APPLICATION OF MOLECULAR METHODS IN DIAGNOSTICS OF BACTERIAL PATHOGENS

By

NAGARAJA RAMAVADHANI THIRUMALAPURA

Bachelor of Veterinary Science
University of Agricultural Sciences
Bangalore, India
1995

Master of Veterinary Science in Immunology
Indian Veterinary Research Institute
Izatnagar, India
1998

Submitted to the Faculty of the Graduate College
of the Oklahoma State University in partial
fulfillment of the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 2005

APPLICATION OF MOLECULAR METHODS IN DIAGNOSTICS OF BACTERIAL PATHOGENS

Dissertation Approved:

Dr. Jerry Malayer

Dissertation Adviser

Dr. Andrew Mort

Dr. Rebecca Morton

Dr. Jean Clarke

Dr. A. Gordon Emslie

Dean of the Graduate College

ACKNOWLEDGMENTS

It gives me immense pleasure to record my sincere gratitude to my mentor, Dr. Jerry Malayer, for his invaluable advice, unstinted help and support. He always had a word of encouragement throughout the course of this study. I thank members of my advisory committee Dr. Andrew Mort, Dr. Rebecca Morton and Dr. Jean Clarke for their help, encouragement and guidance. A word of special appreciation is due to Akhilesh Ramachandran, Hemant Naikare and Ms. Leanne Weir for their help, support and friendship. I thank members of our lab Mr. David Goad, Ms. Amy Hurst, Ms. Lori Vandeventer, Dr. James Flinchbaugh, Ms. Sheena Roles, Mr. Ryan Holland and Dr. Mahesh Mohan for their help.

I am grateful to my parents, brothers and sisters for their love, care, encouragement and support.

TABLE OF CONTENTS

Chapter **Page**

LIST OF TABLES

Chapter III

Table **Page**

LIST OF FIGURES

Chapter II

Figure **Page**

Chapter III

Figure **Page**

Chapter IV

Figure **Page**

Chapter V

Figure **Page**

Chapter VI

Figure **Page**

CHAPTER I

Introduction

Bacterial infections continue to affect human and animal health contrary to the notion that the advent of antibiotics would result in their decline or eradication. It has been estimated that about 1.5 billion people contract bacterial infections each year worldwide and that 70% of these infections result from ingestion of contaminated food. In the US alone, food-borne bacteria affect about 33 million people annually resulting in about 10000 deaths and costing the US economy $9.3-12.8 billion every year (Deisingh & Thompson 2004).

Apart from food borne bacterial infections, there has been an increase in number of cases of tuberculosis, sexually transmitted diseases and meningitis (Deisingh & Thompson 2004). Further, opportunistic bacteria contribute to a significant number of infections in individuals immunocompromised due to HIV infection. In addition, many bacterial pathogens are considered to be potential biological warfare/bioterrorism agents due to their extreme infectivity and are included in the category of select agents by the Centers for Disease Control and Prevention. These agents include *Bacillus anthracis* (anthrax), *Clostridium botulinum* toxin (botulism), *Francisella tularensis* (tularemia), *Yesinia pestis* (plague) and many other bacteria. Recent anthrax attacks through

contaminated postal mails in the US reaffirm their potential use in bioterrorism (Valiante *et al.* 2003).

The control and prevention of bacterial infections depends on our ability to detect bacteria from environmental samples and identify persons exposed to pathogenic bacteria. It is essential to rapidly and accurately detect and identify a bacterial agent used in case of an act of bioterrorism to prevent adverse consequences. On the other hand, constant monitoring and surveillance of bacterial infections in a population will help in their prevention and eradication. Currently, various methods are available for the detection of bacteria or diagnosis of bacterial infections. These include isolation and identification of bacteria, detection of bacterial nucleic acids (PCR and DNA microarrays) and bacterial antigens/whole bacteria (immunoassays and biosensors) and disease diagnosis based on indirect methods such as demonstration of specific antibodies (immunoassays). Isolation and identification of bacteria is very laborious and time-consuming and some bacteria are fastidious in nature requiring special growth conditions and grow slowly. Although the detection of nucleic acid using either PCR or microarrays is more sensitive and rapid, the methods are expensive. Antibodies used in biosensors and immunoassays for the detection of bacteria are expensive to produce and are not stable. These limitations of current methods necessitate either improving the existing methods or developing novel methods for the detection of bacteria and diagnosis and monitoring of bacterial infections.

Antibodies have been in use in a wide range of applications as analytical agents. Polyclonal sera were used as the source of antibodies before the development of hybridoma technology (Kohler & Milstein 1975). Monoclonal antibodies produced through the hybridoma technology have found applications in research, diagnostics, biosensors and therapeutics. Monoclonal antibodies are superior to polyclonal antibodies and can be produced in relatively large quantities. However, they suffer from some limitations such as high cost of production, variation from batch to batch, sensitivity to temperature, etc. *In vitro* selected nucleic acid probes known as aptamers have potential applications as analytical reagents that are based on molecular recognition. Aptamers (Latin 'aptus' meaning 'to fit') are single stranded oligonucleotides that are selected through an *in vitro* process known as SELEX (Systematic Evolution of Ligands by EXponential enrichment) for specific binding to a target of interest (Ellington & Szostak 1990; Tuerk & Gold 1990).

Enizyme linked immunosorbent assay (ELISA) is a sensitive and rapid method and has been employed in a wide range of applications such as detection and quantification of proteins, tumor markers, antibodies and other analytical applications. However, in the present form microtiter plate-based ELISA suffers from limited throughput and requires a large quantity of reagents and samples. The advent of microarray technology has revolutionized the field of genomics. The technology is being rapidly expanded into other areas such as proteomics, glycomics and metabolomics and even clinical medicine. Microarray based immunoassays are multiplex in nature and

require small quantities of reagents and samples. Antigen microarrays will allow rapid screening of clinical sample against multiple antigens and require small quantity of samples (Bacarese-Hamilton *et al.* 2004).

The objectives of the present study are

1. To select ssDNA aptamers against a surface target of *Francisella tularensis* for possible use in diagnostics and biosensors.
2. To develop lipopolysaccharide microarrays for the detection of antibodies.
3. To develop intact whole bacterial cell arrays for the detection and characterization of antibodies to surface antigens.

The latter two objectives are expected to aid in rapid disease diagnosis and seroepidemiological studies.

References

Bacarese-Hamilton, T., L. Mezzasoma, A. Ardizzoni, F. Bistoni, and A. Crisanti, 2004 Serodiagnosis of infectious diseases with antigen microarrays. *J.Appl.Microbiol.* **96**: 10-17.

Deisingh, A. K., and M. Thompson, 2004 Biosensors for the detection of bacteria. *Can.J.Microbiol.* **50**: 69-77.

Ellington, A. D., and J. W. Szostak, 1990 In vitro selection of RNA molecules that bind specific ligands. *Nature* **346**: 818-822.

Kohler, G., and C. Milstein, 1975 Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**: 495-497.

Tuerk, C., and L. Gold, 1990 Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**: 505-510.

Valiante, D. J., D. P. Schill, E. A. Bresnitz, G. A. Burr, and K. R. Mead, 2003 Responding to a bioterrorist attack: environmental investigation of anthrax in New Jersey. *Appl.Occup.Environ.Hyg.* **18**: 780-785.

CHAPTER II

Review of literature

Francisella tularensis

Francisella tularensis, the causative agent of the zoonotic disease tularemia, is a Gram negative, facultative intracellular bacterium. The organism was first isolated in 1911 from rodents affected with a plague-like disease in Tulare County, California and named *Bacterium tularensis* (Ellis *et al.* 2002). Many vertebrates including mammals, birds, amphibians, and fish and even invertebrates are naturally infected with *F. tularensis*. Tularemia is restricted to the northern hemisphere for reasons unknown (Johansson *et al.* 2004b) and is known by many names such as rabbit fever, hare fever, deerfly fever and lemming fever (Ellis *et al.* 2002).

Francisella tularensis belongs to the genus *Francisella* within the family *Francisellaceae*. The strains of *F. tularensis* differ in biochemical properties, geographic distribution and virulence and are accordingly grouped into one of the four now well recognized subspecies: *F. tularensis* subsp. *tularensis* (type A *F. tularensis*), subsp. *holarctica* (type B *F. tularensis*), subsp. *mediasiatica,* and subsp. *novicida.* The

subspecies of *F. tularensis* exhibit a close phylogenetic relationship and are antigenically similar (Broekhuijsen *et al.* 2003). *Francisella philomiragia* the only other member of the genus *Francisella,* is an opportunistic pathogen causing disease in immunocompromised individuals and is often associated with water.

Strains of *F. tularensis* subsp. *tularensis* (type A *F. tularensis*) are highly virulent for humans and are reported from North America and recently from Europe (Ellis *et al.* 2002). It has been reported that as few as ten organisms can cause severe illness and death in humans (Sjostedt 2003). Infections with type A *F. tularensis* resulted in 5 to 30% mortality before the advent of effective antibiotics (Titball *et al.* 2003). Strains belonging to subsp. *holarctica* and *mediasiatica* are moderately virulent for humans, causing a similar infection but without a significant mortality (Titball *et al.* 2003). *F. tularensis* subsp. *holarctica* is mainly reported from North America and Eurasia. *F. tularensis* subspecies *mediasiatica* is prevalent in the former Soviet Union (Ellis *et al.* 2002). Strains of *F. tularensis* subsp. *novicida* are primarily found in North America and reported to cause infections in immunocompromised individuals (Ellis *et al.* 2002; Titball *et al.* 2003).

Tularemia occurs in many forms in humans depending on the route of entry and the strain of *F. tularensis* involved. The ulceroglandular form of tularemia is the most common form and occurs when the organism enters through cutaneous route, either through a wound infection or an insect bite. Involvement of virulent strains of *F.*

tularensis subspecies *tularensis* results in an acute septicemic form of the disease 'typhoidal tularemia'. Ingestion of contaminated food or drinking water containing *F. tularensis* results in oropharyngeal or gastrointestinal form of tularemia. The most severe form of the disease occurs when organisms are inhaled resulting in pneumonic form of tularemia (Ellis *et al.* 2002; Johansson *et al.* 2004b).

Francisella tularensis is considered to be a potential biological weapon owing to its extreme infectivity, ease of dissemination after aerosolisation, and its ability to cause illness and death. The organism is very hardy and survives for weeks at low environmental temperatures, making it an ideal agent for bioterrorism. Aerosol dissemination of *F. tularensis* in a populated area would be expected to cause a large number of acute cases in 3 to 5 days, with overwhelming medical and public health consequences. For these reasons *F. tularensis* is considered as a category A agent (Dennis *et al.* 2001). In the event of use of *F. tularensis* in bioterrorism, rapid detection of organisms in environmental samples becomes crucial for containment and clean-up efforts. Simple, rapid and reliable diagnostic tests need to be developed to screen persons exposed to the pathogen.

The laboratory diagnosis of tularemia is based on isolation and identification of the organism, demonstration of antigen or antibodies using immunoassays, or detection of nucleic acid. Culturing of *F. tularensis* is generally avoided, as the organism is fastidious and slow growing under laboratory conditions and extensive precautions must

be taken to prevent infection of laboratory personnel. Organisms can be cultured directly from ulcer scrapings, lymph node biopsies, or sputum (Ellis *et al.* 2002; Johansson *et al.* 2004b).

Tularemia can be diagnosed by the detection of antigens from clinical samples. An antigen capture ELISA has been described that can detect bacteria using antibodies specific for lipopolysaccharide of *F. tularensis* LVS (Live Vaccine Strain). The antibodies used in the assay specifically recognized type A and type B *F. tularensis* with the detection limit of 10^3 to 10^4 bacteria per ml (Grunow *et al.* 2000). An immunochromatographic hand-held assay has been developed based on antibodies to LPS of *F. tularensis* LVS. The sensitivity of the assay was reported to be 10^6 bacteria per ml in PBS and 10^6 to 10^7 bacteria per ml in spiked human serum (Grunow *et al.* 2000). The time required for this assay is approximately 15 min, but the sensitivity of the assay is not very high. Although LPS is a commonly targeted molecule as it is readily accessible on the surface of the bacterium and present in high copy number, antibodies directed against LPS of *F. tularensis* LVS do not discriminate between highly virulent type A *F. tularensis* and less virulent type B *F. tularensis*. Antigens specific for various subspecies of *F. tularensis* have been recently identified (Hubalek *et al.* 2003).

Tularemia is frequently diagnosed based on the clinical picture and serology as isolation of the organism is difficult. The presence of antibodies against *F. tularensis* is detected by immunoassays such as agglutination tests or ELISA (Carlsson *et al.* 1979;

Koskela & Salminen 1985; Syrjala *et al.* 1986). Specific antibodies against *F. tularensis* appear in serum in about 10 to 14 days after the onset of infection. Tularemia can be diagnosed by detecting antigen in human urine samples, but the method is not routinely used (Tarnvik *et al.* 1987; Johansson *et al.* 2004b).

A number of PCR based methods have been described for the detection of *F. tularensis* and diagnosis of tularemia. These assays are highly sensitive and can discriminate the subspecies of *F. tularensis* (Ellis *et al.* 2002; Johansson *et al.* 2004b). These assays use primers directed against the outer membrane proteins such as *fop*A (Fulop *et al.* 1996) or the 17-kDa outer membrane lipoprotein (Junhui *et al.* 1996; Grunow *et al.* 2000). The detection limit of these assays is 10^2 bacteria per ml in PBS and 10^3 to 10^4 bacteria per ml in spiked serum (Grunow *et al.* 2000). A nested PCR is reported to be capable of detecting 1 CFU (Colony Forming Unit) of *F. tularensis* (Fulop *et al.* 1996). However, some compounds present in clinical samples inhibit PCR affecting the sensitivity of these assays. Also some preparation of sample is required to achieve greater sensitivity with PCR (Fulop *et al.* 1996; Grunow *et al.* 2000).

PCR-based methods show greater sensitivity compared to other methods even in their current form. A PCR assay positively identified 73% of serologically confirmed cases of human ulceroglandular tularemia, whereas bacteria could be isolated from only 25% of these patients (Sjostedt *et al.* 1997). Real-time PCR based methods have also been described for the detection of *F. tularensis* from infected tissues and environmental

samples (Higgins *et al.* 2000; Versage *et al.* 2003; Emanuel *et al.* 2003; Lamps *et al.* 2004). RT-PCR is about 10 times more sensitive than the standard PCR, detecting 10 to 100 bacteria per ml. Also RT-PCR is more rapid than the conventional PCR requiring only minutes to a few hours. The usefulness of PCR based methods is established for clinical diagnosis of the ulceroglandular form but not for respiratory or oropharyngeal forms of tularemia (Johansson *et al.* 2004b).

Many PCR methods have been developed to discriminate the subspecies of *F. tularensis*. A PCR has been developed that can differentiate type A *F. tularensis* from type B *F. tularensis* based on one base difference in the 16S rRNA (Forsman *et al.* 1994). Various genetic typing methods such as pulsed-field-gel-electrophoresis (PFGE), arbitrary primed PCR (AP-PCR), multiple locus sequence typing (MLST), microarray analysis, insertion sequence probed restriction fragment length polymorphism (IS-RFLP), amplified fragment length polymorphism (AFLP) and multiple-locus variable-number tandem repeat analysis (MLVA) have been applied to discriminate *F. tularensis* subspecies (Johansson *et al.* 2004b). Among these techniques MLVA is highly discriminatory and can be used to distinguish individual isolates of *F. tularensis* (Johansson *et al.* 2004a).

Aptamers

Aptamers are single-stranded oligonucleotides that are selected *in vitro* for specific binding to a target of interest. Aptamers have been selected against a variety of targets such as simple ions, small molecules, peptides, single proteins, organelles, viruses, and even whole cells (Wilson & Nock 2002). Aptamers consist of RNA, ssDNA or modified nucleotides and are usually 15 to 60 nucleotides long. The binding affinity of aptamers range from K_D's of 1 ρM to 1 μM with the majority in the range of 1 – 10 ηM. The word aptamer is derived from Latin word "aptus" meaning "to fit". Aptamers are selected from a pool of nucleotide templates consisting of random sequences by an *in vitro* selection process known as SELEX (Systematic Evolution of Ligands by EXponential enrichment). SELEX process was independently developed by three laboratories in 1990 (Robertson & Joyce 1990; Ellington & Szostak 1990; Tuerk & Gold 1990). Aptamers are versatile molecules and have the potential to be developed into novel diagnostic, therapeutic and enzyme (ribozymes) systems.

Aptamers have several advantages over antibodies. The selection of aptamers is completely an *in vitro* process and it does not involve animals, cells or *in vivo* conditions. Therefore the properties of aptamers can be changed as required. Aptamers could be selected to function in non-physiological conditions such as non-physiological pH or temperature. Similarly, the kinetic parameters of aptamers such as on-rate and off-rates can be changed as desired. Unlike antibodies, aptamers can be generated against

molecules such as toxins or molecules that are inherently poor immunogens. Once selected, aptamers can be inexpensively synthesized using nucleic acid techniques with little or no batch-to-batch variations. Reporter molecules such as fluorophores and biotin can be incorporated during synthesis without compromising the function of aptamers. Aptamers are stable and can be stored at room temperature. Aptamers do undergo denatuaration but it is reversible unlike that of antibodies (Jayasena 1999).

SELEX involves screening of a large combinatorial oligonucleotide library by an iterative process of *in vitro* selection and amplification in order to find sequences of desired properties (Figure 1). Typically a SELEX process starts with design and combinatorial chemical synthesis of a random DNA library. These sequences will have a randomized region flanked by fixed regions for PCR amplification during the selection process. The molecular diversity of these libraries is determined by the number of randomized bases in the library. In an oligonucleotide library with 30 randomized nucleotides, the number of possible individual unique sequences would be 10^{18} ($4^{30}=10^{18}$). However, in practice the diversity of these libraries is determined by the scale of solid–phase DNA synthesis and libraries with a diversity of 10^{14} to 10^{15} individual sequences are usually used. Diversity of oligonucleotide libraries used in SELEX experiments exceed the diversity of antibodies that can be generated by the immune system. The chances of finding specific and high-affinity aptamers against a target increases with the diversity of library.

The selection process involves incubation of the library with a target of interest under defined conditions such as buffer, pH and temperature. After incubation, oligonucleotide sequences that specifically interact with the target will be separated from the rest of the library, amplified, and used in the next round of selection. Normally nitrocellulose filter partitioning is used for protein targets to separate the sequences that interact with the target. The whole process of selection and amplification will be repeated typically 8 -15 cycles until the library becomes enriched. The selected pool is tested for its enrichment using binding assays. The enriched library is cloned and sequenced. Individual selected sequences are further characterized for their binding specificity and affinity.

The selection of RNA aptamers is similar to that of DNA aptamers except for minor modifications. A promoter for an RNA polymerase is engineered within a fixed region of the library to allow *in vitro* RNA synthesis. After each selection step with the RNA library, the library is amplified by RT-PCR and later *in vitro* transcribed to generate a pool of RNA for next round of selection. DNA aptamers are relatively stable compared to RNA aptamers. The presence of 2' hydroxyl of groups on ribose renders the RNA susceptible to breakdown by endonucleases such as RNase (Sampson 2003). Alternatively, RNA sequences compared to DNA sequences enrich the diversity of libraries by increasing the variety of folded structural motifs. The presence of 2'-hydroxyls may increase the stability of tertiary structure in RNA by helping in interdomain interactions (Gallo *et al.* 2003).

Aptamers (RNA and ssDNA) have the ability to form three dimensional structural conformations through intramolecular interactions. Unlike nucleic acid-nucleic acid interactions that involve base-base complementarity, aptamers recognize their targets through molecular shape complementarity (Hermann & Patel 2000). This recoginiton is stabilized by forces such as electrostatic interactions, hydrogen bonding, and stacking of flat moieties. Aptamers show a high degree of specificity to their targets sometimes that may exceed antibody specificity (Jayasena 1999). The high specificity is a result of selective pressure in the SELEX experiment. They can discriminate molecules based on subtle changes such as the presence or absence of methyl or hydroxyl groups. The high binding affinity and specificity of aptamers toward their targets is achieved by enclosing large parts of the target by aptamers (Hermann & Patel 2000). Although aptamers have been selected against a wide range of targets, the affinity of aptamers is influenced by the properties of targets. Molecules with hydrogen bond donors or acceptors, flat moieties (planarity), and positively charged groups make better targets for aptamers. Non-planar molecules with a neutral to net negative charge or a highly hydrophobic nature are poor targets for aptamers (Wilson & Nock 2002).

Aptamers as analytical agents could be developed into novel diagnostics and biosensors. Aptamers offer several advantages over antibodies (Jayasena 1999; O'Sullivan 2002). A biosensor was developed for human thrombin using a ssDNA aptamer that could detect as low as 0.7 amol of thrombin using evanescent-wave-induced

fluorescence anisotropy (Potyrailo *et al.* 1998). A DNA aptamer was incorporated in an affinity probe capillary electrophoresis to quantify human IgE with a detection limit of 46pM (German *et al.* 1998). An aptamer specific for L-selectin was incorporated into the stationary phase of an affinity chromatography column and used to purify recombinant L-selectin-Ig fusion protein produced in Chinese hamster ovary cells (Romig *et al.* 1999). Aptamer-based biosensor arrays have been developed by immobilizing fluorescently labeled aptamers onto a glass substrate. The array was used to detect and quantify tumor markers from a complex mixture of biomolecules (McCauley *et al.* 2003). Additionally, aptamers have been engineered into beacons (stem-loop structure with a fluorophore on one arm of the stem and a quencher on the other) for the detection of targets without the need to wash off unbound aptamers (Hamaguchi *et al.* 2001; Li *et al.* 2002). An RNA aptamer was incorporated into an affinity capillary electrophoresis/laser-induced fluorescence assay for detection of reverse transcriptase (RT) of the type 1 human immunodeficiency virus (HIV1). While the detection limit for the assay was 50nM (6µg/ml), presence of undiluted cell culture media interfered with the assay necessitating the dilution of culture media at least 100-fold (Pavski & Le 2001).

Although described as "emerging molecules that rival antibodies in diagnostics" (Jayasena 1999), aptamers have found very few applications in diagnostics and continue to be mainly a subject of academic interest (Hesselberth *et al.* 2000a). Aptamers could be used as capture or reporter molecules or in combination with antibodies for the detection of analytes in a sandwich-type Enzyme linked oligonucleotide assay (ELONA) analogous

to ELISA (O'Sullivan 2002). An aptamer-based magnetic bead-electrochemiluminescence assay has been developed for the detection of anthrax spores with a dynamic range equivalent to <10 to >$6x10^6$ spores (Bruno & Kiel 1999). DNA aptamers against *E. coli* O111:B4 LPS conjugated to quantum dots were employed for the detection of the bacteria. A shift in fluorescence emission was observed upon binding of bacteria to quantum dot-aptamer conjugate. Although this is a homogenous assay without the need for wash steps, the study did not report the detection limit of bacteria (Dwarakanath *et al.* 2004). DNA aptamers were developed against cholera toxin and Staphylococcal enterotoxin B by conjugating these toxins to tosyl-activated magnetic beads. These toxins could be detected in the low nanogram to picogram ranges by employing selected aptamers in electrochemiluminescence and colorimetric microplate assays (Bruno & Kiel 2002). Further a DNA spiegelmer (mirror-image aptamer) was developed against staphylococcal enterotoxin B. Initially, a D-peptide corresponding to a stable domain of 25 amino acids of whole protein (28 kDa protein consisting of 239 amino acids) was used as the target for selection of D-DNA aptamer. The corresponding enantiomeric L-DNA spiegelmer recognized the full-length protein with a binding constant of ~ 420nM (Purschke *et al.* 2003). An RNA aptamer was selected against ricin, a class II ribosome inactivating protein and a potential biological warfare agent. The selected aptamer recognized the catalytic ricin A-chain (RTA) with a binding affinity of Kd = 7.3 nM and could compete with the natural substrate, the sarcin-ricin loop (SRL) of eukaryotic 23-28S rRNA. The aptamer could be used as a diagnostic reagent for the

detection of ricin (Hesselberth *et al.* 2000b). RNA aptamers have been selected to the amyloid peptides of the Alzheimer's disease (Ylera *et al.* 2002).

Aptamers as therapeutics may be useful in the treatment of infectious diseases, cancer, cardiovascular diseases. Currently, three aptamers are being evaluated in clinical trails for inhibition of HIV replication, angiogenesis, and intimal hyperplasia (Nimjee *et al.* 2005). RNA aptamers selected against an invariant surface protein of African trypanosomes may be used as a therapeutic agent against the parasite (Homann & Goringer 1999). Several properties of aptamers make them better therapeutic agents. Apart from exhibiting high specificity and affinity towards their targets, they lack immunogenicity. The activity of aptamers can be controlled using oligonucleotide antidote independent of biological clearance (Rusconi *et al.* 2002). Oligonucleotide antidotes inhibit aptamer binding to its target by altering the aptamer structure through Watson-Crick base paring. Further, aptamers can be modified to alter their bioavailability and pharmacokinetics properties. The use of modified nucleotides increases the stability of aptamers against nuclease degradation. The small size of aptamers results in faster renal clearance. However, modification of aptamers by conjugating to polyethylene glycol or dialkylglycerol or 3'-biotinylation or incorporation of aptamers into liposome slows down aptamers clearance (Wilson & Nock 2002; Rimmele 2003; Nimjee *et al.* 2005). Furthermore, aptamers can be selected with specificity for ortholog proteins from two different species using toggle SELEX. Selection of an aptamer against a target of interest in humans and ortholog target from a species in which pre-clinical trials are

planned allows pre-clinical evaluation of aptamers. This is achieved by alternating the targets between human form and ortholog protein from other species during selection of aptamers.

Some of the limitations associated with aptamers include their susceptibility to nucleases. A number of strategies have been developed to increase the stability of aptamers. The use of modified nucleotides such as pyrimidines with 2'-amino or – fluoro groups or substitutions of O' methyl for OH group at 2' position in purines after selection, enhance the stability of aptamers (Sampson 2003; Nimjee *et al.* 2005). The stability of aptamers can also be increased by modifying the phosphodiester backbone. The use of enantiomeric aptamers known as spiegelmers provides an alternative solution in increasing the stability of aptamers. Aptamers are initially selected against the chemical mirror image of the target (e.g. an unnatural D-amino acid peptide) and the selected aptamers are later converted into chemical mirror image of their own by substituting natural D-ribose with L-ribose to obtain spiegelmers (mirror–image aptamers). The selected spiegelmers recognize the original target because of molecular symmetry (Cerchia *et al.* 2002; Sampson 2003).

The development of aptamers is influenced by the properties of a target. Some molecules are better targets for aptamers than the others. There is no standard selection method that can be applied to all targets, requiring adaptation of methods for each target in most cases. Generally, aptamers function well in the conditions in which they are

selected, and they invariably need certain concentrations of cations to fold into their active state. These properties of aptamers may limit their use in certain applications (Rimmele 2003).

Lipopolysaccharide

Lipopolysaccharides (LPS) are important antigens of Gram-negative bacteria (Jauho *et al.* 2000). They constitute the major components of the outer membrane of Gram-negative bacteria. Lipopolysccharides are amphipathic molecules consisting of three distinct regions: hydrophobic lipid-A, hydrophilic core region and polysaccharide O-antigen (Figure 2). LPS represents 10 to 15 percent of the surface molecules in the outer membrane and may cover up to 75% of the Gram-negative bacterial cell surface (Lerouge & Vanderleyden 2002; Caroff & Karibian 2003). Approximately 2 million molecules of LPS are estimated to be present on the surface of *E. coli* (Raetz 1990; Rietschel & Brade 1992). LPS has been shown to be highly immunogenic and plays a major role in pathogenesis. Lipid-A is highly conserved among Gram-negative bacteria, whereas the core region and O-antigen show genus and species/strain specificity, respectively (Lerouge & Vanderleyden 2002). The diversity and specificity of O-antigen is attributed to its unique sugar composition and number of possible glycosidic linkages. The identification of Gram-negative bacteria is traditionally done by serotyping using antibodies specific for O-antigen.

