBS, pH 7.0), the cells (n = 200, 000) were analyzed using flow cytometry and percent fluorescent cells and fluorescence intensity were recorded. For inclusivity studies, *Listeria* spp. including *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. grayi*, *L. welshimeri* and *L.*

seeligeri were grown to late log phase (10^8 ~ 10^9 CFU/ml) and incubated with 1 μ M FAM-labeled aptamers. After discarding unbound and non-specifically bound aptamers, the first 200,000 cells were analyzed for total number of fluorescent cells after resuspending in 200 μ l of PBS. Non-*Listeria* spp. including *E. coli* O157:H7, *Salmonella enterica*, *Bacillus cereus* and *Brochthrix thermosphacta* were applied as targets in binding exclusivity tests that were conducted in the same manner as the inclusivity studies. The data were analyzed using BD CellQuest™ Pro software (BD Biosciences).

3.3.6. Determination of Equilibrium Dissociation Constants and Predicted Aptamer Structures

Equilibrium dissociation constants were calculated by performing binding assays with target cells using varying concentrations of each of the aptamer candidates. Briefly, a *L. monocytogenes* ATCC19115 culture containing 10^8 - 10^9 cells was washed and incubated with various concentrations (10 nM, 100 nM, 200 nM, 500 nM, 1 μ M, and 2 μ M) of aptamers for 30 min at RT with gentle rotation. Aptamer-bound cells were then centrifuged at 7,000 x g, washed with PBS, and processed by flow cytometry. The equilibrium dissociation constant (K_d) was calculated by fitting the average total percent fluorescent bacterial cells (Y) [due to binding with FAM-labeled aptamers] as a function of the concentration of the aptamer (X) using a non-interacting binding sites model in SigmaPlot (Jandel, San Rafael, CA). In addition, the structural folding (secondary structure) of aptamer sequences displaying binding affinity to *L. monocytogenes* was predicted using the on-line

software DNA Mfold version 3.2 (<http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi>) (Zuker, 2003).

3.3.7. Comparative Recognition Site Studies

To confirm that the different aptamers bound to potentially different target binding sites, a sequential binding experiment was performed using the four top performing aptamers (LM6-2, LM6-116, LM12-6, and LM12-13). A flow diagram outlining this study is provided in Figure 2. For example, target *L. monocytogenes* cells (ca. 10^6 CFU) were first exposed to aptamer LM12-6, after which the non-fluorescent cells were collected by cell sorting (Beckman Coulter MoFlo modular flow cytometer; Beckman Coulter, Inc, Fullerton, CA). These non-fluorescent cells were then exposed separately to the other three aptamers (LM6-2, LM6-116 and LM12-13) in a secondary binding assay that included another flow cytometry round which also read mean fluorescence intensity and percent fluorescent cells (n=200,000). A similar experiment was performed for aptamer LM6-2 as a pre-binder, followed by subsequent binding with the other three aptamers.

3.3.8. Proof-of –Concept of Feasibility of Detection Using FAM-Labeled Aptamer (2-Site Binding Sandwich Format Assay) and Real-Time q-PCR

A schematic diagram of the sandwich format assay is provided in Figure 3. Briefly, magnetic beads conjugated with anti-*Listeria* antibody were applied to capture serially diluted *L. monocytogenes* cells. The captured *L. monocytogenes* cells were then exposed to

FAM-labeled aptamer LM6-116, followed by subsequent washings and heat treatment to facilitate release of the aptamer from the magnetic bead complex. Down-stream detection was done using qPCR targeting the sequence of aptamer LM6-116 as the template for amplification. Specific procedures are explained in greater detail below.

3.3.8.1. Preparation of Antibody-Conjugated Magnetic Beads

Biotinylated anti-*Listeria monocytogenes* antibody (Abcam, Cambridge, MA) was conjugated to streptavidin-labeled magnetic beads (M-280) (Invitrogen Dynal AS) by mixing 25 µg of beads with 2 µg of anti-*L. monocytogenes* antibody with incubation at RT for 30 min. After three subsequent washing steps, the antibody-conjugated beads were blocked overnight with Superblock solution (Pierce Biotechnology, Rockford, IL) and washed three times with PBS-Tween buffer (1x PBS containing 0.05% Tween20). The washed beads were resuspended in PBS-Tween and stored at 4°C until use.

3.3.8.2. Immunomagnetic Capture of *L. monocytogenes* Cells Followed by Sandwich Binding with Aptamer LM6-116

A 25 µg aliquot of antibody-conjugated magnetic beads was used to capture *L. monocytogenes* cells suspended in 500 µl PBS-Tween having cell concentrations ranging from 0.04 to 6.4 log₁₀ CFU/500 µl. The mixture was incubated for 30 min at RT with gentle rotation, followed by subsequent washings and magnetic pull-downs to remove unbound or unspecifically-bound cells. The cell-bound beads were then blocked by the addition of a 5% skim milk-PBS-Tween solution containing 10 µM of ssDNAs (primer pairs of the *glyA* gene

of *Campylobacter jejuni*) (Jensen et al., 2005). After blocking for 2 h at RT with gentle rotation, the blocked beads were washed again in PBS-Tween buffer, concentrated by magnet, and supplemented with 10 nM of aptamer LM6-116. The aptamer binding reaction was carried out for 30 min at RT with rotation, after which the aptamer-cell-bead complexes were washed sequentially (2 times each) with PBS-Tween, followed by PBS and 2x Binding & Washing buffer (10mM Tris/Tris HCl, 1mM EDTA and 2 M NaCl). The washed aptamer-cell bead complexes were resuspended in 50 µl of DEPC-treated water and placed in a hot water bath (90°C) for 5 min to release and denature aptamer LM6-116. The recovered supernatant used as template for subsequent qPCR targeting the aptamer.

3.3.8.3. Downstream Detection of Aptamer LM6-116 Using qPCR

Real-time qPCR was performed using the same primers as used in the SELEX process, without the 5' end labels (Table 1), and a TaqManTM probe designed to be complementary to the unique internal sequence of aptamer Lm 6-116 (5'-TET-TTC GTA GCA CTT TTC CCC AC-BHQ-3') as designed using the online program Primer3, v. 0.4.0 (<http://frodo.wi.mit.edu/>) (Rozen and Skaletsky, 2000, Rodríguez-Lázaro et al., 2004). A 25 µl reaction mixture containing 1x GoTaq buffer (Promega), 0.3 mM GeneAmp® dNTPs Mix (Applied Biosystems), 2 U Go Taq® DNA Polymerase (Promega), 500 nM Forward and Reverse Constant Region primer, and 2.5 µl of template aptamer solution was used in the amplifications. The PCR amplification was performed in a SmartCycler using a two-step thermal protocol of initial denaturation at 95°C for 120 sec followed by 45 cycles of 95°C for

15 sec, 60°C for 60 sec. A standard curve was prepared to quantify the copy number of aptamer LM6-116 captured and detected by sandwich assay. The X-axis represents \log_{10} copy number of aptamer LM6-116, while the Y-axis represents the Ct values corresponding to those aptamer copy numbers (Figure 4. a).

3.3.8.4. ImmunoMagnetic Separation (IMS) and Downstream qPCR Detection

Targeting *L. monocytogenes* Genomic DNA

Simultaneously, we also amplified the genomic DNA associated *L. monocytogenes* cells captured by IMS. In this case, the genomic DNA of *L. monocytogenes* was extracted using an automated nucleic acid extraction system, NucliSENS[®] easyMAG[®] (bioMérieux, Durham, NC) in accordance with manufacturer instructions, with a starting sample volume of 200 μ l and an ending extraction volume of 50 μ l. The downstream detection of the *L. monocytogenes* genomic DNA was done using a Taqman[™] quantitative real-time PCR (qPCR) protocol targeting a 64-bp fragment from the *L. monocytogenes hly* gene (positions 113 to 177). The primer pair and Taqman probe used in the assay are described in Table 1 (Rodríguez-Lázaro et al., 2004). The qPCR reaction was carried out in a SmartCycler system with a 25 μ l PCR reaction volume containing 1 x PCR Buffer, 5 mM MgCl₂, (Invitrogen Life Technologies, Carlsbad, CA, USA), 0.3 mM dNTPMix (Applied Biosystems), 200 nM *hlyQ* forward primer, 200 nM *hlyQ* reverse primer, 120 nM Taqman probe, 1.75 U Platinum Taq DNA Polymerase (Invitrogen) and 2.5 μ l of *L. monocytogenes* DNA. The two-step temperature protocol used in qPCR was as follows: after initial denaturation of 95° for 120

sec, annealing was performed for 45 cycles of 95°C for 15 sec and 63°C for 60 sec. For quantification purposes, a standard curve was prepared using DNA isolated from a *L. monocytogenes* that was serially diluted and simultaneously enumerated by culture-based methods and DNA extraction followed by qPCR. The X-axis represents log₁₀ number of *L. monocytogenes* enumerated by conventional plate counting method while the Y-axis represents the Ct values corresponding to that cell number (Figure 4. b). Capture efficiency was expressed as the ratio of CFU equivalents to CFU (as determined by plate count of stock culture) multiplied by 100.

3.3.9. Statistical Analysis

Results were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to compare each parameter as appropriate. Values of $p < 0.05$ were considered statistically significant. Comparisons between groups were performed using t-tests when the ANOVA test was statistically significant. Values of $p < 0.05$ were considered significant.

3.4. RESULTS

3.4.2. Selection of Aptamer Candidates

After six rounds of SELEX and two rounds of counter-SELEX, the resulting aptamer pools were cloned. A total of 50 transformants (25 transformants each from the 6 h and 12 h cell

SELEX processes) were selected for sequencing. From these transformants, six unique aptamer sequences were obtained from the 6 h culture SELEX clones, and 4 unique aptamer sequences were obtained from the 12 h culture SELEX clones (Table 2). Aptamer candidates LM6-2 and LM6-116 (selected by targeting 6 h cells); and LM12-6 and LM12-13 (selected by targeting 12 h cells) were chosen for further characterization based on their relatively high binding affinities observed in preliminary flow cytometry analysis (Figure 5). Specifically, these four selected aptamers showed binding affinities between 32-44% when evaluated using overnight cultures of target *L. monocytogenes* ATCC 19115, while the other aptamer candidates showed binding affinities of only 10-25%. Aptamer LM12-6 demonstrated the highest binding affinity (44%), followed by LM12-13 (37%), LM6-2 (35%) and LM6-116 (32%), respectively.

3.4.3. Ability of Aptamers to Recognize Multiple *Listeria* spp. (Inclusivity Studies)

The top four aptamer candidates (LM6-2, LM6-116, LM12-6 and LM12-13) were subjected to binding inclusivity analysis using various representatives of the *Listeria* genus. When a 1 μ M concentration of each aptamer was exposed to 10^8 - 10^9 CFU of six members of the *Listeria* spp. including *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. grayi*, *L. welshimeri*, and *L. seeligeri*, subsequent flow cytometry analysis (n = 200, 000) suggested binding efficiencies between 32-45% for cells of *L. monocytogenes*, and 20-55% for cells of other members of the *Listeria* genus. Aptamer LM12-6 showed the highest binding efficiency for *L. monocytogenes* as well as other *Listeria* spp., followed by LM12-13, LM6-2 and LM6-

116, respectively (Figure 6). For all of the aptamer candidates, the binding affinity for two members of the *Listeria* genus (i.e. *L. grayi* and *L. ivanovii*) was even higher than that observed for *L. monocytogenes*, while the binding affinity for *L. innocua* and *L. welshimeri* was lower than that of *L. monocytogenes*. The binding affinity for *L. seeligeri* was similar to that of *L. monocytogenes* for all four aptamer candidates.

3.4.4. Binding Specificity (Exclusivity) Studies

One μM solutions of FAM-labeled aptamers (LM6-2, LM6-116, LM12-6, and LM12-13) were used for separate binding assays with 10^8 - 10^9 CFU of non-*Listeria* spp. including *S. Enteritidis*, *E. coli* O157:H7, *B. thermosphacta* and *B. cereus* (all in separate experiments), and the first 200,000 cells were analyzed for fluorescence intensity by flow cytometry. All four aptamers showed low apparent cross-reactivity with *S. enterica* subsp. *enterica*, *E. coli* O157:H7, and *B. thermosphacta*, for which binding efficiencies never exceeded 13%. Minimal cross-reactivity (14-19% of cells fluorescent) was observed for *B. cereus*. Specific % binding exclusivities are displayed in Table 3. Overall, LM6-2 showed the lowest binding exclusivity against *B. cereus* (14%), followed by LM12-13 (16%), LM6-116 (17%), and LM12-6 (19%).

3.4.5. Comparative Recognition Site Studies

In an effort to determine if the four candidate aptamers targeted different cell surface binding sites, a comparative study was undertaken. Initially, an overnight culture (ca. 10^8 CFU) of *L.*

monocytogenes was exposed to aptamer LM12-6 (or LM6-2). After binding, the non-fluorescent cells were collected by cell sorting. These cells were then exposed to the other aptamers in separate binding assays with subsequent analysis done by flow cytometry (n=200,000). The overlapping histogram of sequential aptamer binding assays is shown in Figure 7. Overall, except for aptamer LM6-116, all the other aptamers bound less than 5~8% of the cells recovered after pre-exposure to alternative aptamers. However, LM6-116 demonstrated a binding efficiency exceeding 30% after pre-exposure to either aptamer LM12-6 (Figure 7-a) or LM6-2 (Figure 7-b), suggesting that this aptamer may bind to a different *L. monocytogenes* surface site than do the other three aptamers.