LPS is also known as endotoxin as it is responsible for the endotoxemia or sepsis associated with Gram-negative bacterial infections. The lipid-A part of LPS is responsible for its endotoxic activity. The structure of Lipid-A typically consists of a β-D-GlcN-(1-6)-α-D-GlcN disaccharide backbone with two phosphoryl groups at positions

1 and 4'. Up to four acyl chains are attached to the disaccharide backbone of lipid-A by amide or ester linkage. Further, these acyl chains may carry additional fatty acids so that their number can go up to seven in some LPS molecules. Lipid-A anchors LPS molecules into the outer membrane. The number of acyl chains, their length and phosphorylation state of the disaccharide backbone influence the endotoxicity of lipid-A. The structure of lipid-A containing disaccharide backbone with six acyl chains, as in case of *E. coli* LPS, along with the two phosphate groups appeared to be essential for optimum recognition by mammalian receptors for expression of endotoxic activity (Erridge *et al.* 2002).

The core oligosaccharide consists of a small number of sugars that link lipid-A and O-polysaccharide. The core polysaccharide can be divided into inner core and outer core. The inner core is proximal to lipid-A and consists of unusual sugars such as 3-deoxy-D-manno-octulosonic acid (Kdo) and L-glycero-D-manno heptose (Hep). Kdo is seen in almost all LPS examined to date and is essential for the viability of bacteria. Kdo links the core to lipid-A through an acid-labile ketosidic linkage. Additionally, three to six sugars are present in the inner core. The outer core consists of more common sugars such as hexoses and hexosamines and is known as hexose region. The structure of the core region is very much conserved within a genus.

The O-polysaccharide, the outermost part of LPS, is attached to the core region and extends into the environment. O-polysaccharide consists of repeating subunits of hexoses and the number of sugars in each subunit varies from one to eight among

different bacteria. The number of subunits present in O-polysaccharide is between 0 and 50. Lipopolysaccharides are heterogeneous molecules due to incomplete addition of O-polysaccharide subunits. The variable number of subunits is responsible for the characteristic ladder-like pattern of LPS observed on SDS-PAGE. The O-polysaccharide is responsible for the diversity and specificity observed in LPS. The structure and chemical composition of O-polysaccharide varies among strains and in some cases even within a strain. O-polysaccharide is the target for host antibody response to LPS and hence O-polysaccharide is also known as O-antigen. O-antigen serology has been used for typing Gram-negative bacteria. O-antigen also plays a role in the inhibition and activation of the complement system of the host (Erridge *et al.* 2002). In some bacteria such as *Salmonell,* O-antigen protects the bacteria by preventing the formation of membrane attacking complex (MAC) by the complement. The high structural diversity exhibited by O-antigen may possibly help bacteria to evade the immune system (Raetz 1990). The presence of O-polysaccharide gives a smooth appearance to bacterial colonies on agar plates resulting in so-called 'smooth' LPS (S-LPS) or LPS I. Mutant bacteria having a 'rough' phenotype or R phenotype produce LPS II (lipid-A and core) that lacks O-polysaccharide.

Lipopolysaccharide of *F. tularensis* has unusual biological properties unlike LPS from other bacteria. It does not exhibit endotoxic activity and is a poor inducer of proinflammatory cytokine responses (Sandstrom *et al.* 1992). This prompted the recent structural analysis of LPS of *F. tularensis*. The LPS contains a β-GlcN-(1-6)-GlcN lipid-

A backbone with a number of unusual features. The lipid-A apparently has reducing glucosamine endgroup with no phosphate substituents and βGlcN does not contain substituents at O-3 and O-4. Some lipid-A in LPS preparations are found to be unsubstituted with Kdo. There is no substituent at O-4 of Kdo. The lipid-A possesses longer chain fatty acids compared to the highly toxic lipid-A of *E. coli*. The LPS has a small core and the inner part of the core contains mannose rather than more common heptose. The unusual structural features of *F. tularensis* LPS could be responsible for the weak endotoxicity associated with the LPS (Vinogradov *et al.* 2002).

The O-antigen structure of *F. tularensis* strain 15 and the Live Vaccine Strain (LVS) contains repeating tetra-saccharide subunits: 4-(α-D-Gal*p*NAcAN-(1-4)-α-D-Gal*p*NAcAN-(1-3)-β-D-Qui*p*NAc-(1-2)-β-D-Qui*p*4NFo-1) (Vinogradov *et al.* 1991; Conlan *et al.* 2002). Both the strains are empirically derived from type B *F. tularensis*. Lipopolysaccharides from type A and type B *F. tularensis* show serological cross reactivity and appear to be structurally related (Grunow *et al.* 2000; Conlan *et al.* 2002). The repeating units of the O-antigens from type A *F. tularensis* Schu S4 strain and LVS were presumed to be the same based on MALDI-MS analysis (Prior *et al.* 2003). LPS of *F. tularensis* subspecies *novicida* is biologically more active and immunochemically distinct from of LPS of type A and type B *F. tularensis*. The lipid-A from *F. novicida* LPS does not contain phosphate substituents and apparently contains a free reducing end. The core polysaccharide of *F. novicida* has an additional α-Glc residue compared to the core of *F. tularensis* (Vinogradov & Perry 2004). The tetrasaccharide subunit of O-

antigen of *F. novicida* shares a common disaccharide unit with that of *F. tularensis* (Vinogradov *et al.* 2004).

Microarray Technology

The advent of microarray technology that allows the high-throughput study of molecule-molecule interactions has revolutionized the field of biomedical research. The technology is rapidly being expanded into many areas including clinical medicine. Microarrays were first used in immunological assays (Ekins 1989). However, the advent of genome sequencing coupled with high-throughput oligonucleotide synthesis and PCR resulted in its extensive use in the study of nucleic acid–nucleic acid interactions. DNA microarrays allow the gene expression study of thousands of genes in a single experiment. The technology is being increasingly used in the study of cell biology, cancer, inflammatory and metabolic disease and pathogenic microorganisms. Further, DNA microarrays have potential applications in disease diagnosis and epidemiological studies.

The level of mRNAs is not directly correlated to the amount or function of protein in a cell. Factors such as mRNA half-lives, efficiency of translation and post-translational modifications of proteins affect the amount and function of proteins in a cell. Therefore, it is difficult to draw meaningful conclusions on the amount or function of proteins in a cell based on the relative abundance of mRNAs (Howbrook *et al.* 2003). Protein and peptide microarrays allow the study of enzyme-substrate, DNA-protein and protein-protein interactions (Templin *et al.* 2002). Protein and peptide arrays have been

generating vast amounts of information in the area of proteomics and have been helpful in overcoming some of the limitations of DNA microarrays.

Many proteins undergo post-translational modifications that influence their functions. Some proteins and other biomolecules are conjugated to sugars and these sugar moieties are involved in molecular interactions. Further, the development of carbohydrate arrays benefit the study of interactions between sugars and proteins (Fukui *et al.* 2002; Wang *et al.* 2002; Willats *et al.* 2002). Carbohydrate microarrays have potential applications in functional glycomics, drug discovery and diagnois (Shin *et al.* 2005). Recently, a carbohydrate array has been developed to study the interaction between carbohydrate and cells and detect pathogens. Bacteria could be detected based on known carbohydrate binding epitope present on the surface of pathogens. Further, the study suggested the use of carbohydrate arrays for screening potential antiadhesion therapeutics against bacteria (Disney & Seeberger 2004).

Transfected-cell microarrays allow the high-throughput identification of the cellular functions of genes. The microarrays are fabricated by printing cDNA in expression vectors in gelatin on microarray slides. The slides are briefly exposed to a lipid transfection reagent and placed in dishes; cells are then added onto the slides and allowed to form a confluent lawn. The cells that land on printed cDNA will get transfected and each spot corresponding to the printed cDNA will have a cluster of 30 to 80 cells expressing a gene of interest as the cells divide. The resulting living cell arrays

contain the transfected cells with in a background of untransfected cells. The function of genes such as apoptosis, cell adhesion, and signal transduction can be identified using transfected cell arrays (Ziauddin & Sabatini 2001; Wu *et al.* 2002). Furthermore, transfected cell microarrays can also be used to monitor the presence of biological agents and toxicants (Delehanty *et al.* 2004). Eukaryotic cell microarrays were developed by printing whole intact cells on poly-L-lysine coated glass slides, and the arrays were used of characterize cell surface-specific antibodies (Schwenk *et al.* 2002).

Further, the development of tissue microarrays helps in high throughput *in situ* analysis of specific molecular targets in a large number of tissue specimens (Kononen *et al.* 1998). Tissue microarrays are constructed from needle biopsies taken from paraffin embedded tissue specimens. These biopsies are then embedded into a master paraffin-block. The master block containing tissue specimens is sectioned into slices and transferred onto slides. Tissue microarrays can be probed for molecular targets such as proteins, DNA or RNA employing immunohistochemistry, fluorescence *in situ* hybridization, or other detection methods (Kallioniemi *et al.* 2001).

Antibodies immobilized on a microarray substrate constitute antibody arrays. Antibody arrays have been employed for the study of relative abundance of proteins in diseased versus normal tissues (Haab *et al.* 2001). Antibody arrays were used to study cancer markers (Miller *et al.* 2003) and to profile proteins in cancer tissues (Knezevic *et al.* 2001; Miller *et al.* 2003). Antibodies targeting the surface markers of cells have been

used on a microarray to classify leukemias. Sandwich antibody microarrays have been used to measure cytokines and chemokines (Schweitzer *et al.* 2002). A microarray immunoassay has been described for simultaneous detection of bacteria and proteins. Antibodies immobilized on slides were used to capture analytes under flow conditions and the captured analytes were detected with labeled antibodies. Assays could be completed with in 15 minutes. The detection limits for cholera toxin, staphylococcus enterotoxin B, ricin, and *Bacillus globigii* whole cells were 8 ng per ml, 4 ng per ml, 10 ng per ml, and $6.2x10^4$ CFU per ml respectively (Delehanty & Ligler 2002).

In addition, antigen arrays have been developed for the detection and characterization of antibodies. Antibodies against infectious agents such as *Toxoplasma gondii*, rubella virus, cytomegalovirus and herpes simplex virus types 1 and 2 have been detected in human serum using antigen arrays (Mezzasoma *et al.* 2002; Bacarese-Hamilton *et al.* 2004). Autoantigen microarrays were developed for the characterization of autoantibody responses and to define autoantigens in human autoimmune diseases (Joos *et al.* 2000; Robinson *et al.* 2002). A protein microarray chip was developed for the serologic diagnosis of hepatitis virus C infection (Yuk *et al.* 2004).

Figure 1. Systematic Evolution of Ligands by EXponential enrichment (SELEX). The process of *in vitro* selection of aptamers involves the incubation of a target of interest with a random single stranded oligonucleotide library. After incubation, the sequences that do not interact with the target will be partitioned from those that interact. The sequences that bind the target will be eluted and amplified for use in the next of selection. The selected pool is tested for enrichment of sequences that bind the target after a few rounds of selection. The enriched pool is cloned and sequenced. Individual selected sequences are further characterized for binding specificity and affinity (See text for details).

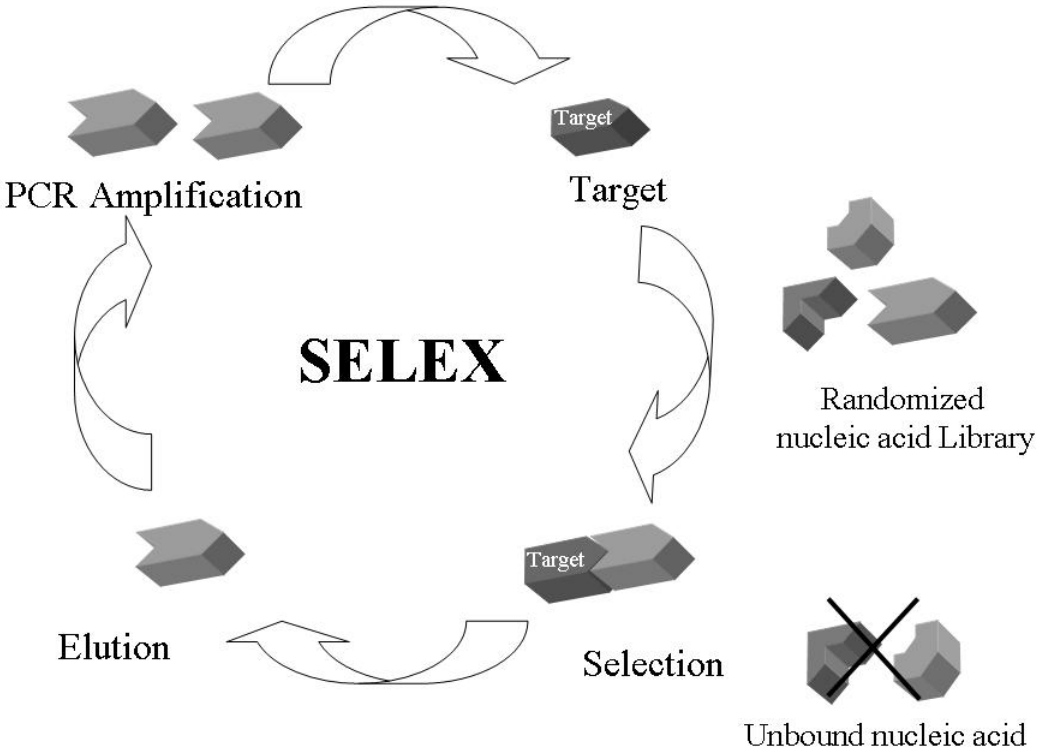
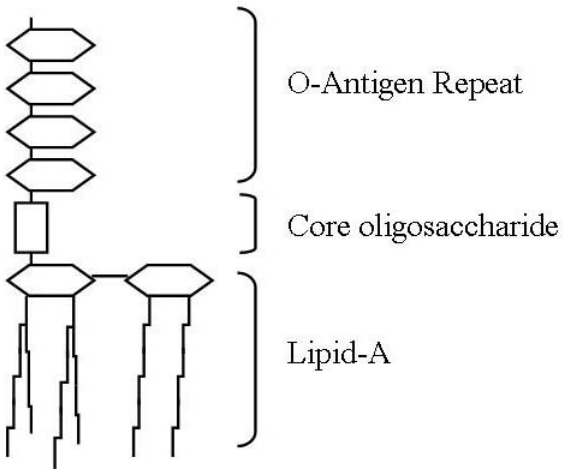

Target
PCR Amplification
Target
SELEX
Randomized
nucleic acid Library
Target
Elution
Selection
Unbound nucleic acid

Figure 2. Schematic diagram of bacterial lipopolysaccharide structure. LPS is an amphipathic molecule and consists of three regions – a hydrophobic lipid-A, hydrophilic core oligosaccharide and an outer polysaccharide O-antigen.


O-Antigen Repeat
Core oligosaccharide
Lipid-A

References

Bacarese-Hamilton, T., L. Mezzasoma, A. Ardizzoni, F. Bistoni, and A. Crisanti, 2004 Serodiagnosis of infectious diseases with antigen microarrays. *J.Appl.Microbiol.* **96**: 10-17.

Broekhuijsen, M., P. Larsson, A. Johansson, M. Bystrom, U. Eriksson *et al.* 2003 Genome-wide DNA microarray analysis of Francisella tularensis strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent F. tularensis subsp. tularensis. *J.Clin.Microbiol.* **41**: 2924-2931.

Bruno, J. G., and J. L. Kiel, 2002 Use of magnetic beads in selection and detection of biotoxin aptamers by electrochemiluminescence and enzymatic methods. *Biotechniques* **32**: 178-3.

Bruno, J. G., and J. L. Kiel, 1999 In vitro selection of DNA aptamers to anthrax spores with electrochemiluminescence detection. *Biosens.Bioelectron.* **14**: 457-464.

Carlsson, H. E., A. A. Lindberg, G. Lindberg, B. Hederstedt, K. A. Karlsson *et al.* 1979 Enzyme-linked immunosorbent assay for immunological diagnosis of human tularemia. *J.Clin.Microbiol.* **10**: 615-621.

Caroff, M., and D. Karibian, 2003 Structure of bacterial lipopolysaccharides. *Carbohydr.Res.* **338**: 2431-2447.

Cerchia, L., J. Hamm, D. Libri, B. Tavitian, and F. de, V, 2002 Nucleic acid aptamers in cancer medicine. *FEBS Lett.* **528**: 12-16.

Conlan, J. W., H. Shen, A. Webb, and M. B. Perry, 2002 Mice vaccinated with the O-antigen of Francisella tularensis LVS lipopolysaccharide conjugated to bovine serum albumin develop varying degrees of protective immunity against systemic or aerosol challenge with virulent type A and type B strains of the pathogen. *Vaccine* **20**: 3465-3471.

Delehanty, J. B., and F. S. Ligler, 2002 A microarray immunoassay for simultaneous detection of proteins and bacteria. *Anal.Chem.* **74**: 5681-5687.

Delehanty, J. B., K. M. Shaffer, and B. Lin, 2004 Transfected cell microarrays for the expression of membrane-displayed single-chain antibodies. *Anal.Chem.* **76**: 7323-7328.

Dennis, D. T., T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher *et al.* 2001 Tularemia as a biological weapon: medical and public health management. *JAMA* **285**: 2763-2773.

Disney, M. D., and P. H. Seeberger, 2004 The use of carbohydrate microarrays to study carbohydrate-cell interactions and to detect pathogens. *Chem.Biol.* **11**: 1701-1707.

Dwarakanath, S., J. G. Bruno, A. Shastry, T. Phillips, A. John *et al.* 2004 Quantum dot-antibody and aptamer conjugates shift fluorescence upon binding bacteria. *Biochem.Biophys.Res.Commun.* **325**: 739-743.

Ekins, R. P., 1989 Multi-analyte immunoassay. *J.Pharm.Biomed.Anal.* **7**: 155-168.

Ellington, A. D., and J. W. Szostak, 1990 In vitro selection of RNA molecules that bind specific ligands. *Nature* **346**: 818-822.

Ellis, J., P. C. Oyston, M. Green, and R. W. Titball, 2002 Tularemia. *Clin.Microbiol.Rev.* **15**: 631-646.

Emanuel, P. A., R. Bell, J. L. Dang, R. McClanahan, J. C. David *et al.* 2003 Detection of Francisella tularensis within infected mouse tissues by using a hand-held PCR thermocycler. *J.Clin.Microbiol.* **41**: 689-693.

Erridge, C., E. nett-Guerrero, and I. R. Poxton, 2002 Structure and function of lipopolysaccharides. *Microbes.Infect.* **4**: 837-851.

Forsman, M., G. Sandstrom, and A. Sjostedt, 1994 Analysis of 16S ribosomal DNA sequences of Francisella strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. *Int.J.Syst.Bacteriol.* **44**: 38-46.

Fukui, S., T. Feizi, C. Galustian, A. M. Lawson, and W. Chai, 2002 Oligosaccharide microarrays for high-throughput detection and specificity assignments of carbohydrate-protein interactions. *Nat.Biotechnol.* **20**: 1011-1017.

Fulop, M., D. Leslie, and R. Titball, 1996 A rapid, highly sensitive method for the detection of Francisella tularensis in clinical samples using the polymerase chain reaction. *Am.J.Trop.Med.Hyg.* **54**: 364-366.

Gallo, M., J. M. Montserrat, and A. M. Iribarren, 2003 Design and applications of modified oligonucleotides. *Braz.J.Med.Biol.Res.* **36**: 143-151.

German, I., D. D. Buchanan, and R. T. Kennedy, 1998 Aptamers as ligands in affinity probe capillary electrophoresis. *Anal.Chem.* **70**: 4540-4545.

Grunow, R., W. Splettstoesser, S. McDonald, C. Otterbein, T. O'Brien *et al.* 2000 Detection of Francisella tularensis in biological specimens using a capture enzyme-linked immunosorbent assay, an immunochromatographic handheld assay, and a PCR. *Clin.Diagn.Lab Immunol.* **7**: 86-90.

Haab, B. B., M. J. Dunham, and P. O. Brown, 2001 Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. *Genome Biol.* **2**: RESEARCH0004.

Hamaguchi, N., A. Ellington, and M. Stanton, 2001 Aptamer beacons for the direct detection of proteins. *Anal.Biochem.* **294**: 126-131.

Hermann, T., and D. J. Patel, 2000 Adaptive recognition by nucleic acid aptamers. *Science* **287**: 820-825.

Hesselberth, J., M. P. Robertson, S. Jhaveri, and A. D. Ellington, 2000a In vitro selection of nucleic acids for diagnostic applications. *J.Biotechnol.* **74**: 15-25.

Hesselberth, J. R., D. Miller, J. Robertus, and A. D. Ellington, 2000b In vitro selection of RNA molecules that inhibit the activity of ricin A-chain. *J.Biol.Chem.* **275**: 4937-4942.

Higgins, J. A., Z. Hubalek, J. Halouzka, K. L. Elkins, A. Sjostedt *et al.* 2000 Detection of Francisella tularensis in infected mammals and vectors using a probe-based polymerase chain reaction. *Am.J.Trop.Med.Hyg.* **62**: 310-318.

Homann, M., and H. U. Goringer, 1999 Combinatorial selection of high affinity RNA ligands to live African trypanosomes. *Nucleic Acids Res.* **27**: 2006-2014.

Howbrook, D. N., d. van, V, M. C. O'Shaughnessy, D. K. Sarker, S. C. Baker *et al.* 2003 Developments in microarray technologies. *Drug Discov.Today* **8**: 642-651.

Hubalek, M., L. Hernychova, J. Havlasova, I. Kasalova, V. Neubauerova *et al.* 2003 Towards proteome database of Francisella tularensis. *J.Chromatogr.B Analyt.Technol.Biomed.Life Sci.* **787**: 149-177.

Jauho, E. S., U. Boas, C. Wiuff, K. Wredstrom, B. Pedersen *et al.* 2000 New technology for regiospecific covalent coupling of polysaccharide antigens in ELISA for serological detection. *J.Immunol.Methods* **242**: 133-143.

Jayasena, S. D., 1999 Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clin.Chem.* **45**: 1628-1650.

Johansson, A., J. Farlow, P. Larsson, M. Dukerich, E. Chambers *et al.* 2004a Worldwide genetic relationships among Francisella tularensis isolates determined by

multiple-locus variable-number tandem repeat analysis. *J.Bacteriol.* **186**: 5808-5818.

Johansson, A., M. Forsman, and A. Sjostedt, 2004b The development of tools for diagnosis of tularemia and typing of Francisella tularensis. *APMIS* **112**: 898-907.

Joos, T. O., M. Schrenk, P. Hopfl, K. Kroger, U. Chowdhury *et al.* 2000 A microarray enzyme-linked immunosorbent assay for autoimmune diagnostics. *Electrophoresis* **21**: 2641-2650.

Junhui, Z., Y. Ruifu, L. Jianchun, Z. Songle, C. Meiling *et al.* 1996 Detection of Francisella tularensis by the polymerase chain reaction. *J.Med.Microbiol.* **45**: 477-482.

Kallioniemi, O. P., U. Wagner, J. Kononen, and G. Sauter, 2001 Tissue microarray technology for high-throughput molecular profiling of cancer. *Hum.Mol.Genet.* **10**: 657-662.

Knezevic, V., C. Leethanakul, V. E. Bichsel, J. M. Worth, V. V. Prabhu *et al.* 2001 Proteomic profiling of the cancer microenvironment by antibody arrays. *Proteomics.* **1**: 1271-1278.

Kononen, J., L. Bubendorf, A. Kallioniemi, M. Barlund, P. Schraml *et al.* 1998 Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat.Med.* **4**: 844-847.

Koskela, P., and A. Salminen, 1985 Humoral immunity against Francisella tularensis after natural infection. *J.Clin.Microbiol.* **22**: 973-979.

Lamps, L. W., J. M. Havens, A. Sjostedt, D. L. Page, and M. A. Scott, 2004 Histologic and molecular diagnosis of tularemia: a potential bioterrorism agent endemic to North America. *Mod.Pathol.* **17**: 489-495.

Lerouge, I., and J. Vanderleyden, 2002 O-antigen structural variation: mechanisms and possible roles in animal/plant-microbe interactions. *FEMS Microbiol.Rev.* **26**: 17-47.

Li, J. J., X. Fang, and W. Tan, 2002 Molecular aptamer beacons for real-time protein recognition. *Biochem.Biophys.Res.Commun.* **292**: 31-40.

McCauley, T. G., N. Hamaguchi, and M. Stanton, 2003 Aptamer-based biosensor arrays for detection and quantification of biological macromolecules. *Anal.Biochem.* **319**: 244-250.

Mezzasoma, L., T. Bacarese-Hamilton, C. M. Di, R. Rossi, F. Bistoni *et al.* 2002 Antigen microarrays for serodiagnosis of infectious diseases. *Clin.Chem.* **48**: 121-130.

Miller, J. C., H. Zhou, J. Kwekel, R. Cavallo, J. Burke *et al.* 2003 Antibody microarray profiling of human prostate cancer sera: antibody screening and identification of potential biomarkers. *Proteomics.* **3**: 56-63.

Nimjee, S. M., C. P. Rusconi, and B. A. Sullenger, 2005 APTAMERS: An Emerging Class of Therapeutics. *Annu.Rev.Med.* **56**: 555-583.

O'Sullivan, C. K., 2002 Aptasensors--the future of biosensing? *Anal.Bioanal.Chem.* **372**: 44-48.

Pavski, V., and X. C. Le, 2001 Detection of human immunodeficiency virus type 1 reverse transcriptase using aptamers as probes in affinity capillary electrophoresis. *Anal.Chem.* **73**: 6070-6076.

Potyrailo, R. A., R. C. Conrad, A. D. Ellington, and G. M. Hieftje, 1998 Adapting selected nucleic acid ligands (aptamers) to biosensors. *Anal.Chem.* **70**: 3419-3425.

Prior, J. L., R. G. Prior, P. G. Hitchen, H. Diaper, K. F. Griffin *et al.* 2003 Characterization of the O antigen gene cluster and structural analysis of the O antigen of Francisella tularensis subsp. tularensis. *J.Med.Microbiol.* **52**: 845-851.

Purschke, W. G., F. Radtke, F. Kleinjung, and S. Klussmann, 2003 A DNA Spiegelmer to staphylococcal enterotoxin B. *Nucleic Acids Res.* **31**: 3027-3032.

Raetz, C. R., 1990 Biochemistry of endotoxins. *Annu.Rev.Biochem.* **59**: 129-170.

Rietschel, E. T., and H. Brade, 1992 Bacterial endotoxins. *Sci.Am.* **267**: 54-61.

Rimmele, M., 2003 Nucleic acid aptamers as tools and drugs: recent developments. *Chembiochem.* **4**: 963-971.

Robertson, D. L., and G. F. Joyce, 1990 Selection in vitro of an RNA enzyme that specifically cleaves single-stranded DNA. *Nature* **344**: 467-468.

Robinson, W. H., C. DiGennaro, W. Hueber, B. B. Haab, M. Kamachi *et al.* 2002 Autoantigen microarrays for multiplex characterization of autoantibody responses. *Nat.Med.* **8**: 295-301.

Romig, T. S., C. Bell, and D. W. Drolet, 1999 Aptamer affinity chromatography: combinatorial chemistry applied to protein purification. *J.Chromatogr.B Biomed.Sci.Appl.* **731**: 275-284.

Rusconi, C. P., E. Scardino, J. Layzer, G. A. Pitoc, T. L. Ortel *et al.* 2002 RNA aptamers as reversible antagonists of coagulation factor IXa. *Nature* **419**: 90-94.

Sampson, T., 2003 Aptamers and SELEX: the technology. *World Patent Information* **25**: 123-129.

Sandstrom, G., A. Sjostedt, T. Johansson, K. Kuoppa, and J. C. Williams, 1992 Immunogenicity and toxicity of lipopolysaccharide from Francisella tularensis LVS. *FEMS Microbiol.Immunol.* **5**: 201-210.

Schweitzer, B., S. Roberts, B. Grimwade, W. Shao, M. Wang *et al.* 2002 Multiplexed protein profiling on microarrays by rolling-circle amplification. *Nat.Biotechnol.* **20**: 359-365.

Schwenk, J. M., D. Stoll, M. F. Templin, and T. O. Joos, 2002 Cell microarrays: an emerging technology for the characterization of antibodies. *Biotechniques* **Suppl**: 54-61.

Shin, I., S. Park, and M. R. Lee, 2005 Carbohydrate Microarrays: An Advanced Technology for Functional Studies of Glycans. *Chemistry.*

Sjostedt, A., 2003 Virulence determinants and protective antigens of Francisella tularensis. *Curr.Opin.Microbiol.* **6**: 66-71.

Sjostedt, A., U. Eriksson, L. Berglund, and A. Tarnvik, 1997 Detection of Francisella tularensis in ulcers of patients with tularemia by PCR. *J.Clin.Microbiol.* **35**: 1045-1048.

Syrjala, H., P. Koskela, T. Ripatti, A. Salminen, and E. Herva, 1986 Agglutination and ELISA methods in the diagnosis of tularemia in different clinical forms and severities of the disease. *J.Infect.Dis.* **153**: 142-145.

Tarnvik, A., S. Lofgren, L. Ohlund, and G. Sandstrom, 1987 Detection of antigen in urine of a patient with tularemia. *Eur.J.Clin.Microbiol.* **6**: 318-319.

Templin, M. F., D. Stoll, M. Schrenk, P. C. Traub, C. F. Vohringer *et al.* 2002 Protein microarray technology. *Drug Discov.Today* **7**: 815-822.

Titball, R. W., A. Johansson, and M. Forsman, 2003 Will the enigma of Francisella tularensis virulence soon be solved? *Trends Microbiol.* **11**: 118-123.

Tuerk, C., and L. Gold, 1990 Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**: 505-510.

Versage, J. L., D. D. Severin, M. C. Chu, and J. M. Petersen, 2003 Development of a multitarget real-time TaqMan PCR assay for enhanced detection of Francisella tularensis in complex specimens. *J.Clin.Microbiol.* **41**: 5492-5499.

Vinogradov, E., W. J. Conlan, J. S. Gunn, and M. B. Perry, 2004 Characterization of the lipopolysaccharide O-antigen of Francisella novicida (U112). *Carbohydr.Res.* **339**: 649-654.

Vinogradov, E., and M. B. Perry, 2004 Characterisation of the core part of the lipopolysaccharide O-antigen of Francisella novicida (U112). *Carbohydr.Res.* **339**: 1643-1648.

Vinogradov, E., M. B. Perry, and J. W. Conlan, 2002 Structural analysis of Francisella tularensis lipopolysaccharide. *Eur.J.Biochem.* **269**: 6112-6118.

Vinogradov, E. V., A. S. Shashkov, Y. A. Knirel, N. K. Kochetkov, N. V. Tochtamysheva *et al.* 1991 Structure of the O-antigen of Francisella tularensis strain 15. *Carbohydr.Res.* **214**: 289-297.

Wang, D., S. Liu, B. J. Trummer, C. Deng, and A. Wang, 2002 Carbohydrate microarrays for the recognition of cross-reactive molecular markers of microbes and host cells. *Nat.Biotechnol.* **20**: 275-281.

Willats, W. G., S. E. Rasmussen, T. Kristensen, J. D. Mikkelsen, and J. P. Knox, 2002 Sugar-coated microarrays: a novel slide surface for the high-throughput analysis of glycans. *Proteomics.* **2**: 1666-1671.

Wilson, D. S., and S. Nock, 2002 Functional protein microarrays. *Curr.Opin.Chem.Biol.* **6**: 81-85.

Wu, R. Z., S. N. Bailey, and D. M. Sabatini, 2002 Cell-biological applications of transfected-cell microarrays. *Trends Cell Biol.* **12**: 485-488.

Ylera, F., R. Lurz, V. A. Erdmann, and J. P. Furste, 2002 Selection of RNA aptamers to the Alzheimer's disease amyloid peptide. *Biochem.Biophys.Res.Commun.* **290**: 1583-1588.

Yuk, C. S., H. K. Lee, H. T. Kim, Y. K. Choi, B. C. Lee *et al.* 2004 Development and evaluation of a protein microarray chip for diagnosis of hepatitis C virus. *Biotechnol.Lett.* **26**: 1563-1568.

Ziauddin, J., and D. M. Sabatini, 2001 Microarrays of cells expressing defined cDNAs. *Nature* **411**: 107-110.

CHAPTER III

In vitro selection of a DNA aptamer against *Francisella tularensis* O-polysaccharide

Abstract

Francisella tularensis, a Gram-negative, facultative intracellular bacterium is the etiological agent of the disease tularemia. The pathogen is highly infectious with a reported infective dose of ten organisms for humans. Tularemia is endemic in many parts of the world. *Francisella tularensis* is considered to be a potential biowarfare/bioterrorism agent owing to its high infectivity and ease of spread after aerosolization. Presently, the identification of the organism or the diagnosis of tularemia is mainly done by antibody-based assays. Special techniques such as antigen detection assays and PCR are limited to reference and research laboratories. Systematic Evolution of Ligands by Exponential Enrichment (SELEX), an *in vitro* technique, offers an alternative and novel tool known as aptamer for diagnostic and biosensor applications. In the present study, we adopted SELEX for *in vitro* selection of specific ssDNA ligands to *F. tularensis* subspecies *tularensis* O-antigen. The selected DNA aptamer Ft1 was found to bind specifically to LPS from *F. tularensis* subspecies *tularensis* and subspecies *holarctica*. This is not unexpected as antibodies raised against LPS of *F. tularensis* LVS

(Live Vaccine Strain derived from subspecies *holarctica*) recognized both subspecies *tularensis* and subspecies *holarctica*. Furthermore, aptamer Ft1 differentiated *F. tularensis* subspecies *tularensis* and subspecies *holarctica* from *F. philomiragia*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Vibrio cholerae*, *Salmonella typhimurium*, *Yersinia* species and *Brucella ovis*. However, aptamer Ft1 did not recognize whole *F. tularensis* cells in binding assays. This could be due to recognition of the core oligosaccharide part of LPS by the aptamer and the core oligosaccharide may not be readily accessible on the surface of bacterium. The use of whole bacterial cells instead of purified O-polysaccharide containing core oligosaccharide as target may help in selecting aptamers against a surface target of the bacteria.

Introduction

Francisella tularensis, the causative agent of the disease tularemia, is a Gram-negative, facultative intracellular bacterium, and the pathogen is one of the most infectious bacteria known. Tularemia is a disease of rodents, lagomorphs and humans and the disease is widely endemic in North America, Europe and Asia.

Francisella tularensis is one of the two recognized species of the genus *Francisella* within the family *Francisellaceae*, the other species being *F. philomiragia* (Ellis *et al.* 2002). *Francisella philomiragia* is an opportunistic pathogen causing disease in immunocompromised individuals and is often associated with water (Titball *et al.* 2003). *Francisella philomiragia* shares more than 98% 16S rRNA sequence homology with *F. tularensis* (Forsman *et al.* 1994). The strains of *F. tularensis* are grouped into one of four subspecies: *tularensis* (type A), *holarctica* (type B), *mediasiatica* and *novicida*. (Ellis *et al.* 2002; Titball *et al.* 2003). Although all the subspecies of *F. tularensis* are genetically and antigenically similar, they differ in virulence and geographical distribution. Strains of *F. tularensis* subspecies *tularensis* are highly virulent for humans and are generally reported from North America, but recently also from Europe (Ellis *et al.* 2002). The infective dose required is very small with as little as ten bacteria reported to cause human infection (Ellis *et al.* 2002). The mortality associated with *F. tularensis* subspecies *tularensis* was reported to be 5-30% prior to the advent of antibiotics (Titball *et al.* 2003). Human infections caused by strains of

subspecies *holarctica* and *mediasiatica* are less severe with low mortality (Titball *et al.* 2003). Strains of *F. tularensis* subspecies *novicida* are less virulent, with infection most often seen in immunocompromised individuals (Titball *et al.* 2003).

Francisella tularensis is considered to be a potential biological weapon owing to its ability to spread rapidly upon aerosolization and a very low infective dose (Dennis *et al.* 2001). The organism is very hardy and can persist in watercourses and at low environmental temperatures making it an ideal agent of bioterrorism. Aerosol dissemination of *F. tularensis* in a populated area would be expected to cause a large number of acute cases 3 to 5 days after the exposure, with overwhelming medical and public health consequences (Dennis *et al.* 2001).

Clinical cases of tularemia are generally diagnosed by antibody-based tests such as agglutination test or ELISA. However, at least two weeks are required for development of antibodies in humans after infection (Koskela & Salminen 1985), and antibodies developed against certain strains of *F. tularensis* have failed to agglutinate commercially available antigens (Clarridge, III *et al.* 1996). Moreover, ELISA procedures are time consuming, involving blocking, several steps of incubations, and washings. *F. tularensis* is a fastidious organism which grows slowly at 37° C and requires special growth conditions (Doern 2000). Culturing of the organism in the laboratory is a potential source of infection to laboratory personnel (Burke 1977), making it a less acceptable method for diagnosis and identification. Special techniques for the

identification of *Francisella* such as antigen detection assays, PCR, ELISA, immunoblotting and pulsed-field gel electrophoresis are generally performed in reference and research laboratories (Dennis *et al.* 2001). In the event of the use of *F. tularensis* as a biological weapon, rapid detection of organisms in environmental samples becomes crucial to containment and clean-up efforts. Additionally, a simple, rapid and reliable diagnostic test is needed to screen persons exposed to the pathogen.

Systematic Evolution of Ligands by Exponential Enrichment (SELEX) offers an alternative and novel tool, known as aptamer, for diagnostic and biosensor applications (Brody *et al.* 1999; Hesselberth *et al.* 2000; O'Sullivan 2002). Aptamers are oligonucleotides (RNA, DNA or modified nucleotides), that are selected *in vitro* through SELEX for specific binding to a variety of targets, ranging from simple ions to whole cells (Ellington & Szostak 1990; Tuerk & Gold 1990; Ellington & Szostak 1992; Famulok & Mayer 1999; Wilson & Szostak 1999). Aptamers have several advantages over antibodies (Jayasena 1999; O'Sullivan 2002). Although, aptamers are described as "emerging molecules that rival antibodies in diagnostics" (Jayasena 1999), so far, the selected aptamers have found very few applications in diagnostic assays and continue to be mainly the subject of academic interest (Hesselberth *et al.* 2000). A DNA aptamer based magnetic bead-electrochemiluminescence assay has been developed for the detection of anthrax spores (Bruno & Kiel 1999). The present study involves the selection of aptamers to O-antigen of *F. tularensis* subspecies *tularensis*. We selected an aptamer,

Ft1, which specifically recognized LPS of *F. tularensis* subsp. *tularensis* and subsp. *holarctica*. The aptamer Ft1 could differentiate *F. tularensis* lipopolysaccharide (LPS) from other closely related Gram-negative bacterial LPS.

Materials and Methods

Bacteria and lipopolysaccharide

Francisella tularensis subspecies *tularensis* strain OSU 10 (feline isolate), *F. tularensis* subspecies *holarctica* strain 3093 (beaver isolate) and *Brucella ovis* – 1 (sheep isolate) are field isolates from our strain collection. *Yersinia enterocolitica*-1 and *Yersinia pseudotuberculosis*-1 are field isolates from animals, kindly provided by the Oklahoma Animal Disease Diagnostic Laboratory. *Yersinia pestis* D-1 (avirulent mutant) was kindly supplied by Dr. Robert Brubaker, Michigan State University. *Salmonella typhimurium* 14028 and *Francisella philomiragia* 25105 were obtained from American Type Culture Collection (Manassas, VA). Lipopolysaccharide (LPS) from these bacteria were isolated using Tri-reagent method described previously (Yi & Hackett 2000). LPS preparations from *Escherichia coli* O111:B4, *Pseudomonas aeruginosa* serotype 10, *Vibrio cholera* Inaba 569B and *Shigella flexneri* serotype 1A were purchased from Sigma (St. Louis, MO).

Isolation of *F. tularensis* subspecies *tularensis* O-antigen

The O-antigen from *F. tularensis* subspecies *tularensis* was isolated from LPS by acid hydrolysis. The LPS was suspended in 1% acetic acid and heated at 100° C for 2.5 hours. The lipid portion was then removed by centrifugation at 12000g for 20 minutes, and the supernatant containing O-antigen was freeze dried. The O-antigen was further purified by gel filtration chromatography. The lyophilized O-antigen was suspended in 50mM ammonium acetate and passed through Toyopearl HW-50F (Supelco, Bellefonte, PA) gel filtration column. Elution of polysaccharide was monitored by refractive index. The three major fractions were pooled separately and freeze dried. The O-antigen fraction, eluting just after the void volume, was identified by its immunoreactivity with monoclonal antibodies (BioDesign, Saco, ME) specific for *F. tularensis* O-antigen and by its ^{1}H NMR spectrum.

SELEX Procedure

SELEX DNA library: Synthetic template DNA containing 30 randomized nucleotides flanked by fixed sequences for PCR amplification was used in the study (Integrated DNA Technologies, Coralville, IA). The sequence of DNA library was 5'-TTGAGCGTTTATTCTGAGCT CCC- $(N)_{30}$ – TTCGACATGAGGCCCGGATC – 3' and the primers used were 5'-TT GAGCGTTTATTCTGAGCTCCC -3' and 5' – *B-GATCCGGGCCTCATGTCGAA – 3' (*B = biotin).

Polymerase Chain Reaction and generation of ssDNA: All reaction mixtures for PCR contained 1 µM each primer, 3 mM $MgCl_2$**,** 200 µM dNTPs, GeneAmp 1X PCR gold buffer (15 mM Tris-HCl, pH 8.0, 50 mM KCl) and AmpliTaq Gold DNA polymerase (1.5 units/ 50 µl; Applied Biosystems, Foster City, CA). Amplifications were carried out in a MJ Research, Inc. thermal cycler (Waltham, MA) at 94 ° C for 5 minutes preheating followed by 20 cycles of 94° C for 30 sec, 55° C for 30 sec and 72° C of 1 minute. The PCR product was gel purified using Nusieve GTG agarose (BioWhittaker Molecular Applications, Rockland, ME). ssDNA was generated by using the Streptavidin MagneSphere paramagnetic particles (Promega, Madison, WI). The gel purified dsDNA (with 5' biotinylated negative sense strand) was incubated with streptavidin-paramagnetic particles in 0.5X SSC buffer for 10 minutes and then washed with 0.1X SSC buffer to remove unbound dsDNA. The bound dsDNA was denatured by incubation with 0.2 N NaOH for 5 minutes. Then the streptavidin-paramagnetic particles were captured on a magnetic stand and the solution containing ssDNA was transferred to a new microcentrifuge tube and the ssDNA was desalted by ethanol precipitation.

Selection procedure: *Francisella tularensis* subspecies *tularensis* O-antigen was immobilized on a circular disc of nitrocellulose membrane (~ 0.5 cm^2, 0.45µM, Bio-Rad laboratories, Hercules, CA) by overnight incubation with 10 mg/ml O-antigen in SELEX binding buffer (20mM Tris, pH 7.5 / 100 mM NaCl / 5 mM $MgCl_2$). The

immobilized O-antigen was then incubated with ssDNA library in binding buffer at room temperature. After incubation the membrane was washed with binding buffer to remove unbound ssDNA. The bound ssDNA was eluted by heating the membrane in 7 M urea at 94 ° C for 10 minutes. ssDNA - O-antigen binding was performed under progressively increasing stringency. The duration of incubation was decreased from 1 hour for initial rounds (1 – 5) of selection to 30 minutes for the closing rounds (6 -10) and similarly, the number of washings was increased from 3 times to 5 times (10 minutes each). The eluted ssDNA was ethanol precipitated and amplified by PCR for the next round of selection. For the first round of selection 10 µg of synthetic ssDNA from the library (~$2.7x10^{14}$ molecules) was used for selection. Counter selections were made against lipopolysaccharides from *Escherichia coli* serotype 0111:B4, *Pseudomonas aeruoginosa* serotype 10, *Shigella flexneri* serotype 1A and whole cells of *Salmonella typhimurium* ATCC14028 prior to the 4^{th}, 5^{th}, 6^{th} and 7^{th} rounds of selection, respectively, using conditions described above.

The enriched DNA library from the 10^{th} round of SELEX was PCR amplified and cloned into the plasmid vector pCRII-TOPO and chemically transformed into competent TOP10 *E. coli* cells (TOPO TA Cloning, Invitrogen, Carlsbad, CA). Clones were selected, plasmid DNA isolated by the alkaline lysis, and sequences of seventy clones determined by standard dideoxy terminator method (Recombinant DNA Core facility, Oklahoma State University). The sequences obtained were arranged into different groups by constructing a dendrogram using SeqWeb software version 2.1 (Accelrys, Inc. San

Diego, CA), and sequences within a group were aligned using AlignX (Vector NTI Suite, v.6, Informax, Bethesda, MD).

Dot-blot assay

Lipopolysaccharides were blotted on to nitrocellulose membranes and the membranes were blocked with 5% non-fat milk in SELEX binding buffer overnight at 4°C. After incubation the membranes were washed in binding buffer for 5 minutes, then incubated with ^{33}P-labeled individual aptamer sequences (~ 5 pmoles) in binding buffer at RT for 30 minutes. DNA was labeled with ^{33}P using [γ-^{33}P] ATP and T4 polynucleotide kinase (DNA 5´ End-Labeling System, Promega, Madison, WI) and unincorporated [γ-^{33}P] ATPs were removed by size exclusion chromatography using Sephadex G-25 Quick Spin column (Roche diagnostics corp., Indianapolis, IN). Ten-fold molar excess of BSA and 100-fold molar excess of tRNA were included in binding reactions as competitors to avoid non-specific binding. The membranes were washed in binding buffer four times (10 minutes each), air dried and subjected to autoradiography.

Microtiter plate based binding assays

96-well microtiter plates (Immulon 1B, Dynex Technologies, Chantilly, VA) were coated with solutions of LPS (0.5 μg/μl, 200μl per well) in SELEX binding buffer at 37° C for 3h followed by overnight incubation at 4° C. The LPS solution was aspirated and

wells were blocked with 1% BSA in binding buffer at RT for 1h. Wells were then washed in binding buffer for 5 minutes and incubated with ^{33}P-labeled individual aptamer sequences (0.8 – 1.2 pmoles/well) at RT for 30 minutes. DNA was labeled with ^{33}P as described above. The binding reactions included 10-fold and 100- fold molar excess of BSA and tRNA, respectively. After incubation the wells were washed (3X 10 min) with binding buffer. The amount of radioactivity retained in each well was determined by liquid scintillation analysis.

Results

Characterization of *Francisella tularensis* subspecies *tularensis* LPS and O-antigen

Isolated *Francisella tularensis* subsp. *tularensis* LPS was analyzed by SDS-PAGE and western blotting. The LPS sample was run on 12.5% acrylamide gels and stained with silver stain. The LPS showed the characteristic ladder pattern on acrylamide gels indicating variation in the polysaccharide side chain length (data not shown). Further, the LPS immunoreacted with monoclonal antibodies (BioDesign, Saco, ME) specific for *F. tularensis* LPS (data not shown).

Selection of DNA aptamers to *Francisella tularensis* subspecies *tularensis* O-antigen

The ssDNA sequences that bind to *Francisella tularensis* O-antigen were selected by established SELEX protocols (Ellington & Szostak 1990; Tuerk & Gold 1990; Ellington & Szostak 1992) with some modifications. The DNA library had 30 randomized bases flanked by fixed sequences for PCR amplification. The starting pool was diverse with approximately 10^{14} individual sequences. Nitrocellulose membrane discs were used as a matrix to immobilize O-antigen for separation of unbound sequences from bound sequences.

Sequencing of 70 clones from the selected aptamer pool resulted in 24 individual sequences (Table 1). The high sequence redundancy of the selected pool indicates the completion or nearing completion of SELEX process with the selection of high affinity and specific aptamers. The length of the randomized region varied from 28 nucleotides to 30 nucleotides, although the starting pool had 30 nucleotides in the randomized region. This is most likely due to insertion and deletion of nucleotides by DNA polymerase during amplification (Yang *et al.* 1998). The selected sequences were rich in guanine content and guanine percentage varied from 10 to 67 percent (Table 1).

The selected sequences could be grouped into different families of aptamers based on sequence homology, with the majority of sequences (57%) falling into one family (Figure 1; Table 1). Ft1 appeared 31 times (44%) and also showed high sequence homology with 5 other selected sequences (Table 1). This prompted us to reason that the

randomized region of aptamer Ft1 could be the motif required for binding (5'-AGGGCGGGAGTGGGTGTGGAAGGTGCGG-3'; aptamer Ft1).

Specific identification of *Francisella tularensis* LPS by aptamer Ft1

Aptamer Ft1 along with other full length selected aptamers (with flanking fixed sequences) Ft1, Ft5, Ft23, Ft37 and Ft79 were tested for their specific binding to *Francisella tularensis* LPS and for the ability to differentiate other closely related Gram-negative bacterial LPS. Among the sequences tested aptamer Ft1 showed high specific binding to *F. tularensis* LPS compared to other sequences tested. Autoradiograph results of the dot-blot assay showed intense binding of aptamer Ft1 to *F. tularensis* LPS (Figure 2). The starting pool showed a faint binding to *F. tularensis* LPS. Further, aptamer Ft1 specifically bound *F. tularensis* LPS in a concentration dependent manner but not *E. coli* LPS immobilized on the same membrane (Figure 3).

Binding of aptamer Ft1 to *Francisella tularensis* LPS was tested in microtiter plate-based assays. Aptamer Ft1 showed higher binding to *F. tularensis* subsp. *tularensis* LPS compared to either the starting pool or an arbitrary control sequence of 28 nucleotides (Figure 4). Further, Ft1 bound strongly to both *F. tularensis* subspecies *tularensis* (type A) and subspecies *holarctica* (type B; Figure 5). However, the aptamer showed only moderate binding to *F. philomiragia*, the other species of genus *Francisella* (Figure 5). Further, aptamer Ft1 differentiated *F. tularensis* LPS from other Gram-

negative bacterial LPS (Figure 4 and 5). Aptamer Ft1 did not bind to LPS from *E. coli*, *Pseudomonas aeruginosa, Shigella flexneri, Vibrio cholerae, Salmonella typhimurium, Yersinia* species and *Brucella ovis*. These results indicate the specificity of aptamer Ft1 to *F. tularensis* subsp. *tularensis* and subsp. *holarctica*.

Discussion

Francisella tularensis has been considered a threat as a possible biological weapon and has acquired new importance with the recent acts of terrorism across the globe. In addition, the pathogen continues to be responsible for human infections in many countries. Presently the identification of the organism or the diagnosis of tularemia is based on isolation of the causative agent, immunoassays, and PCR. In the present study we have selected DNA aptamers against the O-antigen of *Francisella tularensis* subspecies *tularensis* (Strain OSU 10). High affinity and high specificity aptamers have been selected against a variety of targets (Famulok & Mayer 1999; Wilson & Szostak 1999). In the majority of these studies the target for aptamers is protein. A few studies have shown the feasibility of selecting DNA and RNA aptamers to carbohydrates or polysaccharides. Aptamers have been selected against cellobiose (Yang *et al.* 1998), sephadex (Srisawat *et al.* 2001), chitin (Fukusaki *et al.* 2000) and aminoglycosides (Wilson & Szostak 1999).

The identification of gram-negative bacteria is traditionally done by serotyping using antibodies specific for O-antigen. Monoclonal antibodies specific to LPS have been used for identification of *F. tularensis* (Greiser-Wilke *et al.* 1989; Fulop *et al.* 1991; Grunow *et al.* 2000). Targeting of O-antigen for identification of Gram-negative bacteria offers several advantages over other targets such as rRNA, or specific genomic sequences. In the present study, *F. tularensis* O-antigen was used as target for aptamer selection for several reasons: (i) O-antigen provides specificity and is present in high copy number on the cell surface (ii) repeating tetra-saccharide subunits of O-antigen are likely to present multiple targets per molecule, and (iii) O-antigen is expected to be relatively stable during transport of clinical samples or in the environment. LPS preparations from *F. tularensis* and *F. novicida* were found to exhibit species-specific and genus-specific epitopes (Pavlovich *et al.* 2000) and these antigenic determinants of *F. tularensis* LPS were found to be localized on O-polysaccharide and not on the core region (Pavlovich *et al.* 2000). Identification of bacteria based on genomic DNA or rRNA requires lyzing of cells to release the targets and invariably the nucleic acid target has to be amplified to be highly sensitive. The nucleic acid target also poses the problem of secondary structure interfering with probe binding and is susceptible to nucleases.

The use of purified O-antigen instead of LPS as the target was intended to avoid selection of aptamers to the lipid-A portion, which is highly conserved among Gram-negative bacteria and also not readily accessible on the surface. *In vitro* selection of aptamers to a purified single target requires a partitioning matrix or immobilization of the

target to separate the unbound sequences during selection. Adsorption and immobilization of *F. tularensis* O-antigen onto nitrocellulose membrane was tested using monoclonal antibodies specific for *F. tularensis* O-antigen. In addition, high concentrations (10 mg/ml) of the polysaccharide were used to ensure saturation of binding sites on the membrane. It has been shown that microbial polysaccharides can be immobilized on glass slides coated with nitrocellulose without chemical conjugation, and that such immobilized polysaccharides retain their immunological properties to be recognized by specific antibodies (Wang 2003). We also adopted counter selection in order to achieve selection of highly specific ligands, and to hasten the selection process. LPS preparations from related bacteria and whole cells were used for the counter selection. Negative selection and counter selection are employed along with alteration of incubation time for the efficient enrichment of the aptamer library during SELEX process (O'Sullivan 2002).

The selected aptamers are characterized by high sequence redundancy and high guanine content (Table 1). DNA aptamers selected against cellobiose and chitin were also rich in guanine (Yang *et al.* 1998; Fukusaki *et al.* 2000). This suggests that guanine residues might contribute to the recognition of polysaccharide/carbohydrate targets. Aptamer Ft1 showed strong binding to *F. tularensis* LPS immobilized on nitrocellulose membrane compared to the starting pool, indicating the selection of the aptamer Ft1 against the *F. tularensis* O-antigen (Figure 2). The binding of aptamer Ft1 to *F. tularensis* LPS but not to *E. coli* LPS immobilized on the same membrane in a concentration

dependent manner establishes the specificity (Figure 3). Further specific recognition of *F. tularensis* LPS immobilized on the polystyrene surface (microtiter plate) by the aptamer Ft1 suggests its selection against the O-antigen of *F. tularensis*. The binding of starting pool is comparable to that of control sequence (Figure 4). The adsorption of LPS molecules on polystyrene surfaces seems to occur through hydrophobic interactions involving lipid-A, resulting in the orientation of LPS molecules on polystyrene surface similar to that on bacterial surface (Peula-Garcia *et al.* 2002). This makes the microtiter plate-based assays more appropriate for evaluating ligands to LPS molecules. The aptamer Ft1 recognized both *F. tularensis* subspecies *tularensis* (type A) and subspecies *holarctica* (type B) and differentiated the only other species, *F. philomiragia* (Figure 5). This is not surprising as the monoclonal antibodies raised against LPS of *F. tularensis* LVS (ATCC 29684) recognized subspecies *tularensis* and subsp. *holarctica* suggesting a structural similarity of LPS between the two subspecies (Grunow *et al.* 2000). The moderate binding of aptamer Ft1 to *F. philomiragia* suggests a possible close structural relatedness of O-antigen between the two species. It would be interesting to test the binding of aptamer Ft1 to *F. tularensis* subsp. *novicida* and *mediasiatica* along with other isolates. In addition, the aptamer differentiated *F. tularensis* from *E. coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Vibrio cholerae*, *Salmonella typhimurium*, *Yersinia* species and *Brucella ovis* (Figure 5). These Gram-negative bacteria are possibly related to *F. tularensis* (Grunow *et al.* 2000). *Brucella* species, *Haemophilus influenza* and *Yersinia pestis* serologically cross react with *F. tularensis* due to presence of common protein antigens (Greiser-Wilke *et al.* 1989). The structure of *F. tularensis* subspecies *holarctica*

O-antigen is related to those of *Pseudomonas aeruginosa* O6, immunotype1 and II D 1008, and *Shigella dysenteriae* type 7 (Vinogradov *et al.* 1991). The tetrasaccharide subunits of O-antigens from these bacteria differ in one constituent monosaccharide (Vinogradov *et al.* 1991). The serological relationship of these two species with *F. tularensis* is not established.

Although the selected aptamer Ft1 was found to recognize LPS of *F. tularensis* subspecies *tularensis* and subspecies *holarctica* immobilized on either nitrocellulose membrane and on polystyrene surface (microtiter plates), the aptamer did not bind to whole *F. tularensis* cells. Fluorophore labeled aptamer Ft1 did not show significant binding to whole *F. tularensis* cells when analyzed by flow cytometry and confocal microscopy (data not shown). This could be due to recognition of the core polysaccharide part of LPS by the selected aptamer, and the core polysaccharide may not be readily accessible for the aptamer to bind. Mild acid hydrolysis of LPS cleaves the ketosidic bond that links the core polysaccharide with lipid-A. Therefore purified O-polysaccharide does retain core polysaccharide. Alternatively, use of whole bacterial cells in selection process may facilitate selection of aptamers against the surface targets of bacteria.