3.4.6. Determination of equilibrium dissociation constant

Equilibrium dissociation constants were calculated for two aptamers (LM6-116 and LM12-6) that apparently bound to different cell surface sites. These values were determined by performing binding assays with a defined concentration of target cells and using varying concentrations of each aptamer candidate. The percent fluorescent cells (n= 200, 000) on binding analysis of 10^8 - 10^9 *L. monocytogenes* cells with 10 nM, 100 nM, 500 nM, 1 μ M, 2 μ M of aptamer LM12-06 solution were 16.2 ± 1.37 , 22.1 ± 3.79 , 32.4 ± 0.79 , 43.5 ± 1.79 , $50.4 \pm 5.33\%$, respectively. When these data were fitted to a non-linear regression model for one-site saturation using SigmaPlot (Jandel, San Rafael, CA), the resulting equilibrium dissociation constant (K_d) was 106.4 ± 43.91 nM for aptamer LM12-06 (Figure 8. a). In similar experiments with aptamer LM6-116, percent fluorescent cells were 7.1 ± 0.76 , 7.6

± 1.1 , 12.0 ± 0.89 , 16.4 ± 1.02 , $25.2 \pm 3.98\%$ at aptamer concentrations of 1 nM, 10 nM, 50 nM, 100 nM, and 200 nM, respectively. The resulting dissociation constant was 74.4 ± 52.69 nM (Figure 8. b).

3.4.7. Predicted Secondary Structure of Aptamer Candidates

The predicted secondary structures of four candidate aptamers are shown in Figure 9. With a minimum free energy of -21.47 kcal/mol (LM6-2), -20.18 kcal/mol (LM6-116), -17.24 kcal/mol (LM12-6) and -13.83 kcal/mol (LM12-13), all four aptamer candidates showed multiple structural hairpins and loops. Both of the aptamers targeting 6 h cultured cells (LM6-2 and LM6-116) had the four hairpin structures that surrounded a large center circular structure. The hairpin structures of LM6-2 were found between sequences 4-11, 13-39, 43-67 and 69-75, while the hairpins for LM6-116 were found at sequences 2-15, 20-39, 49-61 and 63-77. Although the number of hairpins was similar, the length in between sequences was different. The secondary structures of the two aptamer targeting 12 h cells (i.e. LM12-6 and LM12-13) had little in common.

3.4.8. Proof-of-Concept of Feasibility of Detection Using FAM-Labeled Aptamer (2-Site Binding Sandwich Format Assay) and Real-Time q-PCR

The sandwich assay format was developed as a proof-of-concept that the aptamers reported in this study could be used for capture and/or detection of *L. monocytogenes*. Various concentrations of *L. monocytogenes* cells (ranging from a high of $6.4 \log_{10}$ to a low of 0.04

\log_{10} CFU) were first exposed to antibody-conjugated magnetic beads, washed, and subsequently exposed to a 10 nM concentration of aptamer LM6-116, producing a two-site binding sandwich assay. Subsequently, the bound aptamer sequence was amplified by qPCR. Figure 10 shows the relationship between initial *L. monocytogenes* cell number (prior to the antibody capture step) and the Ct values obtained after amplification of the aptamer sequences. As expected, the Ct values of samples containing high concentrations of *L. monocytogenes* ($>4 \log_{10}$ CFU) were very low (<20) and these Ct values gradually increased as concentration of *L. monocytogenes* decreased. The negative control sample (no *L. monocytogenes* exposure during antibody capture but exposure to the aptamer) had a Ct value of approximately 24, which indicates some degree of non-specific binding of the aptamer to the antibody-coated magnetic beads. However, further analysis of the data show that Ct values associated with samples containing *L. monocytogenes* were statistically significantly different from the Ct values of the control for all samples having a high concentration of *L. monocytogenes* ($6.4 \log_{10}$ CFU) to a low concentration of $0.4 \log_{10}$ CFU; the latter would constitute the assay limit of detection. There was no significant difference in Ct values between the sample containing $0.04 \log_{10}$ CFU *L. monocytogenes* and the negative control.

Figure 11 shows the relationship between the initial numbers of *L. monocytogenes* cells applied in IMS (input), to the total number of aptamers bound in the secondary binding (sandwich) step. Overall, the number of aptamers per single cell increased with decreasing concentration of *L. monocytogenes*. For example, $2.3 \log_{10}$ (200) aptamer molecules were

bound per input *L. monocytogenes* cell when the combined capture-detection assay was applied to $6.4 \log_{10}$ (2.5×10^6) CFU *L. monocytogenes*. On the other hand, when the assay was applied to $1.4 \log_{10}$ (25) CFU *L. monocytogenes*, $6.2 \log_{10}$ (1.5×10^6) aptamer molecules were bound to each input *L. monocytogenes* cell. The general trend was a steady, near exponential, increase in the number of aptamers bound per cell with decreasing \log_{10} cell number.

To better understand the dynamics of the antibody capture step, parallel IMS/qPCR assays were performed. As expected, Ct values were much higher, with a low of 28 when the initial *L. monocytogenes* concentration was $6.4 \log_{10}$ CFU, to a high of 37 when the initial *L. monocytogenes* concentration was $2.4 \log_{10}$ CFU; the latter concentration was the assay limit of detection since samples having $<2.4 \log_{10}$ CFU *L. monocytogenes* were not detectable ($Ct \geq 45$) by qPCR (Figure 12). The capture efficiency of IMS/qPCR (calculated as [*L. monocytogenes* cells detected by IMS-qPCR]/[input *L. monocytogenes* cells] x 100) was 1.7% at $6.4 \log_{10}$, 1.2% at $5.4 \log_{10}$, 0.7% at $4.4 \log_{10}$, 3.8% at $3.4 \log_{10}$ and 10% at $2.4 \log_{10}$ CFU/ 500 μ l. When input cell number was corrected for using capture efficiency, the relationship between *L. monocytogenes* cells captured by IMS to the total number of aptamers bound in the secondary binding (sandwich) step ranged from a low of $4.1 \log_{10}$ to a high of $5.3 \log_{10}$ with this number generally increasing with decreasing *L. monocytogenes* concentration (Figure 13). Note however that, due to the relatively poorer detection limit for the IMS-qPCR assay, it was impossible to calculate these numbers for *L. monocytogenes* input levels $<1.4 \log_{10}$ CFU/500 μ l.

3.5. DISCUSSION

Generally, knowledge of the relevant diagnostic marker on the target cell membrane has been required for successful application of SELEX. This marker/receptor is usually used in SELEX in its isolated and purified form (Pestourie et al., 2006). Alternatively, whole-cell SELEX, in which the entire cell surface is considered a target, can be employed to identify aptamers specific to multiple cell surface targets. An aptamer selected using an isolated or purified single protein/molecule may be useful for capturing that molecule, however when it is applied to an intact bacterial cell, it may perform less optimally because the molecule may present differently when in its native cell-associated form as compared to the purified form (Cao et al., 2009, Dwivedi et al., 2010). For this reason, we chose the whole cell SELEX approach, as it has the potential to yield aptamers having specificity for different targets in their native conformation.

Listeria monocytogenes is able to grow from 4 to 42°C, however flagella development occurs only at growth temperatures of 20°-25°C (Peel et al., 1988). By using an incubation temperature of 37°C for *L. monocytogenes* stock culture propagation, flagella development was minimized with an associated increase in the likelihood of selecting aptamers that bound to cell surface moieties. Besides flagella development, surface proteins and other functional proteins of *L. monocytogenes* are expressed differentially as a function of growth temperature and growth phase. For example, transcription of the *inl* locus, required for the expression of internalin, an 800 amino acid protein that is responsible for the entry of *L. monocytogenes*

into epithelial cells, is regulated by temperature, with higher expression at 37°C versus 25°C and maximum expression during exponential growth phase (Dramsi et al., 1993). Likewise, the expression of the *L. monocytogenes* adhesion protein (LAP, a 104 kDa surface protein) differs by growth temperature and phase, with elevated expression during stationary phase (Santiago et al. (1999). *Listeria monocytogenes* also possesses a growth phase dependent acid tolerance system (Davis et al., 1996). By using *L. monocytogenes* cells representing two different growth phases (i.e. exponential and stationary phase) during SELEX, we hoped to be able to produce aptamers having different cell surface binding moieties. It appears, from the results of the competitive binding sites study, that we were successful in this regard. It would be interesting to determine if these two aptamers (e.g., LM6-116 and LM12-6) could be used in tandem to improve assay detection limits by virtue of a multiple binding sites strategy.

Interestingly, although we developed aptamers specifically targeting *L. monocytogenes*, all the candidates showed significant binding affinity to other *Listeria* spp., suggesting that the binding moieties present on the *L. monocytogenes* cell surface were also present on these other members of the *Listeria* genus. Because we did not include these other *Listeria* spp. as counter-targets during the SELEX procedure, these results are perhaps not unexpected. From an assay development standpoint, this finding may actually be beneficial because the *Listeria* genus, as a screening purpose, is frequently used as an indicator for the potential presence of *L. monocytogenes* (Vazquez-Boland et al., 2001). Clearly, the aptamers tended to have poor cross-reactivity with Gram negative pathogens (e.g., *Salmonella* and *E. coli* O157:H7), but

some affinity for a Gram positive, *B. cereus*. It is possible that other Gram positive bacteria (pathogenic and non-pathogenic) may also have cross-reactivity with these aptamers, and studies to characterize this phenomenon are warranted in the future. Interesting, however, binding affinity against *B. thermosphacta*, a genetically close strain, never exceeded 11%.

The equilibrium dissociation constant (K_d) is used as a measure of the strength of the interaction/binding between an aptamer and its target. The K_d value is inversely proportional to binding strength, such that the lower the K_d , the stronger the binding. Typical K_d values for antibodies are in the 10-500 nM range (Menezes et al., 2006, Ngundi et al., 2006), and those for previously reported aptamers have been in the nM to μ M range (Davis et al., 1998, Pan et al., 2005, Shangguan et al., 2006, Chen et al., 2007, Dwivedi et al., 2010, Nadal et al., 2012). Comparatively speaking, at 106.4 ± 43.91 nM and 74.4 ± 52.69 nM for aptamers LM12-6 and LM6-116, respectively, our aptamer K_d values are rather low and are consistent with those ranges reported by others developing aptamers with potential food-related applications, suggesting relatively strong binding efficiencies. Interestingly, the K_d values of the FAM-labeled *L. monocytogenes* aptamers were lower (better) than those for our biotinylated aptamers reported in the previous chapter. These differences may be a function of methodological differences in measuring K_d values for FAM-labeled aptamers versus biotinylated aptamers, the latter of which requires additional conjugation of a fluorescent molecule (FITC). The fluorescent signal intensity generated by this conjugation step is probably less intense than that achieved by the FAM-labeled aptamer.

Fluorescein (FAM) and Cy5-labeled aptamers have been used in a variety of aptamer detection approaches (Cao et al., 2009, Stanlis and McIntosh, 2003, Ulrich et al., 2002, Vivekananda and Kiel, 2006), including flow cytometry analysis and fluorescent microscopy (Chen et al., 2007, Shanguan et al., 2006, Dwivedi et al., 2010). Aptamers labeled with different dyes and/or chemistry linkers have been used in a variety of detection platforms such as aptamer-linked immobilized sorbent assay (Drolet et al., 1996), calorimetric analysis (Chen et al., 2007), dot blot assay (Vivekananda and Kiel, 2006), proximal ligation assay (Fredriksson et al., 2002), aptamer-based magnetic separation (Bruno et al., 2009, Joshi et al., 2009), and for biosensor analysis (Liss et al., 2002). For example, a fluorescent dye-conjugated DNA aptamer specific to the internalin A protein was used to detect *L. monocytogenes*. When the assay was combined with an 18 h enrichment culture prior to the detection, the lower limit of detection was 10^2 CFU/25 g in an artificially contaminated ready-to-eat meat product (Ohk et al., 2010). In a different study, a two-site binding sandwich assay was used to capture *Francisella tularensis* using two different aptamers, one immobilized on a 96-well plate and used for capture, and the second a biotinylated aptamer that bound to a different recognition moiety (Vivekananda and Kiel, 2006). Detection was achieved at concentrations as low as 1.7×10^3 CFU/ml without prior cultural enrichment.

The above applications were all potential options for the use of the aptamers developed in this study. However, we chose a two-site binding sandwich assay as our prototype assay design, using an antibody as the capture ligand and a FAM-labeled aptamer as the detector ligand. The assay protocol was similar to the format of Lee et al. (2009) which was

previously designed for the capture and detection of *E. coli*. When our sandwich assay was combined with qPCR targeting the detector aptamer, the lower limit of detection was 0.4 log₁₀ CFU in 500 μl buffer (2.5 CFU/500 μl); this was about 2 log₁₀ CFU lower (better) than that of IMS followed by qPCR. Hence, the addition of the secondary aptamer binding step improved the limit of detection by almost 2 log₁₀. Interestingly, our detection limit was better than that reported for *E. coli* by Lee et al. (2009) (i.e., 10 CFU/ml), using an assay design similar to ours. The literature clearly demonstrates that antibodies against *E. coli* are usually highly efficient, displaying capture efficiencies of 50-100% in the range of 10¹ to 10⁷ *E. coli* cells (1 ml sample volumes) (Fu et al., 2005, Varshney et al., 2005, Lee et al., 2009), while the anti-*Listeria* antibody used in our study showed a capture efficiency of only 1-10% in the range of 2.5 x10⁶ to 2.5 cells in 500 μl. Since the *E. coli* antibody used by Lee et al. (2009) had about a 10-fold better capture efficiency than the *L. monocytogenes* antibody used here, it appears that some aspect of our assay design was better than that of Lee et al. (2009), probably the aptamer binding affinity and/or the efficiency of binding/blocking buffer. Unfortunately, the direct comparison of binding affinity of aptamers was not available because the K_d value of the *E. coli* aptamer was not disclosed by the author.

The aptamer-based sandwich assay developed in this study had a detection limit of 0.4 log₁₀ CFU/500 μl, which was 2 log₁₀ lower (better) than that of a parallel IMS/qPCR. Although we did not perform comparative assays with ELISA, most traditional dual antibody sandwich ELISA tests report detection limits ranging from 10⁴-10⁵ CFU/ml (Kim et al., 2005, Magliulo et al., 2007), in which case our assay design is as much as 4 log₁₀ more sensitive. Clearly,

there are advantages to the two-site binding sandwich assay relative to IMS/qPCR and perhaps even traditional sandwich ELISA. For example, aptamers are approximately four times smaller than antibodies, so it is likely that more aptamers can bind to the surface of the target relative to antibodies. Hence, an aptamer-based sandwich assay may inherently be more sensitive than a dual antibody-based sandwich ELISA. In our assay design, we used a qPCR amplification in place of the enzyme-substrate reaction of sandwich ELISA. This may also have provided additional signal amplification, again improving our detection limits.

In fact, with a capture efficiency of $\leq 10\%$, the rate limiting factor of our aptamer-based sandwich assay was the immunomagnetic capture step. Obviously, the capture efficiency and perhaps the assay sensitivity could be increased if a more efficient capture process was used. For example, using a more efficient antibody or different ligands (i.e. bacteriophage, biofunctional peptide or peptide aptamer) could be investigated. Alternatively, use of a second aptamer, particularly if it bound to an alternative cell surface moiety, could be explored.

An additional advantage to our approach is its potential utility as a screening tool. Because both aptamers and whole cells are being collected during the concentration process, a presumptive positive result by qPCR applied to the aptamer could be quickly confirmed by a second qPCR applied to the target (in this case, *L. monocytogenes*). In the case of very low levels of contamination (<1 CFU/sample), which might not be reliably picked up by a target-specific qPCR, the bead-bound cell concentrate could be subjected to a “flash” cultural

enrichment to quickly increase cell numbers to reach the PCR-amplifiable range. Finally, because the capture step is non-destructive and results in intact, live target cells, the analyst would be able to propagate these so as to have a culture for further biochemical and/or molecular characterization.

The classic method to determine the cut-off value of an assay is based on the mean value of the negative control and its standard deviation; two or three times the standard deviation (error inherent in a test) is often used to distinguish positive from negative samples. In our study, the cut-off value ($C_t = 21.9$) was calculated as three times the standard deviation of the negative control (1.8) subtracted from the mean value of negative control ($C_t = 23.7$), yielding a lower detection limit of $0.4 \log_{10}$ CFU *L. monocytogenes*. There have, however, been several studies that have sought to identify more statistically reliable means by which to establish cut-off values for pathogen detection methods (Walker et al., 1990, Johnson et al., 1995, Rha et al., 2000). Optimization of cut-off values is very important because they inherently impact assay sensitivity and specificity. For example, a more stringent (lower) cut off value can result in more frequent false positive results, while a less stringent (higher) cut off value will result in more false negative results. To achieve ideal cut off values, the maximum acceptable level of error should be considered from both food consumer's and food producer's point of view. Producers tends to lean toward less stringent (higher) cut-off values, as this means they have less false positive samples that need to be subjected to additional confirmation testing. On the other hand, consumers are most interested in public health protection, and would lean toward more stringent (lower) cut-off values, minimizing

the opportunity for accepting false negative sample results. The balance between the producer's risk and the consumer's risk are also important considerations when determining assay cut-off values.

Non-specific binding continues to be a recalcitrant problem when designing ligand-based capture and detection assays (Janssen and Rios, 1989). Many, many different approaches to alleviate this problem have been investigated (Kenna et al., 1985, Dietzgen and Francki, 1987, Vogt et al., 1987, Janssen and Rios, 1989, Tomoyasu, 1998, Péterfi and Kocsis, 2000, Lasne, 2001). Because our ultimate goal is to be able to apply our capture and detection approach to complex sample matrices such as food and environmental samples, highly stringent blocking protocols are required. Various blocking buffers (i.e. BSA, skim milk, Tween 20, Superblock) have been evaluated over the years (Gibbs, 2001), but in our hands, none of them were completely successful in eliminating non-specific binding of *L. monocytogenes* to the surface of unconjugated (without antibody) magnetic beads, nor to prevent non-specific binding of the aptamers to the beads. Ultimately, we found it necessary to use two sequential blocking steps prior to each of the two binding steps. The first block was done using a standard blocking agent (Superblock) for extended time at 4°C. The second block was done using 5% skim milk in PBS-Tween to which had been added unrelated DNA sequences (two different ssDNA primers, each having a length of 20 bases). The basic assumption was that these small, unrelated ssDNA molecules were more efficient at blocking sites that had a tendency to attract nucleic acids. In other words, the use of a protein-based blocking buffer results in blocking sites preferred by proteins, while the addition of DNA

provided value added in blocking sites preferred by DNA molecules. In fact, the use of this combined protein-DNA blocking buffer resulted in significant improvements in ΔC_t (differences in C_t values between positive samples and negative control samples), from 0.5 to 2.0. This improvement made it possible to effectively establish an assay cut-off value. It may well be that additional improvements to blocking buffer composition will be necessary as we seek to apply the assay to complex sample matrices in the future.

In conclusion, in this study we selected and characterized aptamers having binding specificity to the *Listeria* genus, then used them in the development of a prototype detection assay. Our study design is unique in its application of whole cell SELEX to bacterial cells of different growth phases, which did result in aptamers having different comparative binding sites, although the significance of this assay design has not yet been investigated. We also provided proof-of-concept that the ssDNA aptamers developed here could be used as detector ligands in a sandwich-type assay, resulting in detection limits up to 100-fold better than would have been obtained had the assay been limited to immunomagnetic capture alone. Although further work is needed to confirm the binding specificity to different serotypes of *L. monocytogenes* and other Gram positives, and apply the assay to more complex sample matrices, ssDNA aptamers provide promise in the development of real-time methods to detect pathogen contamination in foods and environmental sample matrices.

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Table 3.1. DNA aptamer library constructed with a 40 nucleotide random region flanked by two constant regions at the 5' and 3' ends having digestion enzyme sites to facilitate further modifications. Labeled constant region primers were used to amplify and label DNA aptamer library. The primers and TaqMan probe (*hlyQ*) have been used in IMS/qPCR while TaqMan probe (LM6-116) has been used for sandwich assay.

Name	Oligonucleotides
DNA Aptamer Library	5'-GTATACGTATTACCTGCAGC - N ₄₀ - CGATATCTCGGAGATCTTGC-3'
Restriction sites	<i>Sna</i> B I <i>Pst</i> I <i>Eco</i> R V <i>Bgl</i> II
FAM-Forward Constant Region Primer	5'-/56FAM/- AGTATACGTATTACCTGCAGC -3'
Biotin- Reverse Constant Region Primer	5'-/5Biosg/- GCAAGATCTCCGAGATATCG -3'
TaqMan TM probe targeting LM6-116	5'-TET-TTC GTA GCA CTT TTC CCC AC-BHQ-3
<i>hlyQ</i> Forward	Forward 5'-CAT GGC ACC ACC AGC ATC T-3'
<i>hlyQ</i> Reverse	5'-ATC CGC GTG TTT CTT TTC GA-3'
<i>hlyQ</i> TaqMan probe	5'-/56-FAM/CGC CTG CAA GTC CTA AGA CGC CA/BHQ_1/-3'

Table 3.2. Aptamer sequences obtained after six rounds of SELEX followed by two rounds of counter SELEX.

Sequence name	Target cells	%GC	Number of repeat
LM6-2	6 hr cultured <i>L. monocytogenes</i>	62.5	2
LM6-103		67.5	1
LM6-109		55	1
LM6-116		47.5	1
LM6-118		47.5	1
LM6-121		65	1
LM12-2	12 hr cultured <i>L. monocytogenes</i>	57.5	1
LM12-6		45	2
LM12-13		77.5	2
LM12-74		42.5	1

Table 3.3. Binding exclusivity of candidate aptamers. One μM solutions of FAM-labeled aptamers were used for separate binding assays with 10^8 - 10^9 CFU of non-*Listeria* spp., and the first 200,000 cells were analyzed for fluorescence intensity using flow cytometry.

Counter targets	LM6-2	LM6-116	L12-6	LM12-13
<i>S. Enteritidis</i>	6.8 \pm 0.13	5.4 \pm 0.44	6.6 \pm 0.01	12.4 \pm 1.42
<i>E. coli</i> O157:H7	6.9 \pm 1.05	7.0 \pm 0.49	4.9 \pm 0.29	6.9 \pm 0.96
<i>B. thermosphacta</i>	7.3 \pm 0.54	6.6 \pm 0.01	6.8 \pm 0.51	11.8 \pm 1.03
<i>B. cereus</i>	13.9 \pm 1.54	16.6 \pm 1.84	19.2 \pm 1.41	15.7 \pm 1.55

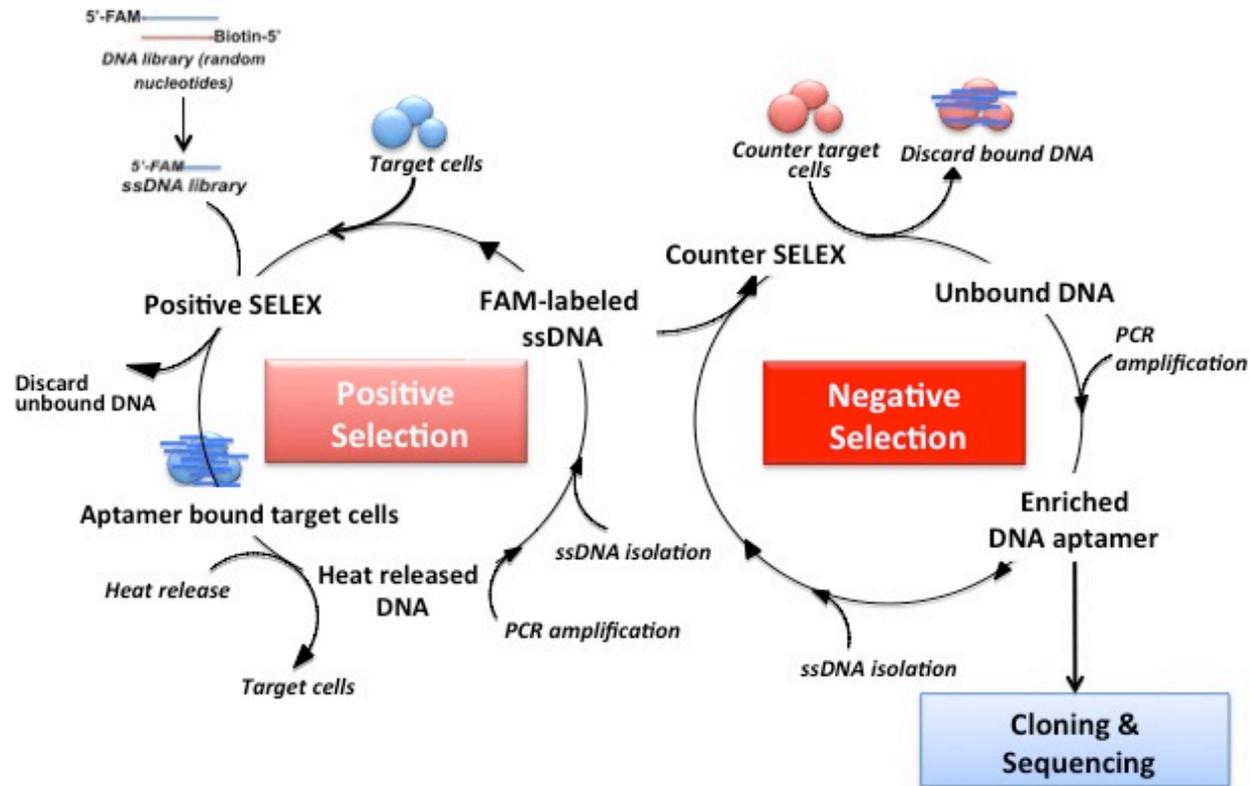


Figure 3.1. Whole-cell SELEX approach used to select DNA aptamers with functional binding specificity to *L. monocytogenes* from a large random sequence library.