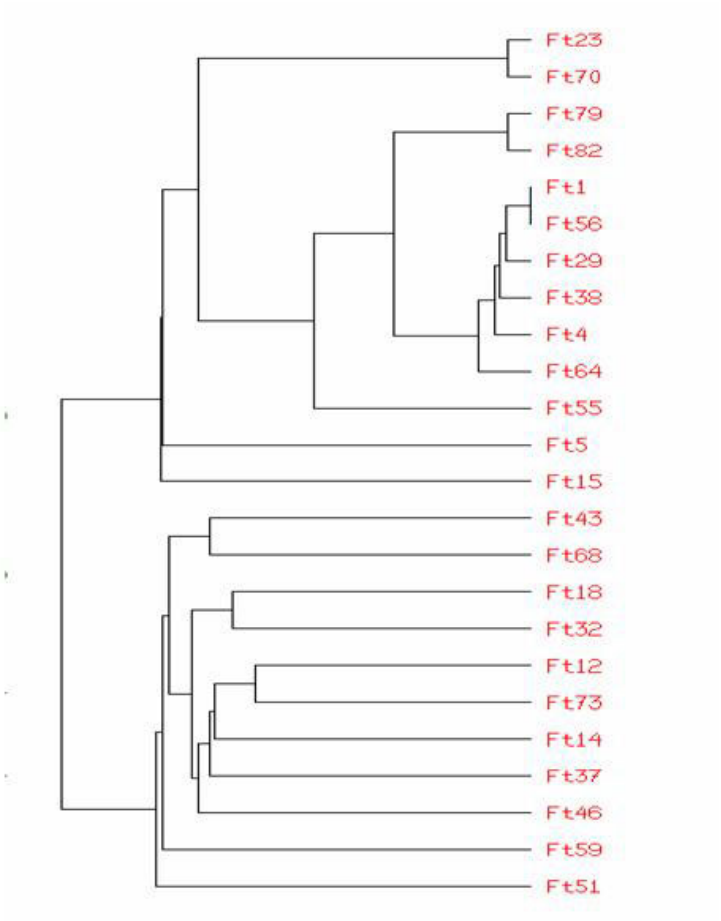
Aptamers are versatile molecules that have the potential to overcome limitations associated with use of antibodies in diagnostic assays (Jayasena 1999; O'Sullivan 2002). Aptamers can be rapidly selected against a wide range of targets by an *in vitro* process, and it is possible to select aptamers of desired properties. Aptamers are also very stable

and reporter molecules can be easily attached. Although, considerable progress has been made on the therapeutic applications of aptamers with many aptamers in clinical trials, their potential use as diagnostics has yet to be realized.

Acknowledgments

This study was supported by Oklahoma Center for Advancement of Science and Technology. We thank Shannon Cowan for culturing bacteria used in this study. We thank Janet Rogers and Dr. Patricia Ayoubi, Recombinant DNA core facility, OSU for sequencing clones and helping with use of SeqWeb software, respectively.

Figure 1. Selected sequence alignment dendrogram. DNA sequences selected against the O-antigen of *Francisella tularensis* subspecies *tularensis* (strain OSU 10) were grouped into different families based on randomized region sequence homology using SeqWeb software version 2.1 (Accelrys, Inc. San Diego, CA).



Multiple Sequence Alignment Dendrogram

Table 1. Sequences of DNA aptamers selected against the O-antigen of *F. tularensis* subsp. *tularensis* (strain OSU 10). Only the randomized regions of the selected sequences are shown along with the number of times sequences appeared (out of 70 clones sequenced) and guanine content. The selected sequences were classified into different groups based on sequence homology. The sequences showed high sequence redundancy with the majority of sequences falling into one group and are rich in guanine.

Aptamer	# Clones	Randomized Region	Guanine %
Ft1	(31)	–AGGGCGGGAGTGGGTGTGGAAGGTGCGG	64
Ft56	(1)	CAGGGCGGGAGTGGGTGTGGAAGGTGCGG	62
Ft29	(2)	-AGGGCGGGAGTGGGTGTGGAAGGTGTGG	64
Ft38	(1)	-AGGGCAGGAGTGGGTGTGGAAGGTGCGG	60
Ft4	(4)	-GGGGCGGGAGTGGGTGTGGAAGGTGCGG	67
Ft64	(1)	-AGGGCGGAAGTGGGTGTGGAAGGTGTGG	60
Ft79	(1)	GCGGCACCGAAGTGGGTGTGGAAGGTGTGC	50
Ft82	(1)	GCGGCACCGAAGTGGGTGTGGAAGGTGTGG	51
Ft23	(4)	GGGGTCCGGGGGGGTCCTAAACGAGGGGTG	56
Ft70	(1)	TGGGTCCGGGGGGGTCCTAAACGAGGGGTG	53
Ft12	(1)	ACGAGCACAGTCATCACATTACCACCCCGG	16
Ft73	(1)	CTGAGCGCAGTC – TGTATTTCCCAACCCTG	20
Ft37	(2)	CGAGCGCAGTCC- - ACCATCTCGTCTACCGG	24
Ft46	(1)	CGAGCACAGTCCTTATCACTTACTCGCCCG-	16
Ft43	(1)	ACGGCAGC-ACAGTACCACCTACCAC-CCCG-	17
Ft68	(1)	- -ACCATCTACCCTACTACCTACCTCGCCCGG	10
Ft18	(1)	- - -TGAGCACATTCACATTCATCTCTCTCCCGG	13
Ft32	(2)	ATGAGCGCACT- ACATTTACACCA-TCTCCG--	13
Ft51	(1)	- - CATCACCTCGTACATT- CTCGCTTTACCGCC	10
Ft59	(2)	AGCATGGGGGC-TACATCACTATCTTCACCG--	23
Ft5	(6)	- - GGCCGATGAACGATAGGATGGCTAGGGGGG-	50
Ft15	(2)	GGTGCTGGTGAAG - GTAG - - TTGTTAGGGGTCC	46
Ft14	(1)	TGCGCAGACTGTTCTTCCTTATCTACCCGG	20
Ft55	(1)	GGCAGATGTCCATGGGTGTGGAAGGTGCGG	50

Figure 2. Binding of aptamer Ft1 to *F. tularensis* LPS immobilized on nitrocellulose membrane. Binding assay was performed with ^{33}P radiolabeled starting pool (SP) and aptamer Ft1. The labeled oligonuclotides (~ 5 pmoles) were incubated with *F. tularensis* subsp. *tularensis* LPS (30 and 50 µg) immobilized on nitrocellulose membrane. Autoradiograph was developed after washing off unbound ssDNA. The aptamer Ft1 showed intense binding to *F. tularensis* LPS, whereas the starting pool showed faint binding.

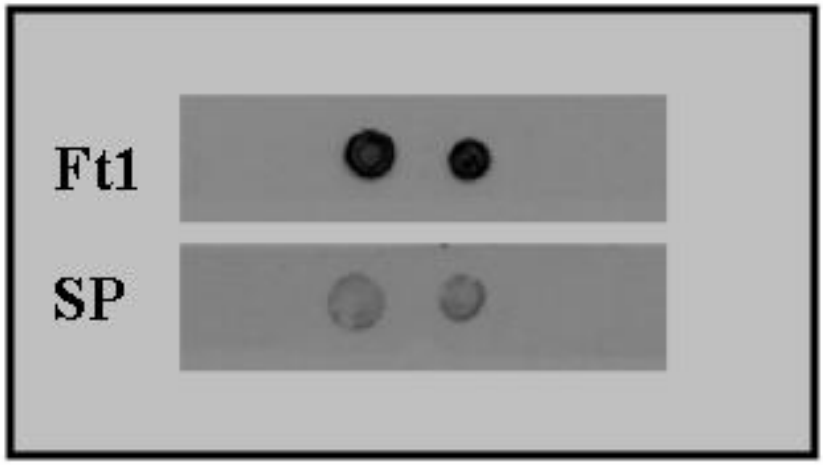
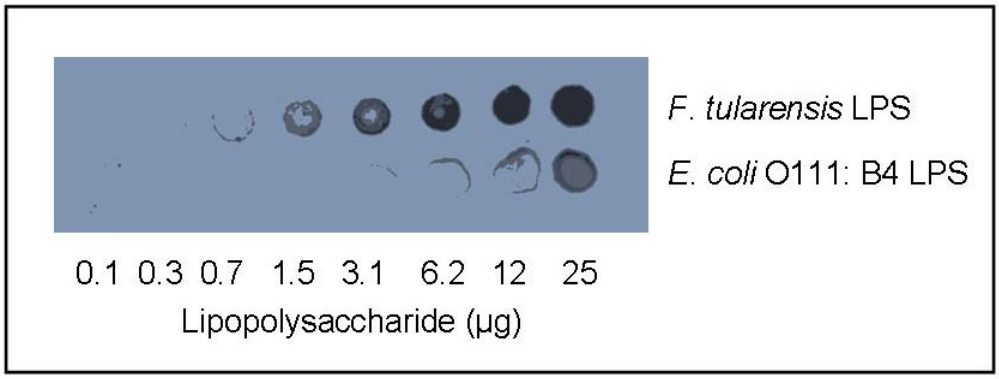

Ft1
SP

Figure 3. Specific binding of aptamer Ft1 to *F. tularensis* LPS immobilized on nitrocellulose membrane in a concentration dependent manner. Different amounts of *F. tularensis* subsp. *tularensis* LPS and *E. coli* O111:B4 LPS in (25 µg to 0.1953 µg) were blotted on a nitrocellulose membrane. The membrane was blocked with non-fat milk and then incubated with with ^{33}P radiolabeled aptamer Ft1. Autoradiograph was developed after washing off unbound ssDNA. The aptamer Ft1 shows concentration dependent specific binding to *F. tularensis* LPS but not to control *E. coli* LPS blotted on the same membrane.


F. tularensis LPS
E. coli O111: B4 LPS
0.1 0.3 0.7 1.5 3.1 6.2 12 25
Lipopolysaccharide (µg)

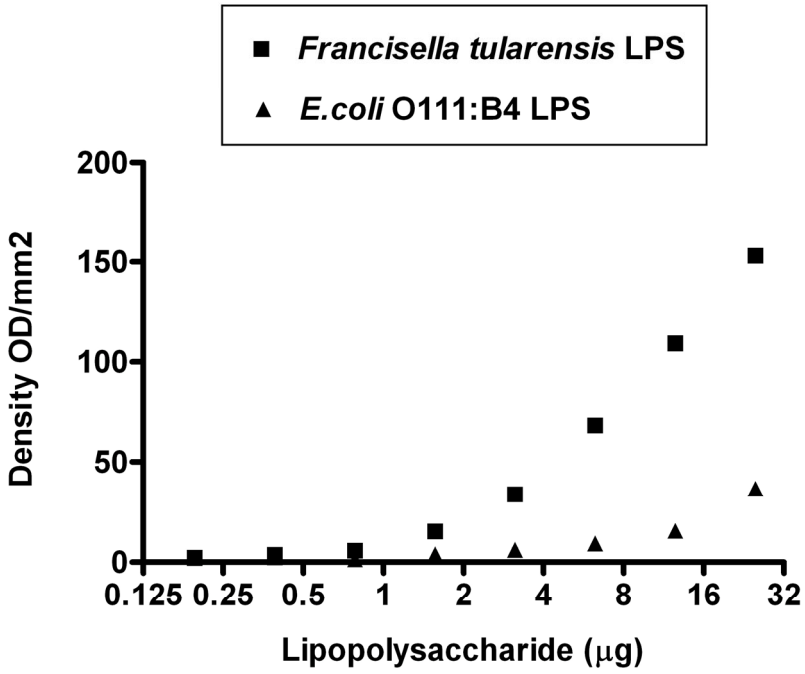

Francisella tularensis LPS
E.coli O111:B4 LPS
Density OD/mm2
200
150
100
50
0
0.125 0.25 0.5 1 2 4 8 16 32
Lipopolysaccharide (µg)

Figure 4. Specific binding of aptamer Ft1 to *F. tularensis* LPS adsorbed onto polystyrene surface of a microtiter plate. Binding of aptamer Ft1 to *F. tularensis* subsp. *tularensis* LPS, *E. coli* O111:B4 LPS and *Pseudomonas aeruginosa* serotype 10 LPS was compared with the starting pool and an arbitrary control sequence of 28 nucleotides. ^{33}P radiolabled ssDNA was incubated with LPS adsorbed onto the polystyrene surface of a microtitre plate. After washings to remove unbound DNA, the radioactivity retained was determined by scintillation counting. The aptamer Ft1 specifically binds to *F. tularensis* LPS but not to *E. coli* and *P. aeruginosa* LPS. Binding of the starting pool is comparable to that of the control sequence. Each bar represents the average CPM ± standard error of three independent experiments with duplicates (n = 6).

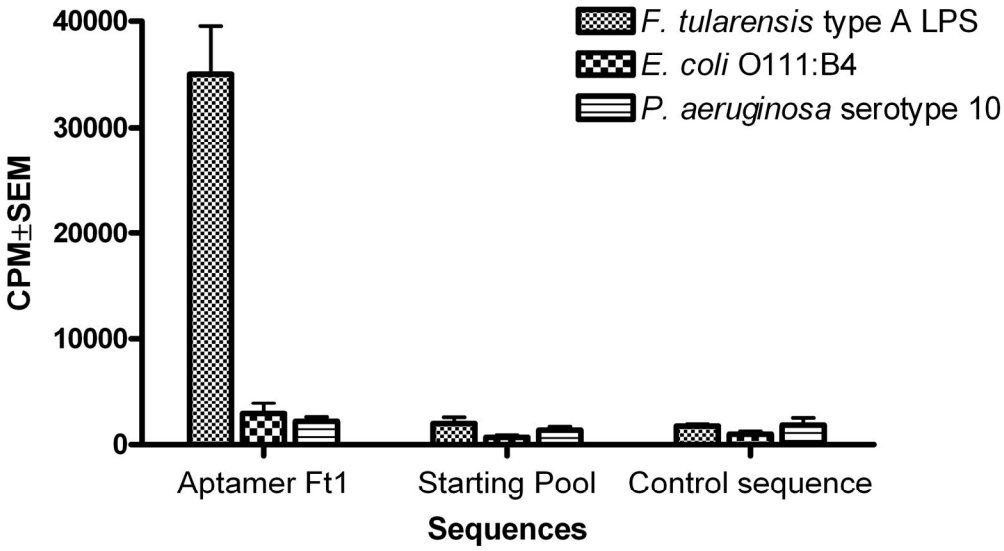
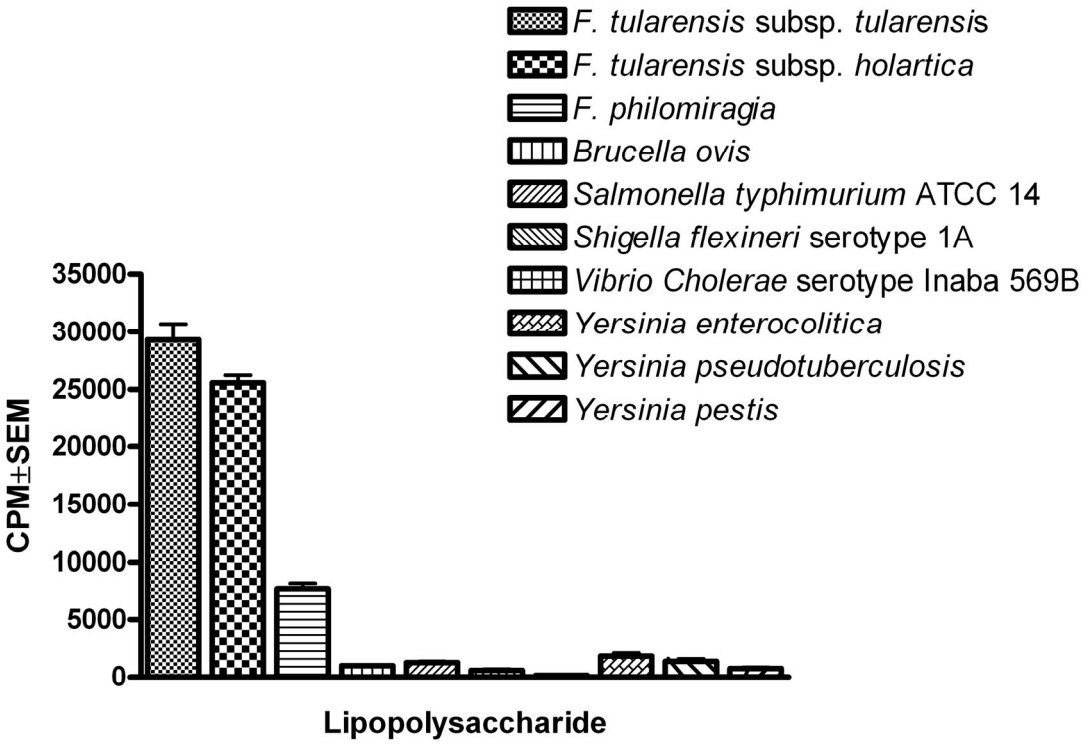

40000
30000
20000
10000
0
CPM±SEM
F. tularensis type A LPS
E. coli O111:B4
P. aeruginosa serotype 10
Aptamer Ft1
Starting Pool
Control sequence
Sequences

Figure 5. Specificity of the aptamer Ft1 to *Francisella tularensis* species. Aptamer Ft1 showed strong binding to *F. tularensis* subsp. *tularensis* and subsp. *holarctica* in microtiter plate binding assays. The aptamer exhibits a moderate binding to *F. philomiragia* LPS suggestive of a possible close structural relatedness of O-antigen between the two species. The aptamer did not cross-react with other possibly related Gram-negative bacteria. Each value represents the average CPM ± standard error of three independent experiments with duplicates (n = 6).


F. tularensis subsp. tularensis
F. tularensis subsp. holartica
F. philomiragia
Brucella ovis
Salmonella typhimurium ATCC 14
Shigella flexineri serotype 1A
Vibrio Cholerae serotype Inaba 569B
Yersinia enterocolitica
Yersinia pseudotuberculosis
Yersinia pestis
35000
30000
25000
20000
15000
10000
5000
0
CPM±SEM
Lipopolysaccharide

References

Brody, E. N., M. C. Willis, J. D. Smith, S. Jayasena, D. Zichi *et al.* 1999 The use of aptamers in large arrays for molecular diagnostics. *Mol.Diagn.* **4**: 381-388.

Bruno, J. G., and J. L. Kiel, 1999 In vitro selection of DNA aptamers to anthrax spores with electrochemiluminescence detection. *Biosens.Bioelectron.* **14**: 457-464.

Burke, D. S., 1977 Immunization against tularemia: analysis of the effectiveness of live Francisella tularensis vaccine in prevention of laboratory-acquired tularemia. *J.Infect.Dis.* **135**: 55-60.

Clarridge, J. E., III, T. J. Raich, A. Sjosted, G. Sandstrom, R. O. Darouiche *et al.* 1996 Characterization of two unusual clinically significant Francisella strains. *J.Clin.Microbiol.* **34**: 1995-2000.

Dennis, D. T., T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher *et al.* 2001 Tularemia as a biological weapon: medical and public health management. *JAMA* **285**: 2763-2773.

Doern, G. V., 2000 Detection of selected fastidious bacteria. *Clin.Infect.Dis.* **30**: 166-173.

Ellington, A. D., and J. W. Szostak, 1990 In vitro selection of RNA molecules that bind specific ligands. *Nature* **346**: 818-822.

Ellington, A. D., and J. W. Szostak, 1992 Selection in vitro of single-stranded DNA molecules that fold into specific ligand-binding structures. *Nature* **355**: 850-852.

Ellis, J., P. C. Oyston, M. Green, and R. W. Titball, 2002 Tularemia. *Clin.Microbiol.Rev.* **15**: 631-646.

Famulok, M., and G. Mayer, 1999 Aptamers as tools in molecular biology and immunology. *Curr.Top.Microbiol.Immunol.* **243**: 123-136.

Forsman, M., G. Sandstrom, and A. Sjostedt, 1994 Analysis of 16S ribosomal DNA sequences of Francisella strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. *Int.J.Syst.Bacteriol.* **44**: 38-46.

Fukusaki, E., T. Kato, H. Maeda, N. Kawazoe, Y. Ito *et al.* 2000 DNA aptamers that bind to chitin. *Bioorg.Med.Chem.Lett.* **10**: 423-425.

Fulop, M. J., T. Webber, R. J. Manchee, and D. C. Kelly, 1991 Production and characterization of monoclonal antibodies directed against the lipopolysaccharide of Francisella tularensis. *J.Clin.Microbiol.* **29**: 1407-1412.

Greiser-Wilke, I., C. Soine, and V. Moennig, 1989 Monoclonal antibodies reacting specifically with Francisella sp. *Zentralbl.Veterinarmed.B* **36**: 593-600.

Grunow, R., W. Splettstoesser, S. McDonald, C. Otterbein, T. O'Brien *et al.* 2000 Detection of Francisella tularensis in biological specimens using a capture enzyme-linked immunosorbent assay, an immunochromatographic handheld assay, and a PCR. *Clin.Diagn.Lab Immunol.* **7**: 86-90.

Hesselberth, J., M. P. Robertson, S. Jhaveri, and A. D. Ellington, 2000 In vitro selection of nucleic acids for diagnostic applications. *J.Biotechnol.* **74**: 15-25.

Jayasena, S. D., 1999 Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clin.Chem.* **45**: 1628-1650.

Koskela, P., and A. Salminen, 1985 Humoral immunity against Francisella tularensis after natural infection. *J.Clin.Microbiol.* **22**: 973-979.

O'Sullivan, C. K., 2002 Aptasensors--the future of biosensing? *Anal.Bioanal.Chem.* **372**: 44-48.

Pavlovich, N. V., N. V. Aronova, N. N. Onoprienko, V. M. Sorokin, and B. L. Mazrukho, 2000 [Species- and genus-specific antigenic epitopes of Francisella tularensis lipopolysaccharides]. *Mol.Gen.Mikrobiol.Virusol.* 7-12.

Peula-Garcia, J. M., J. A. Molina-Bolivar, J. Velasco, A. Rojas, and F. Galisteo-Gonzalez, 2002 Interaction of Bacterial Endotoxine (Lipopolysaccharide) with Latex Particles: Application to Latex Agglutination Immunoassays. *Journal of Colloid and Interface Science* **245**: 230-236.

Srisawat, C., I. J. Goldstein, and D. R. Engelke, 2001 Sephadex-binding RNA ligands: rapid affinity purification of RNA from complex RNA mixtures. *Nucleic Acids Res.* **29**: E4.

Titball, R. W., A. Johansson, and M. Forsman, 2003 Will the enigma of Francisella tularensis virulence soon be solved? *Trends Microbiol.* **11**: 118-123.

Tuerk, C., and L. Gold, 1990 Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**: 505-510.

Vinogradov, E. V., A. S. Shashkov, Y. A. Knirel, N. K. Kochetkov, N. V. Tochtamysheva *et al.* 1991 Structure of the O-antigen of Francisella tularensis strain 15. *Carbohydr.Res.* **214**: 289-297.

Wang, D., 2003 Carbohydrate microarrays. *Proteomics.* **3**: 2167-2175.

Wilson, D. S., and J. W. Szostak, 1999 In vitro selection of functional nucleic acids. *Annu.Rev.Biochem.* **68**: 611-647.

Yang, Q., I. J. Goldstein, H. Y. Mei, and D. R. Engelke, 1998 DNA ligands that bind tightly and selectively to cellobiose. *Proc.Natl.Acad.Sci.U.S.A* **95**: 5462-5467.

Yi, E. C., and M. Hackett, 2000 Rapid isolation method for lipopolysaccharide and lipid A from gram-negative bacteria. *Analyst* **125**: 651-656.

CHAPTER IV

Structural analysis of the O-antigen of *Francisella tularensis* subspecies *tularensis* strain OSU 10

Francisella tularensis, the causative agent of the disease tularemia, is one of the most infectious bacteria known. *F. tularensis* subspecies *tularensis* (type A) and *holarctica* (type B) are the major subspecies, with the former being highly virulent for humans (Conlan *et al.* 2002). A live vaccine strain (LVS) of *F. tularensis* empirically derived from a virulent strain of type B *F. tularensis* provides considerable protection against highly virulent type A strains in humans (Fulop *et al.* 2001; Conlan *et al.* 2002). Although *F. tularensis* LVS is attenuated for humans, it is fully virulent for mice (Conlan *et al.* 2002), and infection with *F. tularensis* LVS in mice has been used as an experimental model of tularemia in a number of studies.

Immunization of mice with lipopolysaccharide (LPS) derived from *F. tularensis* LVS induced protection against intraperitoneal challenge with the live vaccine strain but not against a virulent strain of type A *F. tularensis*. However, the immunization significantly increased the survival time in mice challenged with the virulent strain of

type A *F. tularensis* (Fulop *et al.* 2001). Similarly, mice vaccinated with O-antigen of the LPS from *F. tularensis* LVS chemically conjugated to bovine serum albumin were protected against an intradermal challenge with a highly virulent strain of type B *F. tularensis* but not against a virulent type A strain (Conlan *et al.* 2002). Monoclonal antibodies directed against O-antigen and core polysaccharide of the LPS from *F. tularensis* LVS recognized both type A and type B strains (Fulop *et al.* 1991). This suggests the presence of common epitopes in the LPS of both subspecies. However, the mouse protection studies suggest possible differences in the structure of LPS/O-antigens between the two subspecies (Fulop *et al.* 2001; Conlan *et al.* 2002; Prior *et al.* 2003). Conversely, different mechanisms of protection may be required to resolve infections caused by the two subspecies (Fulop *et al.* 2001).

The O-antigen structure of *F. tularensis* strain 15 (a vaccine strain derived from type B *F. tularensis* in the former Soviet Union) was determined to contain repeating tetra-saccharide subunits: 4-(α-D-Gal*p*NAcAN-(1-4)- α-D-Gal*p*NAcAN-(1-3)- β-D-Qui*p*NAc-(1-2)-β -D-Qui*p*4NFo-1), using ^{1}H- and ^{13}C-NMR spectroscopy (Vinogradov *et al.* 1991). *Francisella tularensis* LVS was also found to express O-antigen identical to that of *F. tularensis* strain 15 (Conlan *et al.* 2002). Studies on the structure of O-antigen from virulent strains of type A *F. tularensis* have been limited to the Schu S4 strain which is a virulent but highly passaged laboratory strain (Prior *et al.* 2003). The repeating units of the O-antigens from Schu S4 strain and LVS were presumed to be the same based on MALDI-MS analysis (Prior *et al.* 2003). In the present study we report the ^{1}H-

and ^{13}C-NMR spectroscopy structural analysis of O-antigen from a field strain of type A *F. tularensis* (strain OSU 10).

Francisella tularensis subsp. *tularensis* (strain OSU 10) was isolated from a cat that died of tularemia and subspecies identification was based on BIOLOG metabolic fingerprinting (Biolog) and PCR (Petersen *et al.* 2004) and confirmed by Centers for Disease Control and Prevention (Atlanta, GA, USA). Lipopolysaccharide from strain OSU 10 was isolated using the Tri-reagent method described previously (Yi & Hackett 2000) and analyzed by SDS-PAGE and western blotting. LPS samples were run on 12 % acrylamide gels followed by silver staining. Isolated LPS showed the characteristic ladder pattern on acrylamide gels indicating variation in the polysaccharide side chain length (Fig.1(a)). Further, the LPS was reactive with monoclonal antibodies specific for *F. tularensis* O-antigen (BioDesign; Fig.1(b)).

The O-antigen was isolated from type A *F. tularensis* strain OSU 10 LPS by acid hydrolysis. Briefly, LPS was suspended in 1 % acetic acid and heated at 100 °C for 2.5 hours, the lipid portion was removed by centrifugation at 12000 g for 20 minutes, and the supernatant containing O-antigen was freeze dried. The O-antigen was further purified by gel filtration chromatography. Lyophilized O-antigen was suspended in 50 mM ammonium acetate and passed through a Toyopearl HW-50F (Supelco) gel filtration column. Elution of polysaccharide was monitored by refractive index. The three major fractions were pooled separately and freeze dried. The O-antigen fraction, eluting just

after the void volume, was identified by its immunoreactivity with monoclonal antibodies specific for *F. tularensis* O-antigen and by its ^{1}H NMR spectrum (Fig.2). A 2D ^{1}H - ^{13}C heteronuclear multiple-quantum coherence (HMQC) spectrum of the O-antigen was obtained on a Varian Inova 600 NMR spectrometer at 30 °C with continuous wave water presaturation using the standard pulse sequence on the instrument.