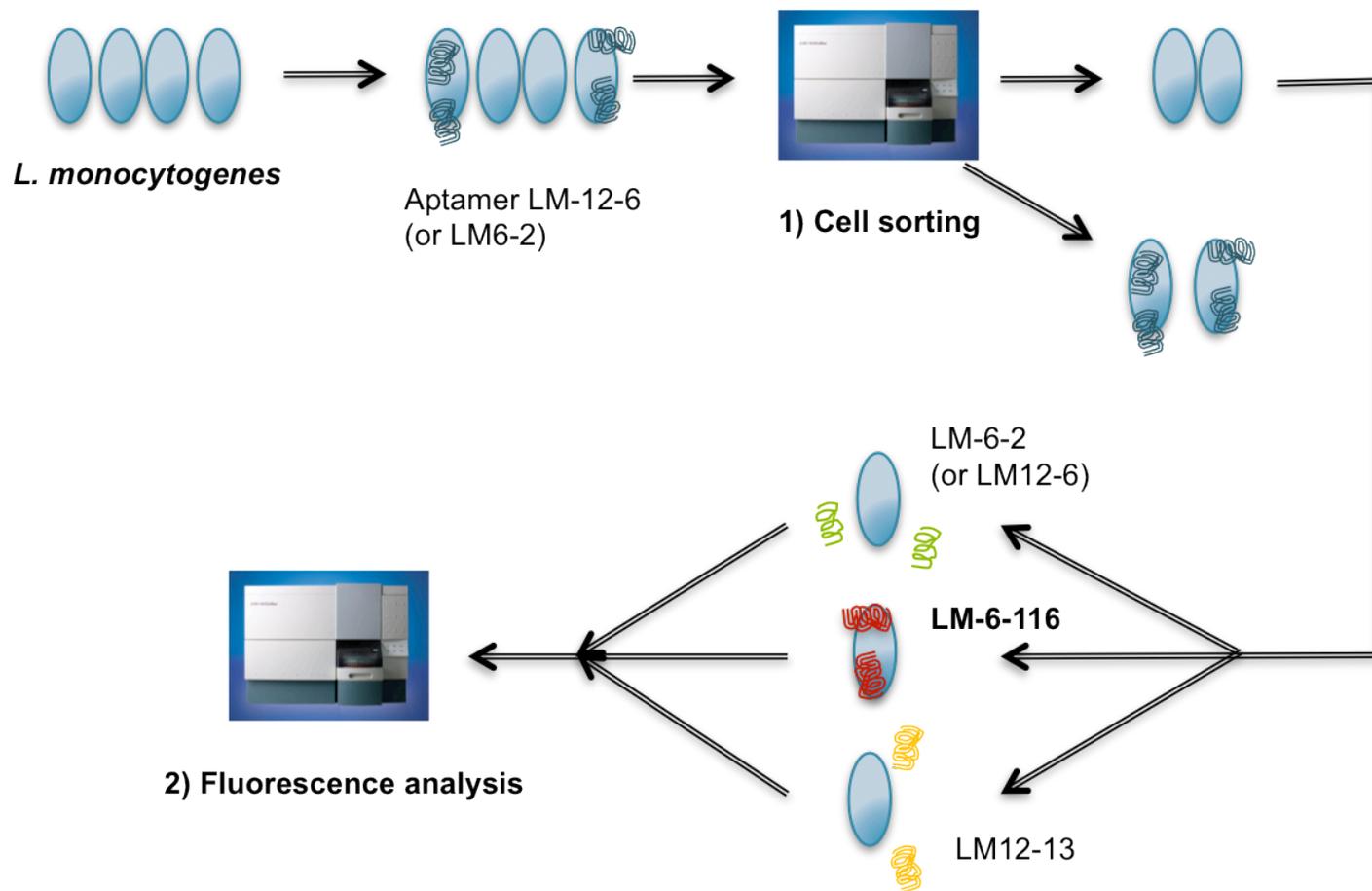


Figure 3.2. Schematic flow diagram of Comparative Recognition Site Studies

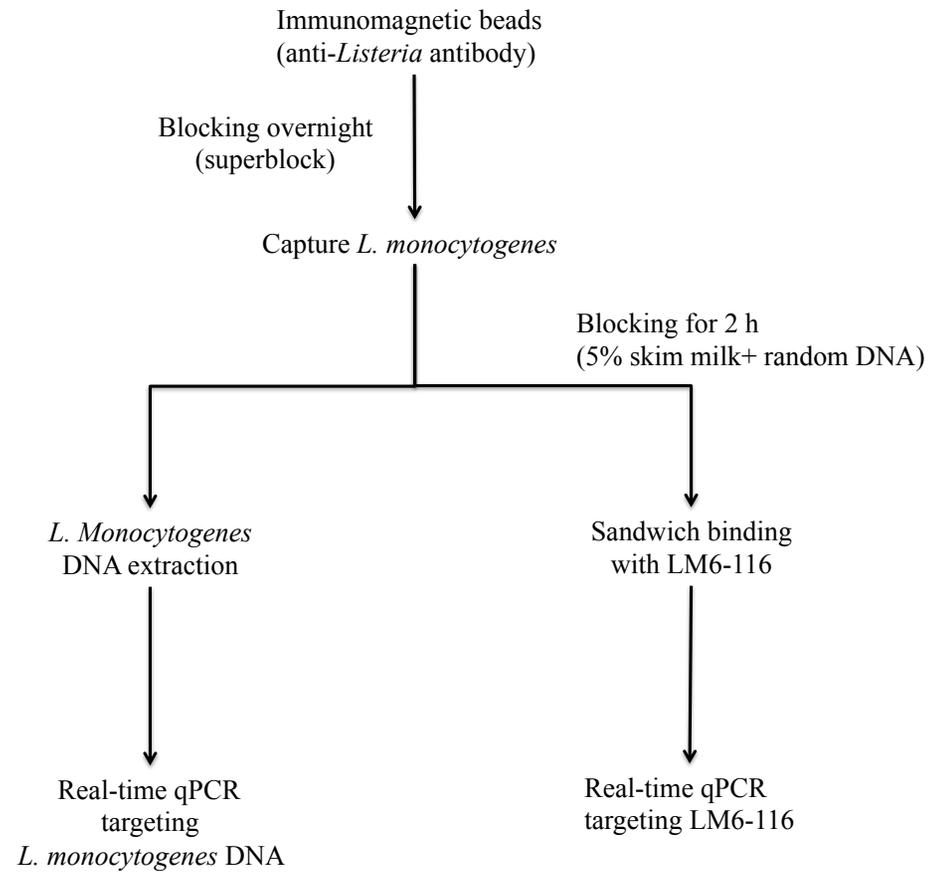


Figure 3.3. A schematic flow diagram of immunomagnetic separation and sandwich assay using two bioaffinity ligand: anti-*L. monocytogenes* antibody (biotinylated) as a capture ligand as well as FAM-labeled aptamer (LM-6-116) as a detection ligand, followed by downstream real-time qPCR analysis

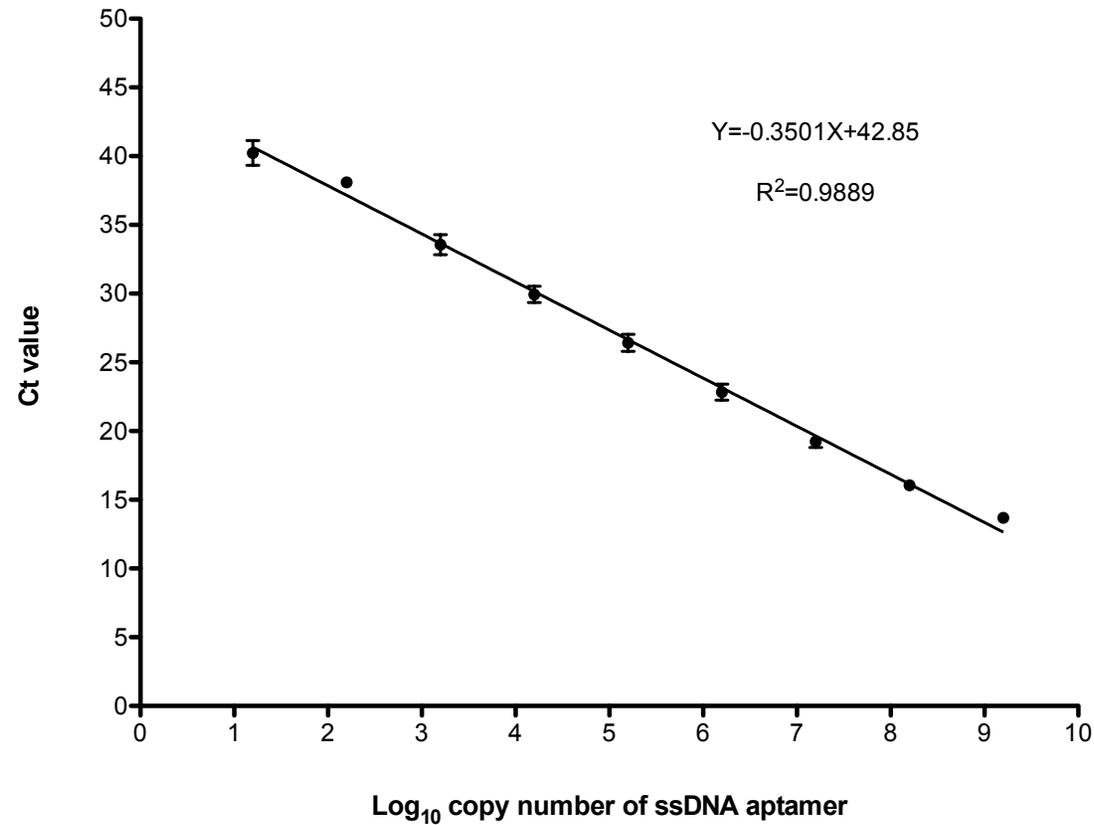


Figure 3.4.a. Standard curve for qPCR amplification of aptamer LM6-116 using the constant region aptamer primers (forward and reverse) and associated TaqMan™ probe (Table 1). The X-axis represents log₁₀ copy number of aptamer LM6-116, while the Y-axis represents the Ct values corresponding to those aptamer copy numbers.

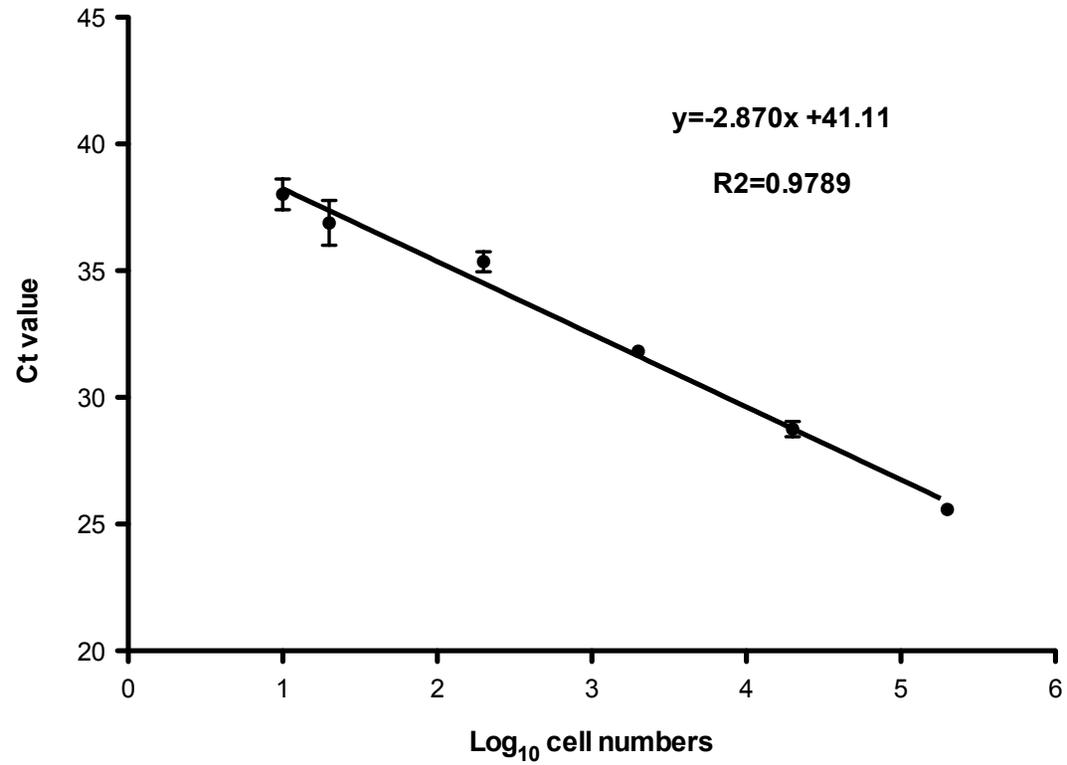


Figure 3.4.b. Standard curve for qPCR amplification of *L. monocytogenes* using the *hly* primers and associated TaqMan™ probe (Table 1). The X-axis represents log₁₀ number of *L. monocytogenes* enumerated by conventional plate counting method while the Y-axis represents the Ct values corresponding to that cell number.

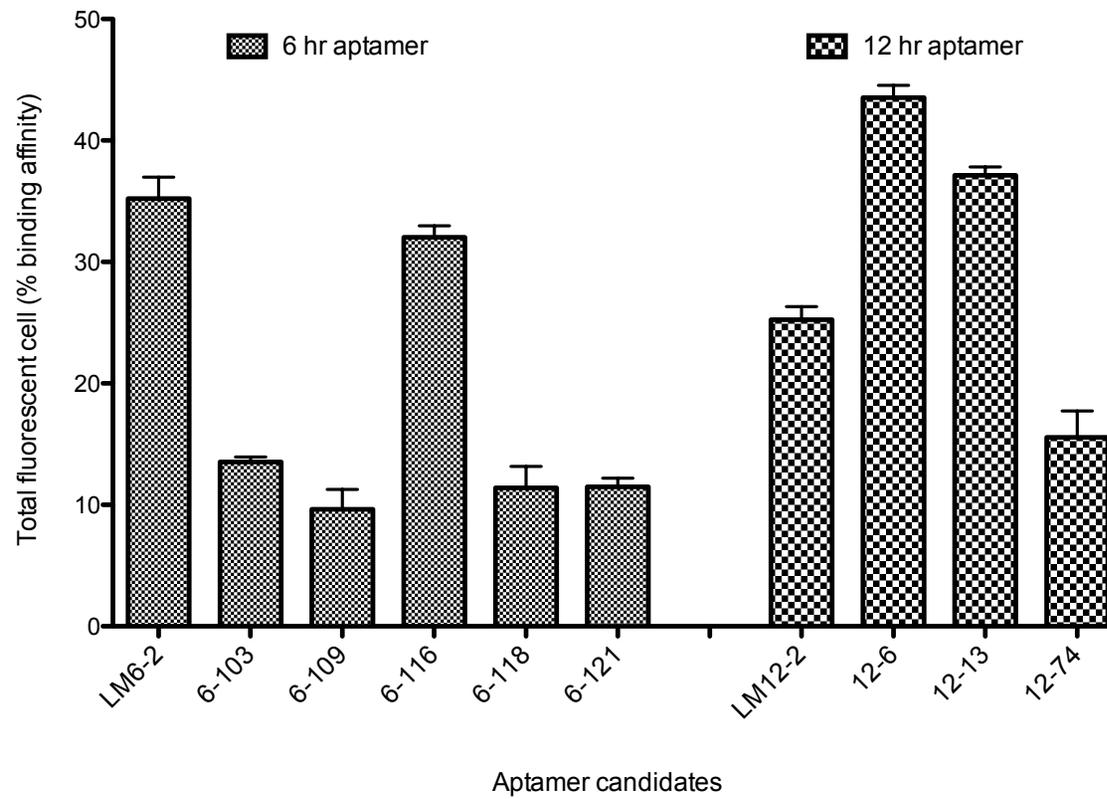


Figure 3.5. Preliminary screen of binding efficiency of 10 candidate aptamer sequences against *L. monocytogenes*.

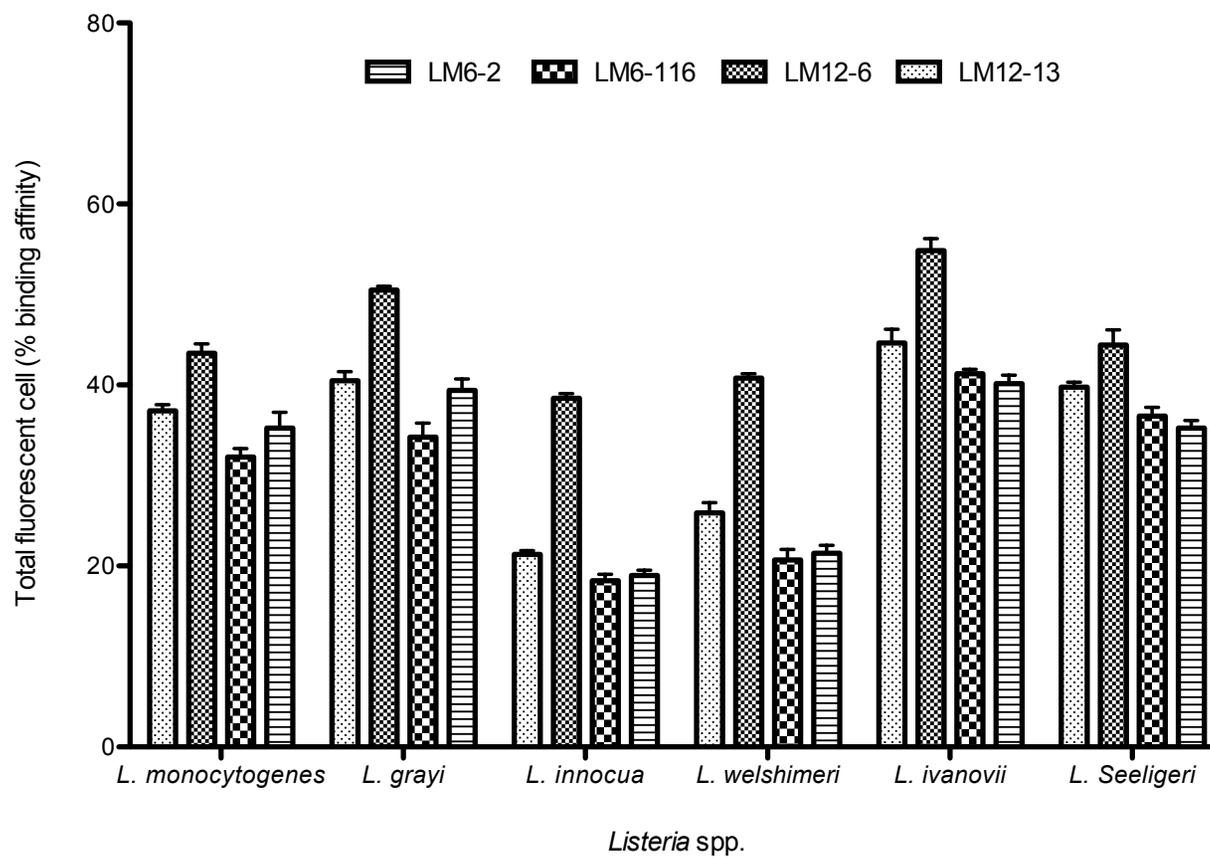


Figure 3.6. Binding efficiency of candidate aptamers (LM12-6, LM12-13, LM6-2 and LM6-116) against 6 different *Listeria* spp.

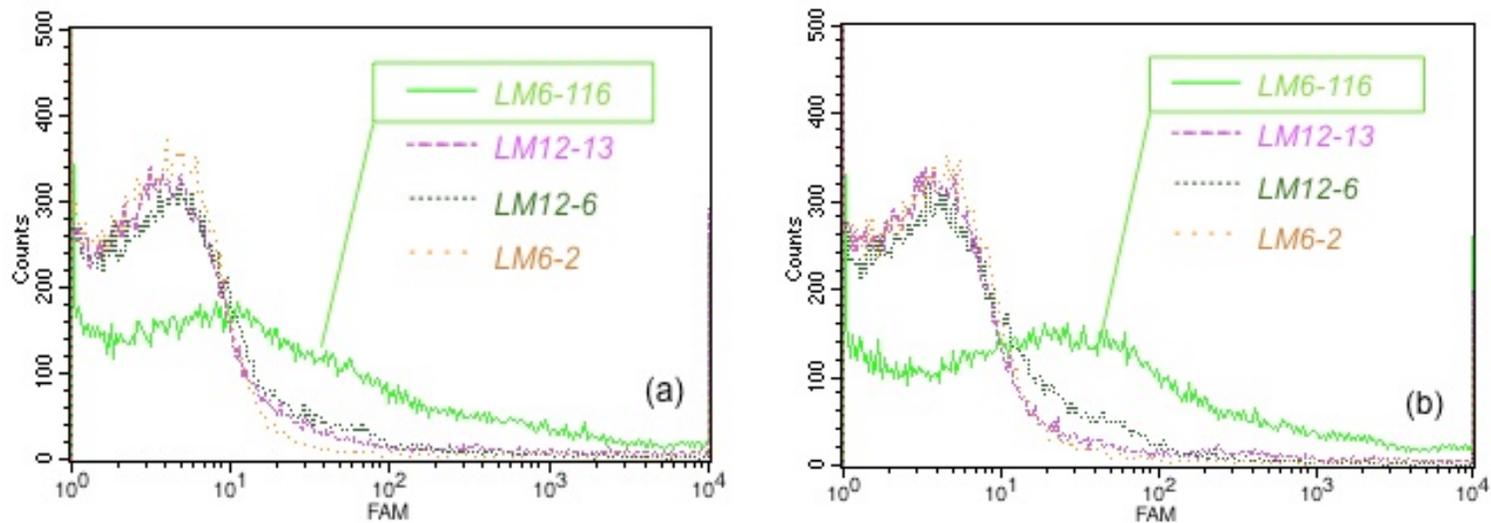


Figure 3.7. Overlapping histogram of sequential aptamer binding after initial binding with aptamer LM12-6 (a) and LM6-2 (b). The initial binding was performed using aptamer LM12-6 (a) or LM6-2 (b). The non-fluorescent cell pool was then collected and exposed to the other aptamers in separate binding assays with subsequent analysis done by flow cytometry (n=200,000).

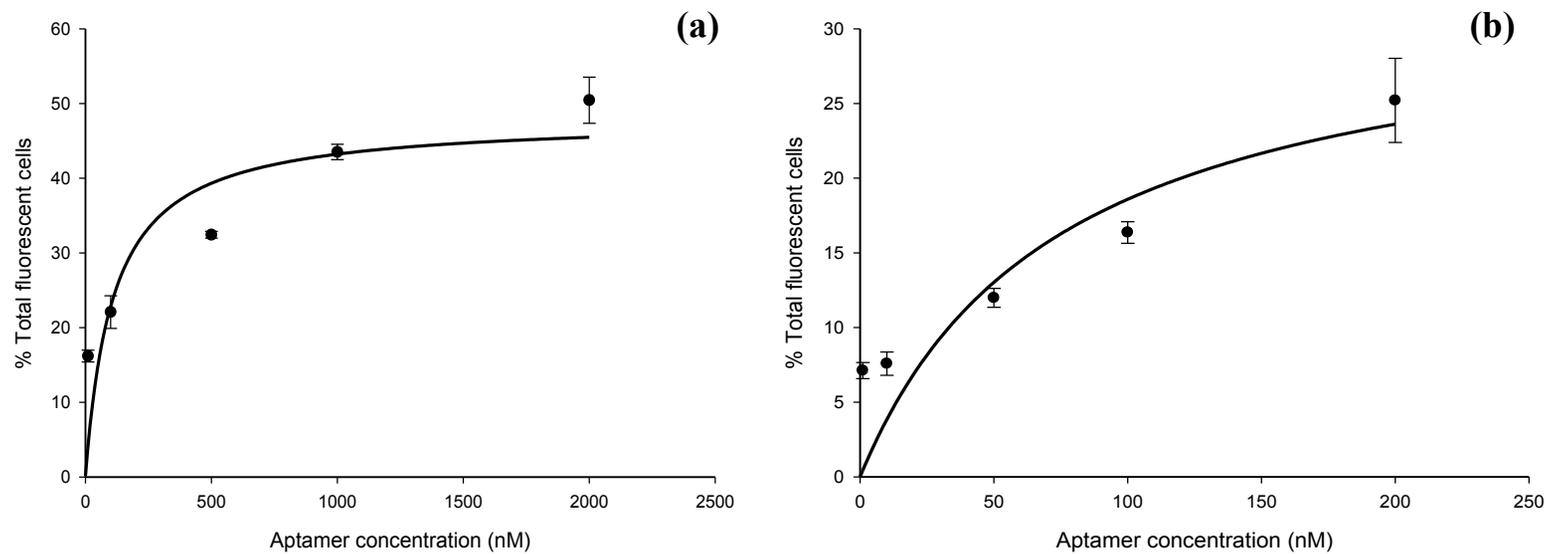


Figure 3.8. Equilibrium dissociation constant of aptamer (a) LM12-6 and (b) LM6-116 calculated using a non-linear regression model for one-site saturation.

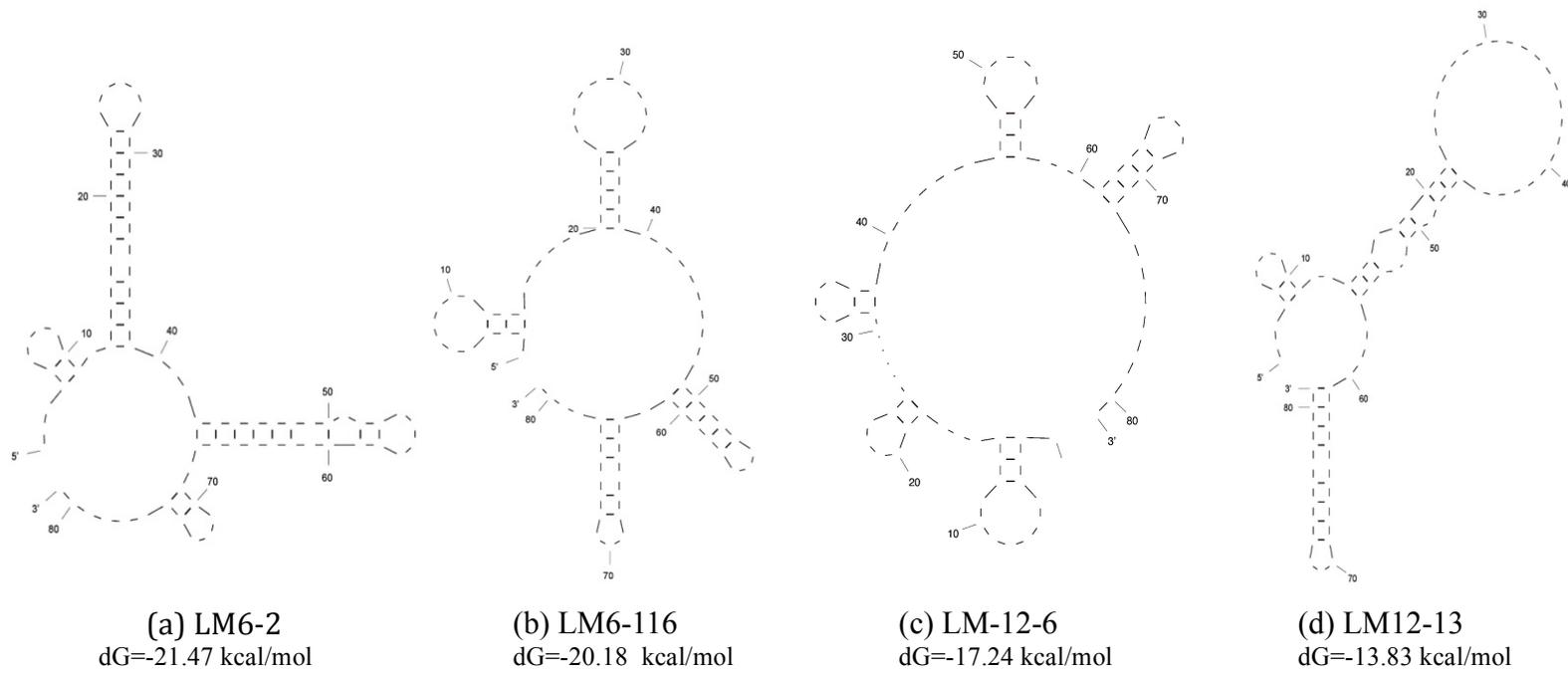


Figure 3.9. Predicted structural folding of DNA aptamers (at 25°C).