The ^{1}H NMR spectrum of O-antigen from type A *F. tularensis* strain OSU 10 was almost identical to that shown for the O-antigen of *F. tularensis* LVS (Fig.2; (Conlan *et al.* 2002), except for some low intensity signals between 3.7 and 4 ppm indicating a small amount of contamination by another polysaccharide (see below). For a more definitive check on the identity between the O-antigen from the two subspecies of *F. tularensis* the HMQC spectrum of the type A *F. tularensis* strain OSU 10 O-antigen was compared to the HSQC spectrum reported for *F. tularensis* LVS (Fig.2; (Conlan *et al.* 2002). Both types of 2D spectra correlate chemical shifts of directly bonded carbons and protons. All of the signals present in the LVS strain were present at the same carbon and proton chemical shifts in the strain OSU 10 indicating that the structure of the two polysaccharides are the same. The signal for the C-1, H-1 of the N-acetyl-quinovosamine was rather weak because it was under the water peak that had been suppressed. Some additional signals were observed in the HMQC spectrum of strain OSU 10. These probably come from a small amount of contamination with the glucans reported earlier (Conlan *et al.* 2003). Judging from the low intensity of the signals in the 1D spectrum

corresponding to the putative C-6, H-6 signal at 61 ppm, 3.9 ppm for glucose the contamination is quite minor.

The NMR data suggest that type A *F. tularensis* strain OSU 10 has an identical O-antigen as that of type B *F. tularensis* strain 15 and *F. tularensis* LVS. The present study supports the theory that strains of both type A and type B *F. tularensis* have identical O-antigen repeats (Conlan *et al.* 2002; Vinogradov & Perry 2004). Further analysis of O-antigen structure from additional strains should confirm this. Thus, it appears that different mechanisms of protection are required to resolve infections caused by virulent strains of type A and type B *F. tularensis* (Fulop *et al.* 2001).

Acknowledgments

This study was supported by Oklahoma Center for Advancement of Science and Technology. We thank Shannon Cowan for culturing the bacterium used in this study. Funds for the 600 MHz NMR spectrometer of the Statewide Shared NMR Facility were provided by the National Science Foundation (BIR-9512269), the Oklahoma State Regents for Higher Education, the M.W. Keck Foundation, and Conoco Inc.

Figure 1. Silver staining of *F. tularensis* subspecies *tularensis* strain OSU 10 LPS separated on a 12 % polyacrylamide gel (a). Western blot of *F. tularensis* subspecies *tularensis* strain OSU 10 LPS developed with monoclonal antibodies specific to *F. tularensis* O-antigen (b).

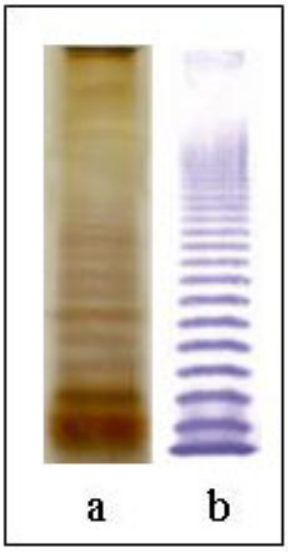
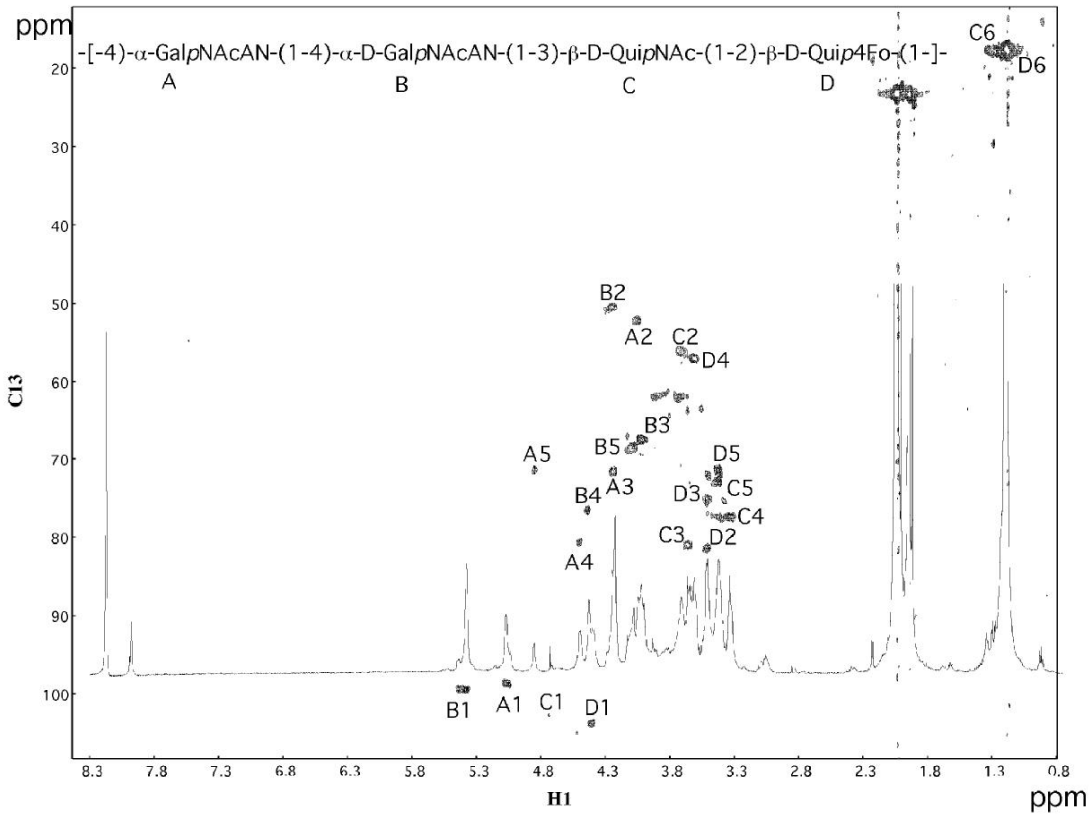

a
b

Figure 2. Superposition of the 1D ^{1}H NMR and 2D $^{1}H - ^{13}C$ HMQC spectrum of the O-antigen of *F. tularensis* subspecies *tularensis* strain OSU 10. Signals are labeled with sugar and proton/carbon number corresponding to the structure shown using the HSQC spectrum for *F. tularensis* LVS (Conlan *et al.* 2002) and the chemical shifts reported for *F. tularensis* strain 15 (Vinogradov *et al.* 1991).


ppm
-[-4)-α-GalpNAcAN-(1-4)-α-D-GalpNAcAN-(1-3)-β-D-QuipNAc-(1-2)-β-D-Quip4Fo-(1-]-
A
B
C
D
C6
D6
B2
A2
C2
D4
B3
A5
B5
D5
A3
B4
D3
C5
C4
C3
D2
A4
B1
A1
C1
D1
C13
H1
ppm

References

Conlan, J. W., H. Shen, A. Webb, and M. B. Perry, 2002 Mice vaccinated with the O-antigen of Francisella tularensis LVS lipopolysaccharide conjugated to bovine serum albumin develop varying degrees of protective immunity against systemic or aerosol challenge with virulent type A and type B strains of the pathogen. *Vaccine* **20**: 3465-3471.

Conlan, J. W., E. Vinogradov, M. A. Monteiro, and M. B. Perry, 2003 Mice intradermally-inoculated with the intact lipopolysaccharide, but not the lipid A or O-chain, from Francisella tularensis LVS rapidly acquire varying degrees of enhanced resistance against systemic or aerogenic challenge with virulent strains of the pathogen. *Microb.Pathog.* **34**: 39-45.

Fulop, M., P. Mastroeni, M. Green, and R. W. Titball, 2001 Role of antibody to lipopolysaccharide in protection against low- and high-virulence strains of Francisella tularensis. *Vaccine* **19**: 4465-4472.

Fulop, M. J., T. Webber, R. J. Manchee, and D. C. Kelly, 1991 Production and characterization of monoclonal antibodies directed against the lipopolysaccharide of Francisella tularensis. *J.Clin.Microbiol.* **29**: 1407-1412.

Petersen, J. M., M. E. Schriefer, L. G. Carter, Y. Zhou, T. Sealy *et al.* 2004 Laboratory analysis of tularemia in wild-trapped, commercially traded prairie dogs, Texas, 2002. *Emerg.Infect.Dis.* **10**: 419-425.

Prior, J. L., R. G. Prior, P. G. Hitchen, H. Diaper, K. F. Griffin *et al.* 2003 Characterization of the O antigen gene cluster and structural analysis of the O antigen of Francisella tularensis subsp. tularensis. *J.Med.Microbiol.* **52**: 845-851.

Vinogradov, E., and M. B. Perry, 2004 Characterisation of the core part of the lipopolysaccharide O-antigen of Francisella novicida (U112). *Carbohydr.Res.* **339**: 1643-1648.

Vinogradov, E. V., A. S. Shashkov, Y. A. Knirel, N. K. Kochetkov, N. V. Tochtamysheva *et al.* 1991 Structure of the O-antigen of Francisella tularensis strain 15. *Carbohydr.Res.* **214**: 289-297.

Yi, E. C., and M. Hackett, 2000 Rapid isolation method for lipopolysaccharide and lipid A from gram-negative bacteria. *Analyst* **125**: 651-656.

CHAPTER V

Lipopolysaccharide microarrays for the detection of antibodies

Abstract

Lipopolysaccharide (LPS) is the major component of Gram-negative bacterial outer membrane. LPS is immunogenic and shows species/strain specificity. The demonstration of anti-LPS antibodies in clinical samples is of diagnostic value in certain Gram-negative bacterial infections. In the present study we explored the possibility of immobilizing LPS isolated from different bacteria in a microarray format for the detection of anti-LPS antibodies. LPS was successfully immobilized on nitrocellulose coated glass slides, preserving the accessibility of epitopes for antibody binding. Specificity of the LPS arrays was established using four different monoclonal antibodies specific for *Escherichia coli* O111, *E.coli* O157, *Francisella tularensis* and *Salmonella typhimurium* O-antigens and a panel of LPS preparations. The detection limit of antibodies was found to be 10 ng/ml, which is about a 100 fold greater sensitivity compared to conventional immunofluorescence assays. Furthermore, using LPS arrays, tularemia positive canine serum samples could be differentiated from negative samples based on the presence of significantly higher levels anti-*Francisella tularensis* LPS

antibodies in positive samples. LPS arrays will facilitate simultaneous screening of samples against multiple antigens and are expected to find applications in diagnostics and seroepidemiology.

Introduction

Gram–negative bacteria represent an important group of human and animal pathogens. These bacteria are characterized by the presence of an outer membrane with unique lipopolysaccharide (LPS) molecules. LPS is an amphipathic molecule and consists of three regions – a hydrophobic lipid-A, hydrophilic core oligosaccharide and an outer polysaccharide O-antigen. LPS plays a major role in pathogenesis (Lerouge and Vanderleyden, 2002) and the O-antigen part of LPS has been shown to be immunogenic (Jauho et al., 2000; Weintraub, 2003). Certain species of Gram-negative bacteria, especially *Salmonella* spp. and *Escherichia coli,* show high diversity in their O-antigen structure (Lerouge and Vanderleyden, 2002) and are identified by classic serotyping using antibodies specific for O-antigen (Poxton, 1995; Lerouge and Vanderleyden, 2002). Different serotypes within a species of Gram-negative bacteria vary in their pathogenicity and epidemiology. Certain serotypes are found to be associated with certain diseases, particularly in case of *E. coli* (Poxton, 1995). Gram-negative bacterial infections can be diagnosed by detecting antibodies specific to LPS (Poxton, 1995). Diagnosis of an infection and establishing the serotype of the bacterium involved is important not only for initiation of proper treatment but also for epidemiological studies (Poxton, 1995). Currently, clinical samples are screened for the presence of specific antibodies using immunological assays such as agglutination tests or ELISA (Jauho et al., 2000) . The presence of a large number of pathogenic Gram–negative bacteria along with numerous

serotypes within certain species warrants a multiplex method for rapid diagnosis of Gram-negative bacterial infections.

In the present study we tested the feasibility of using LPS arrays to detect specific antibodies to bacterial LPS. Microarray-based methods have been described for nucleic acids, proteins, and, recently for carbohydrates (Fukui et al., 2002; Wang et al., 2002). In addition to being multiplexed and rapid, such methods require only small quantities of samples and reagents. The LPS arrays developed were found to be specific and sensitive. The method was also used to detect the presence of specific antibodies against *Francisella tularensis* in canine serum samples.

Materials and Methods

Lipopolysaccharides and Antibodies

Lipopolysaccharide from *Francisella tularensis* subspecies *tularensis* was isolated using the Tri-reagent method described previously (Yi and Hackett, 2000). LPS preparations from *Escherichia coli* O111:B4, *E. coli* O26:B6, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* serotype 10, *Salmonella enteritidis*, *Salmonella typhimurium*, *Shigella flexneri* serotype 1A were purchased from Sigma (St. Louis, MO). *E. coli* O157 LPS was obtained from Nacalai Tesque, Inc. Japan. Commercial preparations of monoclonal antibodies (mABs) of IgG isotype specific for *E. coli* O157, *F. tularensis* and *S. typhimurium* O-antigens were obtained from BioDesign (Saco, ME) and mABs of

IgM isotype specific for *E. coli* O111 O-antigen were obtained from Abcam (Cambridge, MA). Alexa Fluor 488 labeled goat anti-mouse IgG and anti-mouse IgM antibodies (Molecular Probes, Eugene, OR) or FITC-labeled goat anti-dog IgG antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were used as secondary antibodies.

Clinical samples

Five samples of canine serum (PS1 to PS5) collected from clinical cases of tularemia and positive for *Francisella tularensis* antibodies with known microagglutination titer (titer > 20) and two canine serum samples (NS1 and NS2) negative for *F. tularensis* antibodies (microagglutination titer < 20) were obtained from the laboratory of R. J. Morton.

Printing and Scanning of LPS arrays

LPS arrays were printed using a microarray printer (OmniGrid, Gene Machines, CA, USA) on nitrocellulose coated glass slides (SuperNitro microarray substrates, Telechem International Inc., CA, USA). Each LPS sample was resuspended in buffer (Tris 20 mM pH 7.5, NaCl 100 mM and $MgCl_2$ 5 mM) and printed with a spot spacing of 500 µm. The spot size was approximately 200 µm. For LPS immobilization studies, two fold dilutions (1 to 0.031 mg/ml) of fluorescein isothiocyanate (FITC) conjugated *E. coli* O111:B4 LPS were printed in triplicate. For antibody binding experiments, LPS isolated from different bacteria were printed either in a two fold dilution series (1 to 0.031 mg/ml) (Fig. 2) or at a single concentration of 1 mg/ml (Fig. 3, 4, and 6) in replicates of six spots

per dilution. Additionally, mouse immunoglobulins (0.1 mg/ml; Fig. 3) or canine serum (1:10 dilution; Fig. 6) were printed as positive controls. The slides were scanned using a microarray scanner (ScanArray Express, Perkin-Elmer, MA, USA), and the images analyzed using Genepix Pro 5.0 software (Axon Instruments Inc., CA, USA).

Antibody binding experiments

The printed slides were first blocked in 2% BSA in PBS (pH 7.4) for 30 minutes, rinsed twice with PBS containing 0.05% Tween 20 (PBS-T) followed by a rinse in PBS and spin dried. The slides were then incubated with anti-LPS monoclonal antibodies (3 µg/ml) or serum samples (1:50 dilution) in PBS for 30 to 45 minutes at room temperature. After incubation, the slides were washed thrice with PBS-T for 10 minutes each. The slides were flooded with Alexa Fluor488 labeled (unless otherwise specified) secondary antibodies (5 µg/ml in PBS) for 30 minutes and subsequently washed thrice in PBS-T followed by one wash in PBS and dried as described above. The slides were then scanned and the images were analyzed by Genepix Pro 5.0 software.

Microtiter plate based immunoassays

Immunoassays were done as previously described (Fulop et al., 1991; Conlan et al., 2002) with modifications. Briefly, 96-well flat-bottom black microtiter plates (Microfluor1, Nalge Nunc International, Rochester, NY) were coated with LPS (10 µg/ml, 200µl per well) in carbonate buffer pH 9.6 at 37° C for 3h followed by overnight incubation at 4° C. The LPS solution was aspirated and wells were blocked with 2% BSA in PBS at room temperature for 1h. Wells were then washed thrice in PBS-T for five

minutes each and incubated with different concentrations (0.001, 0.01, 0.1 and 1 µg/ml in PBS; 100 µl per well) of anti-LPS mABs in triplicate at room temperature for 1h. After incubation the wells were washed thrice with PBS-T for 10 minutes each. Then, Alexa Fluor488 labeled goat-anti-mouse IgG antibodies in PBS (5 µg/ml; 100 µl per well) was added and incubated at room temperature for 1h. Wells were washed thrice with PBS-T followed by one wash in PBS and fluorescence was recorded using a plate reader (POLARstar Optima, BMG Labtechnologies Inc., Durham, NC).

Statistical analysis

All data presented on LPS arrays were representative of at least three independent experiments. Fluorescence signals were presented as mean ± standard deviation after subtracting the background signal. In order to compare results from different slides, the fluorescence signals were further normalized as ratios of the fluorescence signal values obtained for mouse immunoglobulins (0.1 mg/ml; Fig. 3) or a canine serum (1:10 dilution; Fig. 6) printed on the same slides as positive controls. Data were analyzed using one-way ANOVA and Tukey's test. Means were considered significantly different if $P < 0.05$.

Results

Immobilization of LPS on nitrocellulose coated slides

Printing of FITC labeled *E. coli* O111:B4 LPS on nitrocellulose coated slides produced a concentration dependent fluorescence signal (Fig. 1A & C). Repeated

washings did not result in a significant loss of fluorescence signal (Fig. 1B & D) indicating the stable immobilization of LPS on nitrocellulose coated surface without the need for any additional chemical conjugation. Also, the LPS spot morphology was found to be uniform enabling accurate quantification of the signals.

Antibody binding experiments

Monoclonal antibodies (3 µg/ml) successfully bound to *F. tularensis* LPS immobilized on nitrocellulose coated slides but not *E .coli* O111 LPS (Fig. 2). The fluorescence resulting from binding of antibodies corresponded to the LPS concentrations printed. LPS concentration of 0.0625 mg/ml and above resulted in significantly higher fluorescence compared to controls. Figure 3 shows the specific recognition of O-antigens on LPS arrays by monoclonal antibodies. Monoclonal antibodies of IgG isotype for O-antigens of *E. coli* O157, *F. tularensis* and *S. typhimurium* specifically reacted with the respective LPS but not with other lipopolysaccharides (LPSs) printed on the same slide. Similarly, monoclonal antibodies of IgM isotype specific for *E. coli* O111 O-antigen reacted only with the *E. coli* O111 LPS on arrays.

The sensitivity of LPS arrays for detection of antibodies was compared to that of conventional microtiter plate based immunofluorescence assay using monoclonal antibodies (Fig. 4&5). As low as 10 ng/ml antibodies against *F. tularensis* (Fig. 4A) and *S. typhimurium* LPS (Fig. 4B) could be detected using LPS arrays. The lowest

concentrations of *F. tularensis* and *S. typhimurium* mABs that could be detected in conventional assay was 1 µg/ml (Fig. 5A&B).

LPS arrays were successfully employed to detect antibodies in clinical samples (Fig. 6). Arrays containing a panel of LPSs including *F. tularensis* LPS were exposed to 1:50 dilution of canine serum samples that tested either positive (titer >20) or negative (titer <20) for *F. tularensis* antibodies by microagglutination test. The arrays were developed with FITC-labeled anti-canine secondary antibodies. All five positive samples (PS1, PS2, PS3, PS4 and PS5) tested showed significantly higher level of anti-*F. tularensis* LPS antibodies compared to two negative samples (NS1&NS2; Fig. 6). Certain serum samples (PS3, PS4, NS1 and NS2) showed a slight reaction with *E. coli* O26 LPS as evidenced by the small increase in fluorescence (Fig. 6). Sample PS1 showed higher levels of antibodies against *Klebsiella pneumoniae* and *E. coli* O111 LPS (Fig. 6). This could be due to the presence of low concentrations of serum antibodies against *E. coli* and *K. pneumoniae* LPS.

Discussion

LPS is an important component of the outer membrane of Gram-negative bacteria. It constitutes about 10 - 15% of molecules in the outer membrane and covers up to 75% of Gram-negative bacterial surface (Lerouge and Vanderleyden, 2002; Caroff and

Karibian, 2003). Approximately 2 million molecules of LPS are estimated to be present on the surface of *E. coli* (Raetz, 1990). The structure of lipid-A is conserved among Gram-negative bacteria, whereas the O-antigen is species/strain specific (Lerouge and Vanderleyden, 2002). The diversity and specificity of O-antigen is attributed to its unique sugar composition and number of possible glycosidic linkages (Lerouge and Vanderleyden, 2002; Caroff and Karibian, 2003). LPS are important antigens of Gram-negative bacteria (Jauho et al., 2000; Peula-Garcia et al., 2002) and induce specific antibody responses during infection. The antibodies directed against O-antigen component of LPS have been shown to be species / serotype specific (Jauho et al., 2000).

In the present study, we evaluated the use of LPS microarrays printed on nitrocellulose coated slides as a platform for the detection of antibodies. The adsorption and immobilization of LPS on nitrocellulose coated slides were found to be stable without the need for any additional chemical conjugation (Fig.1). The presence of nitro groups renders the nitrocellulose surface hydrophobic (Wang, 2003). Binding of LPS on nitrocellulose-coated slides is likely to involve hydrophobic interaction between the lipid-A and nitrocellulose surface. The use of nitrocellulose surface will possibly help to overcome the poor reproducibility associated with passive adsorption of LPS onto microplate surface (polystyrene / polypropylene) in ELISA (Poxton, 1995; Jauho et al., 2000).

LPS arrays printed on nitrocellulose-coated slides retained their antigenic properties as evidenced by the binding of antibodies to them (Fig. 2). The specificity of antibody binding and the ability of different antibody isotypes to bind LPS arrays were demonstrated by exposing arrays containing a number of LPSs from different bacteria on the same slide, to O-antigen specific monoclonal antibodies. Specific recognition of immobilized LPS was observed for both IgG and IgM classes of antibodies (Fig. 3). The accessibility of epitopes on immobilized LPS for IgM antibodies is important as antibodies against LPS are biased towards the IgM isotype (Trautmann et al., 1998).

The LPS arrays were capable of detecting antibody concentrations as low as 10 ng/ml (Fig. 4A&B) which is about a 100 fold improvement in sensitivity over conventional immunofluorescence assays (Fig. 5A&B) (Goldsby, 2003). It has been suggested that binding equilibrium is easily achieved with microarrays, as the binding of ligands onto a small spot of immobilized molecules does not significantly alter the concentration of ligands in solution, resulting in greater sensitivity (Wang, 2003).

Experiments were also done to test the intra and inter-slide variability and shelf life of LPS arrays for antibody detection (data not shown). Coefficients of variation (CVs were calculated as the standard deviation of fluorescence value divided by the mean and expressed in percentage) for intra-slide and inter-slide variability were 6.98% (n=18 spots) and 16.21% (n=4 slides) respectively. In order to test the shelf-life, LPS arrays stored at room temperature for 4 weeks were compared with freshly printed arrays by simultaneously developing both the sets with primary and labeled secondary antibodies.

The stored LPS arrays were found to be stable, although there was a slight decrease in the fluorescence signals compared to freshly printed slides (CV of 24.80%; n=4 slides), suggesting some degree of LPS degradation on storage.

Demonstration of anti-LPS antibodies in clinical samples is an indirect indication of infection and has been used in the diagnosis of many infections caused by Gram-negative bacteria such as *Salmonella typhi* (Herath, 2003), *Brucella* spp. (Al Dahouk et al., 2003), enterohemorrhagic *E. coli* O157, O26 and O111 (Ludwig et al., 2002; Tsutsumi et al., 2004), *Chlamydia* spp. (Brade et al., 1994; Priya et al., 2003), *Leptospira interrogans* (Priya et al., 2003), *F. tularensis* (Aronova and Pavlovich, 2000), *Yersinia enterocolitica* (Thibodeau et al., 2001), *Shigella* spp. (Li et al., 1993), *Vibrio cholerae* (Chang and Sack, 2001), *Pseudomonas aeruginosa* (Schaad et al., 1990)and *Bordetella pertussis* (Trollfors et al., 2001). In this study, the suitability of LPS arrays for detection of antibodies from clinical samples was successfully demonstrated using canine serum samples. The serum samples used were known to be either positive (titer>20) or negative (titer<20) for *F. tularensis* antibodies based on microagglutination test. Using LPS arrays, significantly higher levels of anti- *F. tularensis* antibodies could be detected in all five positive samples as compared to the two negative samples (Fig. 6). Generally, clinical samples are screened against one or two antigens chosen based on clinical symptoms and patient history, using conventional assays like agglutination tests or ELISA to arrive at a diagnosis. The LPS array approach allows rapid screening of a clinical sample against multiple Gram-negative bacterial infections with the possible

identification of bacterial serotypes. This could become crucial to initiate an appropriate treatment. Although, technically it may be possible to include most of Gram-negative bacterial LPS on a single slide, the intrinsic limitations of anti-LPS antibody detection may limit the actual number of different LPS that can be included in an array. Some bacterial LPS antigens show cross reaction with other bacterial LPS (Mitov et al., 2003) and it is not possible to diagnose all Gram-negative bacterial infections based on anti-LPS antibodies. However, inclusion of protein and carbohydrate antigens along with LPS may increase the scope of this approach to include other infectious agents.

Other potential areas of application of LPS arrays are in the field of seroepidemiology. The importance of anti-LPS antibodies has been demonstrated in the seroepideomiology of many bacterial diseases, such as *Vibrio cholerae* (Suthienkul et al., 1992), *Klebsiella* spp. (Trautmann et al., 2004), *E. coli* O157 (Evans et al., 2000), typhoid fever (House et al., 2001), *Chlamydia* spp. (Blatz et al., 2001) and *Shigella* spp. (Hyams et al., 1995). LPS arrays allow simultaneous seroepidemiology of a large number of Gram-negative bacterial diseases in a population. This will help in acquisition of valuable information on disease prevalence for well planned disease control programs.

Other promising applications of LPS arrays include evaluation of intravenous immunoglobulin (IVIG) preparations for prophylactic or therapeutic use in sepsis / endotoxemia associated with Gram-negative bacterial infections (Trautmann et al., 1998). Also, LPS arrays may be used to assess the level / quality of anti-LPS antibodies in

patients with sepsis or prior to surgery, as the high level of anti-LPS antibodies is an indicator of favorable prognosis in sepsis patients (Poxton, 1995).

Microarray-based immunoassays offer several advantages over conventional immunological assays. These assays are multiplexed in nature allowing rapid and simultaneous screening of a clinical sample against multiple antigens. In addition, these assays show greater sensitivity compared to conventional assays. Apart from these advantages, microarray-based assays require only small quantities of samples and reagents.

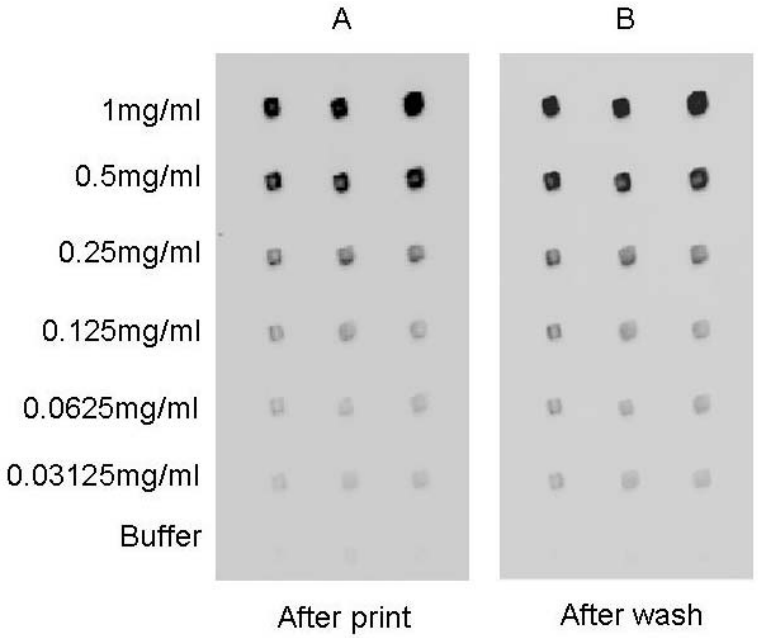
Conclusions

This study demonstrates the applicability of LPS arrays for antibody detection. LPS arrays are likely to find applications in disease diagnosis, seroepidemiology, evaluation of intravenous immunoglobulin preparations and assessment of patients with sepsis / endotoxaemia or prior to surgery. The major advantage of LPS arrays over conventional assays is the ability to simultaneously screen a sample against multiple antigens.