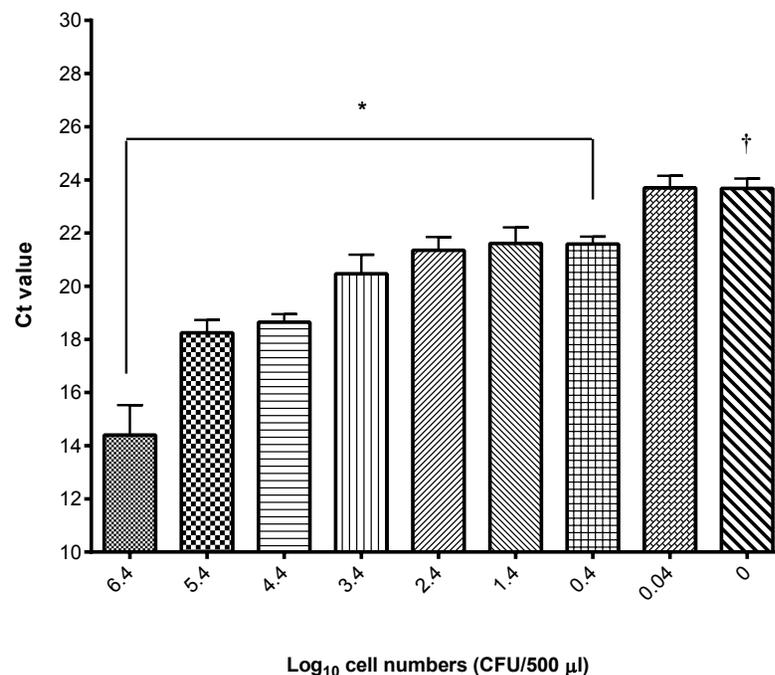


Figure 3.10. Real-time qPCR results of the two-site binding sandwich assay as applied to an overnight culture of serially diluted *L. monocytogenes*. A 25 µg aliquot of antibody-conjugated magnetic beads was applied to capture *L. monocytogenes* cells suspended in 500 µl PBS-Tween. After capturing *L. monocytogenes* by immunobeads, the secondary (sandwich) binding of aptamer LM6-116 to the captured *L. monocytogenes* cells was done. The cell-bound aptamers were then heat released at 90°C for 5 min, and the heat released aptamers were used as a template of PCR amplification. The X-axis represents the initial (input) cell concentration while the Y-axis represents the Ct value as a result of real-time qPCR. [* designates statistical significance ($p < 0.05$) when comparing the Ct values of samples containing *L. monocytogenes* to that of the negative control which did not contain *L. monocytogenes* (buffer control, designated†)].

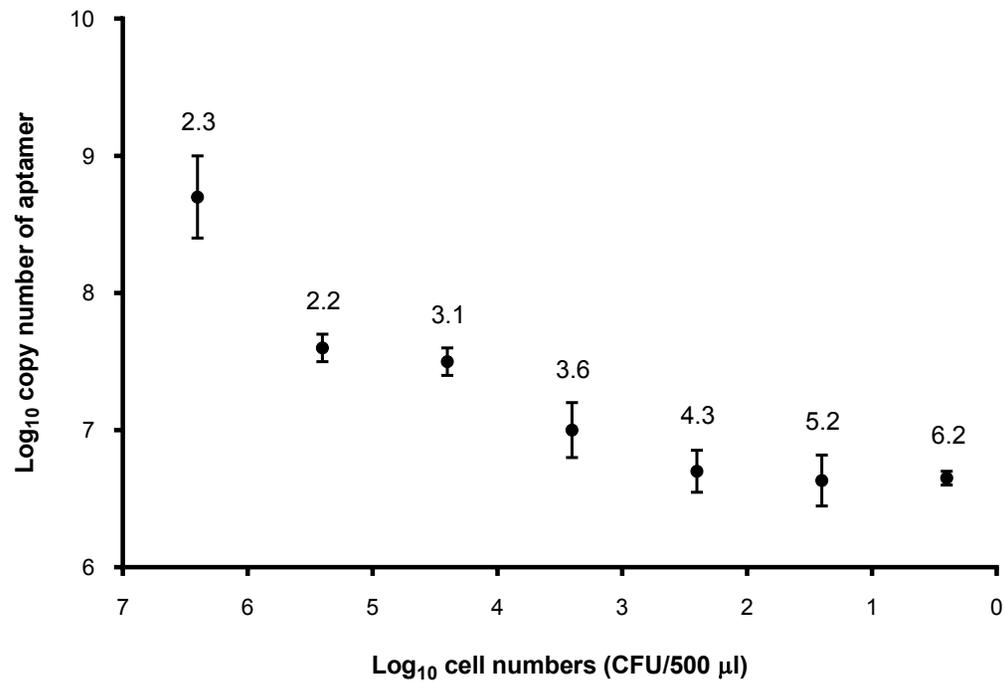


Figure 3.11. Relationship between initial number of *L. monocytogenes* cells provided for the antibody IMS step to the total number of aptamers bound in the secondary binding (sandwich) step (mean \pm standard deviation). The numbers above the points are the predicted ratio of number of aptamers (\log_{10} copy number) bound per input *L. monocytogenes* cell as calculated from the input cell counts and the data provided in Figure 10.

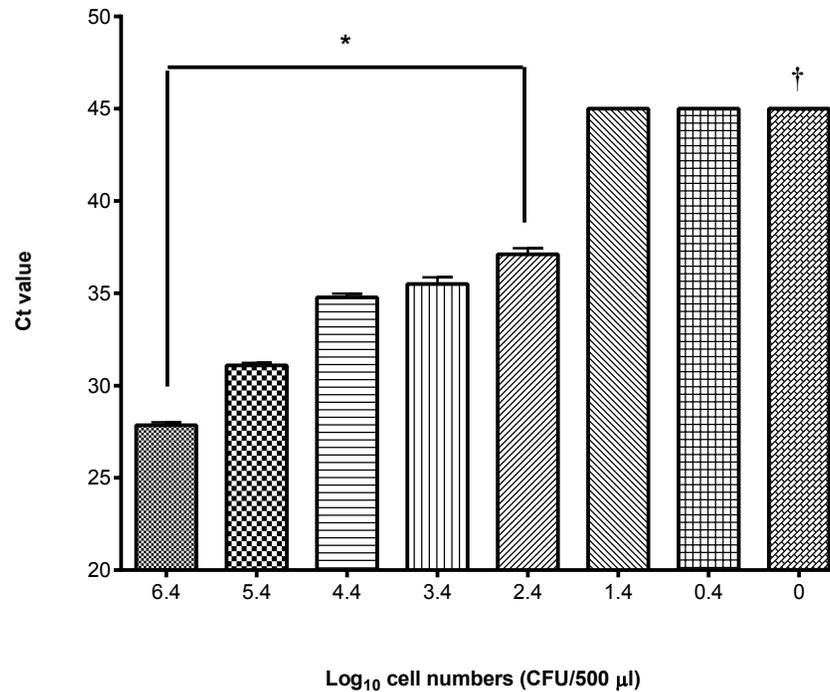


Figure 3.12. Real-time qPCR results of the immunomagnetic separation assay as applied to an overnight culture of serially diluted *L. monocytogenes*. A 25 µg aliquot of antibody-conjugated magnetic beads was applied to capture *L. monocytogenes* cells suspended in 500 µl PBS-Tween. After capturing *L. monocytogenes* by immunobeads, the genomic DNA was extracted from the captured *L. monocytogenes* and used as a template of PCR amplification. The X-axis represents the initial (input) cell concentration while the Y-axis represents the Ct value as a result of real-time qPCR. [* designates statistical significance ($p < 0.05$) when comparing the Ct values of samples containing *L. monocytogenes* to that of the negative control which did not contain *L. monocytogenes* (buffer control, designated†)].

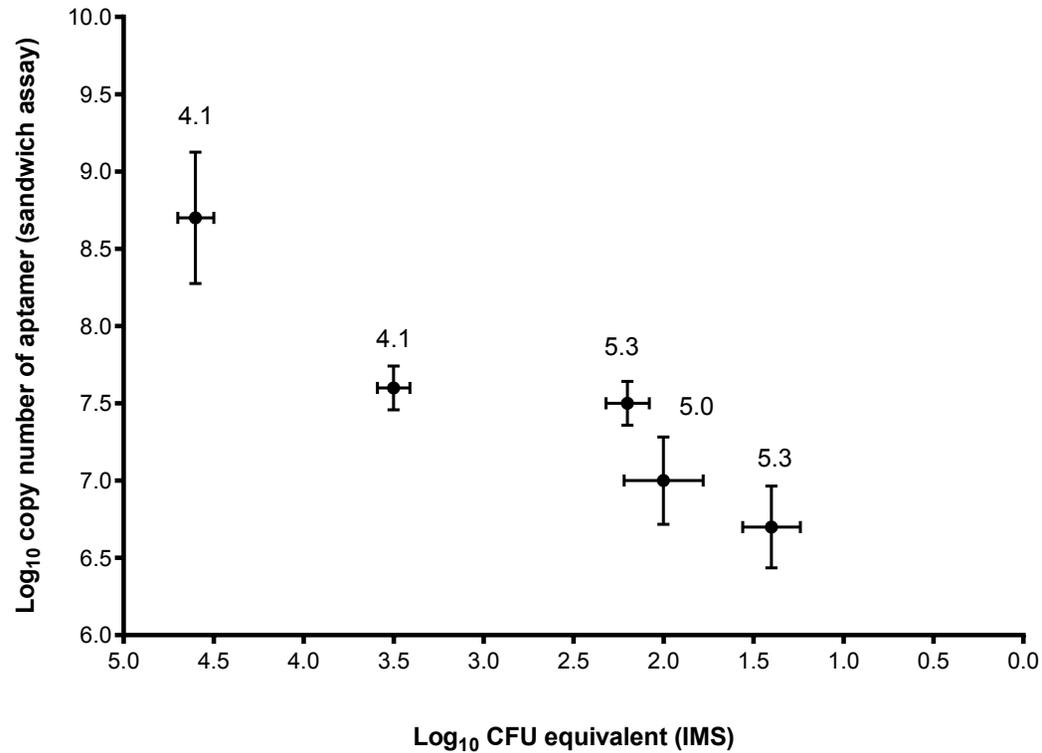


Figure 3.13. Relationship between number of *L. monocytogenes* cells captured by IMS to the total number of aptamers bound in the secondary binding (sandwich) step (mean \pm standard deviation). The numbers above the points are the predicted ratio of number of aptamers (\log_{10} copy number) bound per single *L. monocytogenes* cell captured/detected by IMS/qPCR as calculated from the combined data in Figures 4(a), 4(b) and 12.

APPENDIX

APPENDIX A

The use of DNA aptamers for capture of *Campylobacter jejuni* prior to the application of qPCR for detection

1. ABSTRACT

Campylobacters are one of the most common causes of acute gastroenteritis worldwide. Several methods are available for the detection of *Campylobacter* species but the success of these methods depends on some degree of upstream cultural enrichment of samples. Thus, there is continuing need for improved methods to detect *Campylobacter jejuni* in foods and environmental samples. The purpose of this study was to develop a rapid method to detect *C. jejuni* by combining a DNA aptamer-based capture-concentration step followed by quantitative real-time PCR (qPCR). A previously identified biotinylated DNA aptamer (designated LAJ-229) with binding specificity for *C. jejuni* (A9a) was conjugated to streptavidin-coated magnetic particles for capture of *C. jejuni* cells. The DNA from captured cells was extracted and amplified using a Taqman® qPCR assay targeting the *C. jejuni glyA* gene. To confirm binding specificity of aptamer LAJ-229, a cocktail of *C. jejuni* and non-*C. jejuni* (i.e. *Salmonella enterica* subsp. *enterica*, *Bacillus cereus*, *Shigella flexneri* and *E. coli*

O157:H7) cells was also processed for capture and detection. The sensitivity of the combined aptamer capture-qPCR assay was determined using a serially diluted pure culture of *C. jejuni*; it was also compared to an immunomagnetic separation (IMS)-qPCR assay using anti-*Campylobacter* polyclonal antibodies. The qPCR standard curve demonstrated log linear detection in the range of 1.0 -8.0 log₁₀ CFU *C. jejuni* cells per reaction, with a lower limit of detection of 1.0 log₁₀ cells. When qPCR was preceded by aptamer magnetic capture (AMC), the lower limit of detection (LoD) of the combined method was 1.1 log₁₀ *C. jejuni* cells in 300µl PBS buffer. The capture efficiency (CE) was about 10-13% when applied to 1.1 log₁₀ *C. jejuni* cells in 300 µl PBST buffer; 5% at 2.1 log₁₀ CFU/300 µl; and <1% at higher cell numbers (4.1 – 6.1 log₁₀ cells). Comparatively speaking, the CE of the combined IMS-qPCR assay was 1 - 2.5% at a *C. jejuni* concentration of 2.1- 6.1 log₁₀ CFU/300 µl with an LoD that was one log₁₀ higher (2.1 log₁₀ CFU/300 µl) than that of AMS-qPCR . When a scale-up experiment was performed in 10 ml volumes, the LoD of AMC-qPCR was 2.0 log₁₀ CFU *C. jejuni*/sample with a CE as high as 7% at initial concentrations of 2.0 log₁₀ *C. jejuni* cells/10 ml PBS. This study demonstrates that the AMC assay can be applied in conjunction with qPCR for the detection of *C. jejuni* and might even be a useful alternative to IMS-qPCR based capture-detection assays.

2. INTRODUCTION

Campylobacter jejuni is recognized as one of the leading causes of foodborne illness among the 31 best characterized foodborne pathogens (Scallan et al., 2011). Besides classic bacterial gastroenteritis, *C. jejuni* infection may also result in autoimmune neurological disorders such as Guillain-Barre' syndrome and Miller Fisher syndrome; and less frequently in meningitis, pneumonia, and miscarriage (Yang et al., 2003). Ingestion of as few as 500 *C. jejuni* cells has been reported to cause disease in humans (Robinson, 1981). The main sources of foodborne transmission of *C. jejuni* are poultry and raw milk products as well as untreated water. Based on 2010 FoodNet figures, the incidence of *C. jejuni* infection is 13.6 per 100,000 population (Gilliss et al., 2011).

Rapid and accurate detection and identification of *C. jejuni* is an important tool for the implementation of control strategies for foodborne campylobacteriosis. Currently, culture-based methods are the gold standard, but due to specific temperature and environmental requirements for the growth and recovery of *Campylobacter* spp., culture based methods are often cumbersome and time-consuming. Thus, there is a continuing need for improved methods to detect *C. jejuni* in foods and environmental samples. Detection of foodborne pathogens could be made more accurate, and rapid, if the target pathogens were separated, concentrated, and purified from the sample matrix before detection. This so-called pre-analytical sample processing would facilitate the removal of residual food matrix components with simultaneous concentration of target pathogen cells, hence improving

detection limits. It could also provide reduction of sample size by 10-200 fold (from ml to μ l volumes), making the use of molecular-based methods more feasible.