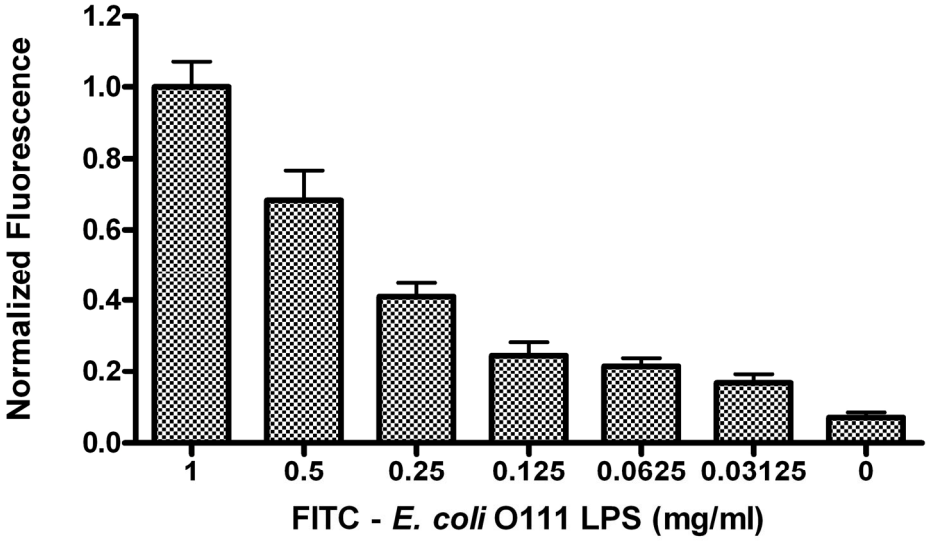
Acknowledgements

We thank Shannon Cowan for culturing bacteria used in this study.

Figure 1. Immobilization of bacterial LPS on nitrocellulose coated slide. Two-fold dilutions (1 to 0.03125 mg/ml) of FITC-labeled *E. coli* O111 LPS in buffer (Tris 20 mM pH 7.5, NaCl 100 mM and MgCl2 5 mM) were printed in triplicate on nitrocellulose coated slides and scanned for fluorescence (A). The slides were washed once in PBS-Tween 20 (0.05%) pH 7.4 followed by two washes in PBS alone and scanned again (B). Fluorescence signals before and after wash did not differ significantly (C & D). The fluorescence signals were normalized as ratios of the highest fluorescence value for each slide. The data points represent mean fluorescence value + SD of three spots for each LPS concentration (three slides).


A
B
1mg/ml
0.5mg/ml
0.25mg/ml
0.125mg/ml
0.0625mg/ml
0.03125mg/ml
Buffer
After print
After wash

C


Normalized Fluorescence
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
0.5
0.25
0.125
0.0625
0.03125
0
FITC - E. coli O111 LPS (mg/ml)

D

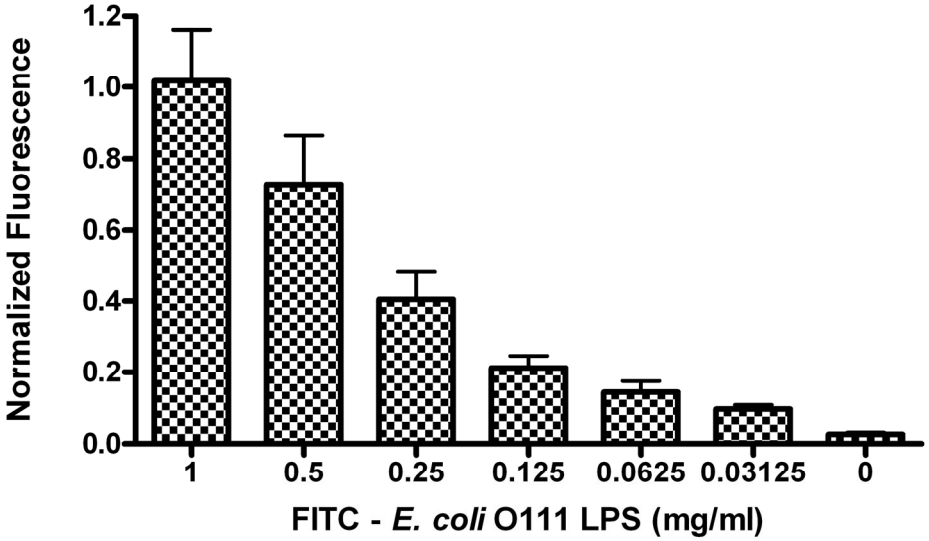

Normalized Fluorescence
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
0.5
0.25
0.125
0.0625
0.03125
0
FITC - E. coli O111 LPS (mg/ml)

Figure 2. Recognition of surface immobilized LPS by monoclonal antibodies. Two-fold dilutions (1 mg/ml to 0.03125 mg/ml) of *F. tularensis* and *E. coli* O111 LPS were printed in replicates of six on nitrocellulose coated slides**.** Slides were incubated with monoclonal antibody (3 µg/ml in PBS) specific for *F. tularensis* O-antigen. The arrays were developed with Alexa fluor 488 labeled goat anti-mouse secondary antibodies (5 µg/ml). *F. tularensis* antibody recognized the *F. tularensis* LPS in a concentration dependent manner. The fluorescence signals were normalized as ratios of the highest fluorescence value for each slide. The data points represent mean fluorescence value ± SD of six replicate spots for each LPS concentration (four slides).

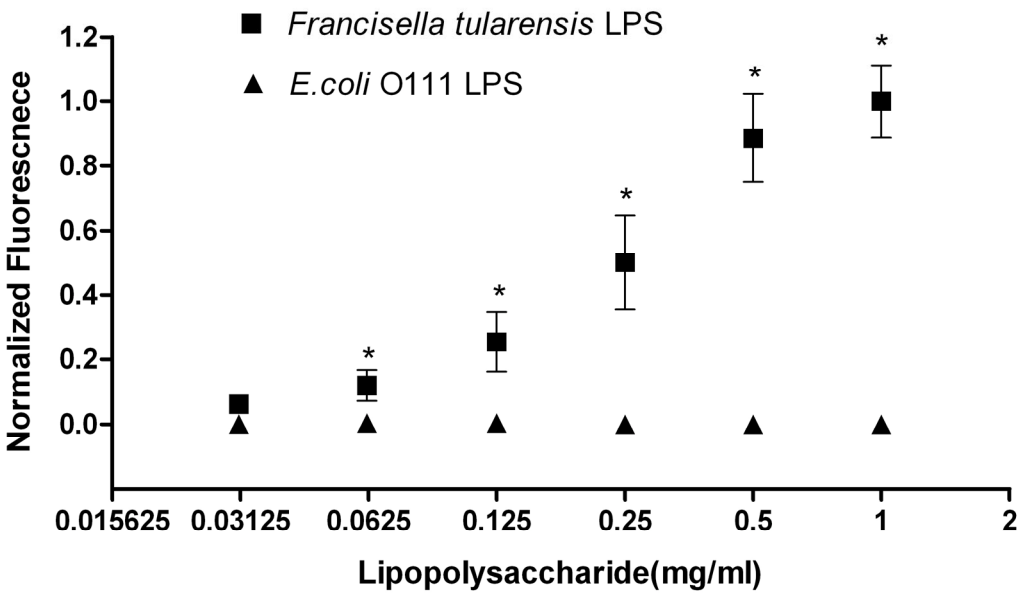

Francisella tularensis LPS
E.coli O111 LPS
Normalized Fluorescnece
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
*
0.015625
0.03125
0.0625
0.125
0.25
0.5
1
2
Lipopolysaccharide(mg/ml)

Figure 3. Binding specificity of O-antigen monoclonal antibodies to LPS arrays. A panel of *nine* LPS preparations isolated from different bacteria was printed in replicates of six at a concentration of 1 mg/ml on nitrocellulose coated slides. The slides were incubated with 3 µg/ml of monoclonal antibodies (IgG isotype) specific for *F. tularensis*, *S. typhimurium*, *E. coli* O157 O–antigens or monoclonal antibody (IgM isotype) specific for *E. coli* O111 O-antigen and developed with Alexa Fluor 488 labeled goat anti-mouse secondary antibody (5 µg/ml). Antibodies specifically recognized the respective O-antigens as evidenced by the significant increase in fluorescence in each array. The fluorescence signals were normalized as ratios of fluorescence signal from mouse immunoglobulins (0.1 mg/ml) printed on the same slide as positive controls after background subtraction. The results are representative of three independent experiments. The data points represent mean ± SD of six replicate spots for each LPS preparation.

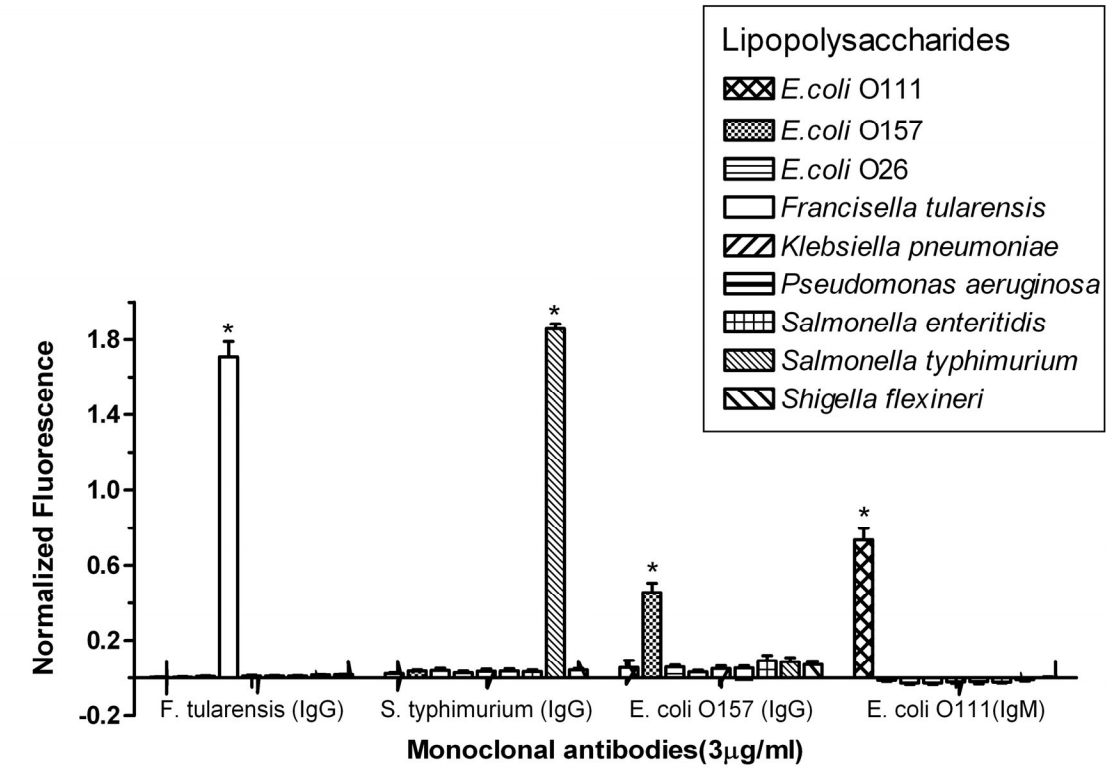
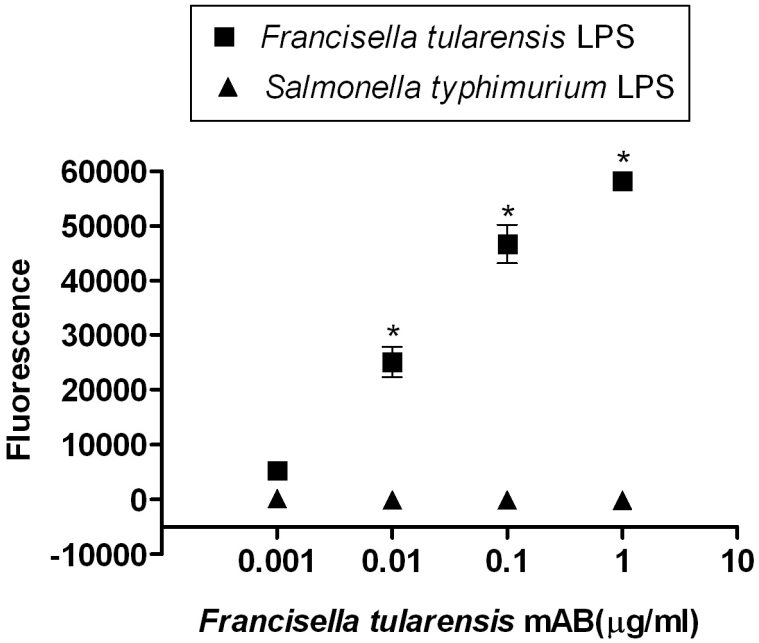

Lipopolysaccharides
E.coli O111
E.coli O157
E.coli O26
Francisella tularensis
Klebsiella pneumoniae
Pseudomonas aeruginosa
Salmonella enteritidis
Salmonella typhimurium
Shigella flexineri
Normalized Fluorescence
1.8
1.4
1.0
0.6
0.2
-0.2
*
*
*
*
F. tularensis (IgG)
S. typhimurium (IgG)
E. coli O157 (IgG)
E. coli O111(IgM)
Monoclonal antibodies(3μg/ml)

Figure 4. Detection limit of antibodies using LPS arrays

Frncisella tularensis and *S. typhimurium* LPS were printed at a concentration of 1 mg/ml in replicates of six on nitrocellulose coated slides**.** Slides were incubated with different concentrations of monoclonal antibodies (1, 0.1, 0.01 and 0.001 µg/ml in PBS) specific for *F. tularensis* (A) or *S. typhimurium* O-antigen (B). The arrays were developed with Alexa fluor 488 labeled goat anti-mouse secondary antibodies (5 µg/ml). The results are representative of three independent experiments. The data points represent mean ± SD of six replicate spots for each LPS preparation.

A


Francisella tularensis LPS
Salmonella typhimurium LPS
Fluorescence
60000
50000
40000
30000
20000
10000
0
-10000
0.001
0.01
0.1
1
10
Francisella tularensis mAB(μg/ml)

B

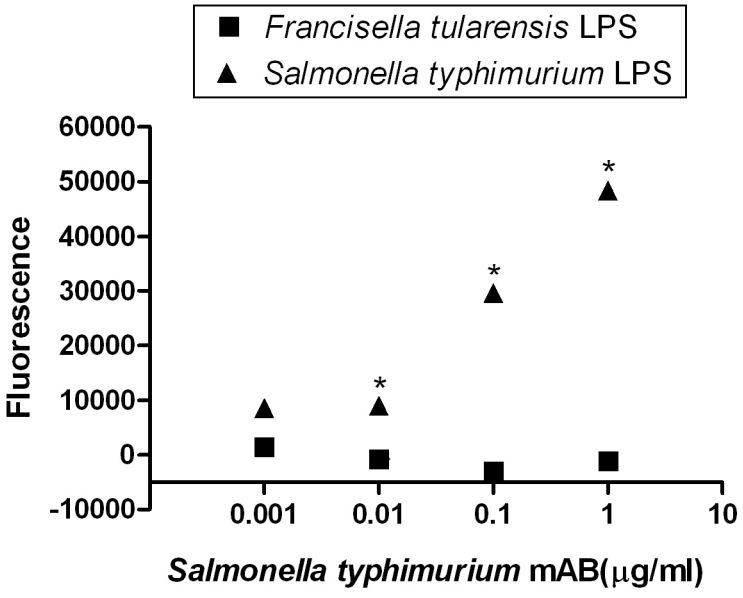
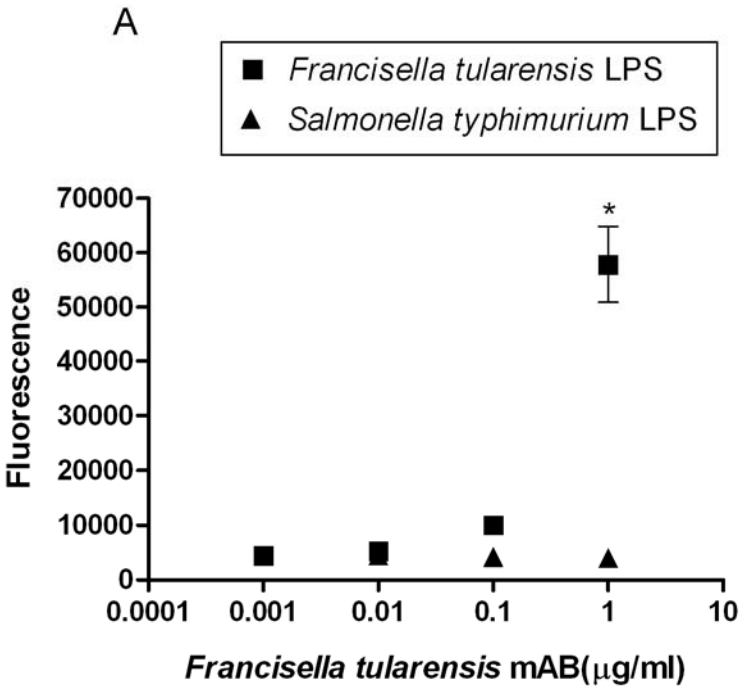

Francisella tularensis LPS
Salmonella typhimurium LPS
Fluorescence
60000
50000
40000
30000
20000
10000
0
-10000
0.001
0.01
0.1
1
10
Salmonella typhimurium mAB(μg/ml)

Figure 5. Detection limit of antibodies for microtiter assays

Microtiter plate wells were coated either with *F. tularensis* or *S. typhimurium* LPS (10 µg/ml) in carbonate buffer. Wells were incubated in triplicate with monoclonal antibodies (1, 0.1, 0.01 and 0.001 µg/ml in PBS) specific for *F. tularensis* (A) or *S. typhimurium* O-antigen (B). Binding of primary antibodies was detected using Alexa fluor 488 labeled goat anti-mouse secondary antibodies (5 µg/ml). The results are representative of two independent experiments. The data points represent mean ± SD of triplicate wells for each antibody concentration.


A
Francisella tularensis LPS
Salmonella typhimurium LPS
Fluorescence
70000
60000
50000
40000
30000
20000
10000
0
*
0.0001
0.001
0.01
0.1
1
10
Francisella tularensis mAB(μg/ml)

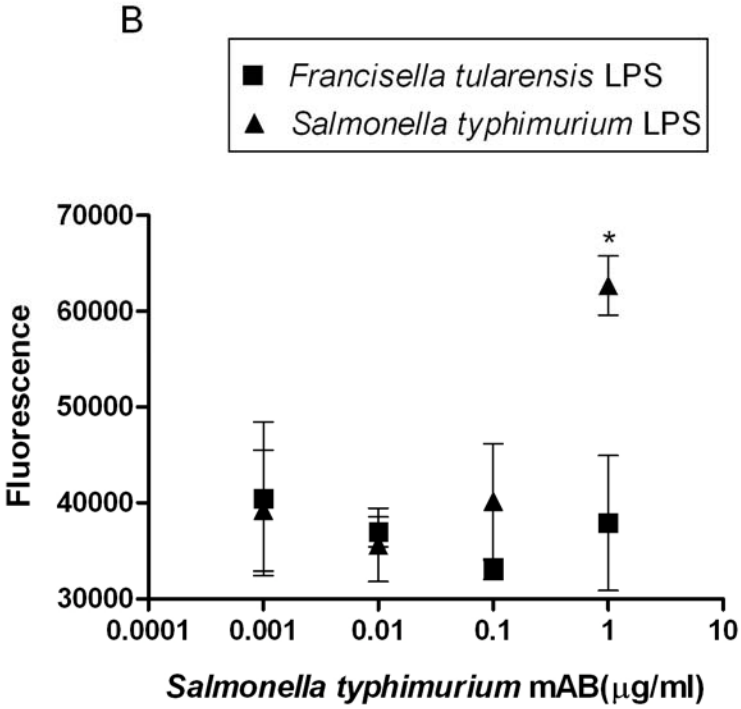
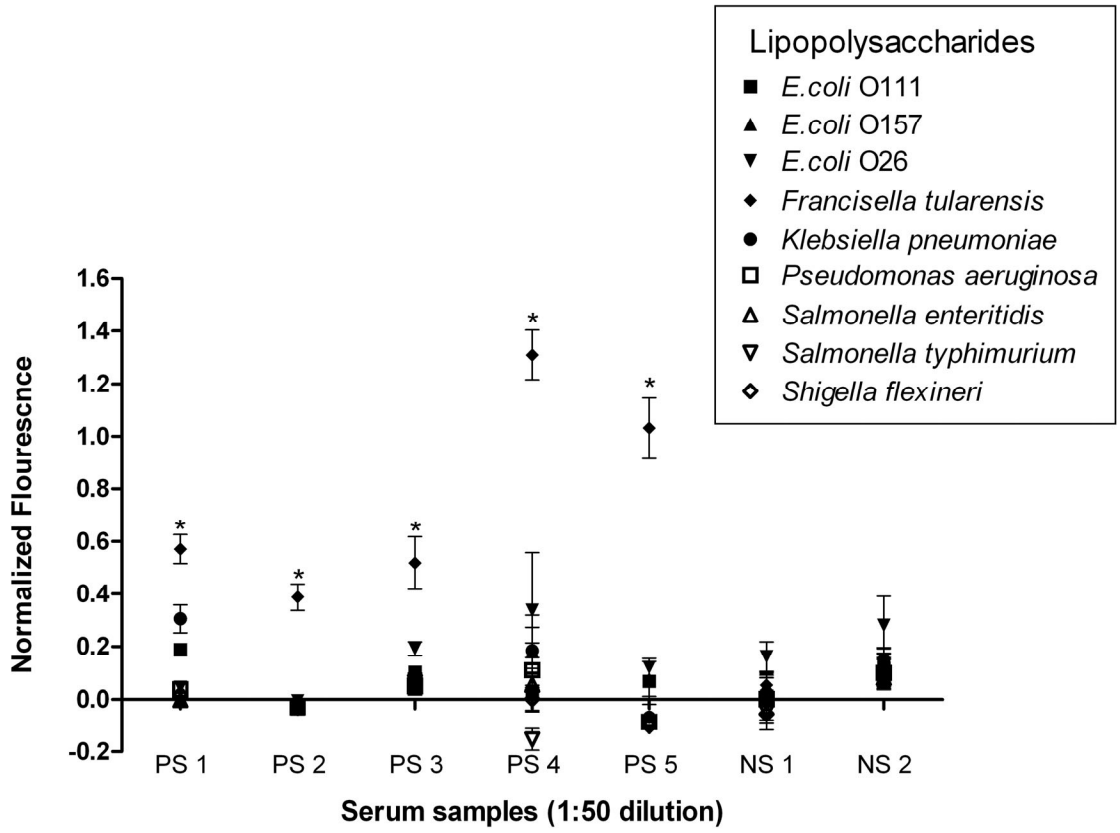

B
Francisella tularensis LPS
Salmonella typhimurium LPS
Fluorescence
70000
60000
50000
40000
30000
*
0.0001
0.001
0.01
0.1
1
10
Salmonella typhimurium mAB(μg/ml)

Figure 6. Detection of *F. tularensis* anti-LPS antibodies in serum samples.

A panel of nine LPS preparations isolated from different bacteria was printed in replicates of six at a concentration of 1 mg/ml on nitrocellulose slides and incubated either with canine serum samples (dilution 1:50) positive for *F. tularensis* antibodies (microagglutination titer >20) or with canine serum samples negative (titer <20) for *F. tularensis* antibodies. Five positive and two negative samples were tested. The slides were developed with FITC labeled goat anti-canine secondary antibodies (5 µg/ml). The level of specific *F. tularensis* antibodies were significantly higher in positive samples (PS1 to 5) compared to negative samples (NS1&2). The fluorescence signals were normalized as ratios of fluorescence signal from canine serum (1:10 dilution) printed on the same slide and developed with FITC labeled anti-canine antibodies after background subtraction. The results are representative of three independent experiments. The data points represent mean + SD of six replicate spots for each LPS preparation.


Lipopolysaccharides
E.coli O111
E.coli O157
E.coli O26
Francisella tularensis
Klebsiella pneumoniae
Pseudomonas aeruginosa
Salmonella enteritidis
Salmonella typhimurium
Shigella flexineri
Normalized Flourescnce
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
PS 1
PS 2
PS 3
PS 4
PS 5
NS 1
NS 2
Serum samples (1:50 dilution)

References

Al Dahouk, S., Tomaso, H., Nockler, K., Neubauer, H. and Frangoulidis, D. (2003) Laboratory-based diagnosis of brucellosis--a review of the literature. Part I: Techniques for direct detection and identification of Brucella spp. Clin Lab 49, 487-505.

Aronova, N.V. and Pavlovich, N.V. (2000) [The use if Francisella tularensis lipopolysaccharide in the dot solid phase enzyme immunoassay]. Zh Mikrobiol Epidemiol Immunobiol, 75-8.

Blatz, R., Aseffa, A., Gedefaw, M., Ruhle, H.J., Forberg, J. and Christian, A.R. (2001) Prevalence of Chlamydia specific antibodies among obstetric and gynecological outpatients in Gondar, north-west Ethiopia. Ethiop Med J 39, 293-303.

Brade, L., Brunnemann, H., Ernst, M., Fu, Y., Holst, O., Kosma, P., Naher, H., Persson, K. and Brade, H. (1994) Occurrence of antibodies against chlamydial lipopolysaccharide in human sera as measured by ELISA using an artificial glycoconjugate antigen. FEMS Immunol Med Microbiol 8, 27-41.

Caroff, M. and Karibian, D. (2003) Structure of bacterial lipopolysaccharides. Carbohydr Res 338, 2431-47.

Chang, H.S. and Sack, D.A. (2001) Detection of anti-lipopolysaccharide antibodies to Vibrio cholerae O1 and O139 using a novel microtiter limulus amebocyte lysate (LAL) assay. Clin Chim Acta 312, 49-54.

Conlan, J.W., Shen, H., Webb, A. and Perry, M.B. (2002) Mice vaccinated with the O-antigen of Francisella tularensis LVS lipopolysaccharide conjugated to bovine serum albumin develop varying degrees of protective immunity against systemic or aerosol challenge with virulent type A and type B strains of the pathogen. Vaccine 20, 3465-71.

Evans, J., Chalmers, R.M., Chart, H., Salmon, R.L., Kench, S.M., Coleman, T.J., Meadows, D., Morgan-Capner, P., Softley, P., Sillis, M. and Thomas, D.R. (2000) Evidence of persisting serum antibodies to Escherichia coli O157 lipopolysaccharide and Verocytotoxin in members of rural communities in England. Eur J Epidemiol 16, 885-9.

Fukui, S., Feizi, T., Galustian, C., Lawson, A.M. and Chai, W. (2002) Oligosaccharide microarrays for high-throughput detection and specificity assignments of carbohydrate-protein interactions. Nat Biotechnol 20, 1011-7.

Fulop, M.J., Webber, T., Manchee, R.J. and Kelly, D.C. (1991) Production and characterization of monoclonal antibodies directed against the lipopolysaccharide of Francisella tularensis. J Clin Microbiol 29, 1407-12.

Goldsby, R.A. (2003) Immunology. W.H. Freeman, New York.

Herath, H.M. (2003) Early diagnosis of typhoid fever by the detection of salivary IgA. J Clin Pathol 56, 694-8.

House, D., Wain, J., Ho, V.A., Diep, T.S., Chinh, N.T., Bay, P.V., Vinh, H., Duc, M., Parry, C.M., Dougan, G., White, N.J., Hien, T.T. and Farrar, J.J. (2001) Serology

of typhoid fever in an area of endemicity and its relevance to diagnosis. J Clin Microbiol 39, 1002-7.

Hyams, K.C., Malone, J.D., Bourgeois, A.L., Hawkins, R., Hale, T.L. and Murphy, J.R. (1995) Serum antibody to lipopolysaccharide antigens of Shigella species among U.S. military personnel deployed to Saudi Arabia and Kuwait during Operations Desert Shield and Desert Storm. Clin Diagn Lab Immunol 2, 700-3.

Jauho, E.S., Boas, U., Wiuff, C., Wredstrom, K., Pedersen, B., Andresen, L.O., Heegaard, P.M. and Jakobsen, M.H. (2000) New technology for regiospecific covalent coupling of polysaccharide antigens in ELISA for serological detection. J Immunol Methods 242, 133-43.

Lerouge, I. and Vanderleyden, J. (2002) O-antigen structural variation: mechanisms and possible roles in animal/plant-microbe interactions. FEMS Microbiol Rev 26, 17-47.

Li, A., Rong, Z.C., Ekwall, E., Forsum, U. and Lindberg, A.A. (1993) Serum antibody responses against shigella lipopolysaccharides and invasion plasmid-coded antigens in shigella infected Swedish patients. Scand J Infect Dis 25, 569-77.

Ludwig, K., Grabhorn, E., Bitzan, M., Bobrowski, C., Kemper, M.J., Sobottka, I., Laufs, R., Karch, H. and Muller-Wiefel, D.E. (2002) Saliva IgM and IgA are a sensitive indicator of the humoral immune response to Escherichia coli O157 lipopolysaccharide in children with enteropathic hemolytic uremic syndrome. Pediatr Res 52, 307-13.

Mitov, I., Haralambieva, I., Petrov, D., Ivanova, R., Kamarinchev, B. and Iankov, I. (2003) Cross-reactive monoclonal antibodies raised against the lipopolysaccharide antigen of Salmonella minnesota Re chemotype: diagnostic relevance. Diagn Microbiol Infect Dis 45, 225-31.

Peula-Garcia, J.M., Molina-Bolivar, J.A., Velasco, J., Rojas, A. and Galisteo-González, F. (2002) Interaction of Bacterial Endotoxine (Lipopolysaccharide) with Latex Particles: Application to Latex Agglutination Immunoassays. J Colloid Interface Sci 245, 230-236.

Poxton, I.R. (1995) Antibodies to lipopolysaccharide. J Immunol Methods 186, 1-15.

Priya, C.G., Bhavani, K., Rathinam, S.R. and Muthukkaruppan, V.R. (2003) Identification and evaluation of LPS antigen for serodiagnosis of uveitis associated with leptospirosis. J Med Microbiol 52, 667-73.

Raetz, C.R. (1990) Biochemistry of endotoxins. Annu Rev Biochem 59, 129-70.

Schaad, U.B., Lang, A.B., Wedgwood, J., Buehlamnn, U. and Fuerer, E. (1990) Serotype-specific serum IgG antibodies to lipopolysaccharides of Pseudomonas aeruginosa in cystic fibrosis: correlation to disease, subclass distribution, and experimental protective capacity. Pediatr Res 27, 508-13.

Suthienkul, O., Poomchart, A., Kositanont, U., Siripanichgon, K. and Vathanophas, K. (1992) ELISA for seroepidemiological study of exposure to Vibrio cholerae of population in Krabi Province, Thailand. Asian Pac J Allergy Immunol 10, 55-60.

Thibodeau, V., Frost, E.H. and Quessy, S. (2001) Development of an ELISA procedure to detect swine carriers of pathogenic Yersinia enterocolitica. Vet Microbiol 82, 249-59.

Trautmann, M., Held, T.K. and Cross, A.S. (2004) O antigen seroepidemiology of Klebsiella clinical isolates and implications for immunoprophylaxis of Klebsiella infections. Vaccine 22, 818-21.

Trautmann, M., Held, T.K., Susa, M., Karajan, M.A., Wulf, A., Cross, A.S. and Marre, R. (1998) Bacterial lipopolysaccharide (LPS)-specific antibodies in commercial human immunoglobulin preparations: superior antibody content of an IgM-enriched product. Clin Exp Immunol 111, 81-90.

Trollfors, B., Lagergard, T., Taranger, J., Bergfors, E., Schneerson, R. and Robbins, J.B. (2001) Serum immunoglobulin G antibody responses to Bordetella pertussis lipooligosaccharide and B. parapertussis lipopolysaccharide in children with pertussis and parapertussis. Clin Diagn Lab Immunol 8, 1015-7.

Tsutsumi, R., Ichinohe, N., Shimooki, O., Obata, F., Takahashi, K., Inada, K., Sasaki, M., Sato, S. and Chida, S. (2004) Homologous and heterologous antibody responses to lipopolysaccharide after enterohemorrhagic Escherichia coli infection. Microbiol Immunol 48, 27-38.

Wang, D. (2003) Carbohydrate microarrays. Proteomics 3, 2167-75.

Wang, D., Liu, S., Trummer, B.J., Deng, C. and Wang, A. (2002) Carbohydrate microarrays for the recognition of cross-reactive molecular markers of microbes and host cells. Nat Biotechnol 20, 275-81.

Weintraub, A. (2003) Immunology of bacterial polysaccharide antigens. Carbohydr Res 338, 2539-47.

Yi, E.C. and Hackett, M. (2000) Rapid isolation method for lipopolysaccharide and lipid A from gram-negative bacteria. Analyst 125, 651-6.

Chapter VI

Bacterial cell microarrays for the detection and characterization of antibodies against surface antigens

Abstract

Bacterial cell surface antigens interact with the host immune system resulting in the production of antibodies. Detection of antibodies against surface antigens has applications in diagnosis of many bacterial infections, assessment of immune status and epidemiological studies. We developed a microarray platform, for antibody detection, by printing Gram-negative and Gram-positive whole bacterial cells on nitrocellulose coated glass substrates. Antibody binding was detected using fluorophore labeled secondary antibodies. The sensitivity of antibody detection was found to be 0.1µg/ml. Using bacterial cell microarrays it was also possible to successfully detect antibodies against *Francisella tularensis* in canine serum samples declared positive for tularemia based on microagglutination antibody titer. Use of bacterial cells as the antigen source in immunoassays has the advantages of simulating *in vivo* presentation of surface antigens and also eliminating the need for antigen purification. The microarray format gives the added advantage of simultaneous detection of antibodies against multiple bacteria employing only small amounts of samples and reagents.

Introduction

Demonstration of antibodies to bacterial surface antigens has found applications in disease diagnosis (Watanabe *et al.* 2001; Itoh *et al.* 2002), epidemiological studies and immune status assessment (Rossi *et al.* 2003). Antibodies produced against surface antigens have a higher probability of neutralizing bacteria, therefore detection of these antibodies can serve as an indicator of the protective immune response (Watanabe *et al.* 2001). Conventional immunoassays usually employ purified antigens for antibody detection. Production of purified antigens is often labor intensive (Itoh *et al.* 2002). The use of whole cells in immunoassays avoids the need for antigen purification and offers the advantage of presenting surface antigens in their native form thereby simulating antigen-antibody reactions *in vivo* (Watanabe *et al.* 2001). Whole cells have been used in the detection of antibodies against various bacteria (Verschoor *et al.* 1990; Marcotte & Lavoie 1993; Lamari *et al.* 1999; Watanabe *et al.* 2001; Itoh *et al.* 2002). However, immobilization of whole cells on microtiter plates has been reported to be variable resulting in poor reproducibility (Poxton 1995).

In the present study, we describe a microarray-based multiplex immunoassay for detection of antibodies to surface antigens of several bacteria. Formalin inactivated bacterial cells were printed on nitrocellulose-coated glass slides using a standard microarray instrument, and used for antibody detection. Sensitivity of this assay was established using monoclonal antibodies and was found to be 0.1µg/ml of antibody.

Successful detection of pathogen specific antibodies against *Francisella tularensis* in canine serum samples was also achieved. The microarray format requires only minimal amount of samples and reagents. It also allows for the simultaneous screening of a sample for the presence of antibodies against numerous bacteria.

Materials and Methods

Bacteria

Gram-negative bacteria *Escherichia coli* (ATCC 25922), *Francisella tularensis* subsp. *tularensis, Klebsiella pneumoniae*, *Salmonella typhimurium* (ATCC 14028), and Gram-positive bacteria *Enterococcus faecalis*, *Staphylococcus aureus* (ATCC 29523), *Staphylococcus epidermidis*, *Streptococcus pyogenes* (ATCC 19615) and *Listeria monocytogenes* were provided by R. J. Morton. Bacteria were grown on suitable agar media and inactivated using 0.5% formol saline. The inactivated cells were washed three times in PBS pH 7.4 and resuspended in PBS. The numbers of bacteria in samples were determined by flow cytometry using a bacteria counting kit (Molecular Probes, Eugene, OR) asper manufacturer's instructions.

Antibodies and serum samples

Monoclonal antibodies to *F. tularensis* O-antigen, Salmonella O-antigen (B group), *Staphylococcus aureus*, and *Listeria monocytogenes* were obtained from a commercial source (Biodesign, Saco, ME). Canine serum samples were obtained from the laboratory of R. J. Morton.

Printing and scanning of bacterial cell arrays

Bacterial cells were suspended in PBS containing 20% Ficoll 400 (Fisher Scientific, Fair Lawn, NJ) and 4% glycerol to get an approximate cell density of $5x10^{11}$cells/ml. In case of *Streptococcus pyogenes*, the cells were suspended in PBS containing 30 % Ficoll 400 and 4% glycerol to get an approximate cell density of $5x10^{9}$ cells/ml. Bacterial cells were printed on microarray substrates using a microarray printer (OmniGrid, Gene Machines, CA, USA). A 1:50 dilution of a canine serum sample was printed as positive control on all slides used in antibody detection from serum samples (Fig. 6) for the purpose of data normalization. After printing, the cell arrays were left on the printer at room temperature and 70% humidity for 30 minutes to enable bacterial cells to adsorb onto the substrate.

The slides were scanned using a microarray scanner (ScanArray Express, Perkin-Elmer, MA, USA), and the images analyzed using Genepix Pro 5.0 software (Axon Instruments Inc., CA, USA).

Evaluation of microarray substrates for bacterial immobilization

Various commercially available microarray substrates - nitrocellulose coated glass slides (SuperNitro microarray substrates), SuperEpoxy slides, SuperAldehyde slides (Telechem International Inc., CA), poly–L-lysine slides (CEL Associates Inc., TX), and glass slides were tested for their suitability for immobilization of whole bacterial cells. *Francisella tularensis* and *Staphylococcus aureus* were stained with BacLight Green fluorescent bacterial stain (Molecular Probes, Eugene, OR) asper manufacturer's instructions and thoroughly washed in PBS to remove unbound dye. Stained cells were resuspended in PBS containing 20% Ficoll 400 and 4% glycerol and printed on microarray substrates. Printed slides were scanned to record fluorescence signals. Then the slides were washed thrice in PBS containing 0.05% Tween 20 (PBS-T) followed by once in PBS, each 10 minutes and spin dried. Slides were again scanned to record the fluorescence signals.

Detection of antibodies to cell surface antigens

The printed slides were first blocked with 2% BSA in PBS (pH 7.4) for 30 minutes, rinsed thrice with PBS-T and spin dried. The slides were then incubated with monoclonal antibodies or serum samples diluted in PBS at various concentrations (as specified in figure legends) for 30 to 45 minutes at room temperature. After incubation, the slides were washed thrice for 10 minutes each with PBS-T. The slides were then incubated with Alexa Fluor 488 labeled (unless otherwise specified) secondary antibodies

(5µg/ml in PBS) for 30 minutes and subsequently washed and dried as described above. The slides were then scanned and the images were analyzed by Genepix Pro 5.0 software.

Statistical analysis

Data were analyzed using one way ANOVA and the means were compared using Tukey's test. Statistical significance was determined at 95% ($P<0.05$). Results presented are representative of two or more independent experiments and are shown as mean ± standard error after background subtraction. Results from experiments using canine serum were normalized to the positive control (canine serum printed at a dilution of 1:50 on the respective slides) before data analysis (Fig. 6).

Results

Both Gram-negative bacteria (*F. tularensis*) and Gram-positive bacteria (*Staphylococcus aureus*) were found to stably adsorb on to the nitrocellulose surface (Fig. 1). Repeated washings did not result in a significant loss of fluorescence signal for *F. tularensis*. However, there was a statistically significant ($P < 0.05$) loss of fluorescence signal after wash in case of *S. aureus* (Fig. 1C). The bacteria did not bind as well to other surfaces tested such as poly-L-lysine coated surface, glass surface, SuperAldehyde,

SuperEpoxy substrates and Hydrogel NHS Ester slides. Results for poly-L-lysine slides are shown in Fig 2.

Fig. 3 shows the recognition of O-antigens by monoclonal antibodies on the surface of Gram-negative bacteria immobilized on nitrocellulose-coated slides. Monoclonal antibodies for O-antigen recognized *F. tularensis* whole cells immobilized on nitrocellulose coated glass slides but not other Gram-negative bacteria *Salmonella typhimurium* and *Escherichia coli* printed on the same slide (Fig. 3A). Monoclonal antibodies against *Salmonella* O-antigen (B Group) specifically bound *Salmonella typhimurium* cells printed on microarray slide (Fig. 3B).

Recognition of Gram-positive bacterial surface antigens by specific monoclonal antibodies on cell arrays was tested (Fig. 4). Monoclonal antibodies specific for peptidoglycan of *Staphylococcus aureus* specifically recognized *S. aureus* cells but not *Streptococci pyogenes* and *Listeria monocytogenes* printed on the same slide (Fig. 4A). Similarly, monoclonal antibodies against *L. monocytogenes* specifically bound *L. monocytogenes* cells on cell arrays (Fig. 4B).

Further the sensitivity of antibody detection using bacterial cell arrays was determined for both Gram-negative and Gram-positive bacteria (Fig. 5). The lowest concentration of monoclonal antibodies directed against the surface antigens of *F.*

tularensis (Fig. 5A) and *L. monocytogenes* (Fig. 5B) that could be detected using bacterial cell arrays was found to be 0.1µg/ml.

Antibody detection in clinical samples using bacterial cell arrays was investigated (Fig. 6). Seven canine serum samples from known clinical cases of tularemia and positive for *F. tularensis* antibodies (microagglutination titer>20) and six canine serum samples negative for *F. tularensis* antibodies (microagglutination titer<20) were tested using bacterial cell arrays printed with *F. tularensis* whole cells and other Gram-negative and Gram-positive bacteria. The level of anti- *F. tularensis* antibodies was significantly higher in tularemia positive samples compared to negative samples.

Discussion

The bacterial cell surface is decorated with an array of molecules such as outer membrane proteins, lipopolysaccharide, flagella (H-antigens), capsules and fimbriae or pili. Surface molecules are involved in bacterial adherence to host cells helping in establishing an infection. Antibodies directed against the bacterial surface antigens can neutralize the bacteria and afford protection particularly against certain extracellular bacteria (Haesebrouck *et al.* 2004).

In the present study we used bacterial cell arrays for the detection of antibodies against cell surface antigens. Bacterial cell arrays were fabricated and analyzed using standard microarray instruments. In order to keep bacterial cells in suspension during printing and prevent the cells from drying once printed, PBS containing Ficoll 400 (20%) and 4 % glycerol was used as printing buffer. Ficoll 400 increases the density of the solution and thereby maintains cells in suspension. The use of glycerol in printing buffer helps prevent total dehydration of cells after printing, preserves native structure of proteins and helps in maintenance of antigenicity of cell surface molecules (Howbrook *et al.* 2003). A similar strategy was used to print eukaryotic cells on microarray slides (Schwenk *et al.* 2002). A higher concentration of Ficoll 400 (30 %) was required to print *Streptococcus pyogenes* cells as these bacteria grow in chains. For the detection of antibodies against surface antigens it was found that printing a high density of bacterial cells (~$5x10^{11}$cells/ml) was most effective. In case of *S. pyogenes,* cells were printed at an approximate density of $5x10^{9}$cells/ml. Due to their chain morphology, there is a possibility that the flow-cytometric determination of the *S. pyogenes* cell count could have been underestimated, and thus the apparent low cell density.

Both Gram-positive and Gram-negative bacteria strongly adsorbed onto nitrocellulose coated slides (Fig. 1). Fluorescently labeled *F. tularensis* and *Staphylococcus aureus* cells showed substantial adsorption onto nitrocellulose-coated slides. The loss of fluorescence signal upon washings was not significant for *F. tularensis* suggesting the stable adsorption of the cells (Fig. 1C). The loss of fluorescence in case of

S. aureus cells may suggest the saturation of spots on nitrocellulose surface (Fig. 1C). Bacterial cells did not bind as well to other microarry substrate surfaces tested such as poly-L-lysine coated slides (Fig. 2), SuperEpoxy Slides, SuperAldehyde slides and glass surface (results not shown). Poly-L-lysine coated slides have been used to immobilize eukaryotic cells (Schwenk *et al.* 2002).

Binding of monoclonal antibodies to surface antigens on immobilized bacterial cells was detected using fluorophore labeled secondary antibodies for both Gram-positive and Gram-negative bacteria (Fig. 3 and 4). The sensitivity of antibody detection was found to be 0.1µg/ml for both S. *typhimurium* and *L. monocytogenes* (Fig. 5). For conventional immunofluorescence assays the sensitivity of antibody detection has been reported to be 1 µg/ml (Goldsby 2003). The affinity of antibodies and the density and distribution of epitopes will influence the sensitivity of detection. Since binding of ligands to small spots of immobilized molecules in microarrays does not significantly change the concentration of ligands in solution, binding equilibrium is easily achieved, resulting in greater sensitivity (Wang 2003).

The variation within the slides and between the slides was determined. The coefficient of variation for intra-slide variability was found to be 7.7% for *S. typhimurium* and 8.96% for *S. aureus* (n=20 spots each). The inter-slide variations were 18.37% and 13.22% for *S. typhimurium* and *S. aureus* respectively (n=6 slides). The cell arrays were found to be stable for at least two weeks when stored at 4ºC.

Using bacterial cell arrays it was possible to detect the higher levels of pathogen specific antibodies against *F. tularensis* (Fig.6) in serum samples obtained from seven known cases of canine tularemia (P1-P7) compared to known negative serum samples (N1-N6). In the serum samples tested, variable levels of antibodies were also observed against other bacteria printed on the slides. Whole organisms have been used as test antigens for diagnosis of brucellosis, legionellosis, leptospirosis, lyme disease, *Mycoplasma* pneumonia, Q fever, tularemia and *Helicobacter pylori* infection, (Evans & Brachman 1998; Watanabe *et al.* 2001). Antibody cross-reactivity among certain bacteria has been well documented (Nielsen *et al.* 2004) and is a major factor to be considered in the development of bacterial cell arrays. The inclusion of protein and carbohydrate antigens along with bacteria may help in this regard. Alternatively, immobilization of surface-engineered bacteria that display heterologous proteins/peptides or antibodies/affibody domains on a microarray substrate could provide novel tools in diagnostics and proteomics research (Samuelson *et al.* 2002; Wernerus & Stahl 2004).

Conclusions

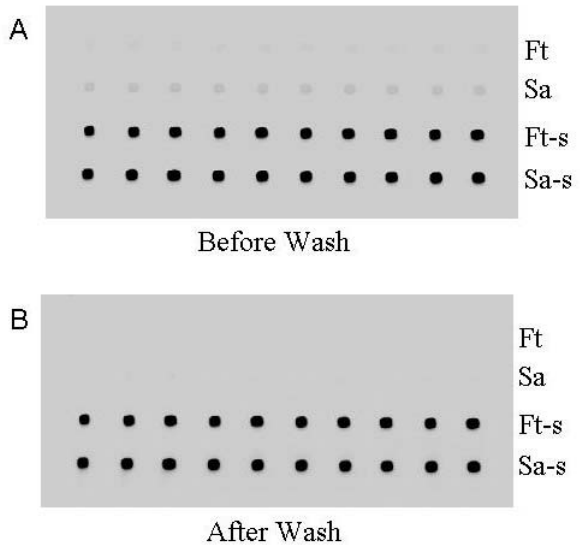
The bacterial cell array-based immunoassays offer several advantages over conventional immunoassays such as agglutination tests or ELISA. Bacterial cell arrays are multiplex in nature and allow simultaneous screening of a sample against multiple

bacteria. These assays show greater sensitivity, require small amounts of samples and reagents and are likely to find applications in disease diagnosis, seroepidemiology, assessment of the immune status (Rossi *et al.* 2003), evaluation of intravenous immunoglobulin preparation (Lamari *et al.* 1999; Lamari *et al.* 2000a; Lamari *et al.* 2000b), screening for potential anti-adhesion therapeutics (Ofek *et al.* 2003; Disney & Seeberger 2004; Thomas & Brooks 2004) and characterization of antibodies / ligands to surface antigens of bacteria.

Acknowledgements

We thank Shannon Cowan for her help in culturing bacteria used in the study

Figure 1. Adsorption of bacterial cells onto nitrocellulose coated slides. *Francisella tularensis* (Ft), *Staphylococcus aureus* (Sa) and fluorescently stained *F. tularensis* (Ft-s) and *S. aureus* (Sa-s) were suspended in PBS containing 20% Ficoll 400 and 4% glycerol at a cell density of ~ $5x10^{11}$ cells/ml and printed in replicates of 10 spots and scanned for fluorescence. Scanned images before and after wash are shown (A and B). Fluorescence signals before and after wash are presented (C). The results are representative of two independent experiments (two slides per experiment). The data points represent mean fluorescence value + SEM.


A
Ft
Sa
Ft-s
Sa-s
Before Wash
B
Ft
Sa
Ft-s
Sa-s
After Wash

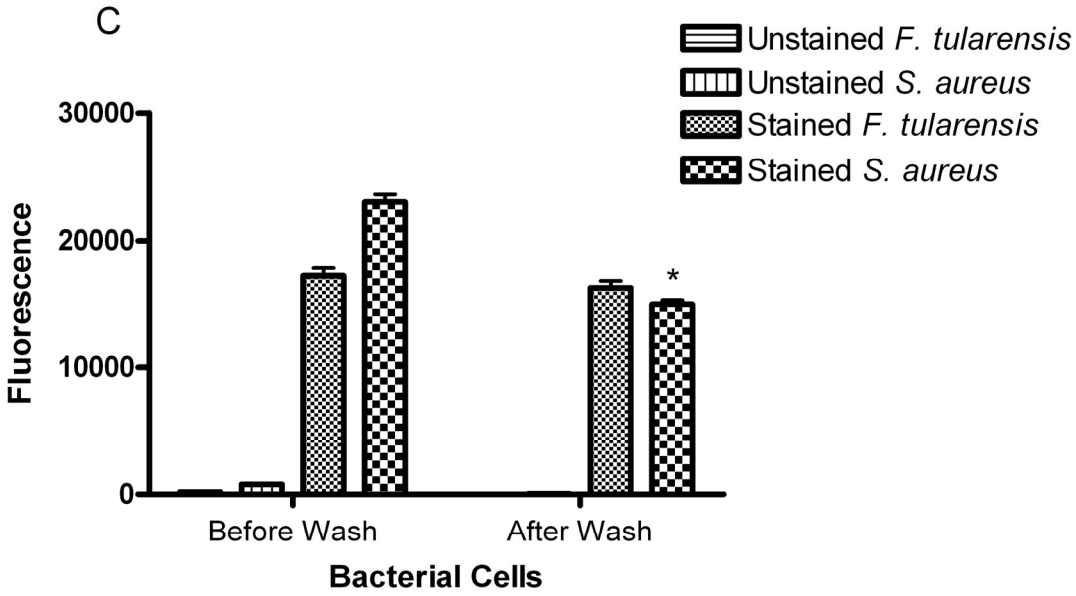
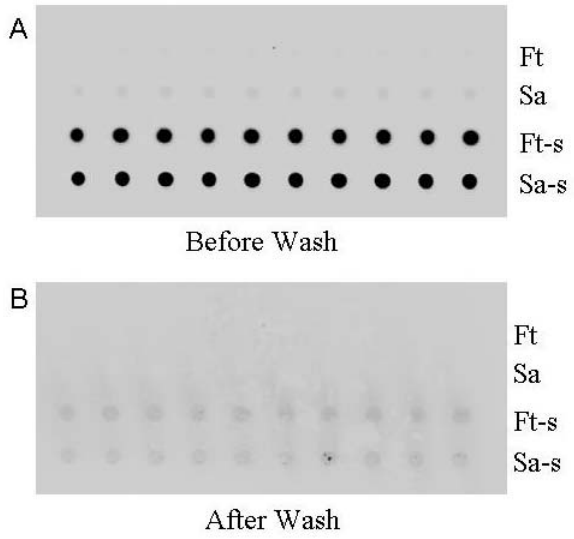

C
Unstained F. tularensis
Unstained S. aureus
Stained F. tularensis
Stained S. aureus
30000
20000
10000
0
Fluorescence
*
Before Wash
After Wash
Bacterial Cells

Figure 2. Adsorption of bacterial cells on poly-L-lysine coated slide. *Francisella tularensis* (Ft), *Staphylococcus aureus* (Sa) and fluorescent stained *F. tularensis* (Ft-s) and *S. aureus* (Sa-s) were suspended in PBS containing 20% Ficoll 400 and 4% glycerol at a cell density of ~ $5x10^{11}$ cells/ml and printed in replicates of 10 spots and scanned for fluorescence. Scanned images before and after wash are shown (A and B). Fluorescence signals before and after wash are presented (C). The results are representative of two independent experiments (two slides per experiment). The data points represent mean fluorescence value + SEM.


A
Ft
Sa
Ft-s
Sa-s
Before Wash
B
Ft
Sa
Ft-s
Sa-s
After Wash

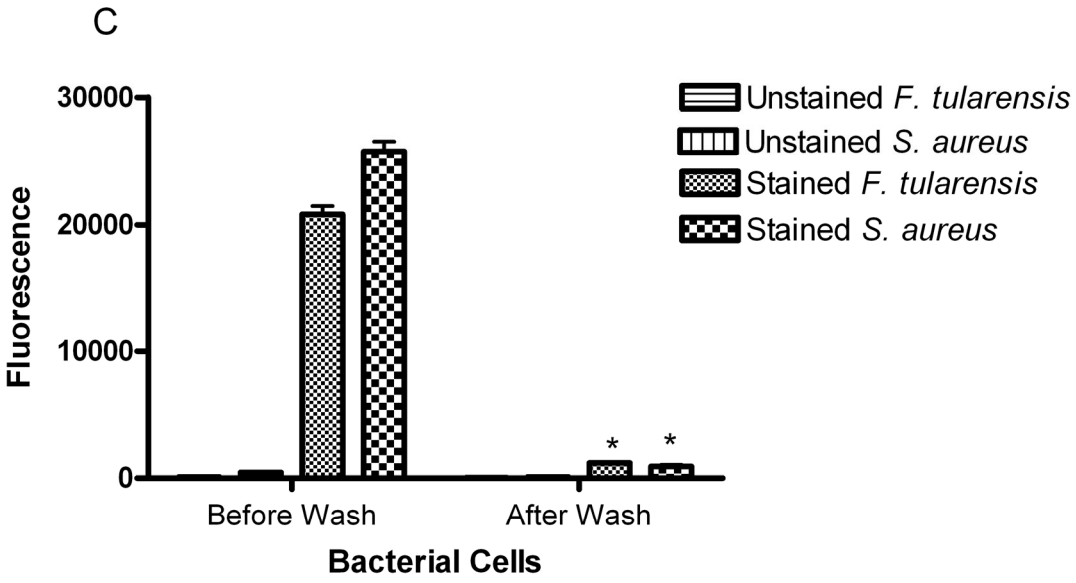
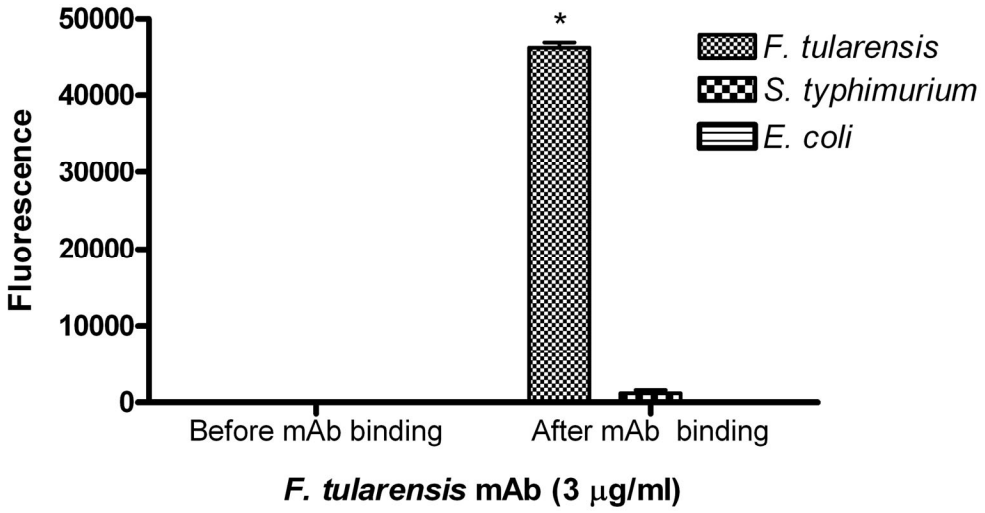

C
30000
20000
10000
0
Fluorescence
Unstained F. tularensis
Unstained S. aureus
Stained F. tularensis
Stained S. aureus
*
*
Before Wash
After Wash
Bacterial Cells

Figure 3. Binding of monoclonal antibodies to surface antigens of immobilized Gram negative bacteria. Cell suspensions of *F. tularensis, S. typhimurium* and *E. coli* were printed in replicates of twenty spots on nitrocellulose coated slides**.** Slides were incubated with monoclonal antibody (3µg/ml in PBS) specific for *F. tularensis* O-antigen (A) or *S. typhimurium* O-antigen (B). The bacterial cell arrays were developed with Alexa fluor 488 labeled goat anti-mouse secondary antibodies (5µg/ml). The results are representative of three independent experiments (two slides for each experiment). The data points represent mean fluorescence value + SEM.