Until recently, antibodies were the most commonly used affinity ligands to capturing and concentrating pathogens from complex sample matrices. In particular, the immunomagnetic separation (IMS) procedure has been widely used (Lamoureux et al., 1997, Waller and Ogata, 2000, Yu et al., 2001). Nucleic acid aptamers, which are single stranded oligonucleotides that can naturally fold into different three-dimensional structures demonstrate target binding capabilities with high affinity and specificity. Aptamers specific to a target are generated using an iterative selection approach called SELEX (Systematic Evolution of Ligands by EXponential enrichment) (Tuerk and Gold, 1990). For diagnostic applications, aptamers provide potential alternatives to antibodies as recognition and capture ligands with added advantages over antibodies in that they are inexpensive, stable, and can be synthetically manufactured and chemically manipulated with relative ease. The purpose of this study was to develop a rapid method to detect *C. jejuni* by combining a biotinylated DNA aptamer-based magnetic capture (AMC) followed by quantitative real-time PCR (qPCR).

3. MATERIALS & METHODS

3.1. Bacterial strains, culture conditions and preparation of cells

Campylobacter jejuni (A9a) served as the target for the SELEX process. Cells were grown in *Brucella* broth (Becton, Dickinson and Co., Sparks, MD under microaerophilic conditions achieved using the GasPak™ EZ Campy Container System (Becton, Dickinson and Co, Sparks, MD) for 48 h at 42°C. The broth culture was centrifuged, washed and diluted in 1X phosphate buffered saline (PBS, pH 7.0) and cell concentrations were determined by plating serial dilutions on Campy Cefex Agar (Hardy Diagnostics, Santa Maria, CA, USA).

Salmonella enterica subsp. Enterica serovar Enteritidis (ATCC 13076), *Bacillus cereus* (ATCC 9789), *Shigella sonnei* (ATCC 25931), and *E. coli* O157:H7 (ATCC 43895) were used in studies to assess the specificity of aptamer 229. All non-*Campylobacter* strains were grown in Brain Heart Infusion (BHI) broth (Becton, Dickinson and Co., Sparks, MD) by overnight incubation at 37°C. The cultured cells were washed, centrifuged, and diluted in PBS and cell concentrations were determined by plating on agar-solidified BHI plates.

3.2. Preparation of aptamer bound magnetic beads

The 81 mer biotinylated aptamer LAJ-229, which was identified and characterized using whole-cell SELEX (Jaykus et al., 2011) was selected for these studies due to its high binding affinity and selectivity to the target. The highly purified biotinylated oligonucleotide was procured from Integrated DNA Technologies (Coralville, IA, USA) and diluted using DEPC-treated water. To prepare aptamer-bound magnetic beads, a solution of biotinylated aptamer

was denatured at 90°C for 5 min and flash cooled for 10 min. on ice prior to its conjugation to streptavidin- coated magnetic particles (Promega, Madison, WI). The aptamer conjugation to magnetic beads was performed as per manufacturer instruction using a concentration of 0.1 nmol aptamer per 50 µg magnetic beads. The aptamer conjugation was done for 30 min at room temperature (RT) with gentle rotation, followed by three consecutive washings with 1X PBS by magnetic pull-down.

For comparison purposes, anti-*Campylobacter* biotinylated polyclonal antibody was obtained from Thermo Scientific (Rockford, IL, USA) and conjugated to streptavidin-coated magnetic beads (Promega) using a concentration of 7 µg antibody per 50 µg beads and the same protocol as described above. To minimize non-specific binding, the aptamer and antibody-conjugated magnetic particles were blocked with 2% bovine serum albumin suspended in 1X PBS containing 0.05% Tween20 (PBST) for 2 h at RT with gentle rotation. After the blocking, the ligand (aptamer or antibody)-conjugated magnetic beads were washed using 1X PBS and magnetic pull-down, and stored at 4°C until used for the assay.

3.3. Aptamer or immunomagnetic capture of *C. jejuni*

The aptamer magnetic capture was done using two different sample volumes (300 µl and 10 ml) (Figure 1). For the small sample volumes (300 µl) studies, a 48 h culture of *C. jejuni* A9a was 10-fold serially diluted in PBST to yield concentrations ranging from 10² to 10⁷ CFU/ml. Nine hundred µl of each 10², 10³, 10⁵ and 10⁷ CFU/ml dilutions was mixed with a suspension (100 µl) of four other foodborne pathogens (i.e. *S. enterica*, *B. cereus*, *S. sonnei*,

and *E. coli* O157:H7) which were held at a concentration of 10^3 CFU/ml. A 250 μ l aliquot of this cocktail was mixed with 50 μ l (50 μ g) of either aptamer-conjugated magnetic beads or antibody-conjugated magnetic beads followed by incubation for 45 min at room temperature with gentle rotation. After incubation, the *C. jejuni* bound beads were pulled-down using a magnetic separation stand (Promega, San Luis Obispo, CA USA) and thrice washed to remove unbound cells using 500 μ l of 1X PBST. The scale-up studies were performed in a similar manner but using 300 μ l of aptamer-bead complex (0.6 nmol aptamer/ 300 μ g beads) as applied to 10 ml buffer system (PBST) containing 10^2 , 10^3 , 10^4 , 10^5 and 10^6 *C. jejuni* CFU/10 ml of buffer. After capture for 45 min, the *C. jejuni* bound magnetic beads were washed and subjected to DNA isolation.

3.4. Quantitative real-time PCR

DNA was extracted from recovered beads using the MasterPure™ DNA Purification kit (Epicentre, Madison, WI) in accordance with manufacturer instructions. Detection of *C. jejuni* was done using a Taqman™ quantitative real-time PCR (qPCR) protocol targeting a 126 bp region of *glyA* gene. Primers (Forward 5'- TAA TGT TCA GCC TAA TTC AGG TTC TC-3'; Reverse 5'- GAA GAA CTT ACT TTT GCA CCA TGA GT -3') and TaqMan probe (5'/56-FAM/AAT CAA AGC CGC ATA AAC ACC TTG ATT AGC/TAMRA_1/-3') used for DNA amplification were those reported by (Jensen et al., 2005). The qPCR was carried out in the SmartCycler PCR system (Cepheid, CA, USA). A 25 μ l PCR reaction containing 1X PCR Buffer, 5 mM MgCl₂, (Invitrogen Life Technologies, CA, USA), 0.4 mM dNTP Mix (Applied Biosystems, CA, USA), 300 nM forward primer, 300 nM reverse

primer, 200 nM Taqman probe, 1.75 U Platinum Taq DNA Polymerase (Invitrogen Life Technologies, CA, USA) and 2.5 µl of *C. jejuni* DNA was used. The two-step temperature protocol used in qPCR was as follows: after initial denaturation of 95°C for 120 sec, annealing was performed for 40 cycles of 95°C for 20 sec and 60°C for 30 sec.

For quantification purposes, a standard curve was prepared using DNA isolated from a *C. jejuni* culture that was serially diluted and simultaneously enumerated by culture-based methods and DNA extraction followed by qPCR. These data were plotted as Ct value (Y axis) vs. CFU equivalent (X axis), where the latter was defined as the initial CFU prior to aptamer (or antibody) capture, DNA extraction, and qPCR. The approximate CFU in unknown samples subjected to magnetic pull-down 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.

3.5. Data Analysis

Statistical comparisons between % CE at each inoculum level were done by one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test using the Statistical Analysis (SAS vr. 9.2, Cary, NC) (* $p < 0.01$).

4. RESULTS

4.1. qPCR Standard Curve

To make the standard curve, *C. jejuni* cells were 10-fold serially diluted to concentrations ranging from 1.0 to 8.0 log₁₀ CFU/ml (Figure 2). The DNA was extracted from each dilution and subjected to qPCR. The term “CFU equivalents” was used to describe the relationship between the input cell concentration subjected to DNA extraction and qPCR amplification, and the Ct value obtained from the corresponding standard curve. The qPCR standard curve demonstrated log linear detection in the range of 1.0 to 8.0 log₁₀ CFU equivalents of *C. jejuni* per reaction, with a lower limit of detection of approximately 1.0 log₁₀ CFU equivalents.

4.2. Aptamer magnetic capture in small volume (300 µl) mixed culture

When the AMC was combined with qPCR for detection of *C. jejuni* in a background of mixed bacterial cells (*S. enterica*, *B. cereus*, *S. sonnei*, and *E. coli* O157:H7), percent CE was < 5% at inoculum levels of 2.1 log₁₀ CFU/300 µl PBST buffer and between 10-13% at levels of 1.1 log₁₀ *C. jejuni* cells/ 300 µl PBST buffer. A progressive increase in percent CE was observed as the target cell numbers decreased. The LoD of the combined AMC-qPCR method was about 1.1 log₁₀ *C. jejuni* cells in 300 µl buffer (Figure 3). For the combined immunomagnetic separation-qPCR, % CE never exceeded 2%. The LoD of the combined IMS-qPCR assay was one log higher than the AMC-qPCR assay, i.e. 2.1 log₁₀ CFU/300 µl. Residual non-specific binding was observed in the negative control (beads lacking ligand

conjugation) at high target cell concentrations ($6.1 - 4.1 \log_{10}$ CFU/300 μ l), but never exceeded 1% CE.

4.3. Scale-up experiment

A scale-up study was performed in which *C. jejuni* cells were suspended in 10 ml PBST and used in aptamer magnetic capture that was performed followed using a simple magnetic pull-down device. The LoD of combined AMC-qPCR for the larger volume studies was $2.0 \log_{10}$ CFU *C. jejuni*/sample. An increase in percent CE was observed with decreasing cell concentration, with a low of <1% and a high of 4-7% at 6.0 and $2.0 \log_{10}$ *C. jejuni* cells, respectively, in 10 ml PBST. Non-specific binding of non-conjugated magnetic beads was apparent at high target cell concentrations, but disappeared at low target cell number ($<3.7 \log/10$ ml) (Figure 4).

5. DISCUSSION

Aptamer technologies have been widely used in numerous fields ranging from development of new drugs and therapeutics (Green et al., 1995, Nimjee et al., 2005, Que-Gewirth and Sullenger, 2007, Keefe et al., 2010) to the diagnostics field (Brody and Gold, 2000); (Tombelli et al., 2005). In recent years, aptamer technology has been applied to the detection of microbial agents including spores, vegetative cells, and metabolic by-products such as mycotoxins (Bruno and Kiel, 1999, Cruz-Aguado and Penner, 2008, Jeffrey and Fischer,

2008). The field of aptamer-based capture and detection technologies for foodborne pathogens is new and emerging (Joshi et al., 2009, Dwivedi et al., 2010, Cao et al., 2009).

Recently, Joshi et al. (2009) used DNA aptamers selected against purified outer membrane proteins of *S. enterica* serovar Typhimurium immobilized on magnetic beads to capture the organism from whole carcass chicken rinse samples. The magnetic pull-down assay combined with qPCR in that study was able to detect *S. Typhimurium* at concentrations as low as 10^1 – 10^2 CFU/9 ml rinsate. When the same aptamer was applied to the capture and detection of *S. Typhimurium* in larger sample volumes using a recirculating magnetic capture device, the investigators observed a lower limit of detection of 10^2 – 10^3 CFU/25 ml chicken rinsate. In another study, DNA aptamers specific to surface proteins of *C. jejuni* linked to magnetic beads were used for capture and subsequent detection of *C. jejuni* using a quantum dot-based fluorescent sandwich assay (Bruno et al., 2009). This assay was able to detect 2.5 CFU equivalents of *C. jejuni* in buffer and 10^1 – 2.5×10^2 CFU/ml in various seeded food matrices without prior cultural enrichment.

Immunomagnetic separation (IMS) is one of the most widely used techniques for capturing foodborne pathogens including *Campylobacter spp.* in food samples. For example, Docherty et al. (1996) reported an IMS technique which on application in chicken samples resulted in 10 to 15% CE for *Campylobacter* cells. The lower limit of detection of the assay as applied to seeded chicken and milk samples first subjected to a 24 h enrichment was 42 CFU/g and 63 CFU/ml, respectively. Similarly, Lamoureux et al. (1997) reported an

immunomagnetic separation method combined with DNA/RNA hybridization to detect of *C. jejuni* from artificially contaminated food samples. The lower limit of detection of this assay was 10^4 CFU/ml without prior enrichment; after a 24-48 h enrichment, detection limits as low as 3 CFU/ml (for milk) and 3 CFU/10 g (for meat) could be achieved. Similarly, Yu et al. (2001) used polyclonal anti-*Campylobacter* antibody-coated magnetic beads for detection of *C. jejuni* from culture suspension and ground poultry meat. A lower limit of detection of 10^3 CFU/ml for culture suspension and 10^4 CFU/g ground poultry meat, without pre-enrichment, was recorded. In our study, the lower limit of detection for IMS/qPCR was about $2.1 \log_{10}$ CFU *C. jejuni* in 300 μ l heterogenous cell suspension (i.e. *S. enterica*, *B. cereus*, *S. sonnei*, and *E. coli* O157:H7), which is similar to that reported by others for immunomagnetic capture (Yu et al., 2001, Lamoureux et al., 1997, Docherty et al., 1996).

When comparing the results of the AMC/qPCR to IMS/qPCR developed in this study as applied to small sample volumes (500 μ l), the LoD for the former was one \log_{10} lower (better) than the LoD of the latter. In addition, the percent CE of AMC ranged between 1% and 13% as compared to a range of 0.5 to 3% for IMS-qPCR. In short, in our comparative studies using small volume samples, the AMC -qPCR assay outperformed IMS-qPCR both in percent CE and LoD. Although the percent CE was relatively low (up to 5%), the AMC/qPCR assay performed relatively well in 10 ml volumes, displaying an LoD of $2.0 \log_{10}$ CFU/10 ml, which is similar to that reported by Joshi et al. (2009). As a general trend, as sample volume increased from 300 μ l to 10 ml, the LoD of AMC/qPCR increased and the percent CE decreased, implying the decreased sensitivity of the assay with larger sample

volumes. Further, optimization of the aptamer-magnetic capture step may be required to enhance the chances of interaction between the aptamers and target cells when sample volumes increase.

Overall, this study provides proof-of-concept that biotinylated aptamers can be employed in a magnetic bead-based capture of *C. jejuni* followed by qPCR detection as applied to small (300 μ l) and larger (10 ml) sample volumes. When compared to an IMS-qPCR assay applied to similar sample volumes, the aptamer-based assay performance was equivalent to (at high target contamination levels), or better than (at low contamination levels) the IMS-qPCR in terms of both assay limit of detection and percent CE. Overall, nucleic acid aptamers may constitute a promising alternative to antibodies for magnetic separation assays designed for selective capture and concentration of target cells. Further experiments are required to assess the feasibility of AMC-qPCR for use in pathogen detection in food and environmental sample matrices.

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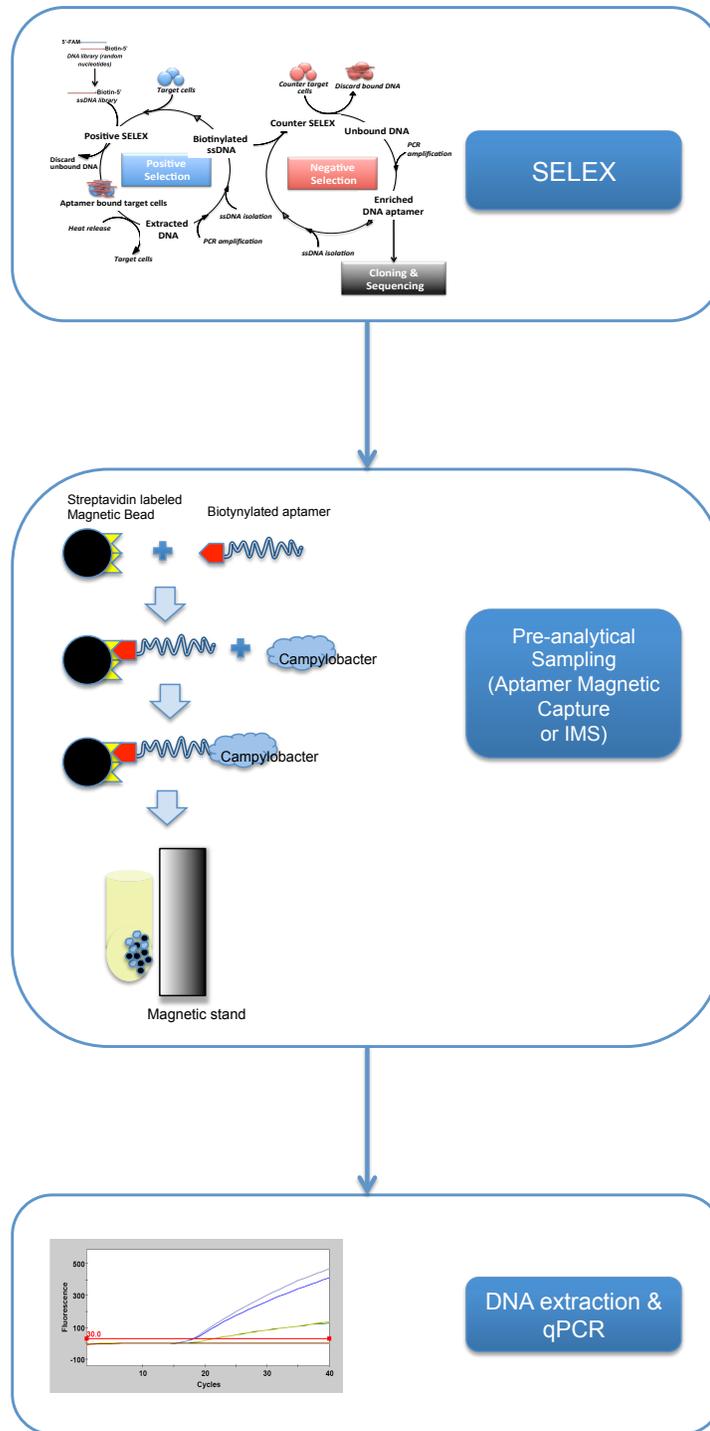


Figure 1. Experimental flow diagram of Aptamer Magnetic Capture (AMC) method

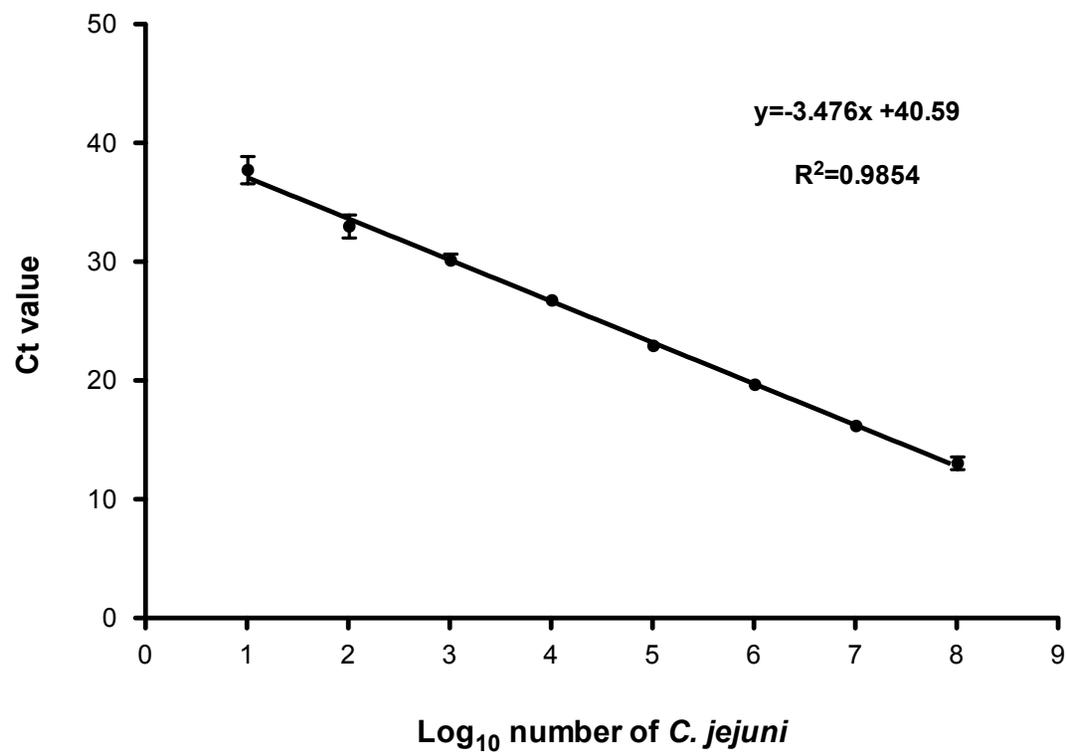


Figure 2. Standard curve for qPCR amplification of *C. jejuni* using the *glyA* primers and associated TaqMan™ probe. The X-axis represents log₁₀ number of *C. jejuni* enumerated by conventional plate counting method while the Y-axis represents the Ct values corresponding to that cell number.

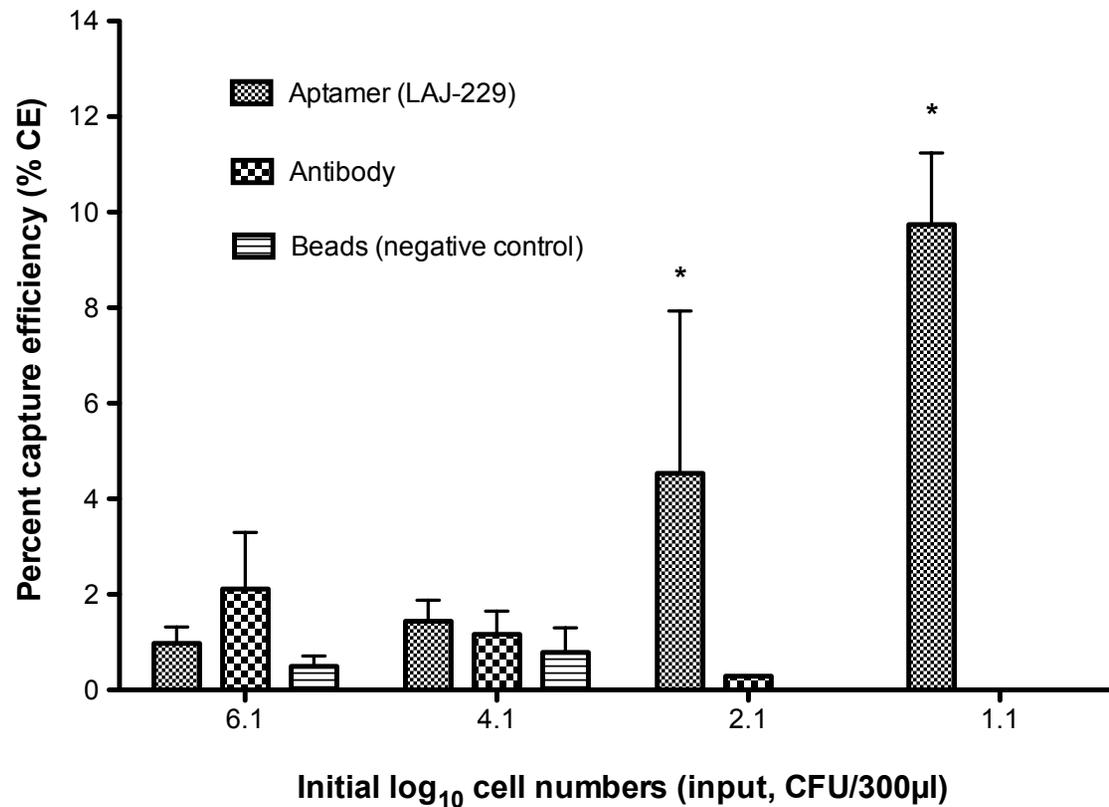


Figure 3. Capture efficiency (% CE) of aptamer and antibody–conjugated magnetic beads as applied to serially diluted *C. jejuni* culture suspended in 300 µl of a bacterial cocktail containing 10³ CFU each of four representative foodborne pathogens. Results are expressed as mean (n=3) ± S.D with Duncan’s multiple range test used to determine statistical significance (**p* < 0.01) when comparing aptamer, IMS, and control beads.

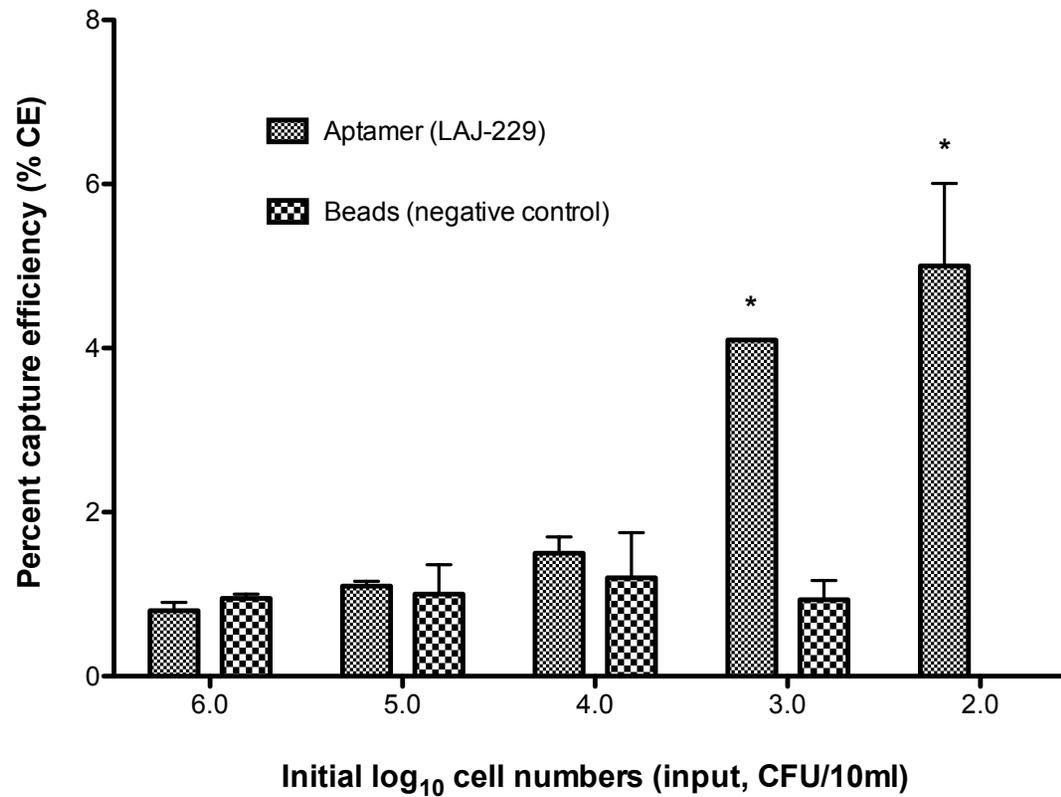


Figure 4. Capture efficiency (% CE) of aptamer–conjugated magnetic beads as applied to serially diluted *C. jejuni* culture suspended in 10 ml PBST. Results are expressed as mean (n=3) ± S.D with Duncan’s multiple range test used to determine statistical significance ($p < 0.01$) when comparing aptamer and control beads.