A


50000
40000
30000
20000
10000
0
Fluorescence
*
F. tularensis
S. typhimurium
E. coli
Before mAb binding
After mAb binding
F. tularensis mAb (3 μg/ml)

B

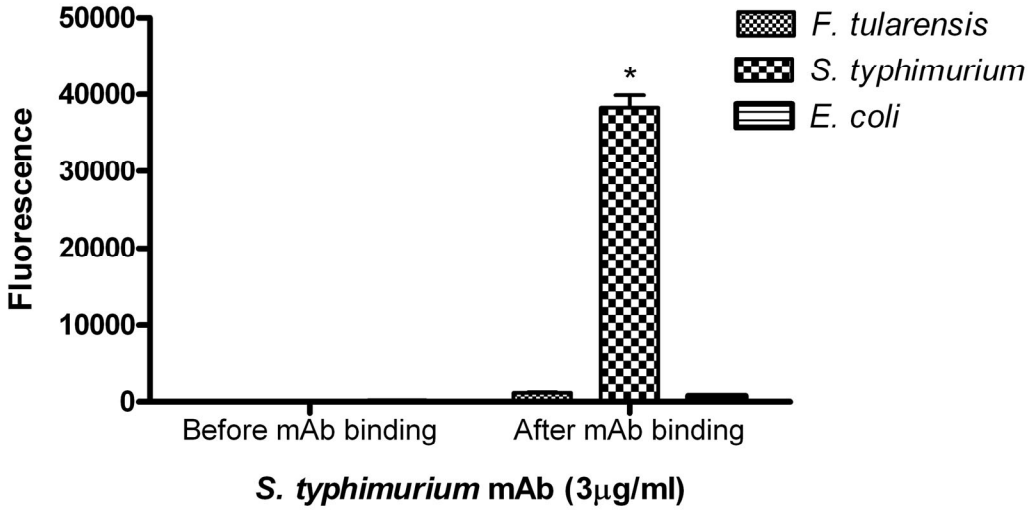
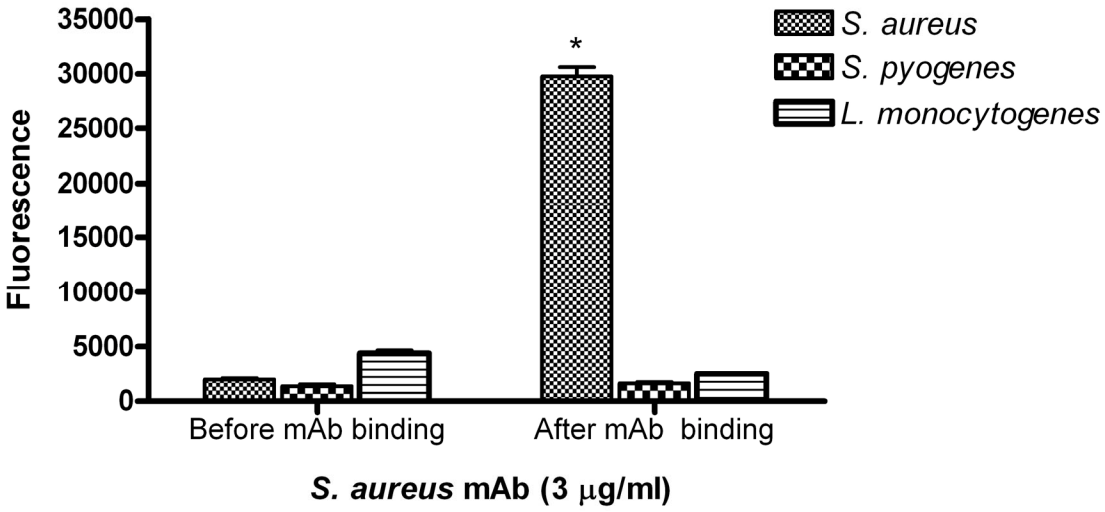

50000
40000
30000
20000
10000
0
Fluorescence
*
F. tularensis
S. typhimurium
E. coli
Before mAb binding
After mAb binding
S. typhimurium mAb (3μg/ml)

Figure 4. Binding of monoclonal antibodies to surface antigens of immobilized Gram-positive bacteria. Cell suspensions of *S. aureus*, *S. pyogenes* and *L. monocytogenes* were printed in replicates of twenty spots on nitrocellulose coated slides**.** Slides were incubated with monoclonal antibody (3 µg/ml in PBS) specific for peptidoglycan of *S. aureus* (A) or *L. monocytogenes* cells (B). The bacterial cell arrays were developed with Alexa fluor 488 labeled goat anti-mouse secondary antibodies (5 µg/ml). The results are representative of three independent experiments (two slides for each experiment). The data points represent mean fluorescence value + SEM.

A


35000
30000
25000
20000
15000
10000
5000
0
Fluorescence
*
S. aureus
S. pyogenes
L. monocytogenes
Before mAb binding
After mAb binding
S. aureus mAb (3 μg/ml)

B

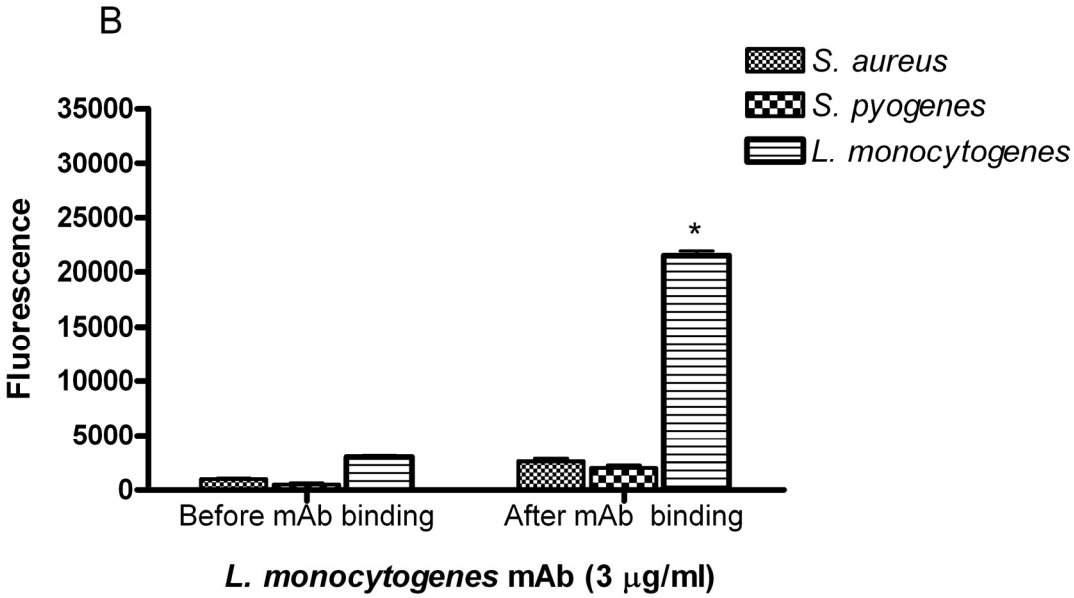
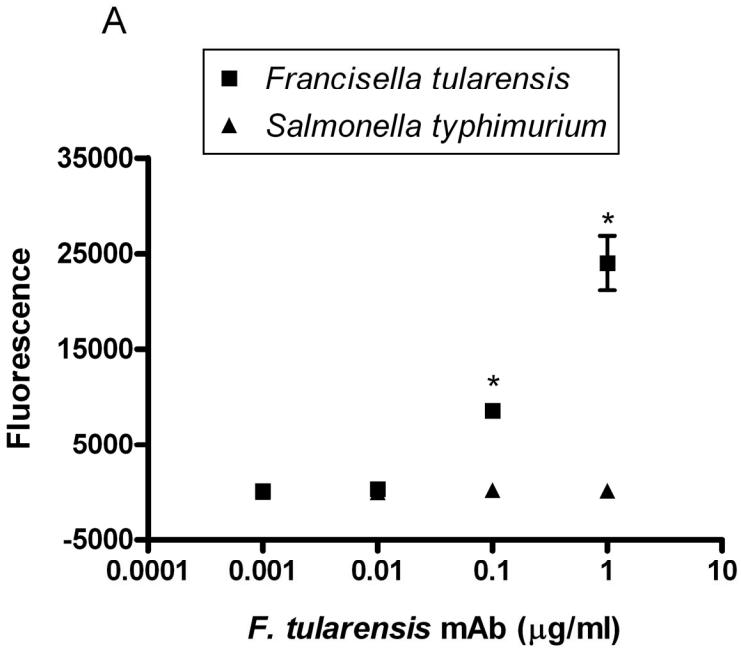

35000
30000
25000
20000
15000
10000
5000
0
Fluorescence
*
S. aureus
S. pyogenes
L. monocytogenes
Before mAb binding
After mAb binding
L. monocytogenes mAb (3 μg/ml)

Figure 5. Antibody detection limit of bacterial cell arrays. Cell suspensions of Gram-negative cells (A; *F. tularensis* and *S. typhimurium*) or Gram-positive cells (B; *S. aureus* and *L. monocytogenes*) were printed in replicates of ten spots on nitrocellulose coated slides**.** Slides were incubated with different concentrations of monoclonal antibodies (1, 0.1, 0.01 and 0.001 µg/ml in PBS) specific for *F. tularensis* O-antigen (A) or *L. monocytogenes* cells (B). The arrays were developed with Alexa fluor 488 labeled goat anti-mouse secondary antibodies (5 µg/ml). The results are representative of three independent experiments. The data points represent mean ± SEM.


A
Francisella tularensis
Salmonella typhimurium
35000
25000
15000
5000
-5000
Fluorescence
*
*
0.0001
0.001
0.01
0.1
1
10
F. tularensis mAb (μg/ml)

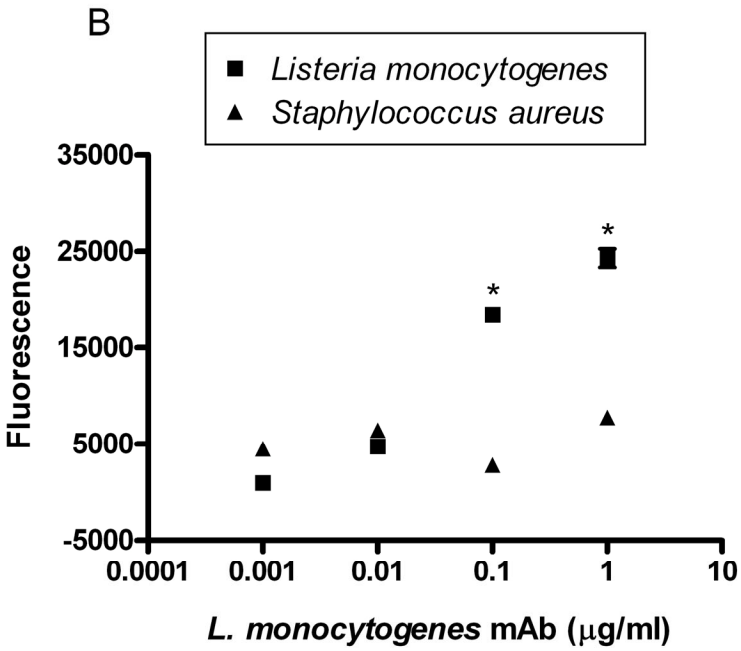
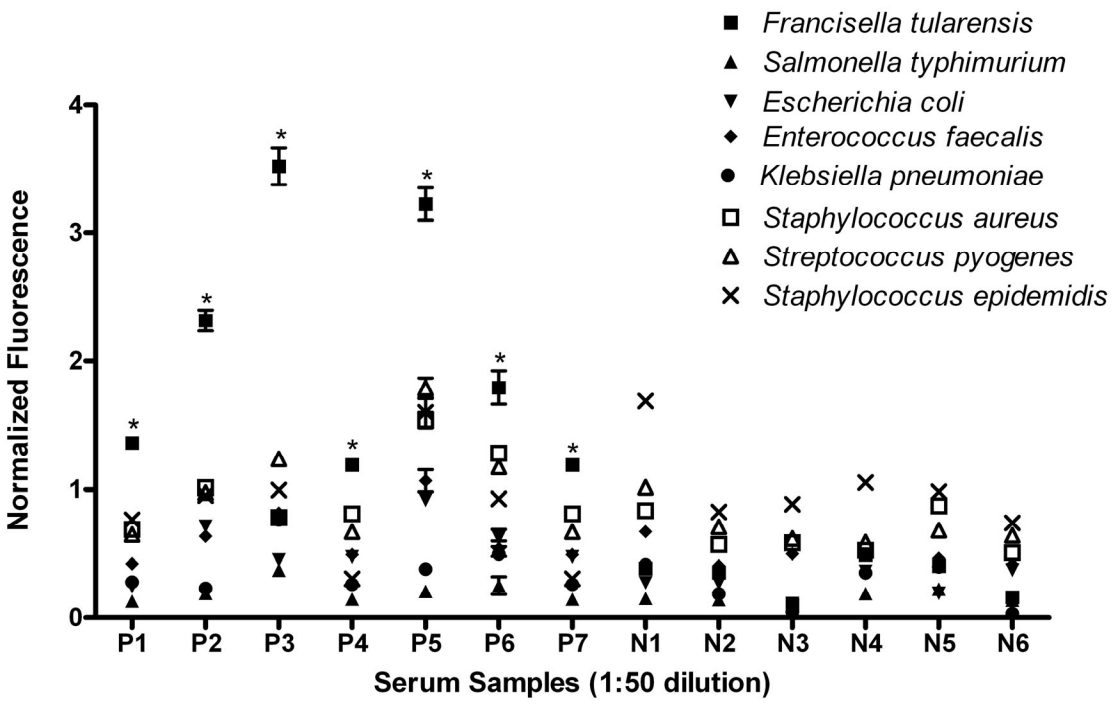

B
Listeria monocytogenes
Staphylococcus aureus
35000
25000
15000
5000
-5000
Fluorescence
*
*
0.0001
0.001
0.01
0.1
1
10
L. monocytogenes mAb (μg/ml)

Figure 6. *Francisella tularensis* specific antibody detection in canine serum samples. An array of eight different bacteria (*F. tularensis, S. typhimurium, E. coli, E. faecalis, K. pneumoniae, S. aureus, S. pyogenes and S. epidermidis*) was printed in replicates of twenty spots on nitrocellulose slides and incubated with canine serum samples (dilution 1:50) having a known microagglutination titer for *F. tularensis* antibodies. Seven positive (P1-P7; titer >20) and six negative serum samples (N1-N6; titer <20) were tested. Goat anti-canine secondary antibodies labeled with FITC were used at a concentration of 5µg/ml. The levels of specific *F. tularensis* antibodies were significantly higher in positive samples (P 1 to 7) compared to negative samples (N 1 to 6). The results are representative of three independent experiments. The fluorescence signals for all samples were normalized as ratios of fluorescence signal from a canine serum sample (1: 50 dilution) printed on the same slide and developed developed with FITC labeled anti-canine antibodies after background substraction. The data points represent mean ± SEM.


Francisella tularensis
Salmonella typhimurium
Escherichia coli
Enterococcus faecalis
Klebsiella pneumoniae
Staphylococcus aureus
Streptococcus pyogenes
Staphylococcus epidemidis
Normalized Fluorescence
0
1
2
3
4
P1
P2
P3
P4
P5
P6
P7
N1
N2
N3
N4
N5
N6
Serum Samples (1:50 dilution)

References

Disney, M. D., and P. H. Seeberger, 2004 The use of carbohydrate microarrays to study carbohydrate-cell interactions and to detect pathogens. *Chem.Biol.* **11**: 1701-1707.

Evans A. S., and P. S. Brachman, 1998 *Bacterial infections of humans epidemiology and control*. Plenum Medical Book Co, New York.

Goldsby R. A., 2003 *Immunology*. W.H. Freeman, New York.

Haesebrouck, F., F. Pasmans, K. Chiers, D. Maes, R. Ducatelle *et al.* 2004 Efficacy of vaccines against bacterial diseases in swine: what can we expect? *Vet.Microbiol.* **100**: 255-268.

Howbrook, D. N., d. van, V, M. C. O'Shaughnessy, D. K. Sarker, S. C. Baker *et al.* 2003 Developments in microarray technologies. *Drug Discov.Today* **8**: 642-651.

Itoh, S., M. Kariya, K. Nagano, S. Yokoyama, T. Fukao *et al.* 2002 New rapid enzyme-linked immunosorbent assay to detect antibodies against bacterial surface antigens using filtration plates. *Biol.Pharm.Bull.* **25**: 986-990.

Lamari, F., E. D. Anastassiou, G. Dimitracopoulos, and N. K. Karamanos, 2000a Immunochemical-based assays in the bioanalysis of immunoglobulins. *J.Pharm.Biomed.Anal.* **23**: 939-946.

Lamari, F., E. D. Anastassiou, T. Tsegenidis, G. Dimitracopoulos, and N. K. Karamanos, 1999 An enzyme immunoassay to determine the levels of specific antibodies toward bacterial surface antigens in human immunoglobulin preparations and blood serum. *J.Pharm.Biomed.Anal.* **20**: 913-920.

Lamari, F., N. K. Karamanos, E. Papadopoulou-Alataki, F. Kanakoudi-Tsakalidou, G. Dimitracopoulos *et al.* 2000b Monitoring of two intravenous immunoglobulin. Preparations for immunoglobulin G subclasses and specific antibodies to bacterial surface antigens and relation with their levels in treated immunodeficient patients. *J.Pharm.Biomed.Anal.* **22**: 1029-1036.

Marcotte, H., and M. C. Lavoie, 1993 Evaluation of mouse salivary IgA directed against indigenous oral bacteria. *J.Immunoassay* **14**: 63-81.

Nielsen, K., P. Smith, J. Widdison, D. Gall, L. Kelly *et al.* 2004 Serological relationship between cattle exposed to Brucella abortus, Yersinia enterocolitica O:9 and Escherichia coli O157:H7. *Vet.Microbiol.* **100**: 25-30.

Ofek, I., D. L. Hasty, and N. Sharon, 2003 Anti-adhesion therapy of bacterial diseases: prospects and problems. *FEMS Immunol.Med.Microbiol.* **38**: 181-191.

Poxton, I. R., 1995 Antibodies to lipopolysaccharide. *J.Immunol.Methods* **186**: 1-15.

Rossi, G. A., C. Peri, M. E. Raynal, A. C. Defilippi, F. M. Risso *et al.* 2003 Naturally occurring immune response against bacteria commonly involved in upper respiratory tract infections: analysis of the antigen-specific salivary IgA levels. *Immunol.Lett.* **86**: 85-91.

Samuelson, P., E. Gunneriusson, P. A. Nygren, and S. Stahl, 2002 Display of proteins on bacteria. *J.Biotechnol.* **96**: 129-154.

Schwenk, J. M., D. Stoll, M. F. Templin, and T. O. Joos, 2002 Cell microarrays: an emerging technology for the characterization of antibodies. *Biotechniques* **Suppl**: 54-61.

Thomas, R., and T. Brooks, 2004 Common oligosaccharide moieties inhibit the adherence of typical and atypical respiratory pathogens. *J.Med.Microbiol.* **53**: 833-840.

Verschoor, J. A., M. J. Meiring, W. S. van, and K. Weyer, 1990 Polystyrene, poly-L-lysine and nylon as adsorptive surfaces for the binding of whole cells of

Mycobacterium tuberculosis H37 RV to ELISA plates. *J.Immunoassay* **11**: 413-428.

Wang, D., 2003 Carbohydrate microarrays. *Proteomics.* **3**: 2167-2175.

Watanabe, K., T. Joh, K. Seno, M. Sasaki, I. Todoroki *et al.* 2001 Development and clinical application of an immunoassay using intact Helicobacter pylori attached to a solid phase as an antigen. *Clin.Biochem.* **34**: 291-295.

Wernerus, H., and S. Stahl, 2004 Biotechnological applications for surface-engineered bacteria. *Biotechnol.Appl.Biochem.* **40**: 209-228.

CHAPTER VII

CONCLUSIONS

The purpose of the present study was to apply recent advances in molecular biology in developing novel methods for the detection of bacteria and diagnosis of bacterial infections. Specifically, the study attempted to adopt an *in vitro* selection process SELEX (Systematic Evolution of Ligands by Exponential Enrichment) for developing ssDNA aptamers against a surface target of *Francisella tularensis* as an alternative to antibodies. Further, microarray-based immunoassays were developed for the detection of antibodies to lipopolysaccharide (LPS), and bacterial surface antigens. The salient findings of the present study are

- DNA aptamers were selected against the O-antigen of *F. tularensis* subspecies *tularensis* (Strain OSU10). A selected aptamer Ft1 showed specific binding to LPS of *F. tularensis* subspecies *tularensis* and subspecies *holarctica.* However, the aptamer did not show significant binding to whole cells of *F. tularensis*. This could be due to recognition of the core oligosaccharide part of LPS by the aptamer. The core oligosaccharide may not be readily accessible on the surface of bacteria for aptamer binding. Alternatively, use of whole cells in the selection process may help in developing aptamers to surface targets of bacteria.
- The structure of the O-antigen of *F. tularensis* subspecies *tularensis* (strain OSU 10) was analyzed by ^{1}H- and ^{13}C-NMR spectroscopy. The NMR data suggest that the O-antigen of *F. tularensis* subspecies *tularensis* strain OSU 10 has an

identical O-antigen as that of *F. tularensis* subspecies *holarctica* strain 15 and *F. tularensis* LVS. The O-antigen of these subspecies consist of repeating tetra-saccharide subunits: 4-(α-D-Gal*p*NAcAN-(1-4)- α-D-Gal*p*NAcAN-(1-3)- β-D-Qui*p*NAc-(1-2)-β -D-Qui*p*4NFo-1).

- Lipopolysaccharide microarrays were developed for the detection of antibodies. LPS arrays will likely to find applications in disease diagnosis and seroepidemiology of Gram-negative bacterial infections. LPS arrays could be used in evaluation of intravenous immunoglobulin preparations for prophylactic or therapeutic use in endotoxemia/sepsis associated with Gram-negative bacterial infections and assessment of patients with sepsis/endotoxemia or patients prior to surgery. LPS arrays may be useful in characterization of anti-LPS antibodies and other ligands.
- Bacterial cell arrays were developed for the detection and characterization of antibodies to surface antigens. Bacterial arrays have potential use in disease diagnosis and seroepidemiology of bacterial infections. Further, bacterial arrays could find applications in screening of intravenous immunoglobulin preparations for the presence of pathogen specific antibodies for use in the treatment of bacterial infections and screening potential anti-adhesion therapeutics.

Aptamers offer several advantages over antibodies. Although, a considerable progress has been made in case of aptamers as therapeutics, their potential in diagnostics yet has to be realized. Microarray based immunoassays are multiplex in nature allowing

simultaneous screening of a sample against multiple targets and require a small amount of reagents and samples. These assays have many potential applications including disease diagnosis and seroepidemiology.

VITA

Nagaraja Thirumalapura

Candidate for the Degree of

Doctor of Philosophy

Thesis: APPLICATIONS OF MOLECULAR METHODS IN DIAGNOSTICS OF BACTERIAL PATHOGENS

Major Field: Veterinary Biomedical Sciences

Biographical:

Education:

- Graduated from Kalpataru Science College, Bangalore University, Karnataka, India in May 1990
- Received Bachelor of Veterinary Science degree from the University of Agricultural Sciences, Bangalore, India in December 1995
- Master of Veterinary Science degree in Immunology from Indian Veterinary Research Institute, Izatnagar, India in July 1998
- Completed the requirements for the Doctor of Philosophy degree with a major in Veterinary Biomedical Sciences at Oklahoma State University in May 2005.

Experience:

- Research and Development Assistant at Jawaharlal Nehru Center for Advanced Scientific Research, Bangalore, India in 1999
- Teaching Faculty at the University of Agricultural Sciences, Bangalore, India in 2000
- Research Associate at Indian Council of Agricultural Research, Bangalore, India during 2000-2001
- Assistant Executive in the National Dairy Development Board, Hubli, India in 2001
- Graduate Research Assistant at Oklahoma State University, Stillwater, Oklahoma during 2001-2005

Professional Memberships: American Society for Microbiology

Name: Nagaraja Thirumalapura Date of Degree: May, 2005

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: APPLICATION OF MOLECULAR METHODS IN DIAGNOSTICS OF BACTERIAL PATHOGENS

Pages in Study: 159 Candidate for the Degree of Doctor of Philosophy

Major Field: Veterinary Biomedical Sciences

Scope and Method of study: The present study investigated the applications of recent advances in molecular techniques for developing novel diagnostics of bacterial pathogens. The study adapted an *in vitro* selection method known as systematic evolution of ligands by exponential enrichment (SELEX) for generation of ssDNA aptamers against *Francisella tularensis* subspecies *tularensis* (Strain OSU 10) lipopolysaccharide (LPS). The study also reports the ^{1}H- and ^{13}C-NMR spectroscopy structural analysis of O-antigen of *F. tularensis* subspecies *tularensis* (strain OSU 10). Further, microarray based immunoassays were developed for the detection of antibodies to bacterial antigens.

Findings and Conclusions: Single stranded DNA aptamers were selected against the purified carbohydrate part of LPS (containing core oligosaccharide and O-polysaccharide) from *F. tularensis* subspecies *tularensis* (Strain OSU 10). A selected aptamer Ft1 showed specific binding to LPS from *F. tularensis* subspecies *tularensis* and subspecies *holartica*. However, the aptamer did not show significant binding to whole cells of *F. tularensis*. This could be due to recognition of the core oligosaccharide part of the LPS by the aptamer and the core oligosaccharide may not be readily accessible on the surface of the bacterium. Alternatively, use of whole bacterial cells as target in the selection process could help in developing aptamers to surface targets of bacteria. Structural analysis suggested that the O-antigen of *F. tularensis* subspecies *tularensis* strain OSU 10 is identical to that of subspecies *holarctica* strain 15 and *F. tularensis* LVS and consists of repeating tetra-saccharide subunits: 4-(α-D-GalpNAcAN-(1-4)- α-D-GalpNAcAN-(1-3)- β-D-QuipNAc-(1-2)-β -D-Quip4NFo-1). A microarray based immunoassay was developed for the detection of antibodies to lipopolysaccharides. The assay showed a hundred-fold greater sensitivity compared to conventional immunoassays. Further, bacterial cell arrays were developed for the detection and characterization of antibodies to cell surface antigens. These microarray-based immunoassays are multiplex in nature and have many potential applications including disease diagnosis and seroepidemiology.

ADVISOR'S APPROVAL: Dr. Jerry Malayer